Immunization with *Treponema pallidum* Outer Membrane Vesicles Induces High-Titer Complement-Dependent Treponemicidal Activity and Aggregation of *T. pallidum* Rare Outer Membrane Proteins (TROMPs)

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Immunization with *Treponema pallidum* Outer Membrane Vesicles Induces High-Titer Complement-Dependent Treponemical Activity and Aggregation of *T. pallidum* Rare Outer Membrane Proteins (TROMPs)¹

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The purpose of this study was to determine whether immunization with purified outer membrane vesicles (OMV) from *Treponema pallidum* (T.p.) could elicit Abs capable of killing this organism. It is well established that the immunization of rabbits or mice with killed T.p. or with recombinant T.p. Ags has failed to generate serum killing activity comparable with that of infection-derived immunity. Because of the small amount of T.p. OMV obtainable, a single mouse was immunized with purified OMV. The mouse anti-OMV serum and infection-derived immune rabbit serum (IRS) were compared by reactivities on two-dimensional T.p. immunoblots and by the T.p. immobilization test, a complement-dependent killing assay. Whereas IRS detected >40 Ags, the anti-OMV serum identified only 6 Ags corresponding to proteins identified previously in the outer membrane. T.p. immobilization testing showed that IRS had a 100% killing titer of 1:44 and a 50% killing titer of 1:662. By comparison, the mouse anti-OMV serum had a significantly greater 100% killing titer of 1:1,408 and a 50% killing titer of 1:16,896. Absorption of the anti-OMV serum to remove Ab against outer membrane-associated lipoproteins did not change the 100% killing titer. Freeze-fracture analysis of T.p. incubated in IRS or anti-OMV serum showed that T.p. rare membrane-spanning outer membrane proteins were aggregated. This is the first demonstration of high-titer killing Abs resulting from immunization with defined T.p. molecules; our study indicates that the targets for these Abs are T.p. rare outer membrane proteins. *The Journal of Immunology*, 1999, 163: 2741–2746.

Syphilis, caused by the noncultivatable spirochete *Treponema pallidum* (T.p.)¹ (1, 2), ultimately results in latent infection in both humans and experimental animals. Chronic infection has been attributed to an unusual property of the T.p. outer membrane, which contains 100-fold less membrane-spanning protein compared with outer membranes from typical Gram-negative bacteria (3, 4). These T.p. rare outer membrane proteins, termed TROMPs after their identification by freeze-fracture analysis (5), have been shown by this procedure to be the only recognized surface exposed proteins of this organism as evidenced by their aggregation following the incubation of T.p. with serum from syphilitic rabbits immune to challenge reinfection (5).

*Abbreviations used in this paper: T.p., *Treponema pallidum*; TROMP, T.p. rare outer membrane protein; OMV, outer membrane vesicles; IRS, immune rabbit serum; TPI, T.p. immobilization; NRS, normal rabbit serum; NMS, normal mouse serum.*

We have reported previously the development of a procedure to isolate the outer membrane from T.p. and have identified a limited set of outer membrane proteins including those of molecular mass 17, 28, 31, 45, and 65 kDa (6). The 17- and 45-kDa proteins are lipoproteins (7, 8) and are primarily associated in greater amounts with the T.p. inner membrane protoplasmic cylinder complex (6, 9). These outer membrane lipoproteins are not membrane-spanning and are apparently not surface exposed; rather, they are anchored to the inner leaflet of the outer membrane (6). By comparison, the 28- and 31-kDa proteins are markedly enriched in outer membrane preparations and have been shown to be hydrophobic (6, 10, 11). The 31-kDa protein is also not found with the protoplasmic cylinder complex following gentle detergent treatment of T.p., which completely solubilizes the outer membrane (10). Both purified native and an *Escherichia coli* recombinant outer membrane form of the 31-kDa protein, designated Tromp1, exhibit porin activity (10, 12). The 28-kDa protein, designated Tromp2, has also been expressed in *E. coli*, where it targets exclusively to the *E. coli* outer membrane (11). Tromp1 and Tromp2 are antigenic, using infection-derived immune serum; however, both of these proteins are weakly immunogenic compared with the lipoproteins.

There is evidence that the protective immunity acquired during latent syphilitic infection in humans and experimental animals results from specific Ab in immune serum (13). The development and persistence of acquired immunity in experimental rabbit syphilis has been shown to correlate with the presence of high-titer (range of 1:16–1:128) complement-dependent treponemical Ab (14). Humans exhibiting latent infection have also been shown to have similar levels of high-titer (range of 1:8–1:128) serum treponemical activity (13). Of particular significance is the demonstration that rabbits, hamsters, and guinea pigs can be passively
protected from challenge infection using serum from immune donors (15–22). These observations have provided compelling evidence that Ab plays a key role in the protective immunity that develops during syphilitic infection. However, the specific T.p. target(s) of high-titer treponemicidal Ab and of protective immunity has not been identified. In addition, immunizations with either killed whole organisms, fractionated organisms, or recombinant proteins have failed to elicit serum treponemicidal activity comparable with that of immune serum. Because the outer membrane contains the likely surface targets for killing Ab, we immunized a mouse with the maximum amount of purified T.p. outer membrane that can be reasonably obtained in an effort to elicit high-titer complement-dependent treponemicidal Ab and identify the target molecules of this activity.

**Materials and Methods**

**T.p. outer membrane isolation**

For immunization and immunoblot analysis, \(-7\times10^{10}\) T.p. equivalents of purified outer membrane were prepared as described previously (6). Because the recovery of purified outer membrane using this procedure is <5% efficient, outer membrane isolations were performed on \(-1.4\times10^{12}\) organisms; these isolations were accomplished over several months by extracting organisms from seven groups of 10 rabbits infected intratesticularly with a total of \(4\times10^{12}\) T.p. (a total of 70 rabbits). In addition, the previous use of octyl-decyl rhodamine chloride to label and visualize membrane was not used in these isolations, resulting in no difference in the visualized membrane banding position following outer membrane purification through sucrose density gradients or in the protein composition of the purified membrane (data not shown).

**Mouse immunization with T.p. outer membrane**

For the mouse immunization with purified T.p. outer membrane vesicles (OMV), a 12-wk-old female BALB/c mouse (Charles River, Raleigh, NC) was injected s.c. in two sites with OMV derived from \(1\times10^{11}\) T.p., containing \(10\)–\(50\) ng of each outer membrane protein, mixed 1:1 with titermax adjuvant (CytRx, Norcross, GA). At 2 and 5 mo, the mouse was boosted with a similar amount of outer membrane material by direct intrasplenic injection while under anesthesia (2.5 mg/mouse of ketamine HCl (Fort Dodge Laboratories, Fort Dodge, IA) and 0.5 mg/mouse of Rompun (Miles, Shawnee Mission, KS). A final i.v. boost with a similar amount of OMV material was given at 8 mo. After 5 days, the mouse was exsanguinated by cardiac puncture with the blood processed into \(-400\) \(\mu\)l of serum. Nonautologous control serum came from a syngeneic littermate. Serum was acquired from syphilitic rabbit immune to challenge (immune rabbit serum (IRS)); rabbits were infected for 6 mo following intratesticular injection with \(4\times10^{12}\) T.p.

**One- and two-dimensional SDS-PAGE and immunoblotting**

T.p. Ag and purified T.p. outer membrane were prepared for two-dimensional immunoblot analysis as described previously (6). Briefly, \(1\times10^{10}\) Ficoll purified T.p. or outer membrane from \(5\times10^{10}\) whole organism equivalents were solubilized for 1 h at room temperature in sample buffer containing 9 M urea, 2% Nonidet P-40, and 2% carrier ampholytes at a pH of 9.5. Isoelectric focusing was conducted for 16 h at a constant voltage of 600 V in polyacrylamide tube gels containing 2% (pH 5–7) and 0.8% (pH 3–10) Ampholines (Bio-Rad, Richmond, CA), 2% Nonidet P-40, and 9 M urea. The second dimension consisted of standard SDS-PAGE as described previously (6). After electrophoresis, gels were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA) (23) and stained with 1% amido black.

Recombinant 31-kDa Tromp1 was expressed and purified by fast performance liquid chromatography (Pharmacia LKB Biotechnology, Uppsala, Sweden) as described previously (12). Approximately 5 \(\mu\)g of purified recombinant Tromp1 was electrophoresed by standard SDS-PAGE and transferred to polyvinylidene difluoride membranes for immunoblot analysis as described previously (12). For immunoblot analysis, the mouse anti-T.p. OMV serum and IRS were each diluted 1/1000 in PBS containing 5% nonfat dry milk (Carnation, Los Angeles, CA) and 0.1% Tween-20 (Sigma, St. Louis, MO) (MT-PBS). The diluted sera were incubated on blots for 1 h; Ab-Ag binding was detected using an enhanced chemiluminescence system (Amersham, Little Chalfont, U.K.). Blots were incubated for 1 h in either anti-mouse or anti-rabbit Ig conjugated to HRP (Amersham) diluted 1/2500 in MT-PBS. Blots were subsequently washed in PBS containing 0.1% Tween-20, incubated for 1 min in the enhanced chemiluminescence developing reagents (Amer- sham), and chemilumigraphed with Kodak X-AR5 film.

**T.p. immobilization (TPI) testing**

To assay for serum complement-dependent killing Ab against T.p., the TPI test was used as described previously (24). Heat-inactivated (56°C/30 min) IRS and heat-inactivated mouse anti-T.p. OMV serum were tested quantitatively using 2-fold serial dilutions in heat-inactivated normal rabbit serum (NRS) with a nonreactive venereal disease research laboratory (VDRL) test (24). Samples (50 \(\mu\)l) of 2-fold diluted test serum were added to a final volume of 350 \(\mu\)l (final test serum dilution for each tube is 1/11) containing 250 \(\mu\)l of Nelson’s medium (24, 25), 200 \(\mu\)l of fresh guinea pig serum, and 200 \(\mu\)l of heat-inactivated and unheated as a source of active complement, and 50 \(\mu\)l of a T.p. suspension containing \(5\times10^{4}\) organisms/ml. The mixtures were incubated in an atmosphere of 95% \(\text{N}_2\) to 5% \(\text{CO}_2\) for 16 h at 34°C, at which time samples were observed by darkfield microscopy for the presence of motile or nonmotile treponemes. Percent motility was determined by randomly counting 25 organisms. Organisms immobilized under similar conditions have been shown to be killed based upon virulence testing using intradermal injection of rabbits (14). The 100% and 50% endpoint killing titers were determined based on the final serum dilutions capable of immobilizing 100% and 50% of the treponemes counted, respectively.

**Absorption of anti-OMV serum**

To remove Ab from the mouse anti-OMV serum directed against subsurface T.p. lipoproteins, the serum was absorbed three times with T.p. protoplastic cylinders, devoid of outer membrane, which were prepared as follows. Approximately \(2\times10^{12}\) T.p. were extracted from 10 intratesticularly infected rabbits in 400 ml of PBS (pH 7.2) containing 6 U/ml of sodium heparin. The suspension was centrifuged twice at \(400 \times g\) to remove gross tissue debris and subsequently at \(20,000 \times g\) for 20 min to pellet the treponemes. The treponemal pellet was resuspended in 18 ml of PBS, to which 2 ml of 10% Triton X-100 was added (1% final Triton X-100 concentration). The suspension was then incubated for 4 h at 4°C to solubilize the outer membrane. The suspension was divided into three equal volumes and centrifuged at \(10,000 \times g\) for 20 min. Treponemal pellets were washed once in 10 ml of PBS and subsequently reconstituted as described previously. The resulting protoplastic cylinders were shown to be free of outer membrane by the failure to detect Tromp1 using specific anti-Tromp1 serum following immunoblot analysis with \(1\times10^{8}\) protoplastic cylinders (data not shown). Absorbed and nonabsorbed anti-OMV sera were compared for their ability to detect T.p. Ags on one-dimensional immunoblots of whole T.p. and by the TPI test as described above.

**Freeze-fracture electron microscopy**

Both mouse anti-OMV serum and IRS were tested for their ability to aggregate TROMPs in the outer membrane as follows: NRS (undiluted), IRS (undiluted), normal mouse serum (NMS) (diluted 1/64 in NRS), and anti-OMV serum (diluted 1/64 in NRS) were incubated with T.p., in the absence of active complement, under the conditions of the TPI test as described above. Following incubation for 16 h, the suspensions were centrifuged at 9000 \(\times g\) for 10 min to pellet the treponemes. The treponemal pellets were resuspended in 0.5 ml of 2% glutaraldehyde and 0.1 M sodium cacodylate (pH 7.2) and were fixed for 1 h. Following fixation, the suspensions were centrifuged at 9000 \(\times g\) for 10 min, and the treponemal pellets were re-suspended in 50 \(\mu\)l of 20% glycerol in 0.1 M sodium cacodylate (pH 7.2). The sample was prepared for freeze-fracture electron microscopy as described previously (5).

**Results**

T.p. Ags identified with mouse anti-T.p. OMV serum

To determine the specificity of the mouse anti-OMV serum, two-dimensional immunoblots of purified T.p. outer membrane and T.p. whole organisms were probed with the anti-OMV serum (Figs. 1 and 2). As shown in Fig. 1, the anti-OMV serum at a 1/1000 dilution used against \(5\times10^{10}\) treponemal equivalents of outer membrane detected both isomeric forms of the 31-kDa protein Tromp1 and weakly detected the 28-kDa protein Tromp2. In addition, several proteins at 15 kDa and 17 kDa were strongly detected as well as a vertical migrating 34-kDa protein and a 45-kDa protein.
A similar pattern of reactivity was also observed when the anti-OMV serum at this same dilution was used to probe a two-dimensional immunoblot containing a total extract of T.p. (1 × 10^9 organism equivalents) (Fig. 2B). In this case, only single reacting species of 15- and 17-kDa proteins were detected. The molecular mass and isoelectric point of the 17- and 45-kDa proteins correspond to lipoproteins (7, 8) that we have shown previously to be constituents of the T.p. outer membrane (6). The molecular mass and isoelectric point of the 15- and 34-kDa proteins also correspond to lipoproteins of T.p. (26–29); however, these lipoproteins were not detected previously in our outer membrane preparations.

In contrast to the outer membrane immunoblot, no detection of Tromp1 or Tromp2 was observed on the two-dimensional immunoblot of whole organisms (Fig. 2B); this finding was not surprising given that 1 × 10^9 whole organism equivalents were probed as compared with the outer membrane immunoblot containing 5 × 10^10 organism equivalents and the relatively weaker immunogenicity of the TROMPs compared with the lipoproteins. It was also noted that neither the immunoblot of purified outer membrane (Fig. 1) nor the total extract of T.p. probed with the anti-OMV serum (Fig. 2B) detected the 65-kDa outer membrane-associated protein previously identified in our outer membrane preparations (6).

By comparison, a 1/1000 dilution of IRS used to probe a total extract of T.p. from 1 × 10^8 organism equivalents resulted in the detection of >40 T.p. Ag spots including the major 47-kDa lipoprotein and those comprising the endoflagellar filament (Fig. 2C). Again, no detection of Tromp1 or Tromp2 was observed using IRS at this dilution. We have observed, however, that IRS can detect Tromp1 and Tromp2 on two-dimensional immunoblots when using 3 × 10^10 T.p. equivalents of purified outer membrane (data not shown).

Detection of Tromp1 by mouse anti-T.p. OMV serum and IRS

Because 1 × 10^8 T.p. organisms, which were used above for two-dimensional analysis, only contain ~10–50 ng of each TROMP, 5 μg of purified recombinant Tromp1 (12) was used as a target Ag to further confirm the detection of Tromp1 by the anti-OMV serum and IRS at a 1/1000 dilution. As shown in Fig. 3, both anti-OMV serum and IRS readily detected 5 μg of purified recombinant Tromp1, confirming the presence of Ab against Tromp1 in the anti-OMV serum and following experimental syphilitic infection.
As also seen, the anti-OMV serum reacted with greater intensity compared with IRS.

**TPI activity of mouse anti-T.p. OMV serum compared with IRS**

To compare the anti-OMV serum with that of IRS for complement-dependent treponemicidal activity, the sera were tested quantitatively by TPI. As shown in Table I, IRS from a rabbit immune to challenge reinfection showed high-titer treponemicidal activity, having a 100% killing endpoint titer of 1:44 and a 50% killing endpoint titer of 1:662. This level of killing activity is consistently observed for IRS when tested by TPI (14, 16). By comparison, the mouse anti-OMV serum showed a strikingly higher level of treponemicidal activity, having a 100% killing endpoint titer of 1:1,408 and a 50% killing endpoint titer of 1:16,896; these titers were 32 and 25 times greater, respectively, than those of IRS. The killing activity for both IRS and the mouse anti-OMV serum was complement-dependent, because the lowest dilutions of test samples containing heat-inactivated complement had no treponemicidal activity. In addition, the syngeneic mouse serum as well as NRS containing heat-inactivated complement had no treponemicidal activity, having a 100% killing endpoint titer of 1:44 and a 50% killing endpoint titer of 1:1,408. This level of killing activity is consistently observed for the noninfected animal showing no treponemicidal activity in the presence or absence of active complement.

**Table I. Comparison of complement-dependent treponemicidal activity of mouse anti-T.p. OMV serum and IRS as measured by the TPI test**

<table>
<thead>
<tr>
<th>Test Serum</th>
<th>100% Killing Endpoint Titer$^a$</th>
<th>50% Killing Endpoint Titer$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>IRS</td>
<td>1:44</td>
<td>1:662</td>
</tr>
<tr>
<td>Anti-OMV</td>
<td>1:1,408</td>
<td>1:16,896</td>
</tr>
<tr>
<td>Absorbed anti-OMV$^d$</td>
<td>1:1,408</td>
<td>ND$^e$</td>
</tr>
</tbody>
</table>

$^a$ The 2-fold serial dilutions of test sera were combined with guinea pig serum, heat-inactivated (56°C/30 min) and unheated as a source of complement, and T.p. The mixtures were incubated for 16 hours at 34°C, at which time the percentage of immobilization was determined by darkfield microscopy.

$^b$ The 100% killing endpoint titer was determined as the final serum dilution that showed 100% immobilization of the organisms counted.

$^c$ The 50% killing endpoint titer was determined from extrapolation and is the dilution that would correspond to 50% immobilization of the organisms counted.

$^d$ Anti-OMV serum was absorbed with T.p. protoplasmic cylinders devoid of outer membrane.

$^e$ Not done.

**Effect upon TPI activity following removal of antilipoprotein Abs from mouse anti-OMV serum**

To determine the contribution to TPI activity in the anti-OMV serum of the antilipoprotein Abs, these Abs were removed by absorption with T.p. protoplasmic cylinders, devoid of outer membrane, with exposed inner membrane-anchored lipoproteins. As shown by immunoblot analysis (Fig. 4), absorption resulted in the complete removal of detectable Ab against the 15-, 17-, 34-, and 45-kDa lipoproteins, but not against Tromp1. When analyzed by the TPI test at the highest dilution that kills 100% of the treponemal suspension (1:1,408), the absorbed serum showed no difference compared with the nonabsorbed serum in its ability to kill 100% of the treponemes (Table I).

**Aggregation of TROMPs in the outer membrane of T.p. by mouse anti-OMV serum and IRS**

To determine whether the anti-OMV serum could aggregate TROMPs when incubated with virulent T.p., as reported previously for IRS (5), organisms were incubated under TPI conditions in the absence of complement before freeze-fracture electron microscopy. Organisms incubated in IRS or in a 1/64 dilution of NMS (Fig. 5, A and C) showed little aggregation of particles in their outer membranes. The few aggregates detected under these conditions never exceeded two particles per aggregate. By comparison, organisms incubated in undiluted IRS or in a 1/64 dilution of the anti-OMV serum (Fig. 5, B and D) showed marked aggregation of outer membrane particles. In some cases, outer membrane particle aggregates following anti-OMV serum incubation were observed to contain as many as seven particles.

**Discussion**

Because the outer membrane of T.p. contains TROMPs that have been shown to be surface exposed (3–5), purified outer membrane enriched for TROMPs was used for immunization in an attempt to artificially generate the high-titer complement-dependent treponemicidal Ab that has only been elicited following infection-derived immunity. Due to the limited amount of T.p. outer membrane that is obtainable from this noncultivatable organism (6, 30) and the...
The mouse, an animal also susceptible to T.p. infection (31, 32), was used as a model for the development of a vaccine against syphilis. Immunization with purified T.p. outer membrane has been shown to result in high-titer treponemicidal antibody activity in mice or rabbits that has resulted in treponemicidal activity that is significantly greater than that of infection-derived immune serum (5). This finding indicates that OMV immunization results in an Ab response against surface epitopes on these rare proteins and suggests that TROMPs may be an important factor in their ability to elicit high-titer treponemicidal activity.

To further characterize the serum following OMV immunization, anti-OMV serum and IRS were used for immunoblot analysis against purified T.p. outer membrane and total T.p. proteins. In contrast to IRS, which detected >40 T.p. proteins by two-dimensional immunoblot analysis, anti-OMV serum at the same dilution identified only a limited set of proteins, including Tromp1 (31 kDa), Tromp2 (28 kDa), and Ags corresponding to lipoproteins of 15, 17, 34, and 45 kDa. We have shown previously that the 17- and 45-kDa lipoproteins are constituents of the outer membrane (6). The presence of Ab against the 15- and 34-kDa lipoproteins was unexpected, because these proteins have not been detected by either gold staining or by immunoblot analysis in our previous outer membrane preparations (6). The possibility that this resulted from a small amount of inner membrane lipoprotein contamination is also unlikely given the complete absence of Ab against the 47-kDa lipoprotein, the most abundant inner membrane-anchored lipoprotein of T.p. (Fig. 2A) and one that is known to be highly immunogenic in mice, rabbits, and humans (26, 33, 34). These findings indicate that trace amounts of the 15- and 34-kDa lipoproteins, previously undetected in our outer membrane preparations, were present, suggesting that they may be either differentially expressed or may simply be a rare contaminant. In either case, our OMV immunization results indicate that Ab against this 65-kDa protein does not appear to be necessary for high-titer serum treponemicidal activity.

In an effort to further identify TROMPs as the targets of high-titer treponemicidal Ab, the anti-OMV serum was absorbed to remove Ab directed against both subsurface and outer membrane-associated lipoproteins. Although immunoblot analysis showed the complete removal of detectable antilipoprotein Abs from the absorbed anti-OMV serum, no loss of anti-Tromp1 Ab was observed. Furthermore, no change in the titer of this absorbed serum was observed in its ability to kill 100% of a T.p. suspension. These findings indicate that the antilipoprotein Abs do not contribute to the high-titer treponemicidal activity elicited by OMV immunization and further suggest that TROMPs are the primary targets of this activity.

Our studies suggest that Tromp1 may be an outer membrane target for bactericidal Ab. However, recent immunizations with purified recombinant Tromp1 have not resulted in significant serum killing activity. It is pertinent to note that the purified recombinant form of Tromp1 used for these immunizations does not possess properties that we have demonstrated previously for native Tromp1, including hydrophobicity, porin activity, and oligomeric conformation (10). The finding that immunization with purified OMV, presumably containing TROMPs in a native conformation, can elicit high-titer killing Ab suggests that native conformation of TROMPs may be an important factor in their ability to elicit killing Ab.

In summary, this is the first demonstration of an immunization that has resulted in high-titer complement-dependent serum treponemicidal Ab; in addition, our study suggests that outer membrane proteins are responsible for this activity. It is tempting to speculate that this level of killing Ab may contribute to protective immunity. Although the mouse model of syphilis does not show an initial skin lesion following challenge, disseminated infection involving the lymphatics, blood, spleen, and brain is well documented (31). We
believe that murine immunization with purified T.p. OMV, as described in this study, provides a heretofore unavailable opportunity to address whether the outer membrane of T.p. can elicit protective immunity against challenge infection. We are hopeful that such future OMV immunization studies will provide further insight into the surface molecules responsible for the generation of protective host immunity during syphilitic infection.

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References


