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Molecular characterization of the pore-forming toxin, pyolysin, a major virulence determinant of *Arcanobacterium pyogenes*

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Abstract

Arcanobacterium pyogenes is a common inhabitant and opportunistic pathogen of domestic animals. The pathogenesis of this organism in a range of suppurative diseases is not well understood. However, the development of genetic techniques to study this organism has allowed advances in the analysis of *A. pyogenes* virulence factors. A major step in this analysis was the identification and cloning of the *A. pyogenes* hemolytic exotoxin, pyolysin (PLO). PLO is the most divergent member of the cholesterol-binding pore-forming family of toxins. PLO is also divergent in a C-terminal undecapeptide motif which is almost invariant among other members of the family. This divergent undecapeptide motif is required for the full cytolytic activity of PLO and is also responsible for its oxygen-resistant nature. Insertional inactivation of the *plo* gene results in a significant reduction in virulence in an intraperitoneal mouse model of infection. The virulence of the *plo* mutant can be restored by providing PLO *in trans*, suggesting that PLO is a major virulence factor in *A. pyogenes* pathogenesis in mice. Results of previous vaccination trials with crude antigens against *A. pyogenes* infection in domestic animals and mice have been equivocal at best. However, a recombinant PLO-based subunit vaccine protected mice from experimental *A. pyogenes* infection, indicating that PLO is also an important host protective antigen. These results provide promise that the dogma that domestic animals are recalcitrant to vaccination against *A. pyogenes* infection may prove false. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: *Arcanobacterium pyrogenes*; Pyolysin; Antigens; Vaccination; Virulence

1. Introduction

The recent reclassification of the gram positive, facultative anaerobe, *Arcanobacterium pyogenes*, on the basis of rRNA sequence data appears to have clarified its taxonomic

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position (Ramos et al., 1997). However, the designations *Actinomyces pyogenes*, and even *Corynebacterium pyogenes*, are still frequently used in clinical veterinary medicine. This organism is a normal inhabitant of the upper respiratory and urogenital tracts of cattle, sheep, swine and other domestic animals (Carter and Chengappa, 1991). Its isolation from the rumen of cattle (Narayanan et al., 1998a,b) and the stomach of pigs (Jost et al., unpublished data), suggests that it may also be a common inhabitant of the gastrointestinal tract of these species. *A. pyogenes* is also an important opportunistic pathogen, responsible for suppurative infections in a variety of domestic animals (Addo and Dennis, 1977; Lechtenberg et al., 1988; Timoney et al., 1988; Semambo et al., 1991), avian species (Barbour et al., 1991; Brinton et al., 1993; Sokkar et al., 1998) and man (Jootar et al., 1978; Kotrajaras et al., 1982; Kotrajaras and Tagami, 1987; Gahrn-Hansen and Frederiksen, 1992; Drancourt et al., 1993). However, infections in man are rare and *A. pyogenes* does not appear to be part of the normal flora of humans. The involvement of *A. pyogenes* in economically significant diseases and syndromes such as, liver abscess (Lechtenberg et al., 1988), mastitis (Hillerton and Bramley, 1989), abortion and infertility (Semambo et al., 1991) and postpartum uterine infections (Ruder et al., 1981) ranks it as one of the most important bacterial pathogens of cattle.

The transition from a commensal to a pathogenic state in *A. pyogenes* is not well understood. However, it usually requires some predisposing condition such as microbial or physical trauma to the underlying tissue, to allow dissemination of the organism. Despite its common isolation and involvement in a range of diseases, the study of factors involved in *A. pyogenes* pathogenesis has been largely neglected. *A. pyogenes* produces a number of extracellular or surface-exposed proteins which have generally been considered putative virulence factors. These include a hemolytic exotoxin, pyolysin (PLO) (Ding and Lämmler, 1996; Funk et al., 1996; Billington et al., 1997), at least two cell-anchored neuraminidases (Schaufuss and Lämmler, 1989; Jost et al., unpublished data), multiple proteases (Schaufuss et al., 1989; Takeuchi et al., 1995) and a DNase (Lämmler, 1990). Until recently, molecular studies of these putative virulence factors have been hampered by the lack of a genetic system for *A. pyogenes*. However, with the development of such a system (Jost et al., 1997) and the identification of plasmid vectors which can replicate in this organism (Jost et al., 1997; Billington et al., 1998), analysis of these factors, and their role in pathogenesis, is now in progress.

β-Hemolysis, due to PLO expression, is a characteristic of the *A. pyogenes* species (Billington et al., 1997; Ramos et al., 1997; Jost et al., 1999). PLO expression appears to be regulated, at least in broth culture, where it is produced in early stationary phase (Ding and Lämmler, 1996; Gilbert et al., unpublished data). PLO is lytic for red blood cells of a variety of species, as well as being dermonecrotic and lethal to laboratory animals via the intravenous (Lovell, 1939, 1944) and intraperitoneal (Jost et al., unpublished data) routes.

2. Cloning and analysis of the *A. pyogenes plo* gene

In order to investigate the toxic nature of PLO and its role in pathogenesis, we cloned the gene for this hemolysin, selecting for *E. coli* clones that produced a zone of hemolysis on

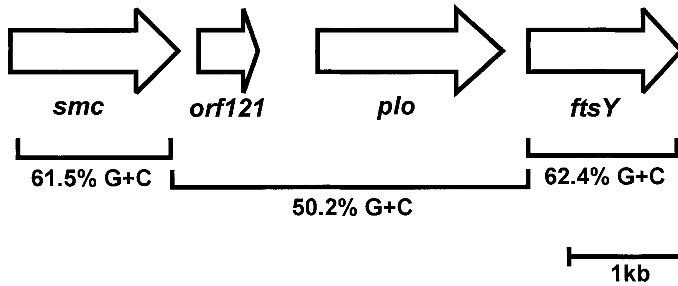

Fig. 1. Schematic representation of the *plo* gene region. ORFs are indicated by the box arrows and are labeled. The G + C content (%) of the DNA delineated by the lines is shown. A 1 kb bar is provided for scale.

blood agar plates (Billington et al., 1997). The *plo* gene is 1605 bp in length and encodes a protein of 534 amino acids, with an apparent signal sequence whose cleavage results in the secretion of the 55 kDa PLO protein (Billington et al., 1997). The *plo* gene is located between two housekeeping genes, *smc* and *ftsY*, that with few exceptions, are found adjacent to each other in most bacterial species (Billington et al., unpublished data; Fig. 1). The region containing the *plo* gene and a small upstream open reading frame (ORF), *orf121*, has a much lower G + C content (%) in comparison with the surrounding housekeeping genes (Fig. 1). This region of DNA does not contain many of the features characteristic of pathogenicity islands, such as association with tRNA genes or insertion sequences (Hacker et al., 1997) and its small size does not lend itself to the term. However, the region clearly carries a virulence gene (see later) and its differential G + C content (%) suggests that it may have originated outside of *A. pyogenes*.

3. PLO is a novel member of the cholesterol-binding pore-forming toxin family

The PLO protein shows 35–41% amino acid identity with a group of toxins historically known as the thiol-activated cytolysins (Table 1), which includes toxins such as

Table 1
Amino acid sequence similarity between PLO and members of the CHOP toxin family

	PLO	
	Identity (%)	Similarity (%)
Alveolysin (ALY)	35.8	72.5
Intermedilysin (ILY)	39.0	77.2
Ivanolysin O (ISO)	39.1	74.0
Listeriolysin O (LLO)	39.1	78.3
Seeligerilysin O (LSO)	39.9	77.2
Perfringolysin O (PFO)	36.0	71.8
Pneumolysin (PLY)	35.4	71.4
Streptolysin O (SLO)	36.1	74.2
Suilysin (SLY)	40.6	75.9

listeriolysin O (LLO), perfringolysin O (PFO), pneumolysin (PLY), and streptolysin O (SLO). The cytolytic activity of these toxins is reversibly inactivated by oxygen and can be restored by reducing agents, hence the term thiol-activated (Billington et al., 2000). However, not all members of the family exhibit this property and we have recently proposed the nomenclature cholesterol-binding pore-forming (CHOP) toxins (Billington et al., 2000), although additional terms such as cholesterol-dependent cytolysins (Shatursky et al., 1999), cholesterol-binding toxins (Gilbert et al., 1999) and Streptolysin O family (Nagamune, 1997) have also recently appeared in the literature.

CHOP toxins are produced by more than 20 species of Gram positive bacteria, and exert their cytolytic effects by forming large oligomeric pores in eukaryotic cell membranes (Billington et al., 2000). Their activity is sensitive to free cholesterol, which appears to be the major host cell receptor for these toxins. The binding of cholesterol appears to be an important step in the structural transition required to convert the soluble monomer toxin to a membrane pore (Gilbert et al., 1999). CHOP toxins possess a characteristic undecapeptide sequence which is located near the C-terminus of the protein and is almost invariant among members of the family (Billington et al., 2000). This undecapeptide forms an extended loop at one end of proposed three dimensional structures (Rossjohn et al., 1997) and has been implicated in the initial binding and penetration of the target membrane by these toxins (Rossjohn et al., 1997; Jacobs et al., 1999). Many CHOP toxins are also important virulence factors for their host organisms (Portnoy et al., 1992; Awad et al., 1995; Mitchell and Andrew, 1997).

While clearly a member of this family of toxins, PLO shows some important differences. Phylogenetically, it is the most diverse member of the family (Fig. 2; Billington et al., 1997). Other members of this family can be grouped according to their nearest neighbors, which for most members follows genus lines, but PLO branches early in the tree and is not grouped with any other member of the family. In addition, PLO along with intermedilysin (ILY), have C-terminal undecapeptide sequences which diverge significantly from the consensus for this family (Fig. 3; Billington et al., 1997; Nagamune, 1997). In the case of

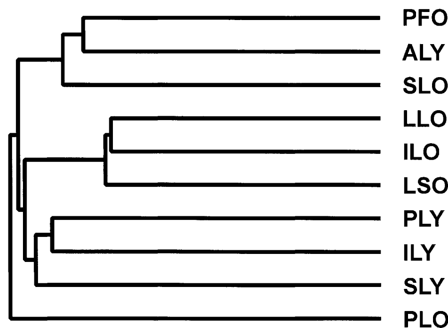

Fig. 2. Phylogenetic relationship between members of the CHOP toxin family. An unrooted dendrogram of the complete amino acid sequences of perfringolysin O (PFO), alveolysin (ALY), streptolysin O (SLO), listeriolysin O (LLO), ivanolysin O (ILO), seeligerilysin O (LSO), pneumolysin (PLY), intermedilysin (ILY), suilysin (SLY) and pyolysin (PLO) is shown. The dendrogram was compiled using the protein parsimony program in the PHYLIP package.

SLO	529	E	C	T	G	L	A	W	E	-	W	W	R
PLY	427	E	C	T	G	L	A	W	E	-	W	W	R
SLY	455	E	C	T	G	L	A	W	E	-	W	W	R
LLO	483	E	C	T	G	L	A	W	E	-	W	W	R
PFO	458	E	C	T	G	L	A	W	E	-	W	W	R
ALY	460	E	C	T	G	L	A	W	E	-	W	W	R
PLO	491	E	A	T	G	L	A	W	D	P	W	W	-
ILY	485	G	A	T	G	L	A	W	E	P	W	-	R

Fig. 3. Amino acid alignment of the undecapeptide sequences of CHOP toxins. Undecapeptide sequences are shown from streptolysin O (SLO), pneumolysin (PLY), suilysin (SLY), listeriolysin O (LLO), perfringolysin O (PFO), alveolysin (ALY), pyolysin (PLO), and intermedilysin (ILY). Amino acids in PLO and ILY which diverge from the consensus undecapeptide sequence are boxed.

PLO, the most significant of these changes appear to be the insertion of a proline residue, and the lack of the terminal arginine residue in the motif, which are likely to affect both the local charge and structure of the region. In addition to a fairly conserved glutamic acid to aspartic acid substitution, PLO and ILY, lack a conserved cysteine residue in the undecapeptide, which is responsible for the thiol-activated nature of this group of toxins (Fig. 3; Billington et al., 1997; Nagamune, 1997). As a result, neither PLO nor ILY exhibit the property of thiol-activation (Funk et al., 1996; Nagamune et al., 1996; Billington et al., 1997).

4. The divergent undecapeptide motif of PLO is required for full cytolytic activity

Like PLO, the activity of a recombinant PLO molecule containing a six histidine tag (HIS-PLO) is unaffected by reducing agents, presumably due to the lack of the cysteine residue at position 2 of the PLO undecapeptide. Substitution of alanine with the conserved cysteine at this position results in a molecule that is subject to oxygen inactivation and can be restored to 30–40% of wildtype HIS-PLO activity by reducing agents (Fig. 4; Billington et al., unpublished results). This mutation was previously reported to be insensitive to oxygen (Billington et al., 1997), probably because this protein is inactivated more slowly by oxygen than other CHOP toxins. However, these data prove that PLO is not thiol-activated simply due to the lack of the conserved cysteine residue.

Since PLO is the most phylogenetically divergent member of the CHOP toxins, the variant PLO undecapeptide sequence may simply reflect this sequence divergence. However, insertion of a consensus undecapeptide sequence into HIS-PLO resulted in a mutant protein with only 1% of wildtype HIS-PLO activity, even in the presence of reducing agents (Fig. 4; Billington et al., unpublished data). This result confirms that the variant undecapeptide of PLO is required for full cytolytic activity of this molecule. Deletion of the proline residue, or substitution with either phenylalanine or glycine, resulted in substantial reductions in hemolytic activity compared to wildtype (Fig. 4; Billington et al., unpublished data), suggesting that the proline residue plays an essential role in the cytolytic activity of PLO. The proline residue is likely to impose a bend in the

		% wildtype HIS-PLO hemolytic activity		
			+BME	+DTT
HIS-PLO	E A T G L A W D P W W -	100	100	100
A492C	E C T G L A W D P W W -	4.4	41	31.4
CONSENSUS	E C T G L A W E - W W R	0.1	1	1.2
ΔP499	E A T G L A W D - W W -	0		
P499F	E A T G L A W D F W W -	1.3		
P499G	E A T G L A W D G W W -	0.3		
W497F	E A T G L A F D P W W -	0.3		
W500F	E A T G L A W D P F W -	3.0		
W501F	E A T G L A W D P W F -	6.3		

Fig. 4. Hemolytic activity of site-specific mutant HIS-PLO proteins. The sequence of the undecapeptide motif is shown for all proteins. Amino acids changed from the HIS-PLO sequence are boxed. The hemolytic activity of each protein as a percentage of HIS-PLO activity is shown on the right. The hemolytic activity in the presence of β-mercaptoethanol (BME) or dithiotheitol (DTT) is also shown for mutants in which the conserved cysteine has been incorporated.

undecapeptide loop structure, which may realign the PLO undecapeptide, compensating for structural differences in other regions of the molecule.

The three hydrophobic tryptophan residues within the undecapeptide motif play a pivotal role in the cytolytic activity of many CHOP toxins (Michel et al., 1990; Boulnois et al., 1991; Sekino-Suzuki et al., 1996; Korchev et al., 1998; Nakamura et al., 1998), and this is also true in PLO (Fig. 4; Billington et al., unpublished data). Substitution of any of these tryptophan residues with phenylalanine results in a substantial reduction in hemolytic activity compared to wildtype (Fig. 4), suggesting that the role of the PLO undecapeptide is similar to that of other members of the family. The role of the undecapeptide sequence in the initial binding and penetration of the target membrane has yet to be proven. However, the position of the first undecapeptide tryptophan residue at the very tip of the undecapeptide loop (Rossjohn et al., 1997) implies a prominent role for this amino acid in the initial interaction of the toxin with the target cell membrane. The marked effect of mutations at this position in PLO (Fig. 4) and other CHOP toxins (Michel et al., 1990; Boulnois et al., 1991; Sekino-Suzuki et al., 1996; Korchev et al., 1998), supports the importance of this residue in cytolytic activity.

5. Characterization of a PLO-deficient mutant of *A. pyogenes*

Members of the CHOP toxin family are often important virulence factors of their host organisms (Portnoy et al., 1992; Awad et al., 1995; Mitchell and Andrew, 1997), suggesting that PLO may play a role in *A. pyogenes* pathogenesis. This hypothesis was assessed by constructing an isogenic *plo* mutant, PLO-1, of the wildtype *A. pyogenes* strain BBR1 (Jost et al., 1999). An erythromycin resistance cassette, *erm*(*X*) from the *Corynebacterium diphtheriae* plasmid pNG2, was inserted into the cloned *plo* gene disrupting the ORF (Fig. 5a). This construct was introduced into the wildtype *A. pyogenes* strain BBR1 by electroporation, and non-hemolytic, erythromycin-resistant recombinants were obtained.

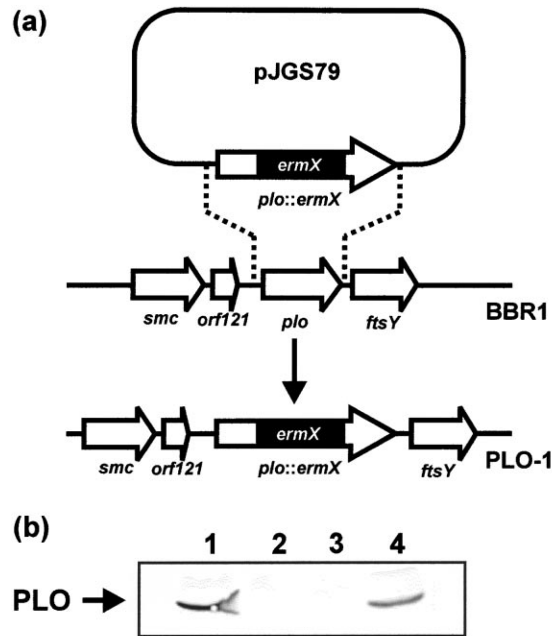

Fig. 5. Construction and analysis of the PLO-1 mutant. (a) Schematic diagram of the construction of PLO-1. The plasmid pJGS79, which contains the *plo* ORF disrupted by an *erm*(*X*) cassette, was introduced into *A. pyogenes* strain BBR1 by electroporation. The dotted lines represent potential regions of homologous recombination which through a double reciprocal crossover event would replace the wildtype copy of *plo* with the inactivated copy, leading to the construction of PLO-1. (b) Western blot analysis of PLO production. Culture supernatant fluid from (1) wildtype BBR1, (2) PLO-1, (3) PLO-1 containing the plasmid vector pEP2, and (4) PLO-1 containing the plasmid pJGS75, which has the *plo* gene cloned into pEP2, were subjected to Western blot analysis with antiserum specific for the PLO protein. Only the appropriated region of the blot is shown and the PLO band is indicated by the arrow on the left.

A double crossover event, replacing the wildtype copy of *plo* in the BBR1 chromosome with the insertionally inactivated copy in PLO-1, was confirmed by Southern blot (Jost et al., 1999). This was the first demonstration of allelic exchange in *A. pyogenes*.

PLO-1 is non-hemolytic on blood agar plates and no hemolytic activity could be detected in culture supernatant fluid of stationary phase cells, indicating that PLO is the only hemolysin expressed by *A. pyogenes*, at least under the conditions tested (Jost et al., 1999). In addition, PLO protein could not be detected in the culture supernatant fluid of PLO-1 cultures by Western blot (Fig. 5b, lane 2), confirming that PLO-1 is deficient in PLO production. The production of PLO and hemolytic activity could be restored by providing the *plo* gene *in trans* on a replicating plasmid (Fig. 5b, lane 4).

6. PLO is a major virulence factor in *A. pyogenes* infections

The virulence of the PLO-1 mutant was assessed using an intraperitoneal mouse model of *A. pyogenes* infection (Jost et al., 1999). Mice were inoculated with 4×10^8 cells of log

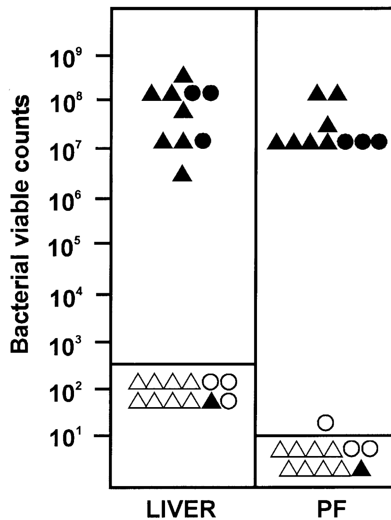

Fig. 6. PLO-1 has reduced virulence in an intraperitoneal mouse model. Viable bacterial counts per gram of liver and per milliliter of PF from mice inoculated with either BBR1 (▲), PLO-1 (△), PLO-1 (pEP2) (○) or PLO-1 (pJGS75) (●) are shown. Each point represents an individual mouse. The bar in each column represents the limits of detection for each tissue.

phase wildtype strain BBR1 or PLO-1 mutant bacteria. Mice were necropsied on day 7 or when moribund, and blood, liver and peritoneal fluid (PF) were cultured for viable bacteria (Fig. 6). The definition of infection in this model is based on numbers of bacteria found in the liver and PF on necropy, but mice infected with *A. pyogenes* generally show characteristic clinical signs of infection such as hypoactivity, ruffled appearance and anorexia, as well as the presence of pus, serofibrinous exudate and abscesses in the abdominal cavity. Seven of the eight mice inoculated with wildtype bacteria became infected, showing characteristic clinical signs, and large numbers of bacteria were recovered in the liver and PF. None of the mice receiving PLO-1 became infected at this dose, and no bacteria were recovered from any site, indicating a defect in virulence of the PLO-1 mutant compared to the wildtype strain, BBR1 (Fig. 6). The virulence of the PLO-1 mutant was restored by supplying the *plo* gene *in trans* (Fig. 6), confirming that the defect in PLO expression is responsible for the reduction in virulence observed with PLO-1. In vivo survival data from the PLO-1 mutant indicated that the mutant is cleared fairly rapidly from the host, within 48 h post-challenge (Jost et al., 1999). Inoculation of mice with 10-fold serial dilutions of wildtype or PLO-1 mutant bacteria indicated that much higher numbers of PLO-1 were required to establish infection, with an approximate 1.8 $\log_{10}$ increase in the ID_{50} compared to the isogenic wildtype strain (Jost et al., 1999). This decrease in virulence is similar to the effects of CHOP toxin knockouts in other bacteria such as *Streptococcus pneumoniae* (Berry et al., 1989) and *Clostridium perfringens* (Awad et al., 1995) and is consistent with the hypothesis that *A. pyogenes* virulence is multifactorial. Therefore, PLO appears to play a major role as a virulence factor in the establishment of *A. pyogenes* infection.

Since PLO is an extracellular protein and likely exerts its effects by interacting with host cell membranes, the ability of wildtype bacteria to rescue PLO-1 in vivo was assessed. Co-infection of mice with equal numbers ($\sim 10^8$) of wildtype and PLO-1 mutant bacteria resulted in establishment of infection, and approximately equal numbers of each strain were recovered from the liver and PF (Jost et al., 1999). Both wildtype and PLO-1 were also isolated from peritoneal abscesses. Thus, extracellular PLO secreted by wildtype cells allows persistence of PLO-1 in *A. pyogenes* infections and implies a function for PLO that does not require proximity to its host organism.

The precise function of PLO in *A. pyogenes* infections is still not fully understood. In addition to its hemolytic activity, PLO is also cytolytic for a kangaroo kidney cell line (Ding and Lämmler, 1996) as well as immune cells, murine peritoneal macrophages, and bovine and ovine PMNs (Ding and Lämmler, 1996; Jost et al., 1999). This cytolytic activity may provide protection for the invading organism against the immune response. However, the activities associated with many CHOP toxins suggest that the function of PLO in virulence may also involve modulation of the immune response through the upregulation of expression of various cytokines (Hackett and Stevens, 1992; Houldsworth et al., 1994; Konig et al., 1994; Nishibori et al., 1996; Ruiz et al., 1998; Kayal et al., 1999) and other inflammatory substances (Mitchell and Andrew, 1997; Ruiz et al., 1998).

7. PLO is an effective protective antigen

Previous vaccination experiments using whole cells or culture supernatant were largely unsuccessful in protecting domestic animals from *A. pyogenes* infections (Lovell et al., 1950; Cameron, 1966; Hunter et al., 1990), although some therapeutic effects on bovine mastitis cases were observed (Brown and Stuart, 1943). Even mouse vaccinations have, at best, given equivocal results (Derbyshire and Matthews, 1963; Cameron et al., 1976), leading some researchers to suggest only a remote possibility of successful immunization of animals against *A. pyogenes* infections (Derbyshire and Matthews, 1963).

However, the recent identification of PLO as an important virulence factor, provided a candidate for a potential subunit vaccine. The serum from mice immunized twice with 30 μg formalin-inactivated HIS-PLO exhibited high neutralizing titers to PLO hemolytic activity (Jost et al., 1999). These mice were protected from challenge with 4×10^8 wildtype *A. pyogenes* strain BBR1 (Fig. 7). A small number of bacteria were isolated from the liver and PF of some of the vaccinated mice, and represented bacteria in the process of being cleared from the host. In contrast, five of the six unvaccinated mice developed characteristic *A. pyogenes* infections with large numbers of bacteria in the liver and PF. These results indicate that PLO is an important antigen in the protection of mice against experimental *A. pyogenes* infection.

Similar protection was obtained with mice passively immunized with anti-PLO goat serum (Billington et al., 1997), suggesting that humoral immunity is important in protection, at least in this model system. However, unlike laboratory mice, domestic animals are naturally colonized with *A. pyogenes*, and have serum antibodies to *A. pyogenes* and PLO (Jost et al., unpublished data). Therefore, it is uncertain how these

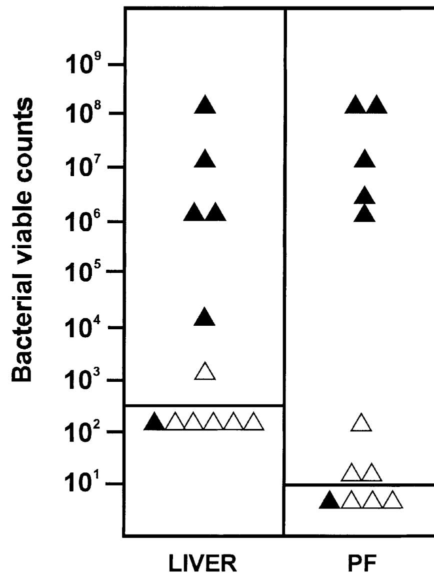

Fig. 7. Immunization with PLO protects mice against intraperitoneal challenge. Viable bacterial counts per gram of liver and per milliliter of PF from unvaccinated mice (▲) and mice vaccinated with HIS-PLO (△), following challenge with 4×10^8 wildtype *A. pyogenes* strain BBR1. Each point represents an individual mouse. The bar in each column represents the limits of detection for each tissue.

results in mice will translate to domestic species in which *A. pyogenes* is a common inhabitant.

The failure of crude *A. pyogenes* vaccines (Lovell et al., 1950; Cameron, 1966; Hunter et al., 1990) suggests that protection of domestic animals may be more complex. It is possible that cell mediated immunity may play a greater role in animals which acquire *A. pyogenes* through natural infections. Alternatively, culture supernatant may be a poor antigen for vaccination, and its deficits may be overcome by the use of purified recombinant PLO antigen. Therefore, the efficacy of a PLO-based subunit vaccine needs to be assessed for its ability to prevent *A. pyogenes* infections in domestic animals.

8. Conclusions and future directions

With the development of genetic techniques for the introduction of DNA into *A. pyogenes*, molecular analysis of *A. pyogenes* pathogenesis is now in progress. The first step in these investigations has now been taken with the identification of PLO as a major virulence factor in experimental *A. pyogenes* infections in mice. Much information has been collected about the role of toxins such as PLY, SLO, PFO and LLO in pathogenesis and the similarity of PLO to these toxins suggests that it may act through modulation of the immune response as well as direct cytotoxicity. While it is clear that PLO is cytolytic for

immune cells, its effect on cytokine induction and the complement cascade are current areas of investigation in our laboratory.

The efficacy of a PLO-based vaccine in the protection of mice, provides promise for the use of similar vaccines in diseases of domestic animals where *A. pyogenes* plays an appreciable role, such as liver abscess in feedlot cattle, mastitis in diary cattle and post-partum uterine infections. It is still unclear whether similar results can be obtained in domestic animals that carry *A. pyogenes* as normal flora, especially if immunity is stimulated through the humoral response as suggested by passive immunization experiments. Definitive studies with purified antigens are required to assess the response and protection of cattle and other domestic animals to *A. pyogenes* vaccines.

The discovery that PLO is a member of the CHOP toxin family, also has implications for research into this family of toxins given its divergent amino acid sequence, particularly within the undecapeptide region which has been assumed to play a critical role in toxin binding and insertion due to its high level of conservation within the family. It is clear that the changes in the PLO undecapeptide sequence are required for the cytolytic activity of this toxin, but whether these changes reflect a realignment of the sequence forced on the undecapeptide by sequence divergence elsewhere in the PLO protein is unknown.

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