Correlation of Immunity in Experimental Syphilis with Serum-Mediated Aggregation of *Treponema pallidum* Rare Outer Membrane Proteins

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We have previously shown by freeze-fracture electron microscopy that serum from infection-immune syphilitic rabbits aggregates the low-density membrane-spanning *Treponema pallidum* rare outer membrane proteins (TROMPs). The purpose of this study was to determine if a relationship could be demonstrated between acquired immunity in experimental rabbit syphilis, serum complement-dependent treponemicidal antibody, and antibody directed against TROMPs as measured by the aggregation of TROMP particles. Three groups of *T. pallidum*-infected rabbits were treated curatively with penicillin at 9 days, 30 days, and 6 months postinfection to generate various degrees of immunity to challenge reinfection. Sera from rabbits completely susceptible to localized and disseminated reinfection possessed a low titer of treponemicidal antibody (≤1:1 in killing ≥50% of a treponemal suspension) and showed a correspondingly low level of TROMP aggregation (16.5% of the total number of outer membrane particles counted) similar to normal serum controls (13.4%); the number of particles within these aggregates never exceeded three. Sera from partially immune rabbits, which were susceptible to local reinfection but had no evidence of dissemination, showed an increase in the titer of treponemicidal antibody (1:16) compared to the completely susceptible group (≤1:1). Although no significant increase was observed in the total number of TROMP particles aggregated (18.9%) compared to the number in controls (13.4%), approximately 15% of these aggregates did exhibit a significant increase in the number of particles per aggregate (4 to 5 particles) compared to controls (≤3 particles), indicating a measurable increase in anti-TROMP antibody. Finally, sera from rabbits completely immune to both local and disseminated reinfection possessed both high titers of treponemicidal antibody (1:128) and significant aggregation of TROMP (88.6%); approximately 50% of these aggregates contained four to six particles. The results indicate that complete immunity in experimental rabbit syphilis correlates with antibody that kills *T. pallidum* and aggregates TROMPs, suggesting that TROMPs are molecules which contribute to the development of acquired immunity.

Syphilis, caused by the noncultivatable spirochete *Treponema pallidum* subsp. *pallidum*, continues to be a significant worldwide venereal disease. The chronicity of infection in both human and experimental rabbit syphilis and the slow development of immunity to reinfection have been well-established (10, 11, 19, 20, 21, 24, 38). Several lines of evidence support a major role for humoral immunity, including immune serum passive protection (4, 6, 13, 29, 33, 35–37, 40), inhibition of *T. pallidum* adherence and invasion of cultured cell monolayers by immune serum (12, 14, 34), immune serum-mediated phagocytosis of *T. pallidum* by rabbit peritoneal macrophages (18), and immune serum complement-dependent treponemicidal antibody (5, 6, 8, 27, 28). It has been demonstrated that a close quantitative correlation exists between the development of acquired resistance and the level of treponemicidal antibody (5, 6), suggesting that killing antibody plays a key role in the acquisition of protective immunity.

One of the more fascinating physical features of *T. pallidum* is its strikingly low density of membrane-spanning outer membrane proteins (31, 39) (*T. pallidum* rare outer membrane proteins [TROMPs]) which are believed to contribute to the pathogenic properties of this organism, including its ability to cause chronic infection and elicit a relatively slow-developing protective immune response. The aggregation of TROMPs in serum from infected and immune syphilitic rabbits (7, 9), as viewed by freeze-fracture electron microscopy, has shown that TROMPs have surface exposure and therefore represent the most likely surface targets for complement-dependent treponemicidal antibody.

In order to measure complement-dependent treponemicidal antibody directed solely against surface targets on *T. pallidum*, such as TROMPs, we developed a procedure termed the "washed-killing" assay (15). In this system, organisms are preincubated in heat-inactivated test serum and then washed to remove unbound antibody prior to the addition of complement. These studies, which utilized animals with various degrees of resistance to challenge reinfection, showed a quantitative correlation between the titer of killing antibody and level of acquired immunity, suggesting that killing antibody against surface-exposed molecules is a key mechanism of acquired host resistance.

In the present study, we tested whether a direct relationship between the status of acquired immunity in experimental rabbit syphilis and antibody against TROMPs can be demonstrated. In order to most closely relate serum killing activity and TROMP aggregation to the degree of protective immu-

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nity, sera analyzed in this study were obtained from postchallenge test animals at the time of lesion appearance in the control animals. The results show that when syphilitic lesions appeared in the control animals, complete immunity in the test animals correlated with the presence of high-titered treponemicidal antibody and antibody which significantly aggregates TROMPs. These findings suggest that TROMPs are the primary targets of treponemicidal antibody and are the molecules responsible for eliciting protective immunity.

Syphilitic infection and curative therapy of rabbits. Fifty rabbits were infected intratesticularly with *T. pallidum* Nichols and treated with curative doses of penicillin G at various times postinfection in order to generate different degrees of immunity to challenge reinfection as previously described (15). Each animal received 2.5×10^7 *T. pallidum* cells per testis. The animals were divided into two groups of 17 rabbits each (groups A and B) and one group of 16 rabbits (group C). At 9 days (group A), 30 days (group B), and 6 months (group C) after intratesticular infection, each animal was treated with 25,000 U of aqueous procaine penicillin G/kg of body weight administered intramuscularly twice daily for 10 days (total of 500,000 U/kg of body weight). Ten days after therapy was completed, serum from each of the treated rabbits was shown to be free of penicillin levels capable of killing *T. pallidum*, based on the ability of this serum to support the viability of the organisms for 16 h in vitro. The efficacy of the treatment was determined 14 days after the completion of therapy with an infectivity test (23), in which a single popliteal lymph node and testis from each animal were removed under anesthesia, suspended in 50% heat-inactivated (56°C for 30 min) normal rabbit serum (NRS) in phosphate buffered saline, and inoculated intratesticularly into normal, serologically nonreactive rabbits. The sensitivity of this assay has been shown to be capable of detecting one to four virulent *T. pallidum* cells in a transferred tissue inoculum (23). Each of the treated animals was found to be free of infection based on dark-field microscopy-negative aspirates from the testes and nonreactive venereal disease research laboratory and *T. pallidum* immobilization tests (25, 26) on serum obtained from the recipient rabbits over 6 months.

Immune status of infected and penicillin-treated rabbits. To determine susceptibility to reinfection, each penicillin-treated rabbit and five serologically nonreactive control rabbits were challenged 35 days after therapy with 10^3 *T. pallidum* cells at each of the four intradermal sites as previously described (15). Each rabbit was examined daily for 90 days for lesion appearance and development. All lesions in the test and control animals were observed to appear 11 to 17 days postchallenge. Erythematous, indurated, well-circumscribed lesions progressing to ulceration were considered typical, and atypical lesions were characterized as pale, soft, flat, irregular, and nonprogressive. Aspirates from representative lesions more than 5 mm in diameter were taken from each rabbit at the time of peak lesion development and examined by dark-field microscopy for the presence of motile treponemes. At the end of the 90-day observation period, the animals were euthanized and the second popliteal lymph node and testis were assayed for treponemes by the infectivity test as described above (23). Rabbits in which dark-field microscopy-positive lesions developed within the same incubation period as the controls and which exhibited disseminated infection by infectivity testing were considered susceptible to reinfection. Rabbits were characterized as partially immune if they exhibited dark-field microscopy-negative lesions without disseminated infection or completely immune if lesions and disseminated infection did not develop.

Sera. Serum from infected, treated, and challenged test rabbits, described above, was obtained 17 days postchallenge when lesions appeared in the control animals at all inoculated sites (11 to 17 days). NRS was obtained from animals with negative venereal disease research laboratory and *T. pallidum* immobilization tests (25, 26). Immune rabbit serum (IRS) from animals immune to challenge infection with 10^3 treponemes at four sites was obtained from animals infected for 6 months following their intratesticular injection with a total of 4×10^7 cells of *T. pallidum*.

Washed-killing treponemicidal assay. Complement-dependent treponemicidal antibody in sera from two representative rabbits each from groups A, B, and C was measured quantitatively by using the washed-killing assay as described previously (15). The treponemicidal endpoint (TE) was defined as the reciprocal of the highest dilution that exhibited ≥50% treponemal immobilization. Treponemes immobilized under similar conditions have been shown to be killed based upon virulence testing by intradermal injection of rabbits (5). Undiluted sera exhibiting differences in motility of 21 to 49% between the test (with complement) and control (without complement) tubes were considered to have a TE of ≤1. Control criteria included a quantitative IRS with a previously established endpoint. Assays were considered valid when the IRS titers were within 1 dilution of the established endpoint and when residual complement activity could be demonstrated as previously described (41). Each serum was run in duplicate on different days, and tests were considered valid when the endpoints were within 1 dilution of each other. The TE was recorded as the reciprocal of the average of two valid assays.

Freeze-fracture electron microscopy. Sera from the representative rabbits that were tested for treponemicidal antibody were further analyzed for their ability to aggregate TROMPs following incubation with live *T. pallidum* cells. Each test serum was set up in quadruplicate as follows. One hundred microliters of a suspension containing approximately 5×10^7 treponemes/ml extracted in heat-inactivated (56°C for 30 min) NRS was combined with 900 μl of heat-inactivated test serum (1:9 ratio of treponemal suspension to test serum as used above for the washed-killing assay). The serum-treponeme mixtures were equilibrated in an atmosphere of 95% N_2 and 5% CO_2 and incubated at 34°C for 16 h to allow antibody against treponemal surface molecules to bind in the absence of complement (100% of treponemes were observed by dark-field microscopy to be actively motile following the incubation). The suspensions were then centrifuged at $8,000 \times g$ for 10 min to pellet the treponemes, and the treponemal pellets were washed by suspension in 1 ml of phosphate-buffered saline and then centrifuged as described above. Each of the four treponemal pellets from an individual test serum was then fixed for 1 h at room temperature by suspension in 500 μl of 0.1 M sodium cacodylate buffer (pH 7.4) containing 2.5% glutaraldehyde. After fixation, the suspensions were centrifuged at $10,000 \times g$ to pellet the treponemes, and the treponemal pellets were then combined by suspension in 20 μl of 0.1 M sodium cacodylate buffer (pH 7.4) containing 20% glycerol. Freeze-fracture electron microscopy was then performed as previously described (9, 39). Particle enumeration was made by counting the total number of individual and aggregated particles from 20 to 24 concave outer membrane fracture faces (a total of approximately 1 μm²) in sera from each immune-status group, NRS, and IRS. Particle aggregation was defined as two or more adjacent particles. Numbers of particles within an aggregate were determined by both counting the particles and determining the surface area of the aggregate in comparison to the surface area of an individual particle. Standard error compar-

TABLE 1. Immunity correlates with serum treponemicidal activity and TROMP aggregation

Rabbit serum (n)	Immune status[a]	TE[b]	OM fracture-face observations	Total no. of particles/μm^2	% Aggregation[c]
Group A (17)	Susceptible	≤1:1	24	54	16.5 ± 5.6
Group B (17)	Partial	1:16	20	41	18.9 ± 6.5
Group C (16)	Complete	1:128	20	79	88.6 ± 5.4[d]
Normal	Susceptible	0	21	48	13.4 ± 4.2
Immune	Complete	1:128	20	53	53.9 ± 7.8[d]

[a] Immune status of infected, treated, and challenged rabbits. Susceptible, dark-field microscopy-positive lesions and disseminated infection; partial, dark-field microscopy-negative lesions and no disseminated infection; complete, no lesions or disseminated infection.

[b] The TE was determined as the final serum dilution which immobilized ≥50% of a treponemal suspension incubated for 16 h in the presence of complement.

[c] Percent aggregation (mean ± standard error) was determined from the number of aggregates consisting of two or more particles and the total number of particles counted.

[d] $P < 0.0001$, compared with the results for the susceptible animals and normal control animals.

ison was used for all particle enumeration and percent particle aggregation analyses. Significances were based upon Student's t test.

Status of immunity correlates with antibody that kills *T. pallidum*. In order to determine the complement-dependent treponemicidal-antibody level at a time of symptomatic infection following challenge, sera from infected, penicillin-treated, and challenged animals were obtained during the time of lesion appearance in the control animals (17 days). As shown in Table 1, animals remaining susceptible to both symptomatic and disseminated challenge reinfection, as determined from the presence of typical, dark-field microscopy-positive lesions, showed only low-titered (≤1:1) serum treponemicidal activity. Similarly, no treponemicidal activity was present in the serum of normal control animals which showed identical lesions. In comparison, animals which exhibited partial immunity, characterized by atypical dark-field microscopy-negative lesions and the absence of disseminated reinfection following challenge, showed a corresponding increase in the titer (1:16) of treponemicidal activity. Finally, sera from animals completely immune to both local (no lesion appearance) and disseminated

challenge reinfection had high-titered (1:128) treponemicidal activity, as did sera from immune control animals (1:128).

Status of immunity correlates with antibody that aggregates TROMPs. In order to correlate immune status with antibody directed against TROMPs, freeze-fracture electron microscopy was used to view antibody-mediated TROMP aggregation following the incubation of test and control sera with live *T. pallidum* cells. As shown in Table 1 and Fig. 1, sera from animals susceptible to challenge reinfection and having low-titered (≤1:1) treponemicidal activity as described above showed only a low level of total particle aggregation (16.5% ± 5.6%) similar to the NRS controls (13.4% ± 4.2%). As further shown in Fig. 2, the number of particles within these aggregates never exceeded three particles, again similar to those in the normal serum controls. While sera from animals which exhibited partial immunity showed a corresponding increase in treponemicidal activity as described above (titer of 1:16), no significant increase in total particle aggregation (18.9% ± 6.5%), compared to the susceptible group (16.5% ± 5.6%), was observed (Table 1). However, sera from the partially immune group did show a significant increase in the number of
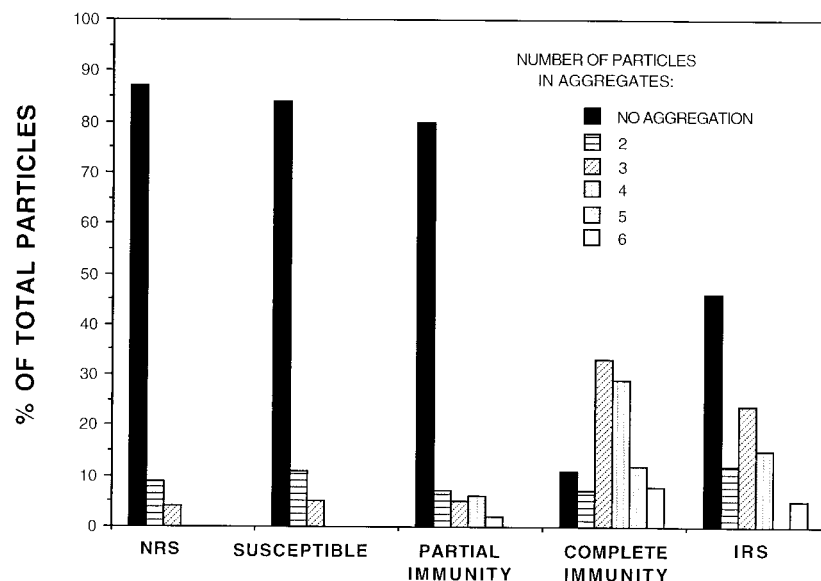

FIG. 2. Determination of the number of particles in aggregates following incubation of *T. pallidum* in sera from animals with various degrees of immunity to challenge reinfection, in NRS, and in IRS. The total number of particles from 20 to 24 concave outer membrane fracture faces was determined. Numbers of particles within an aggregate were determined by directly counting the particles and by determining the surface area of an aggregate in comparison to the surface area of an individual particle.
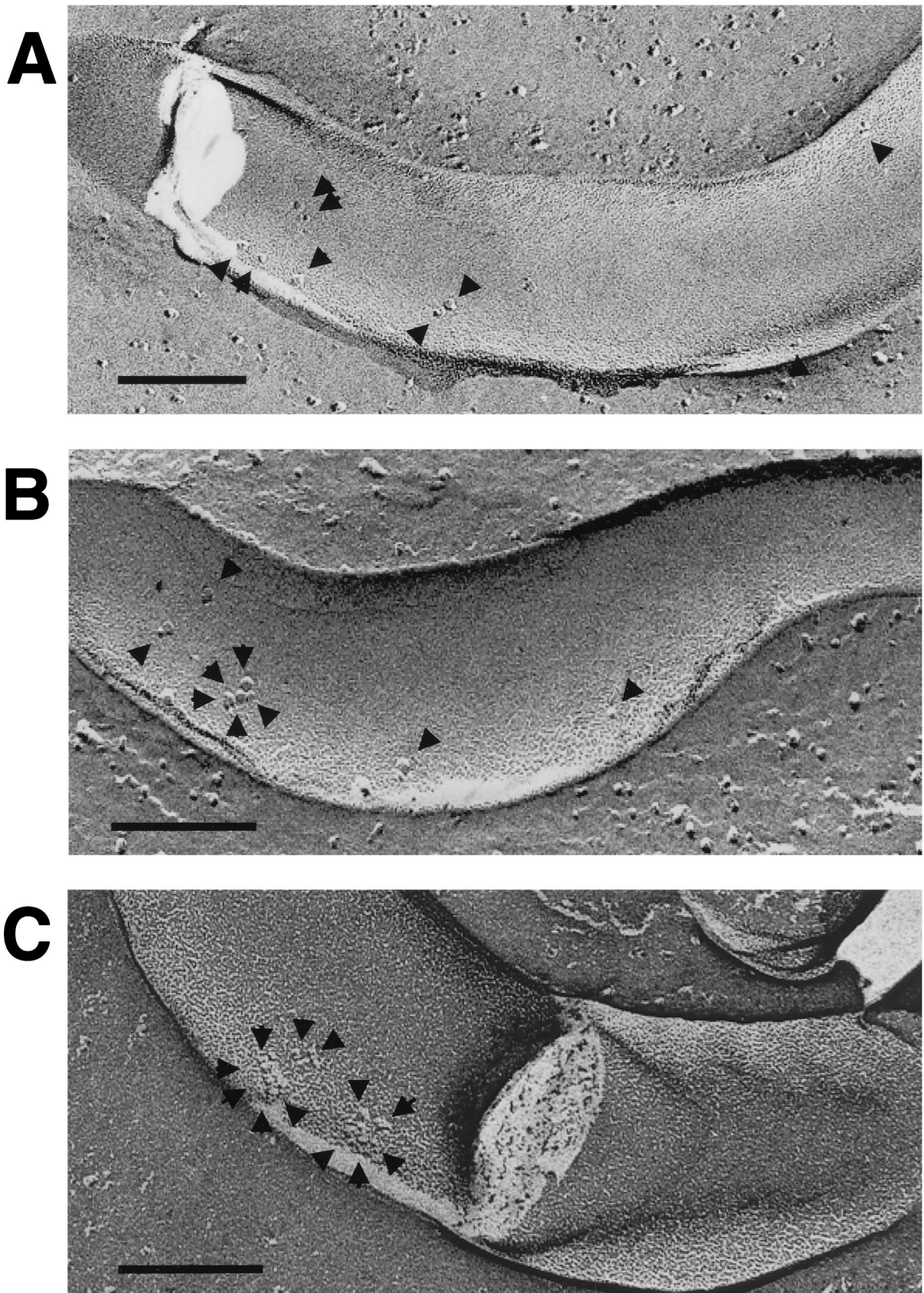
FIG. 1. Freeze-fracture electron microscopy of *T. pallidum* following incubation in sera from infected and curatively treated rabbits with various degrees of immunity to challenge reinfection. *T. pallidum* was incubated for 16 h in the absence of complement with serum from a rabbit susceptible to challenge reinfection (A), a rabbit showing partial protection against challenge reinfection (B), and a rabbit completely immune to challenge reinfection (C). Arrows show nonaggregated (A and B) and aggregated (B and C) TROMPs. Bar, 0.1 μm.

particles within aggregates ($P < 0.01$), which was found to be four to five particles in approximately 15% of the aggregates observed (Fig. 1 and 2). Finally, sera from animals completely immune to challenge reinfection and having high-titered (1:128) treponemicidal activity as described above showed significant aggregation of the total number of particles observed (88.6% ± 5.4%; $P < 0.0001$) (Table 1 and Fig. 1). In addition, approximately 50% of these aggregates contained as many as four to six particles (Fig. 2). A similar, significantly high level of particle aggregation (53.9% ± 7.8%; $P < 0.0001$) and numbers of particles within aggregates (approximately 20% containing four to six particles) was observed with sera from the immune control rabbits (Table 1 and Fig. 2).

Our previous findings that TROMPs have surface exposure, based on their aggregation in immune serum (9) and the apparent absence of surface exposure of other *T. pallidum* antigens previously identified (30), prompted the present investigation to address whether a relationship could be established between TROMPs, killing antibody, and host immunity in experimental rabbit syphilis. Killing antibody in this study was detected by the washed-killing assay (15), a procedure developed to measure antibody directed solely against surface-exposed targets on *T. pallidum*, which presumably would be only TROMPs. A further consideration in this study was the testing of sera from challenged animals at a time when symptomatic infection was observed to occur in the control animals. We believe that sera obtained from the test animals at this time provide the best measurement for establishing a relationship between anti-TROMP antibodies and protective challenge immunity.

The results of the washed-killing assay and freeze-fracture analysis, using sera from infected and cured rabbits that were either susceptible, partially immune, or completely immune to challenge reinfection, demonstrated a positive correlation between the status of immunity and the presence of antibody which kills *T. pallidum* and aggregates TROMPs. Rabbits that showed complete immunity to challenge reinfection had serum antibody that resulted in both high-titered complement-dependent treponemicidal activity (titer of 1:128) and significant TROMP aggregation, shown by the presence of 88.6% of the outer membrane particles existing in an aggregated state. It was also noted that 50% of the aggregates observed were found to contain four to six particles per aggregate. Both the high-titered killing activity and significant TROMP aggregation was similar to that of sera from immune control animals. It was noted, however, that serum from immune control animals aggregated TROMPs slightly less effectively (53.9%) than that from immune test animals (88.6%). One possible explanation for this observation is that serum from the immune test animals was taken after challenge, whereas no challenge was employed in the immune control animals. Thus, the increase in aggregation found in the sera from the immune test group may reflect an increase in antibody and/or antigen-antibody avidity from a booster immunization after challenge.

Rabbits which were only partially immune to reinfection, as determined by atypical lesion development and absence of disseminated infection after challenge, showed a markedly lower level of serum complement-dependent killing activity (titer of 1:16) than sera from the immune animals (titer of 1:128). While no significant increase in total TROMP aggregation was observed (18.9% aggregation of total particles counted), it was found that approximately 15% of these aggregates contained a significant increase in the number of particles per aggregate (four to five particles per aggregate) compared to the susceptible and normal control groups (two to three particles per aggregate). Thus, in accordance with the

low-level but detectable killing antibody against *T. pallidum*, an increase in antibody directed against TROMPs was present in sera from these partially immune animals.

Rabbits which were completely susceptible to reinfection were found to have serum antibody which neither significantly killed *T. pallidum* (titer of ≤1:1) nor aggregated TROMPs (16.5% aggregation of total particles counted), a finding similar to that obtained with sera from normal control animals. It was also noted that of the 16.5% of particles which were aggregated, none of the aggregates contained more than three particles, again similar to the results in sera from normal control animals.

While a low level of aggregation was observed to occur consistently in sera from susceptible and normal control animals, an obvious question is, why did any aggregation occur under these conditions? It is important to stress that *T. pallidum* cells used for these experiments were acquired from 10-day-infected rabbits, a time just before infection is normally cleared (14 days) by specific immune mechanisms (2, 16, 17). It has been observed that *T. pallidum* cells obtained from 14-day-infected rabbits are susceptible to killing with only the addition of complement in the absence of added immune serum antibody (22). It is therefore conceivable that the low level of TROMP aggregation observed in normal control sera is the result of a low level of prebound anti-TROMP antibody on organisms extracted from the infectious rabbit milieu. These observations suggest that this level of anti-TROMP antibody present at or before 10 days after infection, while causing some aggregation, is not sufficient to result in significant complement-dependent killing of *T. pallidum* or resolution of the local infection. We have found that *T. pallidum* extracted from 14-day-infected rabbits shows outer membranes with greater amounts of aggregated TROMPs than *T. pallidum* from 10-day-infected animals. Taken together, these observations suggest that anti-TROMP antibody may have a key role in the resolution of the local primary infection in addition to its likely role in the development of acquired protective immunity.

The implication from this study that TROMPs are the likely targets of high-titered treponemicidal antibody is further supported by a recent study where we found that immunization with purified *T. pallidum* outer membranes elicits the highest titer of killing activity we have measured to date (7). Moreover, we have found that this antiserum to the *T. pallidum* outer membrane, when incubated with live organisms, also results in the aggregation of TROMPs. The possibility that molecules other than TROMPs are targets for killing antibody is unlikely given the absence of detectable *T. pallidum* surface proteins (30). In addition, Radolf et al. (32) have shown that *T. pallidum* outer membrane lipids are not antigenic in syphilitic human serum, which also possesses high-titered killing activity. Thus, these findings are again consistent with the idea that TROMPs are the primary targets of antibody which kill *T. pallidum*.

In summary, the results presented in this study provide compelling evidence that TROMPs are the major targets of protective immunity that develop during the course of experimental syphilitic infection. Notwithstanding cell-mediated immunity, our findings support the idea that specific antibody directed against TROMPs is central to the development of protective immunity against challenge reinfection. While the results show that this antibody, in combination with complement, effectively kills *T. pallidum*, it is certainly conceivable that other antibody-mediated mechanisms, such as opsonization (1, 3, 18) or antibody-dependent cellular cytotoxicity, may play key roles in an antibody protective response. We are hopeful that the future recombinant expression of all TROMP

candidates will allow the ultimate identification of those immunogens responsible for protective immunity against syphilis.

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