Quantitative Assessment of Protection in Experimental Syphilis

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Protection in experimental rabbit syphilis has been previously assessed by lesion development following intradermal challenge with *Treponema pallidum*. We have recently reported that passive immunization using monoclonal antibody M131 conveys partial protection as evidenced by significant lesion delays following intradermal challenge (D. R. Blanco et al., *Infect. Immun.* 73:3083–3095, 2005). To determine whether such delays in time to lesion appearance corresponded to decreases in the numbers of spirochetes, we used real-time PCR to quantitate *T. pallidum* genomic DNA copy numbers in lesion biopsies taken throughout the course of lesion development. Three groups of animals were given one prechallenge passive immunization with immune rabbit serum (IRS), M131, or control monoclonal antibody (CMAb) and then challenged with treponemal admixtures of IRS or monoclonal antibody in normal rabbit serum (NRS). As compared to the CMAb NRS controls, delays in the mean time to lesion appearance of 5.8 days for IRS and 8.8 days for M131 were observed. At the earliest time point (10 days postchallenge), real-time PCR showed a mean *T. pallidum* DNA copy number per μg of rabbit DNA in the CMAb NRS group of 7.65×10^3 copies, while no *T. pallidum* DNA could be detected in the M131 group. At approximately the mean time to lesion appearance in the IRS and M131 groups (17 and 20 days, respectively), the numbers of *T. pallidum* DNA copies were still 5- and 30-fold less, respectively, than those in the control group at these times. By 30 days postchallenge, the *T. pallidum* DNA copy numbers were similar in all three groups. These findings indicate that the delays in appearance of syphilitic lesions conferred by IRS and M131 corresponded to a marked decrease in treponemal numbers during the course of lesion development.

Treponema pallidum subsp. *pallidum* is the etiologic agent of venereal syphilis. While infection-derived immunity is a hallmark of experimental syphilis in the rabbit model, the molecular basis of this acquired protective immunity has not been elucidated. The outer membrane of *T. pallidum* has a remarkably low content of membrane-spanning protein (27, 33) and lacks lipopolysaccharide (3, 17, 18). Certain treponemal proteins have been advanced as outer membrane protein candidates based upon properties such as channel-forming activity (6), the induction of antibodies that result in opsonophagocytosis of *T. pallidum* (10, 12), or their representation in a paralogous gene family believed to represent antigenic variants (12–14, 17). None of these proteins has been definitively localized to the *T. pallidum* surface by physical means (1, 19, 28). Recently, using a monoclonal antibody termed M131, we identified a bone fide surface antigen of *T. pallidum* that is a target of bactericidal antibody and partial protective immunity (5, 7). The surface antigen bound by M131 was found to be membranous phosphorylcholine-containing lipid, not a protein (5). M131 administered to rabbits by passive immunization caused a significant delay in lesion appearance compared to controls (5).

The rabbit model of experimental syphilis has been used for many decades to assess protective immunity (32). Lesion delays following challenge of animals passively immunized with

immune rabbit serum (IRS) have been interpreted to represent partial protection (4, 26, 29, 31). This is based upon studies demonstrating that a 10-fold difference in challenge inoculum size results in a 3- to 4-day delay in time to lesion appearance (21, 31, 32). Over the past 20 years, numerous active immunization studies in rabbits, using native and recombinant *T. pallidum* proteins, have been conducted in an effort to identify targets of protective immunity (8–12, 15, 23). Lesion delays were not observed in any of these studies but rather in all cases, lesions developed either in the same time frame as that in controls or in an accelerated time frame. These lesions were judged to be atypical in terms of being smaller, less erythematous, less prone to ulcerate, and containing fewer treponemes, based upon dark-field microscopy examination of lesion aspirates. However, the accuracy of dark-field microscopy for enumerating treponemes in dermal lesions has never been established. Nonetheless, in some studies the development of atypical lesions has been interpreted to represent partial protection (9, 10, 12, 23). Whether such atypical lesions represent actual protective immunity has been controversial because bone fide quantitative microbiology has not been provided. In contrast to the study of other spirochetal pathogens, including *Borrelia burgdorferi* and *Leptospira interrogans*, where quantitative PCR has been employed to quantitate infection during experimental disease (20, 24, 30), the utilization of quantitative PCR has not been used in experimental syphilis.

In this study, we employed real-time PCR to quantitate *T. pallidum* DNA copy number and address whether delays in lesion appearance reflect a reduction in the numbers of *T. pallidum* at dermal injection sites. We found that delays in the time to lesion appearance, conferred by IRS and in particular

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M131, corresponded to marked differences in *T. pallidum* DNA copy numbers. The use of real-time PCR provides an accurate assessment of protective immunity as it relates to lesion development in experimental syphilis.

MATERIALS AND METHODS

Source of *T. pallidum*. *T. pallidum* subsp. *pallidum*, Nichols strain, was maintained by testicular passage in New Zealand White rabbits as described previously (22). IRS was acquired from rabbits infected intratesticularly for a period of at least 6 months and shown to be immune to challenge reinfection. Normal rabbit serum (NRS) was acquired from animals with nonreactive venereal disease research laboratory (VDRL) serology (25). All experiments using animals in this study were approved by the UCLA Animal Research Committee.

Passive immunization followed by treponemal admixture challenge. Rabbits with nonreactive VDRL serology were divided into three groups, each containing three rabbits, and were given a single passive intravenous immunization at 18 h before challenge. Animals in each group received either 10 ml of heat-inactivated (56°C for 30 min) IRS (H-IRS), 10 ml of mouse ascites containing 100 mg of an irrelevant control monoclonal antibody (CMAb) (QED Biosciences, Inc.), or 10 ml of mouse ascites containing 100 mg of M131. For preparation of the challenge inocula, *T. pallidum* was extracted from infected rabbit testes, as described above, and resuspended into heat-inactivated (56°C, 30 min) NRS (H-NRS) or H-IRS to a concentration of 1×10^4 organisms/ml. To the H-NRS suspensions, M131 or CMAb was added to a final concentration of 3 mg/ml. All suspensions were incubated for no longer than 30 min at room temperature in the inoculating syringes prior to challenge. For each of the three groups, animals were challenged intradermally on their shaved backs with 100 μ l of the respective admixture inoculum at 10 sites per rabbit (1×10^3 organisms/site). One representative site from each animal was biopsied at days 10, 13, 15, 17, 20, 24, and 30 postchallenge for quantitation of treponemes by real-time PCR. Four sites on each animal were dedicated exclusively for the observation of lesion appearance and development up to day 30 postchallenge.

Real-time PCR. Real-time PCR was performed as previously described (5, 16) on syphilitic dermal lesions from the passively immunized test and control animals. Genomic DNA acquired from biopsied tissue samples was prepared using the Easy-DNA kit from Invitrogen, Carlsbad, CA. Primers and probes were selected from the *flaA* gene of *T. pallidum* (GenBank accession no. M63142). The *flaA* forward primer runs from base 121 to base 141 (5'-GGAGGTATGA CGCATAATCGG-3'), and the reverse primer runs from base 202 to 180 (5'-A TGCCTTCTGCTCGTCAGTGAC-3'). The probe corresponds to base 146 to base 171 (5'-CCGTTCTGGACTATGCTTCTCTGGCG-3'). The collagenase 1 precursor gene (*MMP-1*) (exon 2) was selected for rabbit tissue quantitation (GenBank accession no. M17820). The forward primer for *MMP-1* runs from base 4220 to base 4237 (5'-CCGTCTACCTGGGTGCC-3'). The reverse primer runs from base 4274 to base 4296 (5'-ATGGATTTCCTTGCTTGATT CTG-3'). The probe corresponds to base 4243 to base 4270 (5'-TGTGCAGAC CACAGGAGCACTTGACAAC-3'). The probes were labeled at the 5' end with 6-carboxyfluorescein and at the 3' end with *N,N,N'*-tetramethyl-6-carboxytetramethylrhodamine. Primers and probes were purchased from QIAGEN, Valencia, CA. One hundred nanograms of DNA from infected rabbit tissue was used per reaction in triplicate. Taqman universal PCR master mixture (Applied Biosystems, Foster City, CA) was used for all reactions. Each reaction mixture (25 μ l) contained both primers at concentrations of 900 nM and 250 nM for the probe. A standard curve was plotted for each primer-probe set with C_T values obtained from amplification of known quantities of DNA isolated from *T. pallidum* and rabbit liver (Seegene, Seoul, Korea). Values for the *T. pallidum* standard curve were obtained in the presence of 100 ng of rabbit DNA. Each lesion biopsy was tested three times using real-time PCR for the average number of *T. pallidum* DNA copies per μ g of rabbit DNA. The copy number of each sample was determined by plotting the C_T value versus the log of the copy numbers included in each standard curve. Control reactions without template were included for each assay for both primer sets.

Statistical analysis. Significant differences in mean lesion incubation periods were compared by two-tailed *t* test analysis.

RESULTS

Protection in rabbits conferred by a combination of passive immunization and admixture challenge. We have recently reported that M131 used for passive immunization (5) or used in

TABLE 1. Syphilitic lesion development in rabbits following admixture intradermal challenge of passively immunized rabbits^a

Admixture	No. of lesions/sites	Time to detection (days)	
		Range ^b	Mean $\pm$ SE ^c
CMAb	12/12	10–14	11.0 $\pm$ 0.52
IRS	12/12	10–24	16.9 $\pm$ 1.70 ^d
M131 MAb	12/12	17–27	19.8 $\pm$ 0.87 ^d

^a Groups of three rabbits were given one passive immunization with CMAb, IRS, and M131 prior to challenge with admixtures containing *T. pallidum* and IRS or monoclonal antibody in NRS. Each animal was challenged intradermally with a total of 1×10^3 *T. pallidum* organisms at each of 10 sites. Four sites on each animal were dedicated to lesion observation, while the remaining sites were used for lesion biopsy and real-time PCR quantitation of treponemes.

^b The day to first detection of erythematous and indurated lesions.

^c SE, standard error.

^d $P < 0.001$, compared with the results with CMAb.

the skin protection assay (7) confers significant delays in lesion appearance following intradermal challenge. In view of these findings, we utilized in this study a combination of both methods in an attempt to achieve the greatest degree of lesion delay following challenge. As shown in Table 1, animals given one passive immunization with CMAb and then challenged with a *T. pallidum* admixture suspension containing NRS and CMAb developed typical syphilitic lesions at all sites (12 of 12), with a mean time to appearance of 11.0 ± 0.52 ($\pm$ standard error) days, respectively. This mean time to lesion appearance is within the range expected (11 to 14 days) for a challenge inoculum of 1,000 treponemes and is similar to the time of lesion appearance in our previous study in animals passively immunized with either NRS or CMAb (5). By comparison, significant delays in the appearance of lesions occurred in the animals given one passive immunization with IRS or M131 and then challenged with respective *T. pallidum* admixture suspensions containing IRS or M131. Admixture-inoculated sites with IRS (12 of 12 sites) and M131 (12 of 12 sites) showed mean times to lesion appearance of 16.8 ± 1.70 and 19.8 ± 0.87 days, respectively. Based upon previous challenge inoculation control studies (21, 31, 32), the significant delays in lesion appearance of 5.8 days for IRS and 8.8 days for M131 correspond to at least 90% and 99% reductions, respectively, in the number of virulent organisms following inoculation. Furthermore, the overall appearance of these delayed lesions, as compared to controls, was markedly atypical as these lesions were significantly smaller, less indurated, and less erythematous (data not shown).

Real-time PCR quantitation of *T. pallidum* DNA copy number throughout the course of lesion development. Numbers of *T. pallidum* DNA copies at the injection sites were determined by real-time PCR. The range, mean, and standard deviation and the mean and standard deviation of *T. pallidum* DNA copies per μ g of rabbit DNA for each group are presented in Table 2 and Fig. 1, respectively. At the earliest time point (10 days postchallenge), the mean number of *T. pallidum* DNA copies for the control group that received CMAb and NRS was 7.65×10^3 . The number of DNA copies continued to increase over the course of lesion development, reaching a maximum mean copy number of 7.32×10^6 at day 24 postchallenge. These mean *T. pallidum* DNA copy numbers throughout lesion development are similar to those in our previous study using

TABLE 2. Quantitation of the relative numbers of *T. pallidum* in lesions by real-time PCR

Time point (day)	No. of DNA copies detected	
	Range ^a	Mean $\pm$ SE ^b
M131		
10	No DNA detected	No DNA detected
13	0–1.56 $\times 10^3$	$7.58 \times 10^2 \pm 7.58 \times 10^2$
15	0–1.23 $\times 10^4$	$4.90 \times 10^3 \pm 3.76 \times 10^3$
17	0–2.84 $\times 10^4$	$9.69 \times 10^3 \pm 9.35 \times 10^3$
20	1.11×10^4 – 9.7×10^4	$1.84 \times 10^5 \pm 1.32 \times 10^5$
24	3.44×10^5 – 1.63×10^6	$1.07 \times 10^6 \pm 3.80 \times 10^5$
30	7.61×10^5 – 1.03×10^7	$4.13 \times 10^6 \pm 3.09 \times 10^6$
IRS		
10	0–1.34 $\times 10^4$	$4.52 \times 10^3 \pm 4.43 \times 10^3$
13	6.74×10^1 – 1.85×10^4	$6.46 \times 10^3 \pm 6.03 \times 10^3$
15	1.16×10^3 – 1.85×10^5	$6.60 \times 10^4 \pm 5.94 \times 10^4$
17	6.12×10^3 – 3.68×10^5	$1.27 \times 10^5 \pm 1.20 \times 10^5$
20	1.13×10^5 – 1.98×10^6	$1.06 \times 10^6 \pm 5.40 \times 10^5$
24	5.87×10^5 – 1.19×10^7	$4.68 \times 10^6 \pm 3.61 \times 10^6$
30	1.85×10^6 – 7.83×10^6	$4.87 \times 10^6 \pm 1.73 \times 10^6$
CMAb		
10	0 $\times 10^4$ – 1.3×10^4	$7.65 \times 10^3 \pm 3.93 \times 10^3$
13	6.73×10^4 – 7.59×10^4	$7.07 \times 10^4 \pm 2.65 \times 10^3$
15	1.66×10^5 – 5.62×10^5	$3.42 \times 10^5 \pm 1.16 \times 10^5$
17	2.17×10^5 – 8.14×10^5	$5.16 \times 10^5 \pm 1.72 \times 10^5$
20	3.40×10^6 – 8.20×10^6	$5.48 \times 10^6 \pm 1.42 \times 10^6$
24	5.59×10^6 – 9.69×10^6	$7.32 \times 10^6 \pm 1.23 \times 10^6$
30	1.30×10^6 – 9.23×10^6	$4.23 \times 10^6 \pm 2.51 \times 10^6$

^a Real-time PCR results from three punch biopsies per group.^b SE, standard error.

naïve animals challenged with the same number of treponemes used in this study (5). By comparison, a marked decrease in the mean DNA copy number was found at lesion sites in the animals passively immunized and admixture challenged with

either M131 or IRS. At the earliest time point of 10 days postchallenge, no *T. pallidum* DNA could be detected in the group that received M131. This was a >3-log difference as compared to the control group (7.65×10^3 copies). From days 13 through 24 postchallenge, the numbers of *T. pallidum* DNA copies in the M131 group remained lower than those of the control group, showing 93-, 70-, 53-, 30-, and 6.8-fold differences at days 13, 15, 17, 20, and 24 postchallenge, respectively. For the group that received IRS, the decrease in the mean DNA copy number, while not as large as that determined for the M131 group, was found to range from a 1.7-fold difference to a 10-fold difference over days 10 to 24 postchallenge. However, at day 30 postchallenge, the numbers of *T. pallidum* DNA copies in the M131 and IRS groups were both similar to those in the control group. As a further analysis, biopsies were obtained throughout lesion development for real-time PCR at sites not inoculated. No *T. pallidum* DNA was detected at these sites, indicating that spirochete multiplication was localized only to the sites of lesion development (data not shown).

DISCUSSION

We have recently reported that monoclonal antibody M131, which is directed against a membranous phosphorylcholine surface epitope on *T. pallidum*, can convey significant partial protective immunity in rabbits against challenge following either passive immunization (5) or with the skin protection assay developed by Titus and Weiser (7, 29). This conclusion was based upon significant lesion delays of up to 8 days, as compared to controls, following intradermal challenge with *T. pallidum*. Based upon previous inoculation control studies (21, 31, 32), an 8-day delay would theoretically correspond to a 99% decrease of 1,000 organisms following their inoculation. Delays in lesion appearance in rabbits following challenge has only been reported in previous passive immunization studies using IRS (4, 26, 29, 31) and in an active immunization study using the VDRL antigen as an immunogen (2). Using IRS, the greatest delay observed, using a passive immunization protocol similar to what has been used for M131 (5), has been 3 to 4 days (26, 31). Thus, M131 has been able to convey a level of protective immunity by passive immunization that has exceeded that of IRS.

Over the past 20 years, active immunizations in rabbits with recombinant and native *T. pallidum* proteins, including endoflagella (15), 4D (8), glycerophosphodiester phosphodiesterase (9), Tp92 (10), the 15-kDa lipoprotein (11), and recently the *T. pallidum* repeat protein K (TprK) (12, 23), followed by challenge have resulted in atypical lesions that developed either in the same time frame as controls or in an accelerated time frame, but not in lesion delays. These atypical lesions have been noted to be smaller, less erythematous, less prone to ulcerate, and to contain fewer or no spirochetes based upon dark-field microscopy examination of lesion aspirates. In some of these studies, these observations have been used as the basis for a conclusion of partial protective immunity (9, 10, 12, 23), while in other studies, some of these conclusions have been challenged (19, 28). To date there is no definitive evidence that atypical lesions that develop in the same time frame as controls or in an accelerated time frame represent meaningful protective immunity.

FIG. 1. Quantitation of *T. pallidum* in lesions using real-time PCR. Lesion biopsies from passively immunized and admixture-challenged animals in each group were acquired at each of the postchallenge time points indicated for real-time PCR analysis. The mean number of *T. pallidum* (Tp) DNA copies detected per microgram of rabbit DNA and standard error are plotted. CMAb, ■; IRS, ▲; and M131, ◆.

In this study, we utilized real-time PCR as a method of quantitating the numbers of multiplying *T. pallidum* organisms in syphilitic lesions that were delayed in order to determine whether delayed lesions represent an alteration of the infectious process. Passive immunization of rabbits using IRS or M131 combined with admixture treponemal challenge resulted in significant 5.8- and 8.8-day delays, respectively, in the mean time to lesion appearance, which further confirms our previous M131 passive immunization findings (5). At 10 days postchallenge in the CMAb control group, which was just prior to the mean appearance time of lesions in this group (11.0 days), significant numbers of *T. pallidum* DNA copies could be detected at the challenge sites, showing a mean copy number of 7.65×10^3 . This number of DNA copies was similar to the findings in our recent study using naïve rabbits challenged with *T. pallidum* (5). In contrast, no *T. pallidum* DNA could be detected at 10 days postchallenge in animals that received M131, which represents at least a 3-log difference as compared to the CMAb group. At day 17 postchallenge, when lesions first appeared at some sites on the animals that received M131, and at 20 days postchallenge, which was the mean time to lesion appearance in the M131 group, the *T. pallidum* DNA copy number at these sites was still markedly lower than that of the CMAb group (53- and 30-fold less, respectively). While not as striking as the M131 group, the delayed lesions in animals passively immunized with IRS and challenged with IRS admixtures also showed lower numbers of *T. pallidum* DNA copies as compared to controls. At 17 days postchallenge, which was the mean time to lesion appearance in the IRS group, there was a fivefold decrease in the mean number of DNA copies as compared to that in controls. This decrease in DNA copy number in the IRS group was present over the entire 10- to 24-day postchallenge period of lesion development and ranged from 1.7- to 10-fold. The most likely explanation for the greater decrease in DNA copy numbers at inoculation sites in animals receiving M131, as compared to IRS, is the greater degree of killing afforded by M131 immediately following inoculation challenge, which was also reflected in a greater delay in lesion appearance. Because killing of spirochetes at sites from the animals passively immunized with M131 and IRS was not 100%, eventually the multiplication of these remaining spirochetes reached numbers comparable to those in the controls, which occurred 30 days after challenge. Thus, the findings of this study demonstrate that protection related to M131 and IRS was evident both as a marked reduction in the numbers of *T. pallidum* at the injection sites in the first 24 days after challenge and as a significant delay in the time to lesion appearance.

The findings presented in this study have important implications for drawing a conclusion of protective immunity based solely upon atypical lesion development and/or dark-field microscopy of lesion aspirates. As mentioned above, previous active immunization and challenge studies in rabbits have resulted in atypical lesions that have developed either in the same time frame as controls or in an accelerated time frame relative to controls, but not in delays in lesion appearance (8–12, 15, 23). Again, in several of these studies, a conclusion of partial protective immunity was made based upon the appearance of atypical lesions and fewer treponemes observed by dark-field microscopy of lesion aspirates (9, 10, 12, 23). As

mentioned above, it has not been established that dark-field microscopy of dermal lesion aspirates represents an accurate quantitative assessment of treponemal numbers in lesions. It is possible that these nondelayed or accelerated atypical lesions merely represent an alteration of the disease process without an alteration of the infectious process. In view of the results presented in this study, we believe that the real-time PCR methodology provides an unbiased quantitative assessment of the infectious process occurring during lesion development and should be used in all future challenge studies in order to provide an objective basis for conclusions regarding protective immunity.

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