An *Arcanobacterium (Actinomyces) pyogenes* Mutant Deficient in Production of the Pore-Forming Cytolysin Pyolysin Has Reduced Virulence

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Pyolysin (PLO), the hemolytic exotoxin expressed by *Arcanobacterium (Actinomyces) pyogenes*, is a member of the thiol-activated cytolysin family of bacterial toxins. Insertional inactivation of the *plo* gene results in loss of expression of PLO with a concomitant loss in hemolytic activity. The *plo* mutant, PLO-1, has an approximately 1.8-log$_{10}$ reduction in the 50% infectious dose compared to that for wild-type *A. pyogenes* in a mouse intraperitoneal infection model. Studies involving cochallenge of wild-type and PLO-1 bacteria resulted in recovery of similar numbers of both strains, suggesting that PLO production is required for survival in vivo. Recombinant, His-tagged PLO (His-PLO) is cytotoxic for mouse peritoneal macrophages and J774 cells in a dose-dependent manner. Protection against challenge with *A. pyogenes* could be afforded by vaccination with formalin-inactivated His-PLO, suggesting that PLO is a host-protective antigen, as well as a virulence determinant.

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**MATERIALS AND METHODS**

**Bacteria, growth conditions, and preparation of culture supernatant fluid (CSF).** *A. pyogenes* BBR1 was isolated from a bovine abscess (Marana, Ariz.). A. pyogenes strains were grown on brain heart infusion (BHI; Difco) agar plates, supplemented with 5% bovine blood at 37°C and 5% CO$_2$, or in BHI broth supplemented with 5% bovine calf serum at 37°C. *Escherichia coli* DH5α and DH5α F$^{-}$ lac$^{+}$ strains (Bethesda Research Labs) were grown either on Luria-Bertani (Difco) agar or in Luria-Bertani broth at 37°C. Antibiotics (Sigma) were added as appropriate: for *A. pyogenes* strains, erythromycin (ERM) (15 μg/ml) and kanamycin (KAN) (30 μg/ml) were used; for *E. coli* strains, ampicillin (100 μg/ml) ERM (200 μg/ml), and KAN (50 μg/ml) were used. CSF containing PLO was prepared from liquid cultures of *A. pyogenes* grown overnight to an optical density of 600 nm of approximately 3.0 to 4.0. Cells were removed by centrifugation at 5,000 × g, and the CSF was filtered through a 0.22-μm pore-size filter and dialyzed against 400 volumes of 0.01 M phosphate-buffered saline (PBS), pH 7.2, for 18 h at 4°C.

**DNA techniques.** Preparation of plasmid DNA and electrophoresis-mediated transformation of *A. pyogenes* strains were performed as previously described (22). *E. coli* plasmid DNA extraction, transformation, DNA restriction, ligation, agarose gel electrophoresis, and Southern transfer of DNA to nitrocellulose membranes were performed essentially as described elsewhere (2). Preparation of DNA probes, DNA hybridization, and probe detection were performed with the DIG DNA labeling and detection kit (Boehringer Mannheim).

**Preparation of His-PLO and His-PLO toxoid.** His-PLO, purified from DH5α F$^{-}$ lac$^{+}$ (pJGS59) with TALON metal affinity resin (Clontech), was eluted with 50 mM imidazole–20 mM Tris–HCl–100 mM NaCl, pH 8.0 (7). His-PLO was dialyzed against 0.9% (vol/vol) PBS–10% formalin, pH 7.2, for 72 h at 37°C. The formalin was removed by dialysis against 400 volumes of PBS for 18 h at 4°C. Total protein concentration was determined with Bradford protein assay reagent (Bio-Rad).

**Macrophage cytotoxicity assay.** The murine macrophage-like cell line J774 was cultured in Isco's modified Dulbecco's medium (Life Technologies) supplemented with 10% fetal bovine serum and 100 μg of gentamicin per ml (IMDM–10%) in a humidified, 5% CO$_2$ incubator at 37°C. Mouse peritoneal macrophages were harvested from Swiss Webster mice (Holtzman Sprague Dawley) by peritoneal lavage with 5 ml of ice-cold 0.34 M sucrose. Cells were incubated at 37°C and 5% CO$_2$ for 1 h prior to addition of dilutions of His-PLO in IMDM–10%. Cytotoxicity was assessed after 3 h of incubation at 37°C and 5% CO$_2$, by the CytoTox 96 nonradioactive cytotoxicity assay (Promega), as per the manufacturer’s instructions. The percent toxicity was calculated as 100 × (experimental release – spontaneous release)/total release – spontaneous release. The average spontaneous release was 13.3 and 9.6% of the total release for macrophages and J774 cells, respectively.

**SDS-polyacrylamide gel electrophoresis and Western blotting.** CSF was electrophoresed in a 10% (wt/vol) sodium dodecyl sulfate (SDS)–polyacrylamide gel essentially as described elsewhere (2). Proteins were transferred to nitrocellulose, and Western blots were immunostained as previously described (2) with...
goat anti-His-PLO (7) and rabbit anti-goat immunoglobulin G (heavy plus light chains)-peroxidase conjugate as the primary and secondary antibodies, respectively.

Hemolytic assays. Hemolytic assays of CSF preparations were performed as described by Billington et al. (7). The hemolytic titer was the log, of the reciprocal of the highest dilution which resulted in 50% cell lysis. Antihemolytic titers of antisera were determined as described elsewhere (7). The antihemolytic titer was the log, of the reciprocal of the antibody dilution which completely neutralized hemolytic activity.

Mouse challenge model and immunization of mice with His-PLO toxoid. Female, 6- to 8-week-old, outbred ICR mice were challenged intraperitoneally (i.p.) with log-phase A. pyogenes strains as previously described (7). Mice were euthanized on day 7 or when they became moribund. Peritoneal PE (PF) was obtained by lavage with 3 ml of PBS. Blood and liver were aseptically removed, and the liver was macerated by passage through a 3-ml syringe. Serial dilutions of PF, blood, and liver were incubated on BH blood agar, and bacterial viable counts were determined. Bacterial cells were heat killed by incubation at 70°C for 30 min. Infection was measured as either mortality or the presence of ≥500 CFU of A. pyogenes per g of liver and per ml of PF, on necropsy. The 50% infectious dose ID₅₀ was calculated by the Reed-Muench method (47).

Six female, 6-week-old, outbred ICR mice were immunized i.p. with 29 μg of His-PLO toxoid in MPL-TDM emulsion (Ribi Immunocoreh, Inc.) on days 0 and 17. Six similar mice served as unvaccinated controls. Mice were bled from the orbital sinus on days 17 and 33. The mice were challenged i.p. with 4.4 × 10⁵ CFU of log-phase A. pyogenes BBR1 on day 34 as described above.

RESULTS

Construction and characterization of a plo mutant. To facilitate the construction of an A. pyogenes plo mutant, a 1.7-kb HindIII-BamHI fragment containing the emrA gene from pNG2 (44) was treated with T4 DNA polymerase (Promega). This fragment was cloned into the similarly treated, unique NheI site in pAp350 to generate the recombinant plasmid pJGS79 (Fig. 1). pAp350 contains the 1,605-bp plo gene on a 3.5-kb EcoRI-XhoI fragment in Bluescript SKII (Strategene), and the NheI site is located 783 bp into plo (7). Insertion of emrA truncates the plo open reading frame at codon 261. As pJGS79 was based on a CoE1 replicon, it acted as a suicide plasmid in A. pyogenes (22). pJGS79 plasmid DNA was introduced into A. pyogenes BBR1 cells by electroporation, and recombinants were selected on BH blood agar containing ERM. Fifty-six nonhemolytic, Emr colonies were obtained from this experiment. As cells were incubated for approximately 16 h postelectroporation, this number of recombinants probably arose from two to three recombination events. No hemolytic, Emr colonies were obtained, suggesting that pJGS79 did indeed act as a suicide plasmid and that recombination in all cases was via a double-crossover event.

Southern blotting of A. pyogenes genomic DNA digested with EcoRI and XhoI revealed 3.5- and 5.2-kb bands in BBR1 and a plo mutant (PLO-1), respectively, when probed with a plo-specific probe. The same 5.2-kb band was apparent in PLO-1, but not BBR1 genomic DNA, when an Emr gene-specific probe was used. Neither BBR1 nor PLO-1 genomic DNA hybridized with a Bluescript SKII-specific (vector) probe (data not shown). These data confirm disruption of the plo gene in PLO-1, by the integration of the Emr cassette resulting from a double-crossover event.

Insertional inactivation of plo resulted in loss of expression of PLO with a concomitant loss in hemolytic activity, indicating that PLO is the only hemolysin expressed by A. pyogenes under these conditions. PLO-1 appeared to be unaffected in the expression of other extracellular products such as proteases and DNase (data not shown).

In vitro complementation of PLO-1. In order to perform complementation assays on PLO-1, the 3.5-kb EcoRI-XhoI fragment containing the wild-type BBR1 plo gene from pAp350 was cloned into pEP2 (39), to form the recombinant plasmid pJGS75. PLO-1 was transformed to Km' with pEP2 or pJGS75, and the hemolytic activity of CSF was determined by hemolytic assay. Hemolytic titers from cultures of PLO-1 contained no measurable hemolytic activity (<1). In contrast, average titers of PLO-1(pJGS75), but not PLO-1(pEP2), CSF were restored to wild-type levels (7.0, <1, and 7.5, respectively). In addition, Western blotting with antibodies against His-PLO revealed the presence of an approximately 55-kDa band in BBR1 and PLO-1(pJGS75) CSF, but not in PLO-1 or PLO-1(pEP2) CSF (Fig. 2).

PLO-1 is attenuated for virulence in a mouse model. The relative virulence of wild-type A. pyogenes and PLO-1 was assessed in a mouse i.p. infection model. Groups of eight mice were challenged with 10-fold serial dilutions of wild-type or PLO-1 A. pyogenes, and the mice were monitored over 7 days. On necropsy, the gross pathology of infected mice revealed the presence of one or more of the following features; pale and/or mottled liver, scrobilinous exudate, pus, and abscess(es) within the abdominal cavity. Abscesses were generally encapsulated and yielded pure cultures of A. pyogenes. Mice which succumbed to lethal infections in less than 24 h did not display this typical pathology; instead, there was severe hemorrhaging of all organs, with no scrobilinous exudate or abscesses present.

The infection rates for wild-type or PLO-1 A. pyogenes are shown in Table 1. Bacterial viable counts were performed on samples of blood, liver, and PF (Table 1). Generally, mice infected with A. pyogenes had large numbers of bacteria in the
while three of eight mice receiving 4.1 $\times 10^3$ of disease. The ID$_{50}$ for wild-type bacteria. This finding suggests that mice are able to tolerate large contrast, 4.1 $\times 10^3$ wild-type bacteria were unable to establish an infection. In this model, infection with 3.7 $\times 10^7$ wild-type bacteria was uniformly lethal to mice within less than 16 h (Table 1). Challenge with 3.7 $\times 10^8$ wild-type bacteria resulted in infection within 48 to 72 h in seven of eight mice, while 10-fold less wild-type bacteria were unable to establish an infection. In contrast, 4.1 $\times 10^9$ PLO-1 bacteria were unable to infect mice, while three of eight mice receiving 4.1 $\times 10^9$ PLO-1 bacteria became infected within 72 to 96 h. Challenge with 4.1 $\times 10^{10}$ PLO-1 bacteria was uniformly lethal in less than 16 h. While some mice challenged with 4.1 $\times 10^9$ CFU of PLO-1 developed lethal infections within 72 h. An average of 4.5 $\times 10^5$ CFU of wild-type BBR1 per g and per ml were recovered from liver and PF, respectively. Approximately 50% less PLO-1 was recovered from each site; averages were 2.0 $\times 10^7$ CFU/g and 2.8 $\times 10^7$ CFU/ml from liver and PF, respectively. Both wild-type and mutant bacteria were also recovered from abscesses. This finding suggests that provision of PLO in trans from wild-type cells allowed persistence of PLO-1 in vivo.

**Persistence of PLO-1 in vivo.** In order to determine the persistence of PLO-1 in vivo, eight mice were challenged with 3.9 $\times 10^8$ CFU of PLO-1, two mice were necropsied at 24-h intervals, and bacterial viable counts were performed. The bacteria were rapidly cleared from the peritoneum with approximately 10$^3$-fold fewer bacteria being present 24 h post-challenge (Fig. 3). The bacteria were reduced to undetectable numbers by 96 h. Similar clearance rates were also observed in the liver. Bacteria could be detected in the blood only at 24 h and only in low numbers, indicating the absence of a generalized septicemia following challenge with PLO-1. As noted, infection or intoxication from large amounts of toxic cell wall material, mice were challenged with heat-killed bacteria. Challenge with 4.0 $\times 10^{10}$ CFU of heat-killed A. pyogenes BBR1 cells resulted in no signs of illness or toxicity, and mice were normal on necropsy (data not shown).

**In vivo complementation of PLO-1 with pJGS75.** The virulence of PLO-1 could be restored by supplying pJGS75 on the complementing plasmid, pJGS75 (Table 1), demonstrating that attenuation was due exclusively to the absence of plo, and not other polar effects. Three of three mice challenged with PLO-1(pJGS75) displayed signs typical of wild-type infection, while mice challenged with similar numbers of PLO-1(pEP2) were unaffected (Table 1). To determine the in vivo maintenance of pJGS75, 250 colonies of PLO-1(pJGS75) recovered from infected mice on nonselective medium were patched onto BH$\times$-5% blood agar containing KAN. All these colonies were hemolytic and Km$^\beta$, suggesting that pJGS75 is stable in vivo.

**PLO-1 can be rescued in vivo by wild-type A. pyogenes.** As PLO is an extracellular toxin, it is possible that the presence of wild-type A. pyogenes secreting PLO would allow persistence of PLO-1 bacteria. Six of six mice coinfected with 1.4 $\times 10^8$ CFU of A. pyogenes BBR1 and 1.3 $\times 10^6$ CFU of PLO-1 developed lethal infections within 72 h. An average of 4.5 $\times 10^5$ CFU and 6.0 $\times 10^5$ CFU of wild-type BBR1 per g and per ml were recovered from liver and PF, respectively. Approximately 50% less PLO-1 was recovered from each site; averages were 2.0 $\times 10^7$ CFU/g and 2.8 $\times 10^7$ CFU/ml from liver and PF, respectively. Both wild-type and mutant bacteria were also recovered from abscesses. This finding suggests that provision of PLO in trans from wild-type cells allowed persistence of PLO-1 in vivo.

**TABLE 1. Virulence of wild-type and PLO-1 strains of A. pyogenes.**

<table>
<thead>
<tr>
<th>Challenge strain and CFU</th>
<th>No. infected/total no.</th>
<th>Avg bacterial viable count (CFU) from infected mouse specimen:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>Liver</td>
<td>PF</td>
</tr>
<tr>
<td>BBR1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.7 $\times 10^7$</td>
<td>8/8</td>
<td>3.9 $\times 10^3$</td>
</tr>
<tr>
<td>3.7 $\times 10^8$</td>
<td>8/8</td>
<td>ND$^b$</td>
</tr>
<tr>
<td>PLO-1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.1 $\times 10^9$</td>
<td>8/8</td>
<td>4.0 $\times 10^3$</td>
</tr>
<tr>
<td>PLO-1(pEP2)</td>
<td>2.4 $\times 10^9$</td>
<td>0/3</td>
</tr>
<tr>
<td>PLO-1(pJGS75)</td>
<td>2.8 $\times 10^9$</td>
<td>3/3</td>
</tr>
</tbody>
</table>

* Minima detected are 100 CFU/ml of blood, 500 CFU/g of liver, and 10 CFU/ml of PF.

$^b$ Below the limits of detection.

$^c$ ND, not determined.
challenge with wild-type *A. pyogenes* was rapidly lethal within 48 to 72 h, with large numbers of bacteria present in both the liver and PF (Table 1).

**His-PLO is cytotoxic for macrophages.** One possible role for PLO in *A. pyogenes* pathogenesis is that of a bacterial defense mechanism against host cells, a characteristic common to toxins of the TACY family. The cytotoxicity of His-PLO for macrophages and J774 cells was measured by the release of lactate dehydrogenase into cell culture supernatants, which is a reflection of the loss of plasma membrane integrity. His-PLO was cytotoxic for murine macrophages and J774 cells was measured by the release of lactate dehydrogenase into cell culture supernatants, which is a reflection of the loss of plasma membrane integrity. His-PLO was cytotoxic for murine macrophages and J774 cells in a dose-dependent manner (Fig. 4). Fifty percent cytotoxicity was achieved with 7.5 and 34.4 ng of His-PLO for macrophages and J774 cells, respectively. J774 cells were approximately 4.6-fold more resistant to the effects of His-PLO but displayed very similar dose-dependent kinetics, and so this cell line will be used in place of peritoneal macrophages to further study the effects of His-PLO cytotoxicity. Interestingly, His-PLO preparations frozen at −20°C prior to assessment of cytotoxicity completely lacked cytotoxic activity, even at 100 times the 100% cytotoxic dose. This result is in contrast to the hemolytic activity, which remained constant following freezing.

**PLO is a host-protective antigen.** Administration i.p. of 10 μg of untreated His-PLO was lethal in mice. Therefore, His-PLO was inactivated with formalin prior to its use as an immunogen. His-PLO had a specific activity of 128 hemolytic units/μg (7), while the specific activity of His-PLO toxoid was <1 hemolytic unit/μg. The average antihemolytic titer of sera from immunized mice on day 0 was 0.4, on day 15 was 8.6 (range, 8 to 9), and on day 33 was >11 (range, 9 to 12). Five of six challenged and unvaccinated mice displayed signs of illness by 48 to 72 h, with large numbers of bacteria recovered from the liver and PF (Table 2). In contrast, all the mice vaccinated with His-PLO displayed no signs of illness at any time and had no bacteria or low bacterial viable counts from the liver and PF (Table 2), which were below the criteria for an active infection.

**DISCUSSION**

This is the first report of the construction of a mutant strain of *A. pyogenes*, by insertional inactivation of the *plo* gene with an Em′ cassette. Determination of the ID₉₀ₐₚₘ for wild-type *A. pyogenes* and PLO-1 provides evidence that PLO, a pore-forming cytolysin of the TACY family of toxins, is important for the virulence of this organism in a mouse model. The TACYs are cholesterol-binding, pore-forming toxins found in gram-positive bacteria (32). When in crude form, these toxins require treatment with reducing agents for full activity, a property which is not observed in the purified toxins (32). PLO shares many properties with the TACYs, with the exception that it does not undergo thiol activation due to a lack of cysteine residues in the protein (7).

As has now been demonstrated for PLO, TACYs play an important role in the pathogenesis of the bacteria which express them. Pneumolysin (PLY), expressed by *S. pneumoniae*, activates complement (38), stimulates host cytokine production (19), and inhibits the respiratory burst and bacteriocidal activity of neutrophils (37) and monocytes (34). Not surprisingly, insertional activation of the gene encoding PLY results in a significant decrease in virulence in a mouse model (5).

Similarly, a mutant of *C. perfringens* deficient in perfringolysin O expression has reduced lesion severity in a mouse myonecrosis model (3). In addition, perfringolysin O has been demonstrated to promote dysregulated neutrophil-epithelial cell interactions (9). Listeriolysin O (LLO) contributes significantly to the pathogenicity of *L. monocytogenes*, as the toxin is essential for bacterial escape from the phagolysosome, and mutants in LLO production are avirulent (12). LLO is also cytotoxic for murine macrophages (48) and induces the expression of a variety of host cytokines (26, 36).

Similarly, PLO-1, a *plo* mutant of *A. pyogenes*, has an approximate 1.8-log₁₀ reduction in virulence compared to the wild type in a mouse model. Virulence could be fully restored by complementation with the *plo*-encoding plasmid, pJGS75, indicating that attenuation was due to the absence of PLO and not to other polar effects. Analysis of the pJGS75 insert indicates that the only gene downstream of *plo* in this plasmid is an.

<table>
<thead>
<tr>
<th>Mouse group</th>
<th>Avg bacterial viable count (CFU) for specimen:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Blood</td>
</tr>
<tr>
<td>His-PLO toxoid immunized</td>
<td>—</td>
</tr>
<tr>
<td>Unimmunized</td>
<td>320</td>
</tr>
</tbody>
</table>

* Minima detected are 100 CFU/ml of blood, 500 CFU/g of liver, and 10 CFU/ml of PF.

b = below the limits of detection.
inactive vaccination with His-PLO resulted in neutralizing anti-
A. pyogenes cattle (8, 31), and sheep (20) with formalin-inactivated crude
of nonhemolytic mutants of PLO, currently in progress in our
shown to be important in hemolytic activity (32). Construction
protein following freezing and thawing results in occlusion of a
2
20°C. It is possible that aggregation or oligomerization of the
2
(36, 48). Specific cytotoxicity of PLO, apart from pore forma-
ting and cyto-
genic processes of some infectious diseases (11, 41, 49). Mouse
dendritic cells undergo apoptosis when treated with purified LLO
(18).

One possible role of PLO in pathogenesis may be to deplete
cell death via
ionic disequilibrium, or PLO may specifically trigger mac-
phage apoptosis, which is commonly associated with the patho-
genic processes of some infectious diseases (11, 41, 49). Mouse
dendritic cells undergo apoptosis when treated with purified LLO
(18).

Studies with LLO suggest that the pore-forming and cyto-
kinase-inducing domains may be distinct on the LLO molecule
which can potentially cleave cytokine receptors, leading to
further inappropriate cell signaling events (6).

There have been several attempts to vaccinate mice (13),
cattle (8, 31), and sheep (20) with formalin-inactivated crude
A. pyogenes supernatant. Results of these experiments were
equivalent, probably due to varying amounts of hemolysin in the
immunizing preparation. In the first study using a defined,
purified recombinant PLO immunogen, we demonstrated that
active vaccination with His-PLO resulted in neutralizing anti-
odies, which protected mice against challenge with A. pyo-
genesis, suggesting that PLO is important as a host-protective
antigen. Previously, we demonstrated that passive vaccination
with antisera against His-PLO can protect against A. pyo-
genesis challenge (7), suggesting that protection is mainly hu-
morally mediated. Other TACYs have been successfully used
as experimental vaccines. Vaccination with PLY afforded sig-
ificant protection in a mouse model (1, 30), while sulisyn has
been successfully used to vaccinate pigs against Streptococcus
suis challenge (21).

PLO is an important factor for in vivo survival of A. pyo-
genesis, possibly protecting bacteria from host immune defenses
during the early stages of infection. Therefore, it is not sur-
prising that it also is a host-protective antigen and may be
useful as a vaccine candidate against A. pyogenes infections.
Further vaccination experiments with a more appropriate an-
imal model, such as liver abscess development in feedlot cattle,
will be an important step in determining the efficacy of PLO-
based vaccines for A. pyogenes disease.

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