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

David R. Blanco, Cheryl I. Champion, Michael A. Lewinski, Ellen S. Shang, Stephen G. Simkins, James N. Miller and Michael A. Lovett

*J Immunol* 1999; 163:2741-2746; http://www.jimmunol.org/content/163/5/2741

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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).

Received for publication February 11, 1999. Accepted for publication June 11, 1999.

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1. This work was supported by U.S. Public Health Service Grants AI-21352 and AI-12601 (to M.A.L) and AI-37312 (to J.N.M.).
2. An equal contribution to this study was made by D.R.B. and C.I.C., who should be considered co-first authors of this work.
3. Address correspondence and reprint requests to Dr. David R. Blanco, Department of Microbiology, Immunology, and Molecular Genetics, Center for Health Sciences 43-239, University of California School of Medicine, Los Angeles, CA 90095-1747. E-mail address: dblanco@microimmun.medsch.ucla.edu
4. 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.
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, −7–8 × 10⁹ 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 × 10⁴ organisms; these isolations were accomplished over several months by extracting organisms from seven groups of 10 rabbits infected intratesticularly with a total of 4 × 10⁷ 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 × 10⁵ T.p., containing −10–50 ng of each outer membrane protein, mixed 1:1 with titermax adjuvant (CytRx, Norcross, GA). A final i.v. boost with a similar amount of OMV material was given at 8 mo. After 5 days, the mouse was exsanguinated; rabbits were infected for 6 mo following intratesticular injection with 400 ml of PBS (pH 7.2) containing 6 U/ml of sodium heparin. The suspension was centrifuged two times at 400 × g to remove gross tissue debris and subsequently at 20,000 × 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 × g for 20 min. Treponemal pellets were washed once in 10 ml of PBS and subsequently reconstituted as described previously. The resulting protoplasmic 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 × 10⁷ protoplasmic 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 × 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 × g for 10 min, and the treponemal pellets were resuspended in 50 µl of 20% glycerol in 0.1 M sodium cacodylate (pH 7.2). This 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 × 10¹⁰ 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 $\times$ 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 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 $\times$ 10^9 whole organism equivalents were probed as compared with the outer membrane immunoblot containing 5 $\times$ 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 $\times$ 10^9 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 $\times$ 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 $\times$ 10^9 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 from a noninfected animal showed 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 Titerb</th>
<th>50% Killing Endpoint Titerc</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-OMVe</td>
<td>1:1,408</td>
<td>NDf</td>
</tr>
</tbody>
</table>

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*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.

f 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 (1:1408), 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 treponemical 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
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, are an important factor in their ability to elicit a relatively strong Ab response, which is consistent with the high immunogenicity of lipoproteins (35, 36).

Immunoblot analysis of the anti-OMV serum also showed no detectable Ab against a rare 65-kDa protein that we identified previously in our outer membrane preparations (6). We have subsequently found that the presence of this protein in recent outer membrane preparations has been variable, suggesting that it 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.

Acknowledgments
We thank Drs. Yi-Ping Wang and Xiao-Yang Wu for their excellent technical assistance and Dr. Denise Foley for her valuable and helpful comments.

References