To the Graduate Council:

I am submitting herewith a thesis written by Rajeev V. Nair entitled " Construction of recombinant *Pasteurella multocida* toxin (PMT) fragments and evaluation of their immunogenicity and protective efficacy in mice". I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Comparative and Experimental Medicine.

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CONSTRUCTION OF RECOMBINANT *PASTEURELLA MULTOCIDA TOXIN* (PMT) FRAGMENTS AND EVALUATION OF THEIR IMMUNOGENICITY AND PROTECTIVE EFFICACY IN MICE

A Thesis

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Degree

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DEDICATION

This thesis is dedicated to the memory of my beloved Grand father, Late Sri. V. Madhavan Nair, for his love and affection showered upon me.

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ABSTRACT

Atrophic rhinitis (AR) is an economically important upper respiratory tract disease of pigs, and is of multifactorial etiology. Bacterial agents of importance are toxigenic strains of *Pasteurella multocida* and *Bordetella bronchiseptica* alone or combined. Physical and chemical irritants like dust or ammonia in an animal's environment may also contribute to the severity of disease. Many capsular type D and occasional type A strains of *P. multocida* produce a potent, intracellular, heat labile, 146 kD mitogenic toxin called *Pasteurella multocida* toxin (PMT). PMT is the major virulence factor that correlates significantly with progressively severe AR. A single component *P. multocida* toxoid vaccine was sufficient for inducing immunity against experimentally induced AR. In this study subunit fragments of PMT were genetically constructed and evaluated for immunogenicity. Three C-terminal constructs (685, 302 or 155 amino acids from the C-terminus) and two N-terminal constructs (1130 or 482 amino acids from the N-terminus) of PMT were cloned into expression vectors pET 29b+ and pPROEXHTc. A full-length recombinant PMT was also cloned and expressed in pET 29b+ vector. The recombinant proteins were purified by affinity chromatography. Six to eight week old BALB/c mice were immunized with these recombinant proteins and their immunogenicity was evaluated using ELISA and toxin neutralizing assay on Vero cells. Protective efficacy of immunization by these fragments was determined using lethal challenge with recombinant PMT. Immunization by the 685 amino acid C-terminal fragment and the 1130 amino acid N-terminal fragment protected mice from lethal challenge with rPMT. Recent studies that attempted to identify a biological activity domain of PMT reported

contradictory results. While one study reported the requirement of N-terminus 500 amino acid of the toxin for biological activity, two other studies reported the importance of C-terminus on the biological activity of toxin. However, in this study the protective N - terminal fragment lacked the putative C-terminal activity region and the protective C terminal fragment lacked the putative N-terminal activity region. The results of this study indicate that PMT may be a multifunctional toxin and either one of these regions is sufficient to induce a protective immune response in mice. Further studies are required to clarify the regions involved in binding and activity of the toxin. Correlation of the mouse protection assay with prevention of AR in pigs merits further study. The PMT fragments produced in this study will provide a basis for development of more refined subunit vaccine candidates.

TABLE OF CONTENTS

LIST OF TABLES

TABLE PAGE

LIST OF FIGURES

FIGURE PAGE

CHAPTER 1

INTRODUCTION

Atrophic Rhinitis

Atrophic rhinitis (AR) is an upper respiratory tract disease of pigs. The etiology of AR has been debated for more than 150 years and is still considered complex (31, 38). The occurrence of AR in almost every geographical area with intensive pig production has been well documented during recent years (85). Estimates of economic importance vary, but AR may cause up to 5-10 per cent reduction in growth rate in some populations (81). Each year, atrophic rhinitis costs pork producers $ 17 million in lost weight and delay to market (123). Factors of major impact on the economic significance of AR include incidence of clinically affected pigs, meat inspection rules and regulations, effect on growth rate, and intensity of prophylactic treatments/vaccinations (81). It has been widely agreed that AR results in approximately 0.5 % reduction in the growth rate, when determined as an average for all slaughter pigs (38, 81)

AR is characterized by a chronic progressive rhinitis with degeneration and atrophy of nasal turbinate bones leading to visible distortion and shortening of the snout (97). The clinical signs of AR in pigs include shortening and twisting of the snout with wrinkling of skin covering the snout, sneezing, snuffling, nasal discharges, epistaxis, brachygnathia superior and growth retardation. Severe inflammation of nasal mucosa can be seen in some cases, and it has been proposed that turbinate atrophy is the product of a severe, persistent inflammatory reaction in the nose of rapidly growing, young pigs. The

usual gross pathology observed is atrophy of the turbinate bones varying from slight atrophy of the inferior scroll of one of the ventral turbinates to complete bilateral atrophy of the dorsal and ventral turbinates (97). Histopathology of the lesion revealed atrophy and fibrous replacement of the bony medulla of the nasal conchae (31). The resorbed osseous trabeculae of the turbinate bones were replaced by a shrunken mass of fibrous tissue and in the osseous core the osteocytes and the osteoblasts appeared to be replaced by primitive mesenchymal cells (100). It has been experimentally shown that severe turbinate lesions may be seen without overt signs of inflammation without any signs of inflammation (34, 83, 100). Increased numbers of osteoclasts adjacent to the reduced nasal bones have been reported in some cases (83), but were not observed in other cases (38, 98).

Etiology of Atrophic Rhinitis

Atrophic rhinitis showed a marked difference from most other infectious diseases in several respects. It was initially described as a disease of multifactorial etiology and its severity was said to be influenced by many factors. Several researchers in earlier years attributed the cause to heriditary, nutritional and managemental factors (31, 38). Later, it was clearly understood that bacterial etiologies were involved in the development of the disease (33, 98). Bacterial etiologies identified in AR were toxigenic strains of *Pasteurella multocida* and *Bordetella bronchiseptica* alone or combined along with other environmental factors like dust, ammonia and other physical and chemical irritants (6, 38, 44, 45). Isolation and identification of infectious agents from the nasal cavity of pigs with

AR resulted in demonstration of a wide range of microorganisms (38). Report describing the intranasal instillation of different infectious and non-infectious agents inducing turbinate atrophy in pigs led to a proposal that any agent capable of producing a severe, persistent rhinitis at an early age could cause atrophy of the turbinates (31). Even though several agents have been used alone or in combinations to produce experimental infections, only two bacterial species, *P. multocida* and *B. bronchiseptica*, have been able to consistently produce turbinate atrophy (38, 97).

B. bronchiseptica had been recognized as a respiratory tract pathogen of mammals since 1910 (41). It was widely distributed in pig herds both with and without AR (38, 97). The virulence of *B. bronchiseptica* in gnotobiotic piglets was studied by intranasal infection with different strains and phase variants of this organism. It was found that the development of turbinate atrophy was associated with the ability to produce heavy, persistent colonization in the nasal cavity and the production of a heat-labile toxin, and these properties were collectively associated with phase I organisms (21). Isolates of *B. bronchiseptica* obtained from herds with and without clinical AR resulted in similar moderate turbinate lesions (98, 99) and all the isolates obtained from pig herds were phase I organisms (97).

The main virulence factor of *B. bronchiseptica* in AR is dermonecrotic toxin (DNT), a 162 kD, intracellular, dermonecrotic, thermolabile and mitogenic cytotoxin (76). Isolates of this organism obtained from German herds with and without clinical AR produced DNT and no difference was observed in mouse lethality of the toxin prepared from these strains (99). Current opinion holds that infection with *B. bronchiseptica* is an

important, but not necessary predisposing factor for AR and it does not induce severe, progressive form of the disease.

Pasteurella multocida was associated with AR by several groups, but experimental infection studies gave conflicting results (101). In 1980, it was reported that dermonecrotic strains of *P. multocida* could cause severe atrophy of the nasal turbinates of pigs. Type D toxigenic strain of *P. multocida* was the most common isolate in swine atrophic rhinitis (38, 85). One study had shown that the toxigenic *P. multocida* induced atrophic rhinitis was associated with initial bone resorption accompanied by impaired osteogenesis. This differed from turbinate atrophy induced by *B. bronchiseptica*, which was characterized by impaired osteoid synthesis due to the damage to the osteoblasts (83). Experimental infection of gnotobiotic piglets with *B. bronchiseptica* followed by infection with toxin producing *P. multocida* caused a progressive AR that corresponded to the spontaneous disease whereas inoculation with either agent alone did not resulted in progressive disease (101).

Pathogenic strains of *B. bronchiseptica* cause non-progressive turbinate hypoplasia followed by turbinate degeneration (81, 84). A revised definition for AR was proposed. Progressive AR caused by infection with toxigenic *P. multocida* was defined as a specific disease entity, clinically characterized by shortening and distortion of the snout, sneezing, nasal discharge and epistaxis, and reduced growth rate. Nasal bone atrophy being brought about by *P. multocida* toxin induced osteoclastic bone resorption. Infection with *B. bronchiseptica* was described as an important predisposing factor and the term

progressive atrophic rhinitis was proposed for AR caused by infection with *P. multocida* (81, 84,85)

The intranasal inoculation of 1-week old piglets with *B. bronchiseptica,* before experimental intranasal infection with *P. multocida* resulted in improved colonization by *P. multocida*. This was confirmed by intranasal inoculations in gnotobiotic piglets and quantitative colonization studies (101). When compared to experimental infection with *B. bronchiseptica* alone, the subsequent inoculation with *P. multocida* resulted in exacerbated turbinate lesions at inspection six weeks post inoculation (38, 97). Vaccination of pregnant sows with an adjuvanted filtrate of toxigenic *P. multocida* resulted in significant protection from clinical and pathological lesions of naturally occuring and experimentally induced AR.

***Pasteurella multocida* Toxin (PMT)**

Many capsular type D and occasional type A strains of *P. multocida* produce a potent, intracellular, heat labile, 146 kD, mitogenic toxin called *Pasteurella multocida* toxin (PMT), which is the major virulence factor in AR (38, 54, 55, 65, 114). Before successful purification of the toxin was accomplished, it was not known if toxigenic *P. multocida* produced more than one toxin, and the chemical composition of the toxin was unknown (38). A toxic substance produced by *P. multocida* was supposed to be responsible for the clinical and pathological signs of AR (100). There was a good correlation between the ability of different strains of *P. multocida* to cause AR and the production of a toxin, which causes dermonecrosis in guinea pig skin and lethality in mice (24, 25).

Partial purification and characterization of a heat labile dermonecrotic toxin from *P. multocida* was described by van der Herjden et al (111). The method involved ammonium sulfate percipitation of culture filtrate, gel filtration and ion exchange chromatography. Nakai et al characterized the dermonecrotic toxin produced by pig isolates of *Pasteurella* and compared this with dermonecrotic toxin produced by *Bordetella bronchiseptica*. Although biologic and toxic properties of both were similar, cross neutralization tests indicated that DNT from the two bacteriological species were serologically distinct. The proteinaceous nature of PMT was indicated by studies showing that PMT was biologically inactivated by treatment with heat, formaldehyde and trypsin (74, 77, 78).

Crude cell free preparations of toxigenic *P. multocida* were obtained by growing the organism in solid medium or broth at 37°C for approximately 24 hours (98). PMT seems to be located intracellularly during the exponential phase of in vitro growth (49, 102) and remains cell associated in later phases of growth (76). Highest yields of toxic activity were obtained by disruption of cells grown in the exponential phase. Physical and chemical methods of cell disruption have been used effectively (74, 75, 98).

Chemical cell lysis was compared with sonication as a method to release cell associated toxin for biochemical purification. A mixture of EDTA, lysozyme, toluene and the detergent Triton X-100 resulted in a rapid release of PMT and yielded 10 times more protein than sonication method (94). Subsequent purification steps included ammonium sulfate precipitation, gel filtration and anion exchange chromatography, and preparative polyacrylamide gel electrophoresis. Anion exchange chromatography was especially

useful for purification of PMT from crude extracts (38). This method resulted in several fold increases in the purity of PMT, but also caused a loss of total biological activity of PMT (20, 35, 53, 75).

A series of monoclonal antibodies (MAb)with specificity for PMT were produced and characterized, and an affinity chromatographic method for quantitation and purification of PMT using one of these MAb was developed. Application of culture supernatants or crude extracts of toxigenic *P. multocida* to an affinity column containing immobilized anti-PMT MAb, P3F51, resulted in purification of PMT with a yield of 78-93%. The purified PMT had a MW of 143 kD as determined by silver stained SDS-PAGE gels (36)

Three research groups cloned the PMT gene in *Escherichia coli* (54, 63, 88). The methods employed for cloning included isolation of chromosomal DNA from *P. multocida*, fragmentation of the genomic DNA by restriction endonucleases, ligation of the DNA fragments with plasmid vectors (63, 88) or a phage vector (54) and transformation of the resulting vectors into competent *E. coli*. Restriction endonuclease SauIIIA was used for digestion of the genomic DNA, and further fractionation was carried out on a sucrose gradient to obtain fragments of 4-20 kb. The chromosomal library thus established in *E .coli* was screened by a colony immunoblot assay using monoclonal or polyclonal anti-PMT antibodies or by detection of the cytopathic effect on Embryonic Bovine Lung (EBL) cells (88, 102). These selection methods led to the identification of *E. coli* clones producing a protein apparently similar to native PMT (88). Protein profiles and biological activities of the sonic extracts of these clones were tested

by western blotting and EBL cell cytotoxicity. The PMT encoding gene was named *tox A*, with a coding region size of approximately 4 kb, as estimated from apparent moleculr weight of PMT (63, 88).

The *tox A* gene cloned in both orientations directed the expression of an intact functional toxin, which suggested that *P. multocida* transcription and translation directing nucleotide sequences were functional in *E. coli*. It was concluded that *tox A* was localized on the *P. multocida* chromosome and not on plasmids, based on the comparison of the amount of *tox A* bearing DNA obtained by plasmid and chromosomal DNA purification methods. The toxigenic *P. multocida* used for cloning *tox A* gene in one study did not contain any plasmids (88). In vitro hybridization experiments were performed in order to determine whether *tox A* gene could be demonstrated in non-toxigenic bacterial strains. DNA probes covering the entire *tox A* gene, when used in Southern blottings against DNA from reference strains, hybridized only to those of toxigenic strains (56, 88).

Toxin was found in the cell associated fractions of *E. coli* cells as determined by analysis of the cell associated material, periplasmic and growth medium fractions of a stationary-phase culture using SDS-PAGE and Western blots (76, 88). The protein produced by *tox A*-bearing recombinant *E. coli* was named rPMT (88). It has been extensively analysed and compared to native PMT (36). Affinity purified rPMT and native PMT had very similar reaction patterns with a variety of anti-PMT MAbs in an ELISA.

PMT and rPMT produced similar cytopathic effect on EBL cells, dermonecrotic activity in guinea pigs, and lethality in mice (36. 37). The ability to induce turbinate

atrophy and lesions in piglets after i.p. injection were indistinguishable for PMT and rPMT (63). A number of degradation intermediates were observed when PMT was expressed in *E. coli*. These intermediates were also detectable in sonic extracts of *P. multocida* and affinity purified preparations of PMT (88, 94). PMT could be reversibly dissociated into three antigenic polypeptide fragments with approximate sizes of 23,000, 64,000 and 74,000 kD after mild trypsinization, by treatment with 100 mM dithiothreitol and 6 M urea, suggesting involvement of a subunit structure (60, 77, 78). Two additional polypeptide fragments were seen on SDS PAGE gels when the purified toxin is stored for four weeks at 4°C and run on SDS-PAGE gels.

The complete nucleotide sequence of *tox A* gene has been determined for three different strains of toxigenic *P. multocida*. The sequence contains an open reading frame which encodes a protein of 1285 amino acids. The predicted molecular mass of 146 kDa, agreed with the estimated molecular mass of the native toxin determined by SDS-PAGE (64). The open reading frame (ORF) was preceded by a ribosome binding site and followed by a stem loop (63). There was convincing evidence for a promoter region of the *tox A* gene. There was no evidence for a signal sequence at any of the potential start codons, which agrees with the lack of secretion from either the native *P. multocida* or the recombinant *E. coli*. A hydrophobicity plot indictaed that the protein might have several domains; there was a hydrophobic region between amino acids 380 and 470 which was bounded by two hydrophilic domains, and the C-terminal of the protein was also hydrophobic (63, 88). The two hydrophobic domains in the toxin could play a role in the interaction of the toxin with cellular membranes (63).

The *tox A* gene had a low G + C content of 35%, while the G + C content of *P. multocida* genome was estimated as 40.7% (75). It was presumed that the *tox A* gene had been acquired relatively recently and that a family of closely related mitogenic toxin genes might exist in other bacteria (63). It was reported that the toxin gene is flanked by phage elements, which may be carried on a prophage and that some salmonella isolates contain part of the *Pasteurella* toxin gene (89). The deduced protein sequence did not show any significant homology to known sequences with the exception of a motif found in ADP -ribosylating toxins. It is reported that expression of *tox A* gene in *E. coli* is repressed at the transcriptional level. Repression could be abolished either by deletion of a region upstream of *tox A*, or by a putative frame-shift mutation in the same region. The repressor protein encoded within this region was named Txa R (89).

First commercial vaccines for AR consisted of inactivated *B. bronchiseptica*, which was used through out the world (41). Although it has been reported to reduce prevalence of clinical AR in herds where the disease is endemic, the extent of improvements has varied considerably (38, 97). These vaccines have mainly been used for immunizing pregnant sows in order to induce passive immunity in the offspring by transfer of colostral antibodies. Immunization of sows with crude cell-free preparations of toxigenic *P. multocida* either alone or in combination with whole cells of *B. bronchiseptica*, induced partial protection of the offspring against conchal atrophy after experimental infection with *B. bronchiseptica* and thereafter with toxigenic *P. multocida* (85). Several commercial vaccines, based on different combinations of killed *B. bronchiseptica* cells, inactivated toxigenic *P. multocida* cells and inactivated preparations

of partially purified toxin, are widely used in pig herds to reduce the incidence and severity of AR (5, 28, 86).

Purification of large amounts of PMT based on affinity chromatography using a toxin specific MAb (36) lead to studies with inactivated PMT as a vaccine candidate. Detoxification of the PMT can be done by treatment with formaldehyde, glutaraldehyde or heat (37, 74). Treatment with heat or glutaraldehyde resulted in profound reduction of the antigenicity of PMT (37). Treatment with formaldehyde was used for detoxification of commercial AR vaccines. A vaccine prepared by treating native toxin with formaldehyde and adsorbing it on to Alhydrogel adjuant protected pigs against progressive AR. Vaccination of breeding animals twice during pregnancy, with the vaccine provided their progeny with good protection against severe clinical AR caused by combined infection with toxigenic *P. multocida* and *B. bronchiseptica* (8, 37). Monoclonal antibodies were used to evaluate epitopes on PMT that are stable after formaldehyde treatment under different conditions. Two of the MAb's were used in a quantitative immunoassay to monitor vaccine production with formaldehyde detoxified PMT (37). Rats were used as a model for evaluating a potential AR vaccine, containing PMT inactivated by treatment with glutaraldehyde, which provided dose-dependent protection of rats (92).

The protective effects of four commercial vaccines that had different combinations of toxoid from *P. multocida* and bacterins of *B. bronchiseptica* and *P. multocida* were evaluated in a herd with progressive AR (86). This study demonstrated that the mere presence of toxoid in a vaccine does not guarantee protection against

turbinate atrophy, since two bacterin vaccines were as effective as a vaccine containing purified toxoid, and significantly more effective than a vaccine based on crude toxoid. This indicated that the quantity, purity and methods of detoxifying PMT could influence the protection afforded by AR vaccines.

The applicability of PMT protein for vaccine purposes was analysed by a deletion mutagenesis approach, by developing deletion derivatives with impaired toxic potency (90). Pregnant female mice were vaccinated with four purified toxin derivatives lacking different and widely separated regions in the amino acid sequnce, which were characterized by a lack of toxic activity. One non-toxic derivative, which lacked 121 amino acids near the N-terminus induced efficient protection of vaccinated female mice and their offspring against challenge with PMT. This toxin derivative, called the dO protein was further evaluated for its protective efficacy against atrophic rhinitis in swine (80). Piglets from gilts vaccinated with purified dO protein or formalin treated PMT were challenged by dual inoculation with *B. bronchiseptica* followed by toxigenic *P. multocida*. Both vaccines showed efficient protective effect against the clinical, pathological and production limiting effects of progressive AR.

Protective immunity to PMT was developed in rabbits after intranasal immunization with heat-inactivated PMT and the protective response was enhanced by co-administration of cholera toxin (108). A commercial swine vaccine was used for immunization of rabbits to test whether it could produce a similar protective immune response. A commercial swine *P. multocida* bacterin toxoid given by intramuscular inoculation protected rabbits against challenges from PMT (109).

The ability of PMT or crude cell-free preparations of toxigenic *P. multocida* to induce turbinate atrophy and bone resorbing effects were investigated in many studies (1, 14, 30, 34, 114). Intranasal inoculation of newly born piglets with *B. bronchiseptica* induced a transient atrophy of the nasal turbinates, but the severe lesions observed in naturally occuring atrophic rhinitis have not been reproduced experimentally with *B. bronchiseptica* alone (97). Three doses of PMT applied intranasally to three-week-old gnotobiotic piglets resulted in severe turbinate atrophy (29). Pathological changes included degeneration and necrosis of osteoblasts, markedly accelerated osteoclastic osteolysis, replacement of osseous core by a highly cellular mesenchymal stroma and multifocal atrophy of sub mucosal glands. The lack of mucosal inflammation as well as these pathological changes was consistent with earlier descriptions of the histological findings after intra nasal inoculations with broth cultures of toxigenic *P. multocida* in piglets pre-treated with intranasal instillation of acetic acid (33, 83).

Histomorphometric and ultrastructural studies on turbinate osteoporosis in pigs following intranasal inoculation of purified PMT was well-studied (30). Inhibition of bone formation and transient stimulation of osteolysis were reported as major mechanisms. Also, a direct toxic effect of PMT on bone forming cells caused uncoupling of bone formation and bone resorption and contributed significantly to reduced bone volume with time. It was speculated that the PMT- affected osteoblasts, by regulation either through secretion of collagenase promoters or through paracrine signalling, could be the cause of indirect transient stimulation of osteoclastic osteolysis. The study

concluded that PMT stimulated osteoclastic osteolysis and inhibited osteogenesis in turbinates by causing degeneration and death of osteoblasts (30).

In SPF piglets, intranasal instillation of 0.5μg of PMT twice a day for one week resulted in severe unilateral turbinate atrophy (35). The right side turbinates appeared normal compared to untreated controls. The osteolytic activity was further studied in rats by repeated intranasal instillation of PMT, eight times on the same day. The macroscopic examination of the cut surfaces of the nose revealed a slightly larger nasal cavity, and histlogically, the turbinates and parietal bone structures were reduced in size compared with the controls.

An in vitro bone resorption model was used to search for pathogenic mechanisms in AR. PMT had a biphasic effect in this model, low doses slightly stimulated bone resorption whereas high doses were inhibitory (58). Long bone growth prior to puberty depends on chondrocyte proliferation in the physes and the articular epiphyseal complex. Multiple injections of PMT in rats altered physeal chondrocyte proliferation. The change was associated with serum elevations of IL-6 (2). PMT inhibited endochondral bone formation in the proximal portion of the humerus of young pigs by reducing physeal area and chondrocyte proliferation. Hyperimmune serum against PMT neutralized the effect of toxin on weight gain, clinical appearance, physeal area and chondrocyte proliferation (3). The effect of PMT on porcine osteoclast and osteoblast differentiation was studied using in vitro cell culture systems to determine its role in AR (42). The findings demonstrated that PMT exerted its effect through alterations in both osteoblastic and osteoclastic cell populations. Osteoblast necrosis and reduced mineralization, coupled

with increased osteoclast numbers could have contributed to the decreased amount of bone seen in turbinates of pigs with AR.

The effects of PMT after parenteral application in piglets and rats were further studied (68, 69, 100). Intramuscular injection of PMT in piglets caused severe atrophy of conchae and also produced lesions in cartilage and bones. There was total disappearance of cartilage and woven bone as well as disappearance of most lamellar bone. It was concluded that the macroscopic conchae atrophy resulted from histological alterations and subsequent loss of both cartilage and bone (69).

The effect of PMT on the histological, cytochemical and ultrastructural features of the osteoclast population of rats suggested that PMT is able to stimulate osteoclastic bone resorption by increasing osteoclast resorption and by increasing the number of multinucleated osteoclasts. This action was not limited to the osteoclast population of the nasal turbinates, but occurred in the other bones such as long bones (3, 35). The effect of purified PMT on osteoclast formation from hemopoietic progenitor cells was studied using an in vitro system and it was demonstrated that PMT promotes differentiation of osteoclast-like cells from proliferating mouse bone marrow cells (68). A study on the effect of purified toxin on the osteoblastic phenotype of the ROS 17/2.8 rat osteosarcoma cell line postulated that the PMT action upon bone extends to both osteoblastic and osteoclastic cell components. Nanomolar doses of toxin which produced cytopathic changes in EBL cells and in a non-osteoblastic rat osteosarcoma cell line had no cytopathic action in ROS 17/2.8, but caused reduced expression of an osteoblastic

marker, alkaline phosphatase (107). Inhibition of the differentiated cell function of the osteoblast may contribute to the pathologic changes observed in nasal bones.

Parenteral administration of toxin resulted in lesions of various organs including liver, kidney, urinay tract and spleen in pigs (103). Histopathologically, the lesions were extensive and divided into five categories; degenerative, hyperplastic, obstructive, reactive and osteolytic. Changes in the ventral turbinates consisted of epithelial hyperplasia, atrophy of mucosal glands, osteolysis and proliferation of mesenchymal cells. In liver, cell necrobiosis, accumulation of bile pigment, haemopoiesis and hyperplasia of hepatocytes were observed. In the kidneys, hydronephrosis and degeneration of renal tubules were associated with pronounced hyperplasia of the transitional epithelium of ureters and bladder. The pathogenesis of PMT-induced hepatopathy was proposed to be due to interference with hepatocellular metabolism resulting in hepatocyte degeneration and death (32). Six piglets were given 0.1μg of toxin subcutaneously and necropsied at two-hour, 24 hour and 48 hour after toxin administration. The lesions in livers at 24 hour after toxin injection were striking and severe with marked hepatic edema, diffuse swelling and enlargement of Kupffer cells, diffuse endothelial swelling and necrosis with dilation of sinusoids, diffuse degeneration of hepatocytes and random foci of necrosis on liver surface. These lesions indicated that PMT causes hepatic necrosis associated with widespread vascular injury and vacuolar degeneration of hepatocytes (18, 19).

Growth retardation has been documented in pigs given PMT, in low doses changes were quite subtle and detected clinically only by a decrease in rate of weight

gain (7). PMT when given subcutaneously to rats, caused severe liver damage and growth suppression in doses as low as 15.6 ng and was lethal above 31.25 ng. The liver necrosis might have resulted from the effects of vascular injury superimposed upon a direct toxic effect on parenchymal cell metabolism (19). Spleen atrophy was the prominent lesion in mice that survived the acute toxin challenge, spleens were small in size, anemic and reduced to approximately 25% of normal weights (74). Rabbits were inoculated intranasally with purified PMT to study the role of toxin in induction of pneumonia in rabbits infected with *P. multocida*. Pneumonia, pleuritis, acute hepatic necrosis and splenic lymphoid atrophy were observed in those rabbits and one of the five rabbits had bilateral turbinate atrophy (20).

The purified toxin from *P. multocida* is lethal for a wide variety of animal species. The lethal effect has been determined for swine, mice, rats, rabbits, guinea pigs and turkeys (35, 38, 43, 148, 149). The mouse lethality test was widely used to estimate potency of toxin preparations. Four inbred strains and one outbred strain of mice were tested for PMT susceptibility and the BALB/c mice were up to six times more sensitive than others (98). The estimated LD50 for mice ranges from 0.5μg/kg. to 15 μg/kg. The lethal dose for swine was between 0.1 and 0.8μ g when given intradermally (43). Intraperitoneal injection of 500μg of toxin killed piglets within 96 hours and at necropsy, acute liver toxicity and severe jaundice were seen. Piglets given 250μg survived but became lethargic and anorexic for three days after injection and some developed jaundice and edema of the eyelids (100, 103). Six-week-old rats were given PMT in a dose range from 2μg to 0.0156 μg subcutaneously and toxin was lethal in doses as low as 0.031 μg.

Time of survival in acute toxicity, which was above a dose of 250 μg, was less than 24 hours, the survival time was shortened by increasing doses administered (19).

The guinea pig skin test was initially developed as a screening test for differentiation of toxigenic and non-toxigenic *P. multocida* (23, 24). In later studies, it was used as a biological test for PMT. The minimum dermonecrotic dose of different preparations of PMT had been estimated, using criteria of a positive skin lesion with a diameter of 5 to 10 mm and reading the result in 48-72 hours after intradermal injection (35).

Cell culture assays to distinguish between filtrates of culture supernatants from toxigenic and non-toxigenic *P. multocida* were developed utilizing EBL cells and Vero (African green monkey kidney) cells (87, 102). The characteristic morphologic (cytopathic) changes caused in monolayers of these cell lines by the toxin were made use of in these assays. These tests were used in different studies as a sensitive in vitro test for differentiation of *P. multocida* strains and for characterizing native and recombinant PMT purified by different methods. Neutralizing activity was demonstrated in EBL assay with serum from infected gnotobiotic piglets (103). There was complete correlation in results from these assays with those of mouse lethality tests and dermonecrotic tests in guinea pigs. The EBL cell culture assay was 1000 times more sensitive than a lethal assay in BALB/c mice (102). The cytopathic activity of PMT was tested in other cell lines along with EBL cells and it was found that EBL cells were 100 times more sensitive to PMT than others (102). In another study, similar toxin titers were achieved when EBL cells were used in parallel with Vero cells in the cell culture assay (87). The minimum

cytopathic dose (MCD) of PMT in these cell culture assays was estimated from 10 - 179 pico grams in different studies. The dramatic morphological changes caused in Vero cells was not thought to be due to cytotoxicity or cell death (121).

Bacterial toxins have provided novel approaches to elucidate cellular and molecular regulatory mechanisms including signal transduction and cell proliferation. Several studies were done to elucidate the action of PMT and interaction of toxin with host cell surfaces. Both PMT and rPMT are extremely potent mitogens for murine 3T3 cultured fibroblasts (96). Either PMT or rPMT induced DNA synthesis at picomolar concentrations in the absence of any other exogenously added growth-promoting factor. Early, but not late, addition of the lysosomotrophic agent methylamine selectively blocked the mitogenic action of rPMT. Similarly, minutes after exposure to rPMT, the toxin was accessible to a neutralizing PMT antiserum; thereafter, the mitogenic action of rPMT was no longer accessible to PMT antiserum. Exposure of cells to rPMT at 4^0C prevented the mitogenic response. These lines of evidence suggested that toxin is translocated to an intracellular location via an acidic compartment (96). Measurments were carried out on effect of PMT on two major transmembrane signaling systems, cAMP and inositol phosphate production. These revealed that the toxin did not increase intracellular level of cAMP, but induced a dramatic and time dependent increase in the production of inositol phosphates. The later components are involved in signal transduction mechanisms leading to multiple cellular responses including cell growth. Since none of the previously described bacterial toxins that act intracellularly stimulated this transmembrane signaling system, it was proposed that PMT may provide a novel tool

to identify components of the complex cascade of molecular events involved in the initiation of cell proliferation (96, 96, 105)

Prior to stimulation of DNA synthesis, rPMT enhanced the formation of inositol phosphates (105), increased cellular content of diacyl glycerol, caused the translocation of protein kinase C, and stimulated the phosphorylation of an 80 kDa protein (106) that is a major substrate of protein kinase C (96). The binding of epidermal growth factor (EGF) to its receptor was decreased by rPMT, an action attributable in part to protein kinase C activation (105). It was concluded that rPMT stimulates protein kinase C -dependent and -independent protein phosphorylation in Swiss 3T3 cells (105). The stimulation of these early events by rPMT required cellular entry and activation of the toxin. It was demonstrated that the toxin enters the cell to activate inositol phospholipid breakdown causing release of inositol 1;4,5-triphosphate, calcium ion mobilization from intracellular stores, an increase in the level of diacyl glycerol, translocation of protein kinase C, and phosphorylation of an 80 kDa substrate of protein kinase C (105, 106).

It was reported that rPMT induced a potent and dramatic stimulation of anchorage-independent growth in Rat-1 cells (47). This result indicated that the deregulation of signal transduction pathways by an intracellularly acting bacterial toxin could induce a malignant phenotype. Another study provided ultrastructural and biochemical evidence that PMT is internalized by receptor mediated endocytosis in two toxin sensitive cell lines, Canine osteosarcoma and Vero cells (91). This was the first report describing binding and internalization of PMT in host cells and suggested that PMT interacts with a ganglioside-type receptor on Vero cells and is transported to the

cytosol in endocytic vesicles which do not appear to fuse with lysosomes. Purified toxin was conjugated to 20 nm colloidal gold particles to observe binding and internalization at the ultrastructural level. It was reported that rPMT stimulated tyrosine phosphorylation of cytosolic protein kinase p125FAK and the cytoskeleton associated protein paxillin. Also rPMT induced a striking increase in stress fiber formation and focal adhesion assembly in Swiss 3T3 cells (73, 98).

Several lines of evidence suggest that rPMT exerts at least some of its effects by modulating the activity of the $G_{q/11}$ family of heterotrimeric G proteins. Treatment of Swiss 3T3 cells with low doses of rPMT strongly potentiates inositol phosphate production following stimulation of $G_{q/11}$ -coupled receptors, and PMT -induced phosphatidylinositol hydrolysis in permeabilized cells was blocked by guanosine 5'-O-(-thiodiphosphate) (73, 104). In Xenopus oocytes, rPMT induced calcium-dependent chloride currents were blocked by the injection of specific antisera against Phospho Lipase-Cβ1 or the α sub unit of $G_{q/11}$ and by $G\alpha_q$ antisense RNA, and were dramatically enhanced by overexpression of $G\alpha_q$ (104, 119). In a later study the mechanisms of rPMT mediated Erk activation was compared to that employed by endogenous heterotrimeric G-protein coupled receptors in HEK-293 cells. It was found that rPMT activated the Erk cascade via the stimulation of $G_{q/11}$ family of G proteins. Subsequently, both rPMT and endogenous $G_{q/11}$ -coupled α thrombin receptors induce Ras-dependent Erk activation via protein kinase C-independent transactivation of the Epidermal Growth Factor receptor. It was concluded that rPMT represents a novel bacterial strategy for inducing cell proliferation, by usurping heterotrimeric G protein-regulated mitogenic signals (104).

The effects of PMT on the downstream mitogenic response and cell cycle progression in Swiss 3T3 and Vero cells were further characterized (121). PMT treatment caused dramatic morphological changes in both cell lines, a strong mitogenic response occurred only with Swiss 3T3 cells. The mitogenic response was not sustained as revealed by the cell cycle analysis that after initial mitogenic response to PMT both cell lines subsequently arrested in G1 and became unresponsive to further PMT treatment. This transient mitogenic response was in contrast with previous reports which suggested that rPMT treatment caused a sustained and irreversible mitogenic response in fibroblasts (47, 96). In Vero cells, limited multinucleation, nuclear fragmentation and disruption of cytokinesis were also observed.

In agreement with other intracellularly acting toxins, PMT also might be comprised of individual domains for binding, internalization and catalytic activity. There is not much structural information available. Studies to elucidate different domains yielded contrasting results (117, 120). The N-terminal half of the cytotoxic necrotizing factor type 1 (CNF1) and CNF2 from pathogenic *E. coli* showed homology to the N-terminus of PMT. The catalytic domain of CNF1 is located at the C-terminus and receptor binding activity at the N-terminus. While there is homology between the C-termini of the CNFs and the DNT from *B. bronchiseptica*, such a homology is absent with PMT.

Studies to elucidate the postulated domain structure of PMT had contrasting findings. Significance of the eight cysteine residues in PMT was evaluated using site-directed mutagenesis (117). Mutants with individual substitutions for each of these

residues were constructed and the effects of these on the biological activity of the toxin were determined by cultured-cell assays. It was found that the most C-terminal cysteine residue at position 1165 is essential for biological activity of PMT. Replacement of this cysteine (C1165) with any of three different amino acids completely abolished all cytopathic or mitogenic effect on cultured cells. The mutant toxin was completely non-toxic in piglets (117).

Using the *Xenopus* oocyte system to screen toxic activity, it was observed that specific antibodies against an N-terminal peptide of PMT were able to block the PMT mediated response in *Xenopus* oocytes while specific antibodies to a C-terminal peptide of PMT did not (119). This implied a critical role of the N-terminus in intracellular acivity. A deletion mutational analysis of PMT was conducted by generating a series of N-terminal and C-terminal deletion mutants, expressing the His-tagged PMT fragments in *E. coli* and testing them for intracellular activity in the *Xenopus* oocyte system (120). This study found that the N-terminal fragments containing the first 500 residues elicited response in oocytes, but the C-terminal 780 amino acid fragment did not. The proteins were screened for cytopathic activity on Vero cells and only the full-length toxin caused morphological changes. It was postulated that the intracellular activity domain of PMT is at the N-terminal 500 amino acids and the C-terminus is required for entry into cells (120).

A recent study contradicts this finding, and suggested that the C-terminal portion of the toxin is responsible for its biological effect (10). By analyzing different PMT fragments, it was found that a 704 amino acid C-terminal fragment which was inactive in

intact EBL cells after addition to culture medium, caused reorganization of the actin cytoskeleton and rounding of cells when introduced into the cells by electroporation. Also, this fragment induced an increase in total inositol phosphate levels after introduction into the cell by electroporation. Shorter C-terminal and N-terminal fragments were inactive in both ways. The 704 amino acid C-terminal fragment with a mutation made at the cysteine residue at position 1165 was not toxic after electroporation. This residue was found to be important for biological activity of the toxin, a suggestion which agreed with that of a previous study (117). A domain structure of PMT, with N terminus for uptake of the toxin to the target cells and C-terminus for activity was proposed (10).

Statement of purpose

The major focus of this study was to identify a protective antigenic region of PMT, which can be used as a vaccine candidate for protection against progressive atrophic rhinitis in swine. A single component *P. multocida* toxoid vaccine is sufficient for inducing immunity against experimentally induced progressive AR (39). Many of the currently available vaccines for prevention of AR in pigs contain formaldehyde-treated crude extract and whole-cell suspension of toxigenic *P. multocida*. Formaldehyde treatment or other methods of inactivation may result in various levels of denaturation and cross-linking, that would produce inconsistent levels of immunogenecity of the toxoid (37). A recombinant subunit protein needs no inactivation, as it is atoxic, could facilitate production, and provide a reproducible immunogenic effect.

Apart from a study in 1991, in which recombinant derivatives of PMT were evaluated for efficacy as potential vaccine candidates, no further studies were carried out in this regard (90). That study found a recombinant PMT derivative that lacked a 121 amino acid stretch in the region of the N-terminus to be a potential vaccine candidate. It was also found that another derivative which lacked a C-terminal 155 amino acids was neither protective nor immunogenic and proposed that an important immunogenic region might be located at the C-terminus of PMT.

To identify a protective region of PMT, recombinant truncated proteins encompassing the C-terminal or N-terminal region of PMT were cloned in expression vectors, the recombinant proteins purified and used for mouse immunization studies. The fragments were evaluated for their immunogenicity, antigenicity and protective efficacy using ELISA, PMT neutralization assay in Vero cells, and protection against lethal PMT challenge in mice.

CHAPTER 2

MATERIALS AND METHODS

Materials

Toxigenic *Pasteurella multocida* strain NCTC 12178 was purchased from the National Collection of Type Cultures, UK. *P. multocida tox A* gene sequence from accession number X52478 in Genbank was used for designing primers. Primers were obtained from Biosynthesis (Lewisville, TX). Each primer contained a restriction enzyme site either engineered or from the toxin gene sequence. Easy-DNA kit, TA cloning kit, expression vector pPROEX HT and *E. coli* INVF α competent cells were obtained from Invitrogen (Carlsbad, CA). Expression vector pET 29b(+), HisBind kit and *E.coli* BL21 (DE3) were purchased from Novagen (Madison, WI). Protease inhibitors, peroxidase conjugated antibodies and IPTG were obtained from Sigma. Ni2+-nitrilotriacetic acid (NTA)-agarose, PCR purification kit and Gel extraction kit were obtained from Quiagen (Valencia, CA). Restriction enzymes, LA Taq polymerase, and Ex Taq polymerase were purchased from Panvera (Madison, WI). Fast link DNA ligase was obtained from Epicentre (Madison, WI). Vero cells and Hybridoma cell line CRL-1965 were obtained from the American Type Culture Collection (Manassas, VA).

Construction of Plasmids

Genomic DNA from a toxigenic strain of *P. multocida* (NCTC 12178) was isolated using Easy DNA kit (Invitrogen, Carlsbad, CA) as per the manufacturer's

protocol and used as a template for PCR to generate three C terminal constructs of PMT. The *tox A* gene fragment (3588-4055) encoding the C-terminal 155 amino acids was amplified by PCR using forward primer, PMTECOF -(5'-CGCTTACCTCTTTTAGAGAATTCGG-3') and reverse primer, PMTXHR (5'-GGCTCGAGTAGTGCTCTTGTTAAGCG-3'). The PCR product was digested with EcoRI and XhoI, gel purified using Gel Extraction Kit (Quiagen, Valencia, CA) and cloned into EcoRI and XhoI sites of pET 29(b+) (Novagen Inc., Madison, WI) vector. The resultant plasmid is pET-pmt-cex.

To clone the region of *tox A* gene encoding the C-terminal 302 amino acids (base pairs 3148-4055), the *tox A* gene was amplified by PCR using forward primer, PMTHINDF (5' GGATAAAGGAGATATTAATGGTGCTT-3') and reverse primer PMTXHR. The PCR product was digested with HindIII and XhoI, gel purified and cloned into Hind III and XhoI sites of pET 29b+. The resultant plasmid is called pET-pmt-ch.

To construct the third C-terminal construct, *tox A* gene fragment (1999-4055) encoding 685 amino acid was amplified by PCR using the forward primer PMTECOVF (5' – GGGGATATCCGTTATTGGAAAGCCTATTGG – 3'). And reverse primer, PMTXHR. The PCR product was digested with EcoRV and XhoI, gel purified and cloned into respective sites of pET 29b+. The resultant plasmid is called pET-pmt-ce.

LA taq polymerase (PanVera, Madison, WI) was used to clone the full length *Tox A* gene by PCR using forward primer, PMTBAMHF (5'-GGGGATCCGCCCCTTGACCTAGAGGG- 3') and reverse primer PMTBAMHR (5' –

GGGGATCCGTTATAGTGCTCTTGTTAAGCG- 3') using genomic DNA of *P. multocida* (NCTC 12178) as template. The resulting PCR product contained basepairs 42 to 4060 of published PMT sequence (Genbank Accession NO X52478). The PCR product was gel purified and cloned into the TA cloning site of PCR2.1vector (Invitrogen, Carlsbad, CA). The resultant plasmid is called pmt-TA2. This construct was utilized to clone the PMT coding sequence in an expression vector. Forward primer PMTNT, (5' – GGGGAATTCTATGAAAACAAAACATTT- 3'), and reverse primer PMTXHR was used to amplify the full length *tox A* gene by PCR using LA Taq polymerase (PanVera, Madison, WI), and pmt-TA2 as template. To clone the PMT gene in frame in the pET vector, the PCR product was digested with EcoRI, the resulting 3.5-kb fragment was gel purified and cloned into plasmid pET-pmt-cex that had been digested with EcoRI. Orientation of the insert was verified by restriction digestion and agarose gel electrophoresis. The resultant plasmid, which expresses recombinant PMT, is called pET-pmt-w.

The 3.5kb EcoRI restriction fragment used for cloning of PMT in the expression vector was cloned into the EcoRI site of expression vector pET 29(b+) to obtain plasmid pET-pmt-ne, which encodes 1130 amino acids of PMT (base pairs 199- 3588) from the N-terminus. To clone the *tox A* gene encoding 482 amino acids from the N-terminus (base pairs 199-1645), the region was amplified by PCR using forward primer PMTNT and reverse primer PMTX3 (5' – AAGACGTACCAGACGAATTTTATG - 3'). The PCR product was digested with EcoRI and XhoI, gel purified and cloned into EcoRI and XhoI, sites of vector pPROEX- HTc to generate plasmid pPROEX-pmt-nex. Correct, in-

frame insertion of sequences was confirmed by DNA sequencing. The sequencing was done at the University of Tennessee DNA sequencing facility on an Applied Biosystems 373 automated sequencer.

Expression of Recombinant PMT Proteins

Recombinant proteins were expressed in *E. coli* BL21(DE3). Seed flasks containing 50 ml of LB broth with the appropriate antibiotic, kanamycin (30μg/ml) or ampicillin (100μg/ml), were inoculated with the bacterial colonies and incubated at 37°C, with rotary shaking (220 rpm) overnight. Two litre flasks containing 500 ml of LB broth with kanamycin(30 μg /ml) or ampicillin(100 μg /ml) were inoculated with 10 ml of the overnight seed culture and incubated with vigorous shaking (220 rpm) at 37 °C until an optical density at 600 nm (A600) of 0.5 to 0.8 was reached. Expression of the recombinant proteins was induced by addition of isopropyl-b-D-thiogalactopyranoside (IPTG; Sigma, St. Louis, MO) to a final concentration of 1 mM for constructs in pET vectors and 0.4 mM for the construct in pPROEXHTc, cultures were allowed to grow for 3 hours. Cells were harvested by centrifugation at 5000 x g for 10 min at 4°C in a Sorvall RC-5C centrifuge (DuPont, New Town, CT). The pellet was used immediately for purification or stored at –80 °C until further use.

Purification of Recombinant PMT Proteins

Recombinant proteins PMT-CEX, PMT-CH and PMT-NEX were purified under denaturing conditions using Ni-NTA resin (Qiagen, Valencia, CA) according to the

manufacturer's protocol. Recombinant PMT, PMT-CE and PMT-NE were purified under native conditions using His-Bind kit (Novagen Inc., Madison, WI), with modifications to manufacturer's protocol as described below.

Purification of PMT-CEX, PMT-CH and PMT-NEX

Starting culture volume used for induction of these proteins was 500 ml. The cell pellet, which was kept at –70 °C, was thawed for 15-30 minutes on ice and resuspended in Buffer B (100mM sodium phosphate monobasic (NaH2PO4), 100 mM Tris-hydrochloride, 8 M urea, pH 8) at 5 ml per gram wet weight. The suspension was incubated at room temperature for 30-60 minutes with gentle shaking until the solution became translucent. The lysate was centrifuged at 10,000 x G for 30 minutes at room temperature in a Sorval RC-5C centrifuge. Two ml of the 50% Ni-NTA slurry was added to the supernatant and mixed gently by shaking for one hour at room temperature. The resin-lysate mixture was loaded into an empty column (Poly - Prep chromatography columns, 0.8 x 4 cm; Bio-Rad, Hercules, CA) with the bottom cap attached. Bottom cap was removed and the flow through collected. The column was washed with buffer C (100 mM sodium phosphate monobasic, 10 mM Tris-hydrochloride, 8 M urea, pH adjusted to 6.3 using hydrochloric acid) until the absorbance at 280 nm (OD 280) of the wash fractions came down to less than 0.100. The recombinant protein was eluted 4 times with one ml of buffer D (100 mM sodium phosphate monobasic, 10 mM Tris-CL, 8 M urea, pH adjusted to 5.9 using hydrochloric acid), followed by two times with one ml of buffer E (100 mM sodium phosphate monobasic, 10 mM Tris-hydrochloride, 8 M urea, pH

adjusted to 4.5 using hydrochloric acid). All the lysates and the fractions were analyzed by SDS-PAGE. The eluted fractions were pooled and dialyzed against three changes of PBS over night. The samples were concentrated using SlideALyzer (PIERCE, Rockford, IL), by dipping the protein samples in dialysis tubing into the concentrating solution for one hour. Protein concentrations were estimated using the Bicinchonic Acid Protein Assay Kit (Pierce, Rockford, IL) with bovine serum albumin as standard and the proteins were stored at –80 °C until further use.

Purification of PMT-CE, PMT-NE and rPMT.

The starting culture volume for induction of these proteins was 1.5 L. The cell pellet, stored at -70 °C after induction, was thawed on ice. BugBuster protein extraction reagent (Novagen Inc., Madison, WI) was used for cell lysis. The cell pellet was resuspended in 50 ml BugBuster reagent by pipetting and gentle shaking (5 ml BugBuster reagent per gram wet weight of the cell pellet). Protease inhibitors were added to the mixture at this time, 250 μg each of Pepstain, Leupeptin and Aprotinin, 2 mM Benzamidine, 2 mM Phenylmethylsulfonyl flouride (Sigma, St. Louis, WI). After incubating for 30 minutes, the suspension was centrifuged at 20,000 x G for 30 minutes at 4 °C in a Sorval RC5C centrifuge and the supernatant was saved for further purification. HisBind kit (Novagen Inc., Madison, W) was used for protein purification. Two ml of the HisBind resin was transferred into a column (BioRad, Hercules, CA), and equilibrated by sequential washing with 3 volumes of sterile deionized water, 5 volumes of 1X charge buffer and 3 volumes of 1X binding buffer. The charged resin was then

added to the supernatant and incubated in ice with gentle shaking for one hour. The mixture was then centrifuged at 500 x G for ten minutes in an Eppendorf Centrifuge at 4°C to sediment the resin. Twenty five ml of 1 X binding buffer was added to the resin and incubetd in ice for 30 minutes with gentle shaking. The mixture was again centrifuged at 500 x G for 10 minutes to sediment the resin. The resin was then transferred to the column and the column was washed with 20 ml of 1 X wash buffer (60 mM imidazole). The bound protein was eluted with 1 ml elution buffer using an imidazole gradient with imidazole concentrations of 80mM, 100mM, 150mM, 200mM and 400mM. The eluted protein fractions were pooled, dialyzed using SnakeSkin (Pierce, Rockford, IL) dialysis tubing, 10,000 MWCO, against three changes of PBS overnight and concentrated using Centricon concentrators (Millipore, Bedford, MA). Protein concentrations were estimated using the Bicinchonic Acid Protein Assay Kit (Pierce, Rockford, IL) with bovine serum albumin as standard and the proteins were stored at –80 °C until further use.

SDS-PAGE and Immunoblots

All cell fractions and protein preparations were analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) using a Mini Protean gel apparatus (Bio-Rad, Hercules, CA). PAGEr Gold precast gels (BMA, Rockland, ME), 8 % or 10 % were used for running the protein samples. Protein preparations were adjusted to the desired concentration in PBS and 20 µl samples were mixed with equal volumes of 2 X sample buffer (4% SDS, 20% glycerol, 10 % 2-mercaptoethanol, 0.004%

bromphenol blue and 0.125 Tris-HCl, pH approximately 6.8;Sigma, St. Louis, MO), incubated in a boiling water bath for five minutes, and loaded into the ready-gel wells. The samples were then electrophoresed for approximately 90 minutes at 150 volts. The gel was removed from the gel casette, washed in deionized water three times for 5 minutes each and then stained with Bio-Safe Coomassie (Bio-Rad, Hercules, CA). After 6-24 hours of staining, the gel was placed in deionized water for an hour, and visualized. A molecular weight marker, Kaleidoscope prestained standard (Bio-Rad, Hercules, CA) was also run along with the protein samples for estimating the approximate molecular weight of the proteins.

Western Blotting

The gels after SDS-PAGE were subsequently soaked in transfer buffer (0.025 M Tris, 0.192 M Glycine; Tris/Glycine buffer; ICN Biomedicals, Aurora, Ohio) and the proteins within the gels were then transferred to nitrocellulose membranes (Immobilon - NC nitrocellulose transfer membrane; Millipore, Bedford, MA) using a Mini-Protean apparatus (Bio-Rad, Hercules, CA) at 50 volt for 90 minutes. After transfer, the membrane was washed three times for 5 minutes each with PBS- 0.05 % polyoxyethylene sorbitan monolaurate (Tween-20; Sigma, St. Louis, MO). The membrane was then incubated in 4 % non-fat dry milk in PBS- 0.05 % Tween-20 (PBS-T) for 30 minutes. The membrane was then washed with PBS-T 3 times for 5 minutes each and treated with the primary antibody, CRL 1965 MAb (1:1000) or rabbit polyclonal antibody (1:100) diluted in PBS-T. The membrane was incubated for one hour, washed 3 times for 5 minutes each with PBS-T and then treated for one hour with secondary antibody, either

peroxidase conjugated anti mouse IgG or anti rabbit IgG (Sigma.) diluted 1:2000 in PBS-T. The membrane was then washed three times with PBS-T, and developed by the addition of substrate solution (one tablet of 4-Chloro-1-Naphthol (Sigma) dissolved in 10 ml methanol and 40 ml Triethanolamine buffer and 20 µl Hydrogen Peroxide). After 10 minutes, the membrane was washed with deionized water and visualized. All the incubations and washes were done at room temperature on a rocking platform.

Dot Blot

A dot blot was done using a Convertible Filtration Manifold System (Life Technologies, Inc., Gaithersburg, MD) which facilitates transfer of protein solutions onto nitrocellulose membranes using a vacuum source. The protein samples were directly adsorbed onto nitrocellulose membrane using the apparatus. The membrane was then used for dot immunoblotting by the same procedure described for Western blotting.

Production of Monoclonal Antibodies

ATCC cell line CRL-1965 was used for production of monoclonal antibody against PMT. The hybridoma cells were maintained at 37 °C and 10% carbon dioxide in DMEM with 4.5g/L glucose, horse serum 5%; fetal bovine serum 2.5% with fluid renewal in every 2-3 days. The MAb was purified from hybridoma supernatant and also by the production of ascitic fluid in mice.

Production of Ascites in Mice and Purification of MAb from Ascitic Fluid

Four to six week-old female BALB/c mice (Harlan laboratories, Indianapolis, Indiana) were injected with 0.2 ml of 2,6,10,14-tetramethyl decanoic acid (Pristane: Sigma, St. Louis, MO) intraperitoneally on day 0. On day 14, mice were injected with hybridoma cells at a concentration of 5 x 10^6 cells/ ml in 0.5ml 1x PBS, pH 7.4. Within 7-10 days, ascitic fluid developed and was collected by peritoneal puncture using an 18 gauge needle, while animals were anesthetized with Isoflurane (IsoFlo; Abbott laboratories, North Chicago, IL). The collected ascitic fluid was then pooled, centrifuged in a micro centrifuge (Eppendorf, Westbury, NY) at 3000 x G for 10 minutes and the supernatant used for antibody purification using the Immunopure Immobilized Protein A/G Gel (PIERCE, Rockford, IL). The ascitic fluid was diluted 1:1 with Immunopure IgG binding buffer (PIERCE, Rockford, IL), 2 ml of the sample was applied to the protein A/G column pre-equilibrated with the binding buffer and allowed to flow through. The column was washed with 10-15 volumes of binding buffer, and the bound IgG was eluted in one ml fractions with 0.1 M glycine buffer, pH 2.5. The eluted fraction was adjusted immediately to neutral pH with 1M Tris base (Sigma), pH 9.5. The eluted fractions were monitored with measuring absorbance at 280 nm. All fractions with an absorbance (A280) greater than 0.1 were pooled and dialyzed at 4 °C against three changes of 1x PBS in a 2 L flask. The protein concentration was determined by colorimetric analysis using the BCA assay (Pierce, Rockford, IL). Purified antibodies were aliquoted and stored at -80 °C.

Purification of CRL-1965 MAb from Hybridoma Supernatant

The hybridoma cells maintained as described above were seeded into 75cm^2 tissue culture flasks and allowed to reach confluence in two days. In every two days, medium was removed, centrifuged, and supernatant stored at -20 °C. The flasks were reinoculated with one fourth of the hybridoma cells and replenished with medium. Purification was done from one liter of such collected medium. Before purification, the supernatant was centrifuged at 3000 x g for 30 minutes in a Sorval RC-5C to remove any sediment and then filtered with a 0.2 μm filter (Millipore). The supernatant was transferred to a 2-liter flask; a stir bar was placed in the flask and kept on a magnetic stirrer. An equal volume of saturated ammonium sulfate (Fisher, Fair Lawn, NJ) solution was slowly added to the supernatant while being stirred to bring a final concentration of 50 % saturation. The flask was then transferred to 4 °C overnight. The mixture was centrifuged at 3000 x g for 30 minutes and the supernatant discarded. The pellet was drained well, and resuspended in 50 ml PBS. The solution was transferred to dialysis tubing (Pierce) and dialyzed versus three changes of PBS overnight. The solution was removed from the tubing and centrifuged to remove particles. The clarified solution was used for antibody purification using the Immunopure Immobilized Protein A/G Gel (PIERCE, Rockford, IL), as described above.

Production of Anti-PMT Rabbit Polyclonal Serum

One 6 week-old New Zealand White rabbit (Harlan Laoratories, Indianapolis, Indiana) was given 200 μl doses containing 100 μg of heat inactivated rPMT, intramuscularly, on

days 1, 14, 28 and 42. Each dose was administered with equal volumes of Incomplete Freund's Adjuant (IFA; Sigma, St. Louis, MO). On day 51, the rabbit was bleed, serum separated and stored at –70 °C until further use.

Cell Culture

Cultured Vero cells were maintained at 37 °C and 10% CO2 in Dulbecco Modified Eagle Medium (DMEM; GibcoBRL, Grand Island, NY), pH 7.4 supplemented with10% heat inactivated fetal bovine serum and 1% Penicillin Streptomycin Fungizone Mixture (BioWhittaker, Walkersville, MD)

Characterization of rPMT

Determination of the minimal cytopathic dose of rPMT on Vero cells (MCD).

After trypsinization, vero cells were plated onto 96 well plates (Becton Dickinson Labware, Franklin Lakes, NJ) at a density of approximately 12,000 - 15,000 cells per well in 150 µl DMEM medium containing 10 % heat inactivated FBS and 1% Penicillin Streptomycin Fungizone mixture and allowed to grow to confluence. The medium was changed to low serum medium, DMEM-1% FBS containing antibiotics. One day after quiescence with low serum media, the medium was replaced with filter sterilized low serum medium containing serial two-fold dilutions of rPMT in duplicate wells. Heat inactivated rPMT, prepared by heating the sample at 70 °C for 45 minutes, was used as the control. The cells were allowed to grow at 37 °C and 10 % CO2 and observed for morphological changes every 24 hours until the 5th day.

Determination of LD50

Ten to twelve week old BALB/c mice (Harlan Laboratories, Indianapolis, Indiana) were divided into groups of six. Animals were given 100 µl doses, by intraperitoneal injection, of different concentrations of rPMT. All animals in each group received 1000 ng, 500 ng, 250 ng, 125 ng, 62.5 ng, 31.25 ng, 15.625 ng, 7.812 ng or 3.3.9 ng. One group was given 100 ul of sterile PBS, intraperitoneally. Mice were observed for seven days and lethality was recorded. The 50 % lethal dose (LD 50) was determined using the Reed and Muench formula (70). Necropsy was performed on mice that died and gross anatomic lesions were recorded. Two mice from each of the groups that survived were euthanized and examined for lesions. Liver, spleen and kidneys were collected from all necropsied mice and fixed in 10% formalin for histopathology.

Mice Immunizations

Four to six week-old female BALB/c mice (Harlan Laboratories) were maintained by routine procedures in an AALAC accredited laboratory animal facility. Groups of 5-7 mice were immunized with recombinant proteins by intraperitoneal injection. Each animal received three, 100 µl doses, containing 50 µg of the proteins. The proteins were given with an equal volume of Freund's incomplete adjuant (IFA). Booster injections were given on day 14 and day 35. A control group received 3 injections of IFA + PBS. On the 45th day, mice were bled and serum was collected for further analysis.

Evaluation of Antibody Responses

ELISA

In brief, 96 well plates (Immulon 2HB, Dynex) were coated with recombinant PMT (5 μg/ml in PBS) and kept at 4 °C overnight. The plates were washed four times with PBS-0.05% Tween 20(PBS-T) and blocked for 30 minutes at 37 °C in PBS-T. The plates were incubated for 2 hours at 37 °C with two fold serial dilutions of serum from immunized mice. All serum dilutions were made in PBS-T. Plates were washed four times in PBS-T, incubated with secondary antibody (peroxidase conjugated rabbit anti mouse IgG) for one hour at 37 °C, washed three times in PBS-T and once in PBS, and developed by adding 100 μl of substrate solution containing 2,2' –azino di-ethylbenzothiazoline-6-sulfonic acid (ABTS, Sigma) and hydrogen peroxide. The A405 was measured in an Elx800 Universal Microplate Reader (BIO-TEK Instruments Inc., Winooski, VT). The titers were determined as the reciprocal of the highest dilution of serum that gave an absorbance reading three times the conjugate control

Neutralization assay

The ability of mouse serum to neutralize the effect of PMT on vero cells was analyzed using a serum neutralization assay. Cells were maintained at 37 °C and 10% CO2 in Dulbecco modified Eagle medium (DMEM; Gibco/BRL), pH 7.4 supplemented with 10% heat inactivated fetal bovine serum and 1% penicillin-streptomycin-fungizone mixture. After trypsinization, Vero cells were plated onto 96 well plates at a density of approximately 12,000 - 15,000 cells per well in 150 μl medium and allowed to grow to

confluence. The medium was changed to DMEM-1% FBS containing antibiotics and allowed to reach quiescence with low serum medium for one day. The mouse serum was heat inactivated at 56 °C for 30 minutes prior to the assay. The first dilution of mouse serum was 1:5 with low serum medium; it was sterilized by passing through a .45μ filter and then serial two fold dilutions of 150 μl each were made. Recombinant PMT (250 μg/ml) was diluted to 1:1000 in low serum medium (10 times MCD for Vero cells), filter sterilized, 150 μl was added to each diluted serum and incubated at room temperature for 30 minutes. The medium from the the 96-well plates was removed and 150 μl of the toxin serum mixture in low serum medium was added in duplicate to the Vero cells in 96 well plates. The plates were incubated for up to 3 days and observations were recorded every 24 hours.

Evaluation of Protective Efficacy

A mouse lethal challenge model was used to determine protective efficacy. Groups of four to six week old BALB/c mice were immunized with proteins, PMT-CE, PMT-CEX, PMT-NE and heat inactivated rPMT. Fifty micrograms of each protein in 100μl doses, mixed with equal volumes of incomplete Freund's adjuant, were injected intraperitoneally on days 1, 14 and 35. One control group received 100 μl of IFA + PBS. On day 45 the mice were bled, and the next day they all received 1 μg of rPMT (5 LD 50) diluted in 100 μl of sterile PBS by intraperitoneal injection. The number of mice that died in each group within seven days after challenge was registered. Mice surviving on day 7

were euthanized. Necropsy examinations were performed on all mice and tissues were fixed and saved for histopathology.

Cell-Binding Assay

Vero cells maintained as described above were trypsinized, cells were pelleted by centrifugation and washed four times with 1x PBS. Approximately 10^5 cells were added to each well of a 24-well plate. The cells were then incubated by rocking for one hour on ice with protein solutions in one ml blocking buffer (PBS and 4% non fat milk), to reduce nonspecific binding. After incubation, cells were washed four times in one ml PBS, incubated with primary antibody, Mab CRL-1965, 1: 500 in PBS, rocking on ice for one hour. The cells were then washed four times with cold PBS and incubated with FITC conjugated anti-mouse IgG, 1:1000 in PBS, on ice with rocking. After one hour, the cells were washed with PBS four times and resuspended in 0.5 ml PBS. The cell suspension was analyzed in a FACSVantage (Becton & Dickinson, Franklin Lakes, NJ) equipped with a 15 mW air-cooled argon laser set at an excitation wavelength of 488 nm and recorded with a 530-nm emission filter. A total of 10,000 events were acquired for each sample. The mean flourescence intensity for each sample was determined by using CELLQUEST analysis software. The assay was repeated with anti-PMT polyclonal rabbit serum as primary antibody and FITC conjugated anti-rabbit IgG as secondary antibody.

In another method, each of the recombinant proteins was diluted in low serum medium, filter sterilized and added to Vero cells maintained in low serum medium at

37°C in a 12 well plate. The plate was incubated for two hours at 37 °C with the protein fragments. After two hours, the medium was removed and low serum medium containing rPMT (5 times MCD) was added to the cells and incubated at 37°C. The morphological changes in Vero cells were observed for three days.

CHAPTER 3

RESULTS

Construction of Plasmids

The plasmids used and constructed in this study are shown in Table-1 (All tables and figures are in Appendix). The primers designed and used in this study are shown in Table-2. In order to identify a protective subunit region of PMT, three C-terminal and two N-terminal constructs of PMT were cloned into expression vectors, pET 29b(+) (Figure 1) and pPROEX HTc (Figure 2). The restriction enzyme sites in the PMT sequence that were utilized for cloning of the fragments and restriction mapping are shown in figure-3. The three C-terminal constructs were amplified from genomic DNA isolated from *P. multocida* NCTC 12178 by PCR using designed primer pairs. The forward primers used for pET-pmt-cex and pET-pmt-ch utilized the available restriction enzyme recognition sites in the PMT sequence. The forward primer for pET-pmt-ce had an engineered EcoRV restriction enzyme site. The same reverse primer was used for amplifying the three constructs; the reverse primer PMTXHR had an engineered XhoI restriction enzyme recognition site. The three C-terminal constructs contain either 685, 302 or 155 amino acids from the C-terminus. A cysteine residue at position 1165, which was proposed to be essential for biological activity of the toxin (117), was part of all these fragments.

The full-length *tox A* gene with its regulatory and coding sequences was amplified from genomic DNA by PCR using LA taq polymerase and cloned into TA cloning vector to get plasmid pmt-TA2. The LA Taq DNA polymerase is suitable for amplification of long DNA templates with high fidelity. Restriction digestion mapping of pmt-TA2 plasmid DNA is shown in figure 4. A full-length recombinant PMT toxin was cloned into expression vector pET29b(+). First, the coding sequence was amplified using a forward primer with an engineered EcoRI restriction enzyme site and PMTXHR reverse primer with pmt-TA2 as template. The PCR product was digested with EcoRI so as to cut at the N-terminus and the other available site at base pair 3588 near the C-terminus. The digested product was used for cloning the recombinant toxin by ligating to plasmid pET-pmt-cex, digested with EcoRI. The orientation of the insert was verified by restriction digestion mapping. Also, the same digested product was cloned into pET 29b(+) to make the plasmid, pET-pmt-ne. This large, N-terminal construct (pET-pmt-ne) is the whole toxin minus the 155 amino acids from the C-terminus. The second N-terminal construct (pPROEX-pmt-nex) contains 482 amino acids from the N-terminus.

The expression vector pET29b(+) and pPRoEXHTc incorporated a His tag affinity handle at the C-terminus of the proteins and allowed the expression of recombinant proteins under IPTG inducible promoters in *E. coli*. The His tag facilitated one step purification of the protein by affinity chromatography. Since it was previously shown that a C-terminal His tag did not influence the activity of the toxin, the C-terminal His tag was not removed from these proteins (120).

Expression of Recombinant PMT Proteins

Each of the plasmids in expression vectors and the names of proteins expressed by them, with their approximate size, are given in Table-3. A schematic representation of the position of the constructs in relation to full length PMT is shown in Figure. 5. Each of the plasmids expressed recombinant proteins under IPTG-inducible promoters in *E. coli* BL21- (DE-3) after IPTG induction as determined by SDS-PAGE and Western blots. When uninduced and IPTG-induced cultures were run on SDS-PAGE, the induced band was visible after Coomassie staining only in proteins PMT-CEX, PMT-CH and PMT-NEX. In all others, the level of expression was not sufficient to be detectable in SDS-PAGE gels. Western blot or Dot blot of induced cultures with MAb CRL-1965, or a rabbit polyclonal antibody against a C-terminal peptide (kindly provided by Dr. Brenda Wilson) was done to detect the protein expression.

The proteins PMT-CEX, PMT-CH and PMT-NEX were isolated from insoluble cell lysate fractions and required purification under denaturing conditions. Upon dialysis, these proteins formed precipitates. PMT-CE, PMT-NE and rPMT were purified under native conditions. The yields of these proteins purified under native conditions were quite low compared to those purified under denaturing conditions. Approximate yield of each protein from one liter of initial starting volume of LB broth after induction and purification were, 3.5 mg for PMT-CEX, 2 mg for PMT-CH, 1.5 mg for PMT-NEX, 700 μg for rPMT and PMT-CE and 500 μg for PMT-NE. Purified recombinant proteins migrated to their predicted molecular weights in SDS-PAGE gels (Figure 6). Monoclonal antibody CRL1965 reacted only with rPMT and PMT-CE on western blots and dot blots

(Figure 7&8). All the recombinant proteins reacted with rabbit polyclonal antibody on western blots and dot blots (figure 9).

Production of Monoclonal Antibody CRL 1965

Monoclonal antibody was purified from both ascites fluid and hybridoma supernatant. Approximately one milligram of antibody was obtained from one ml of ascites fluid by purification using the Immunopure Protein A/G columns. The hybridoma supernatant yielded very low quantities of antibodies when purified using the same system after ammonium sulfate precipitation. Approximately two milligrams of antibody were purified from one liter of hybridoma supernatant. This MAb was non-neutralizing on the Vero cell neutralization assay.

Production of Anti PMT Rabbit Polyclonal Serum

Heat inactivated rPMT was used as the antigen. The rabbit serum collected on 51st day was ammonium sulfate precipitated and used for further assays. The serum had weak reactivity with PMT on ELISA and neutralization assays. The anti-PMT IgG titer as measured by ELISA was less than 500. The neutralization titer was 20.

Characterization of rPMT

The minimal cytopathic dose (MCD) of rPMT was recorded as the minimal amount of rPMT, which evoked a cytopathic effect on confluent Vero cells in a 96 well tissue culture plate after three days of incubation at 37°C and 10% CO2 with a low serum

media. A dose as low as 70 pg produced morphological changes after 5 days of incubation. The MCD of rPMT for Vero cells was 140pg. Morphologic changes started on the verge of the well, Vero cell monolayer developed foci or patches of dense cell clusters surrounded by enlarged cells and knots developed throughout the monolayer. This uneven distribution of the monolayer progressed and eventually resulted in rolling up and detachment of the monolayer (Figure 10). The cells were viable, as found by trypan blue staining.

The 50 % lethal dose in 10-12 week old BALB/c mice was calculated as follows:

$$\text{Log } LD_{50} = \log D_N + \left(\frac{50 - \%\text{ death at } D_N}{\%\text{ Death at } D_V - \%\text{ death at } D_N} \times \log DF\right)$$

D_N is the dose at which the percentage of deaths is just below 50%, D_v is the dose at which the percentage of death is just above 50%, and $DF = D_v / D_N$

In the group that received 250 ng PMT, there were five deaths (83.33%). In the next low group, those that received 125 ng, there was one death (16.66%). No mice receiving 62.5 ng or less died (Table-4).

$DF = 250 / 125 = 2.$

$$\text{Log LD50} = \log 125 + \left(\frac{50 - 16.66}{83.33 - 16.66} \times \log 2\right)$$

$$= 2.097 + ((33.34 / 66.67) \times 0.30)$$

$$= 2.097 + 0.15 = 2.247$$

LD50 = Antilog of 2.247 = 177ng.

(This value when expressed per body weight is 7 µg/kg bodyweight)

Gross and Histopathology Lesions Caused by rPMT in Mice

Groups of mice which received 1 μg or 500 ng of rPMT died within 24 hours and all other deaths occurred in 48-72 hours. The terminal clinical signs were lethargy, anorexia and lack of movement. Gross pathology lesions in acute cases were minimal. In those that died in 48-72 hours, there was severe atrophy of the spleen and liver necrosis. At the end of the seven day observation period, the surviving mice were euthanized and subjected to necropsy. A dose dependent effect was observed in the size of spleens of mice that received different doses of PMT (Figure 12). The liver from mice that received 500ng and died in 48 to 72 hours had focal areas of congestion and necrosis. The liver from the mouse, that received 250 μg and survived, was reduced in size (figure 13). On histopathological examination, there was lymphoid depletion and red cell destruction and hemosiderosis in affected spleen. Degenerative changes and random foci of necrosis were present in liver tissue.

Antibody Response in Mice

The immunogenicity of different C-terminal and N-terminal constructs were evaluated in mice. IFA + PBS was used as a negative control. Groups of six to seven mice were given 50 μg of each of the recombinant proteins in IFA by intraperitoneal inoculation. The proteins were given on days 1, 14 and 35 and the mice were bled on day 45. The serum was separated and used for evaluation of antibody response. The anti-PMT IgG titers were determined for all individual mice using a direct ELISA. The rPMT was

used as the antigen in the direct ELISA. All these constructs and the recombinant toxin produced significantly higher anti-PMT antibody responses compared to the negative control group ($p<0.01$). The anti-PMT ELISA titers of control mice were <40. The group of mice immunized with PMT-CE had the highest anti-PMT ELISA titers. However, the ELISA titers were not significantly different among groups of mice immunized with the different constructs. (Figure 14)

When the responses of individual mice were analyzed, the group that received PMT-CE showed consistently higher anti-PMT IgG titers ranging from 41,420 to 48,365 (Figure 15). In the group that received PMT-CH, two mice showed a higher titer of 44,590 and 49,995 and four showed a lower titer less than 25,000 (Figure 16). In the PMT-CEX group, one mouse showed a very low titer of 2440, four showed a low titer between 20-25,000 and two showed a high titer of 51,460 and 46,635 (Figure 17). In mice that received the small N-terminal protein, PMT-NEX, four mice showed a low titer less than 25,000 and three showed high titers of 38,810, 41,460 and 55,235 (Figure 18). The group that received the large N-terminal protein, PMT-NE, had only one mouse with a low titer of 20,610 and all others had a high titer above 42,300 (Figure 19). The most variation in titer was found in the group immunized with heat inactivated rPMT, in which individual mice had values ranging from 3430 to 81,420 (Figure 20). All mice in the control group, which received IFA+PBS, had a consistent titer of less than 40 (Figure 21).

The Vero cell Serum Neutralization Assay

Before assaying the serum, the MCD of the recombinant toxin, rPMT, for Vero cells was determined. The MCD was 140 pg. For the toxin neutralization assay, a 250 μg/ml recombinant toxin preparation was diluted in low serum medium to give a concentration of 10 times the MCD.

Individual serum samples were assayed initially after heat inactivation at 56 °C for 30 minutes. The lowest dilution of serum, which neutralized about 10 times the MCD of rPMT, was taken as the serum titer for neutralization. The results are summarized in Table-5. Since the first dilution used for the assay was 1:5 and it required filter sterilization, the quantity of serum was not sufficient to repeat the assays for optimization. Subsequently, pooled mouse serum from each group was evaluated for neutralizing activity on Vero cells. The group immunized with PMT-CE protein gave the highest titer of 80. The smallest C-terminal construct, PMT-CEX gave a titer of 40. Serum from the group immunized with PMT-NE, heat inactivated rPMT and polyclonal anti-PMT rabbit serum gave a neutralization titer of 20. No neutralizing activity was detected in sera from other groups. The results are summarized in Table-6.

Evaluation of Protective Efficacy

The proteins PMT-CE, PMT-CEX, PMT-NE and heat inactivated rPMT were evaluated for protective efficacy in mice against rPMT challenge. Groups of 5-6 mice were vaccinated with the respective proteins in IFA on days 1, 14 and 35 by intraperitoneal inoculation. One group was given IFA+PBS. On day 45, they were bled,

and the next day all were given 1μg of PMT (approximately 5 LD50) of PMT by intraperitoneal inoculation. Mice were observed for seven days and the deaths recorded (Table-7). There was 100% protection in the groups immunized with PMT-CE and PMT-NE. All the mice immunized with PMT-CEX and heat inactivated rPMT died within 36-72 hours. All mice immunized with IFA+PBS died within 24 hours. Histopathologic examination of spleen and liver of mice, which survived the rPMT challenge, did not reveal any lesions. A very small increase in hemosiderin pigments was observed in the spleens of protected mice. A summary showing the relationships between ELISA titers, Vero cell neutralization titer and mouse protection generated by each of the PMT proteins is presented in table-8.

Cell-Binding assay

This was done to see whether it would be possible to find the receptor binding region of the PMT, utilizing the binding of these proteins with Vero cells. The cells were treated with the recombinant proteins while incubating in ice, so that there would not be any internalization after binding. In the first method using flow cytometry, when MAb CRL-1965 was used as the primary antibody, a slight increase in mean flourescence was seen with the large C-terminal fragment PMT-CE (figure. 22), but this result was not consistent. When polyclonal rabbit serum was used as the primary antibody, no difference in mean flourescence was noticed between the fragments. In the second method, none of the fragments prevented morphological changes in Vero cells caused by recombinant PMT. No conclusive reproducible results were obtained from these assays.

CHAPTER 4

DISCUSSION

The major focus of this study was to identify a protective region of PMT, which can be used as a vaccine candidate for protection against progressive atrophic rhinitis. Apart from a study in 1991(90), this is the first study utilizing different C-terminal and N-terminal fragments of PMT to identify a modified non-toxic PMT fragment which can be used as a vaccine component. This study involved construction and evaluation of immunogenicity and protective efficacy of two N-terminal and three C-terminal PMT fragments. Immunization of mice with one C-terminal fragment, PMT-CE (amino acids 600-1285) and one N terminal fragment, PMT-NE (amino acids 1- 1130) elicited protection against lethal challenge with recombinant PMT in mice.

Based on the findings of Petersen et. al (90), it was presumed that an important immunogenic region may be located in the C-terminal part of PMT. This was based on their finding that a deletion protein lacking 155 amino acids from the C-terminus was neither protective nor immunogenic. A cysteine residue at position 1165 was found to be critical for the biological activity of the toxin and it was postulated that this cysteine residue may play a role in the catalytic mode of action of PMT (117). Wilson et. al. postulated that the intracellular activity domain of PMT is localized to the N-terminal 500 amino acids and the C-terminus is required for entry into cells (120). These studies led us to think that a recombinant C-terminal fragment of PMT may induce protective antibody response against PMT. The C-terminal fragments constructed in this study, each

contained the cysteine residue at position 1165. The two N-terminal fragments did not contain this cysteine residue. The shortest C-terminal fragment, PMT-CEX, contains 155 amino acids from the C-terminus and the large N-terminal fragment, PMT-NE lacks these 155 amino acids at the C-terminus.

The recombinant PMT produced in this study was amplified from genomic DNA of *P. multocida* NCTC12178 using designed primer pairs and ligated into a cloning vector. The use of LA Taq polymerase (Panvera), which is suitable for amplification of long DNA templates with high fidelity, enabled one step PCR amplification of the 4.5 kb DNA of PMT. Previous reports of cloning and expression of rPMT were done by a three step cloning (120). A recombinant PMT produced in one lab was qualitatively identical to purified native PMT and a commercially available native PMT on western blots, oocyte assay and vero cell assay (120). Recombinant PMT produced in this study was identical to the previously reported rPMT (received from Dr. Brenda Wilson) on Western blots and Vero cell assays.

When purified rPMT and the fragments PMT-CE (amino acids 600-1285) and PMT-NE (amino acids 1-1130) were run on SDS-PAGE and Western blots, a number of intermediate protein bands were observed. These intermediates were detectable in sonic extracts of *P. multocida* NCTC 12178 and sonic extracts of *E. coli*, pmt-TA2. Petersen et al. observed degradation intermediates during the production of recombinant PMT (88). They found a 70 kDa band especially prominent. In our preparations, we observed fragments of approximately 70 kD and 55 kD. Purified toxin from sonicates of *P. multocida* can be reversibly dissociated into three polypeptides with approximate sizes of

23,000, 64,000 and 74,000 kD, after mild trypsinization, by treatment with 100 mM dithiothreitol and 6 M urea. (77,78). Upon dialysis, these fragments were able to reassociate into native fully toxic PMT. The dissociated fragments were neither toxic nor antigenic. Use of protease inhibitors in the protein purification as described in materials and methods may not be sufficient to prevent degradation. However, the rPMT was very potent. A dose as low as 70 pg produced morphological changes in Vero cells and the LD 50 in BALB/c mice was 7 μg/kg. Previously reported LD50's of PMT for BALB/c mice have ranged from 0.5 μg/kg to 15 μg/kg.

This study provided additional characterization of monoclonal antobody CRL-1965. This hybridoma was prepared from mice immunized with a toxoid, made from toxin purified from the NOR-1 strain of *P. multocida* (65,69). MAb CRL-1965 reacted with rPMT and PMT-CE (amino acids 600-1285) on Western blots and Dot blots, but did not react with the small C-terminal constructs and the two N-terminal constructs. As such, it had only limited use in detection of expressed proteins on western blots and in the binding assay. The binding domain of MAb CRL-1965 may be located within a region involving the C-terminus through amino acid at position 600. The fact that it is not reacting to the same size degradation bands in the large N-terminal fragment is intriguing and further reflect differences in the degradative intermediates. It was previously known and confirmed in this study that this antibody is non- neutralizing in the Vero cell assay.

ELISA and Vero cell serum neutralization assay were used for initial evaluation of serum anti-PMT IgG titers. Anti-PMT IgG titers for individual mice were determined

by a direct ELISA. The rPMT was a very good coating antigen for a direct ELISA. A concentration of 5 μg/ml of rPMT was sufficient for coating the ELISA plates as determined by a checker board titration. Average anti-PMT IgG titers of the fragments ranged from 28,100 for PMT-CEX to 45,830 for PMT-CE. Average titer of mice that received heat inactivated rPMT was 31,505. However, mice received heat-inactivated rPMT showed inconsistent anti PMT antibody responses. The mean titer was 31500, while the standard deviation was 29145. The negative control mice which received IFA+PBS gave a very low titer of 40, which was significantly different from all the other groups. The N-terminal fragment PMT-NE gave a titer of 41,350, which contradicts the finding of Petersen et al (90) that mice immunized with an identical N-terminal fragment was seronegative. In the present study the PMT-NE fragment produced a higher level of anti-PMT antibody response than three other fragments.

When individual mouse serum was analyzed for anti-PMT antibody, mice that received heat-inactivated rPMT showed inconsistent anti-PMT antibody responses. The titers ranged from 3,430 to 81,420. Heat inactivation leads to loss of activity of the toxin. It may be difficult to inactivate toxin's catalytic domain without altering an essential immunogenic epitope. Heat inactivation may be a sub optimal means of producing PMT toxoid antigen (8). The rabbit polyclonal serum obtained after immunizing rabbits with heat inactivated rPMT had low reactivity on ELISA and low titer on neutralization assay.

This is the first report describing the use of Vero cells in a serum neutralizing assay for PMT. The Vero cell assay was originally proposed as a cell culture assay for the detection of PMT in cell-free preparations of *P. multocida* (87). In this study, the assay

was suitably modified to perform as a serum neutralization assay based on a Vero cell assay for verocytotoxigenic *E. coli* and an embryonic bovine lung (EBL) cell assay developed for PMT (102). The EBL cell assay was the preferred cell culture test for detection of toxigenic *P. multocida* as it was 100 times more sensitive to PMT compared to other cell lines (102); however, both the Vero cell assay and the EBL cell assay were able to detect PMT levels down to 10 pg (20). In the EBL cell neutralization assay, serum from gnotobiotic pigs infected with toxigenic *P. multocida* had neutralization titers from 10 to 80 (102).

In the present study serum from mice immunized with C-terminal fragments PMT-CE and PMT-CEX, large N-terminal fragment PMT-NE and heat inactivated rPMT neutralized the cytopathic effect of PMT at titers ranging from 20 to 80. While the study utilized small numbers and experienced technical difficulty in collection of sufficient volume of serum to test each individual, the Vero cell assay may still lack the power to discriminate among protective and non-protective immunogens. Limits of the assay include visual end point and background cytopathic effect by normal mouse serum. A quantitative estimation of the neutralization effect is not possible by this assay. Furthermore, differences in cell receptors and sensitivity may preclude making any correlation between serum Vero cell neutralization activity and protection against mouse lethality. There could be species and/or tissue type differences in the mode of PMT entry into the cell, some requiring the N-terminal domain and others requiring C-terminal domain. The results suggest that the presence of antibodies neutralizing PMT activity in Vero cells may not be a reliable measure of the induced protection against rPMT

challenge in mice. While in vitro serum neutralization may be correlated with protection, the Vero cell assay may not be an ideal tool to establish the relationship. Even though the Vero cell assay is efficient in detecting low levels of PMT and distinguishing toxigenic and non-toxigenic *P. multocida*, it may not be efficient in evaluating vaccine potency using the serum neutralization method. Since EBL cells were reported to be more sensitive in a serum neutralization assay (102), an evaluation of these serum samples on an EBL cell assay is worth pursuing.

The large C-terminal fragment PMT-CE (amino acids 600-1285) and the large N-terminal fragment PMT-NE (amino acids 1-1130) protected mice from lethal challenge with rPMT. In a previous study, an N-terminal PMT fragment having the same number of amino acid residues from the N-terminus did not protect mice from lethal challenge with rPMT (90). That fragment was not protective in their challenge studies conducted in mice. While that study evaluated the protective efficacy in offspring born to pregnant mice vaccinated with the construct, no direct challenge model was used. Based on that study, the N-terminal fragment was included in the protection assay as a negative control, but surprisingly it protected mice from lethal challenge with rPMT in this study. The lack of protection by heat inactivated PMT may be due to an alteration in the conformation of the activity domain, which may be essential for eliciting a protective antibody response. Heat inactivated PMT is unable to induce any morphological changes in Vero cells.

Evidence from previous studies suggests that the mode of action of PMT involves binding to cell surface receptors and internalization via receptor-mediated endocytosis (91, 96), followed by activation of various signaling pathways leading eventually to

morphological and proliferative events (42,47,71,73,119). Since the N-terminal fragment, PMT-NE protected mice from lethal challenge with rPMT, the role of N-terminus in inducing protective antibodies need further study. The small C-terminal fragments and the other N-term fragment were insoluble and were purified under denaturing conditions. The insolubility of these proteins may be related to folding or structural changes that also cause alteration in epitope exposure or structure specificity that leads to failure in evoking a protective antibody response. These fragments have to be modified so as to express them in soluble fractions, which then can be used for further protection studies.

Five LD 50 of rPMT was used for lethal challenge in mice. All the control mice which received IFA+PBS died within 24 hours. The group that received heat inactivated PMT died within 48 hours and that, which received PMT-CEX died within 72 hours. On necropsy, liver of control mice showed small white foci of necrosis and adhesions with diaphragm. This may be due to the effect of IFA since the same type of liver lesion was noticed in mice from other groups. The spleens were normal in appearance. Histopathological examination of liver, spleen and kidney of the survived mice in protection studies did not show any lesions.

The cell binding assay performed in this study by two methods were inconclusive. However, in FACS analysis, a slight increase in fluorescence was noticed with the C-terminal fragment, PMT-CE, suggesting that it might have bound to cells. Inconsistency and lack of reproducibility in the binding assays might have resulted from non-specific binding of the proteins or secondary antibody to the cells and insolubility of three out of the five truncated PMT fragments. In the competitive inhibition assay, even though some

delay in onset of morphological changes in Vero cells was observed when the soluble fragments were added prior to PMT, they did not prevent characteristic morphological changes caused by rPMT in Vero cells. Truncated PMT fragments may be an efficient tool to localize regions or domains required for binding or activity. Further modification in the methods, modification in the protein constructs and use of an FITC conjugated primary antibody reactive to all the constructs may be ideal in optimizing the assay.

The recent studies conducted on the putative biological activity domain of PMT reported contrasting results (10,117,120). While one study reported the requirement of N-terminal 500 amino acids for biological activity, the other two studies reported the importance of the C-terminus for biological activity of toxin. However, these groups utilized different types of cells and methods for evaluating the activity of the toxin. Recombinant PMT differentially modulates cellular responses in different cell types, resulting in different cellular consequence (121). The latest study on functional organization of PMT found that both N-terminal and C-terminal deletion mutants were able to prevent binding and uptake of PMT into Vero cells. Studies involving direct binding interaction between GFP fusions of the PMT fragments and cells using fluorescence microscopy indicate that PMT binds to multiple types of receptors through different portions of the toxin protein (122).

The recombinant protein fragments, which evoked protective responses, were expressed as large and soluble proteins, which might have predictably retained the domain structure leading to greater epitope exposure. The fragment PMT-CE had 685 amino acids from the C-terminus and the fragment PMT-NE had 1130 amino acids from

the N-terminus. By analogy with other intracellularly acting toxins, PMT would be predicted to contain individual domains for binding, internalization and catalytic activity. Since an N-terminal and a C-terminal PMT fragment protected mice from lethal challenge with rPMT, either one of these region are sufficient to induce a protective immune response in mice. PMT may be a multifunctional toxin with different domains for different functions. Antibodies directed against any of the functional domains may be able to neutralize the action of the toxin in vivo and/or in vitro. This may explain the finding that antibodies against both an N-terminal fragment and a C-terminal fragment elicited protection of mice from lethal challenge with rPMT and also were able to neutralize the morphologic changes caused by rPMT in Vero cells.

The C-terminal fragment PMT-CEX used in this study included the essential cysteine residue required for the activity of the toxin (117). However this fragment did not protect the mice from lethal challenge with rPMT. Interestingly, this fragment showed a neutralization titer of 40 in the Vero cell serum neutralization assay. This protein was isolated from insoluble cell lysate fractions and was purified under denaturing conditions. The insolubility of this protein may have resulted in alteration in the epitope exposure or domain structure leading to failure in evoking a protective antibody response. The fact that serum from mice injected with this fragment gave some level of neutralization in Vero cell assay might suggest that an important domain or epitope might be present in this region. Suitable modification to this fragment with regards to expressing the protein in soluble fraction may be ideal to explore this region of the PMT sequence. The N-terminal fragment, PMT-NE, which protected mice from lethal

challenge, did not contain this cysteine residue. Even though the mutation in this cysteine resulted in complete lose of activity of PMT, the residue may not be an essential part of a protective region of the toxin.

Amino acids 600 through 1130 are an overlapping region in those protein fragments, which elicited protection. The domain or epitope responsible for eliciting protective antibody in mice may be located in this region. Based on these observations, the protective region of PMT may be localized to the amino acids between 600 and 1130 of the protein sequence. A recent study postulates that the region covered by amino acids 581 through 700 of PMT is important for the biological activity of a C-terminal fragment (10). Smaller fragments encompassing these amino acids may be expressed and the proteins evaluated for potential vaccine candidates for protection against atrophic rhinitis. Further studies are required to clarify the regions involved in binding and activity of the toxin.

Studying the mechanism of action of bacterial toxins has significantly contributed to our understanding of how these protein toxins mediate their pathogenic effects on host cells. Activity of PMT is dependent upon roles of heterotrimeric G proteins in cellular signaling and the differential regulation of downstream signaling proteins involved in cell cycle progression (122). The pathogenic consequence in bacterial infection is the potential for differential cellular outcome due to their action on different target cells. The disease mechanism and host response involved in AR and mouse lethality model may have important differences. How the protective efficacy of the two fragments in mouse protection experiments correlates with prevention of atrophic rhinitis in swine needs

further investigation. Also, the biological activity of the protective fragments in swine need to be determined.

This study identified two truncated PMT fragments, which can be further evaluated as potential vaccine candidates for prevention of atrophic rhinitis. While immunization with the two fragments elicited protection in mice against lethal challenge with rPMT, further studies are necessary to determine their role in eliciting protection in swine against AR. In a previous study, a deletion fragment of PMT, which elicited protection in mice against lethal challenge with rPMT, was found to be successful in prevention of AR. Evaluation of protective efficacy and biological activity of the two protective fragments in swine may lead to development of a subunit vaccine candidate for prevention of progressive atrophic rhinitis.

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APPENDIX

Table. 1 - Description of plasmids used in this study

Plasmid	Description
PCR 2.1	TA Cloning vector (Invitrogen)
pET 29 b+	Expression vector (Novagen)
pPROEXHTc	Expression vector (Gibco/BRL)
PMT-TA2	*Pasteurella multocida tox A* gene with regulatory and coding sequence in PCR 2.1 TA cloning vector (this study)
pET-pmt-w	PMT gene coding sequence in EcoRI and XhoI sites of pET 29b+ (this study)
pET-pmt-cex	PMT gene sequence encoding carboxy terminal amino acids 1130 - 1285 in EcoRI and XhoI sites of pET 29 b+ (this study)
pET-pmt-ch	PMT gene sequence encoding carboxy-terminal amino acids 979 -1285 in HindIII and XhoI sites of pET 29b+ (this study)
pET-pmt-ce	PMT gene sequence encoding carboxy-terminal amino acids 600-1285 in EcoRV and XhoI sites of pET 29b+ (this study)
PET-pmt-ne	PMT gene sequence encoding amino-terminal amino acids 1-1130 in EcoRI site of Pet 29b+ (this study)
pPROEX-pmt-nex	PMT gene sequence encoding amino-terminal amino acids 1-482 in EcoRI and XhoI sites of pPROEX-HTc (this study)

Table. 2 – Sequence information of primers used in this study

Name of primer	Sequence (5' – 3')
PMTECOF	CGCTTACCTCTTTTAGAGAATTCGG
PMTXHR	GGCTCGAGTAGTGCTCTTGTTAAGCG
PMTHINDF	GGATAAAGGAGATATTAATGGTGCTT
PMTECOVF	GGGGATATCCGTTATTGGAAAGCCTATTGG
PMTBAMHF	GGGGATCCGCCCCTTGACCTAGAGGG
PMTBAMHR	GGGGATCCGTTATAGTGCTCTTGTTAAGCG
PMTNT	GGGGAATTCTATGAAAACAAAACATTT
PMTX3	AAGACGTACCAGACGAATTTTATG

Table. 3 - Description of PMT proteins expressed by plasmid constructs

Plasmid	amino acids encoded	name of protein	Molecular weight
pET-pmt-w	1- 1285	rPMT	146 kD
pET -pmt-ce	600-1285	PMT-CE	80 kD
pET-pmt-ch	979-1285	PMT-CH	35.4 kD
pET-pmt-cex	1130-1285	PMT-CEX	21.6 kD
pPROEX-pmt-nex	1-482	PMT-NEX	64 kD
pET-pmt-ne	1- 1130	PMT-NE	130 kD

Table. 4 - Determination of LD 50 of rPMT in BALB/c mice

Group	[1]Dose of rPMT	Number of mice	[2]Number died	% death
1	1 μg	6	6	100%
2	500 ng	6	6	100%
3	250 ng	6	5	83.33%
4	125 ng	6	1	16.66%
5	62.5 ng	6	0	0
6	31.25 ng	6	0	0
7	15.625 ng	6	0	0
8	7.812 ng	6	0	0
9	3.9 ng	6	0	0

[1] Recombinant PMT was diluted in sterile PBS and 100 μl doses containing the desired concentration was administered intraperitonealy to each mouse.
[2] Mice were observed for 7 days, lethality recorded and the number of mice that survived on day 7 was recorded.

Table. 5 – Vero cell serum neutralization titers of individual mice immunized with recombinant PMT proteins

	Immunization groups [1]						
	CEX	CH	CE	NEX	NE	rPMT	Control
1	160 [2]	<10	20	<10	20	20	<10
2	20	<10	40	<10	20	10	<10
3	10	<10	40	<10	20	10	<10
4.	20	<10	20	<10	<10	<10	<10
5	20	<10	320	<10	<10	<10	<10
6	--	--	320	<10	--	--	--

[1] Individual mice were immunized with 50 μg of the PMT proteins in IFA by IP route on days 1, 14, and 35. Serum was obtained on day 45. PMT proteins consisted of C-terminal fragments (CEX, CH, CE), N-terminal fragments (NEX, NE) and heat-inactivated rPMT. The control group received IFA alone.

[2] Reciprocal of the highest dilution of the serum which neutralized the effects of 10 MCD of rPMT on Vero cells.

Table. 6 – Vero cell serum neutralization titers of pooled mouse serum, from mice immunized with PMT proteins

Groups	Neutralization Titer (pooled mouse serum)
PMT-CEX	40 *
PMT-CH	<10
PMT-CE	80
PMT-NEX	<10
PMT-NE	20
rPMT (heat inactivated)	20
Control (IFA+PBS)	<10

* Reciprocal of the highest dilution of the mouse serum, which neutralized the effects of 10 MCD's of rPMT on Vero cells.

Table. 7 – Effect of immunization with recombinant PMT proteins on protection against lethal challenge in mice

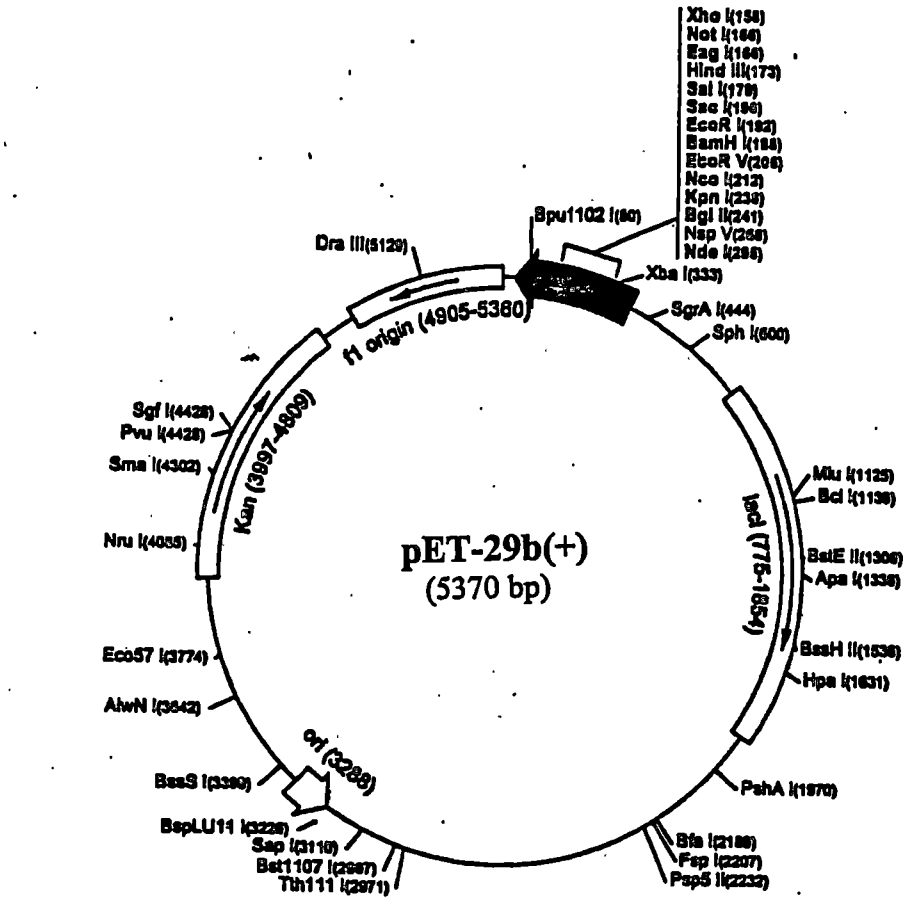
Immunizing antigen	Number of mice vaccinated	Number of mice that survived *						
		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
PMT-CE	6	6	6	6	6	6	6	6
PMT-CEX	6	6	4	2	0	0	0	0
PMT-NE	6	6	6	6	6	6	6	6
rPMT (heat inactivated)	5	4	1	0	0	0	0	0
Control group IFA+PBS	5	1	0	0	0	0	0	0

* Mice were lethally challenged by IP inoculation of 1 μg (~5 LD50) of rPMT.

Table. 8 – A summary showing the results of evaluation of recombinant PMT proteins in mice

PMT proteins [1]	Amino acid encoded	Mean anti-PMT ELISA titer [2]	Vero cell serum neutralization titer [3]	Protection against lethal challenge [4]
PMT- CE	600-1285	45,825	80	Yes
PMT-CH	979-1285	32,700	<10	Not tested
PMT-CEX	1130-1285	28,100	40	No
PMT-NE	1-1130	39,550	20	Yes
PMT-NEX	1-482	32,375	<10	Not tested
rPMT (heat inactivated)	1-1285	31,505	20	No
Control IFA+PBS	--	40	<10	No

[1] Mice were immunized with three successive injections of antigens on days 1, 14 and 35 as described in the text. A control group received IFA+PBS.
[2] Anti-PMT IgG ELISA titers were determined as the reciprocal of the highest dilution of serum that gave an absorbance reading three times the conjugate control.
[3] Reciprocal of the highest dilution of the mouse serum, which neutralized the effects of 10 MCD's of rPMT on Vero cells.
[4] Mice were lethally challenged by IP administration of 1 ug (~5 LD 50) of rPMT



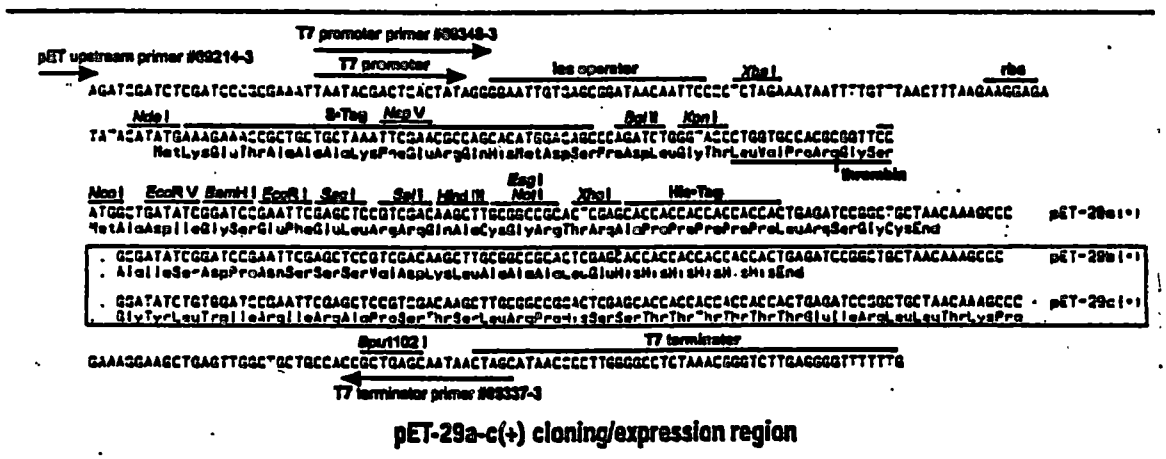


Figure 1 : Vector map of pET-29b(+)
(source: http://www.novagen.com)

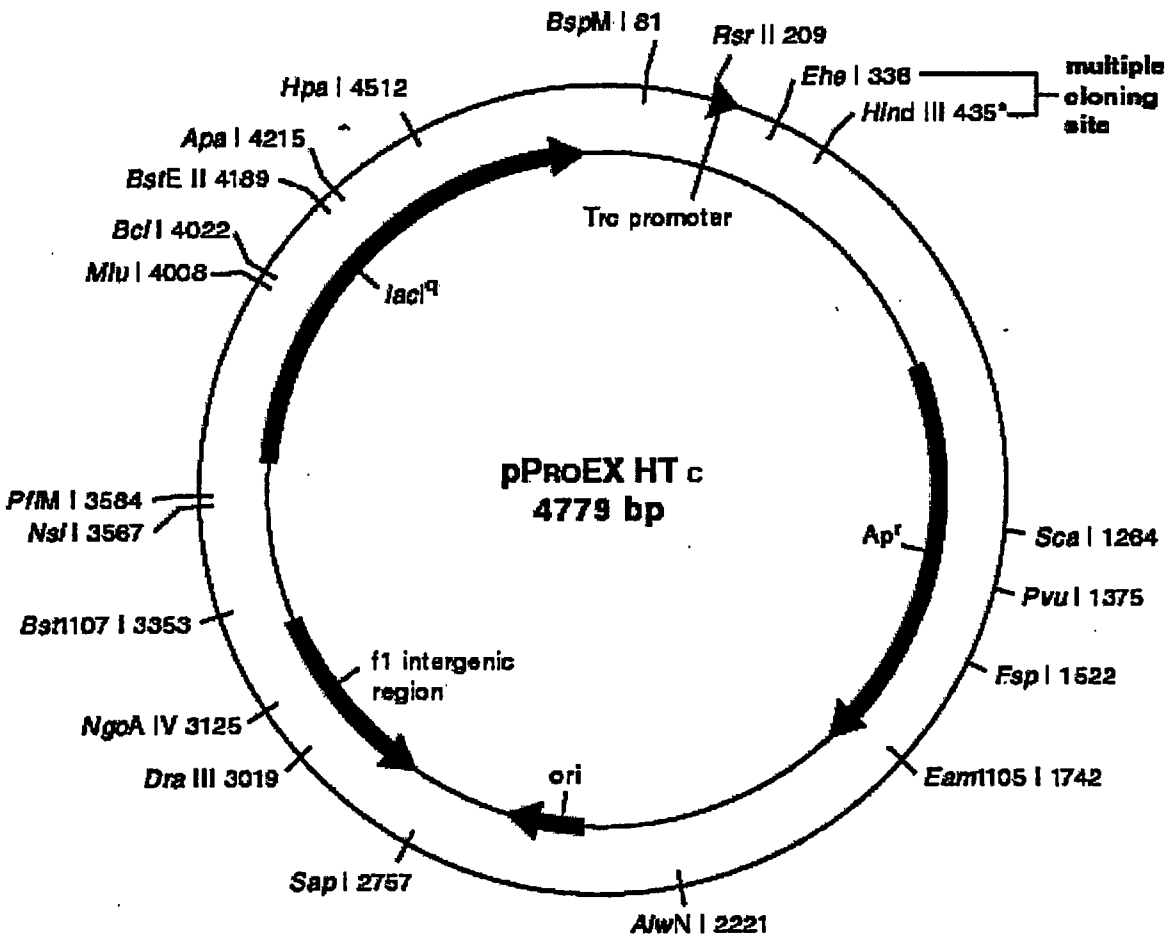


Figure 2 : Vector map of pPROEX HTc
(source: http://www.invitrogen.com)

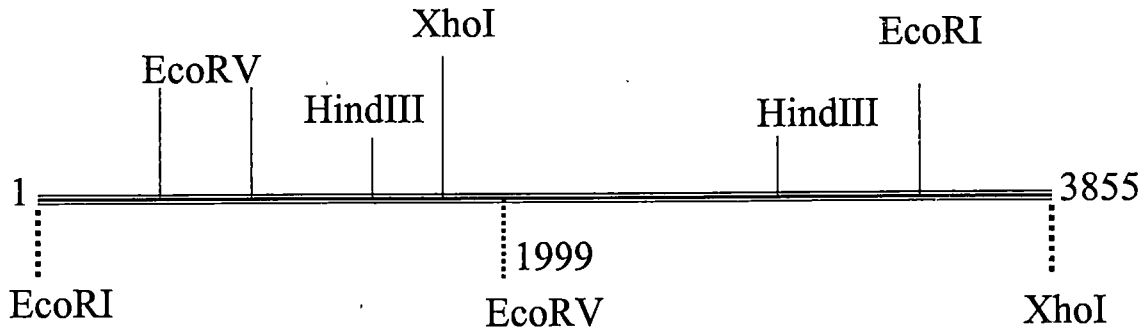


Figure 3: Schematic representation of restriction enzyme sites in the PMT sequence used for cloning and restriction mapping in this study. The sites shown with a dotted line are the restriction sites engineered using designed primers.

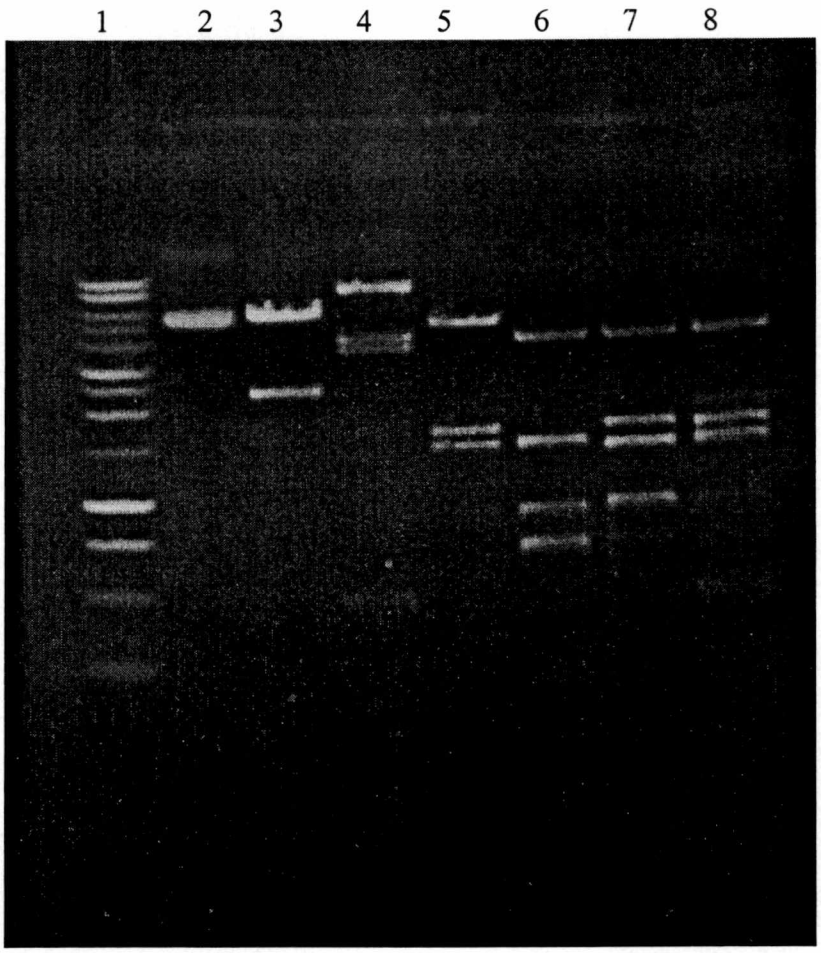


Figure 4: Restriction digestion map of pmt-TA2 plasmid DNA with different enzyme combinations. Lane 1, 1 kb DNA ladder (250 bp to 10 kb)(Promega), lane 2, Plasmid DNA, Lanes 3-8 restriction digestion fragments: lane 3, XhoI; lane 4, EcoRI; lane 5, HindIII; lane 6, HindIII and EcoRV; lane 7, Hind III and XhoI; lane 8, EcoRI and XhoI

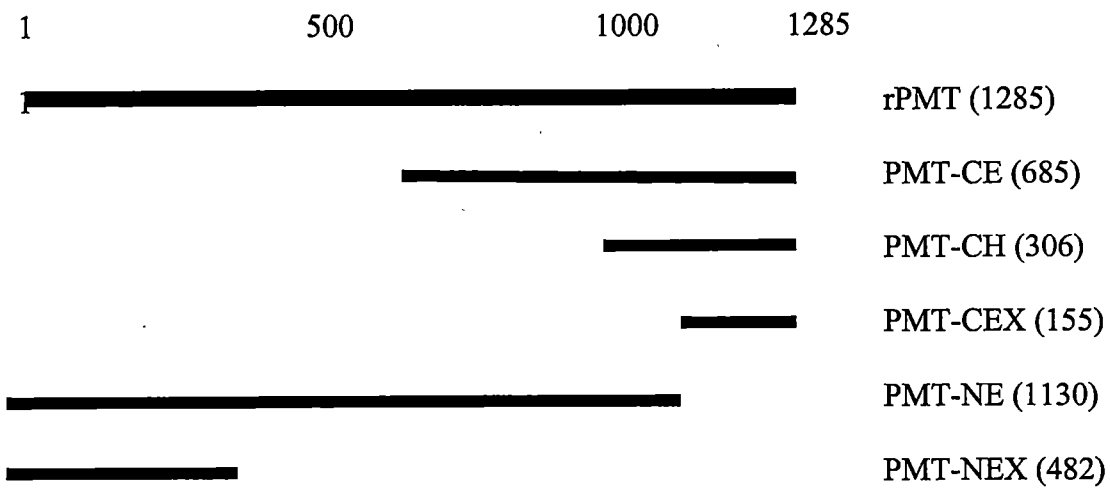


Figure 5: Schematic representation of recombinant proteins used in this study in relation to full-length PMT. (the number of amino acids of PMT included in these recombinant proteins is given in parentheses)

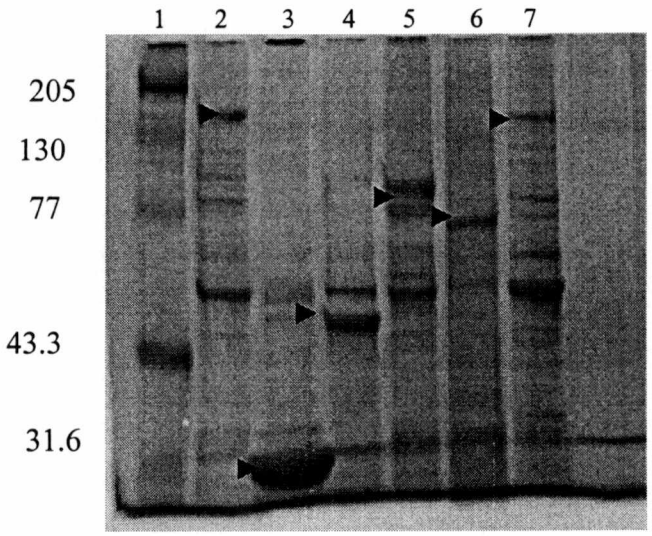


Figure 6: SDS-PAGE of recombinant PMT proteins produced in this study. Recombinant proteins were purified by affinity chromatography. Approximately 5 μg of each protein was electrophoresed on a 10 % polyacrylamide gel and stained with Bio-Safe Coomassie (Bio-Rad). Prestained molecular weight markers in kDa (Bio-Rad) (lane 1), rPMT (lane 2), PMT-CEX (lane3), PMT-CH (lane 4), PMT-CE (lane 5), PMT-NEX (lane 6), PMT-NE (lane 7) The arrows show the respective PMT proteins.

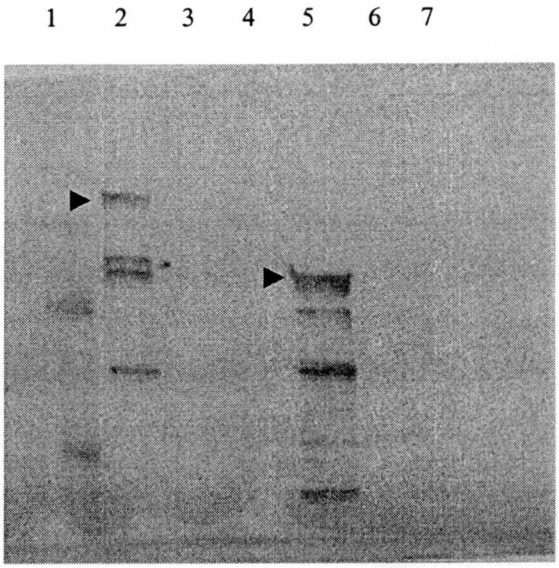


Figure 7: Western blot analysis of recombinant PMT proteins. Approximately 5 μg of each protein were electro-transferred on to a nitrocellulose membrane and probed with the monoclonal antibody CRL-1965. Lanes are in the same order as in figure 6. Arrows indicate the respective size bands of rPMT and PMT-CE.

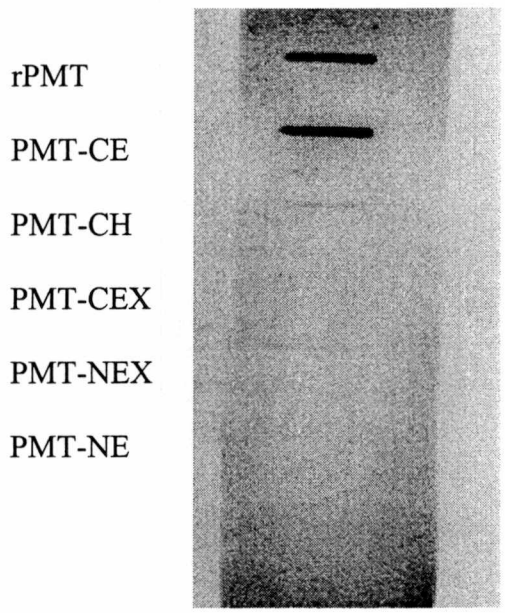


Figure 8: Dot blot of recombinant PMT proteins with monoclonal antibody CRL 1965. Approximately 10 μg of each protein samples were directly adsorbed onto nitrocellulose membrane using a Convertible Filtration manifold System (Life Technologies, Inc., Gaithersburg, MD) and the membrane was probed with MAb CRL-1965. Recombinant PMT and PMT-CE reacted with MAb CRL-1965

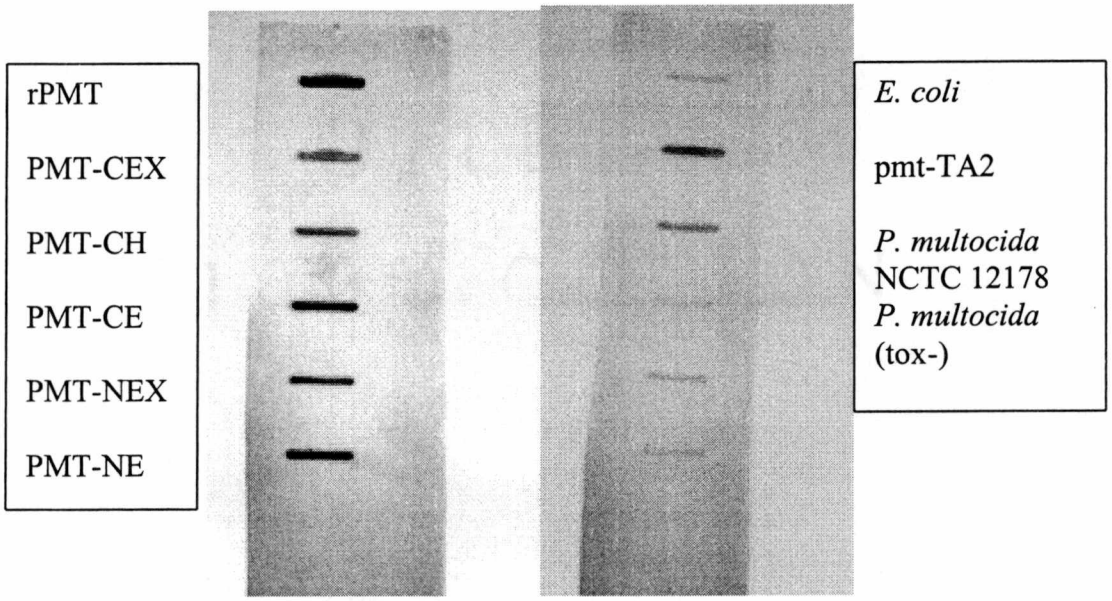
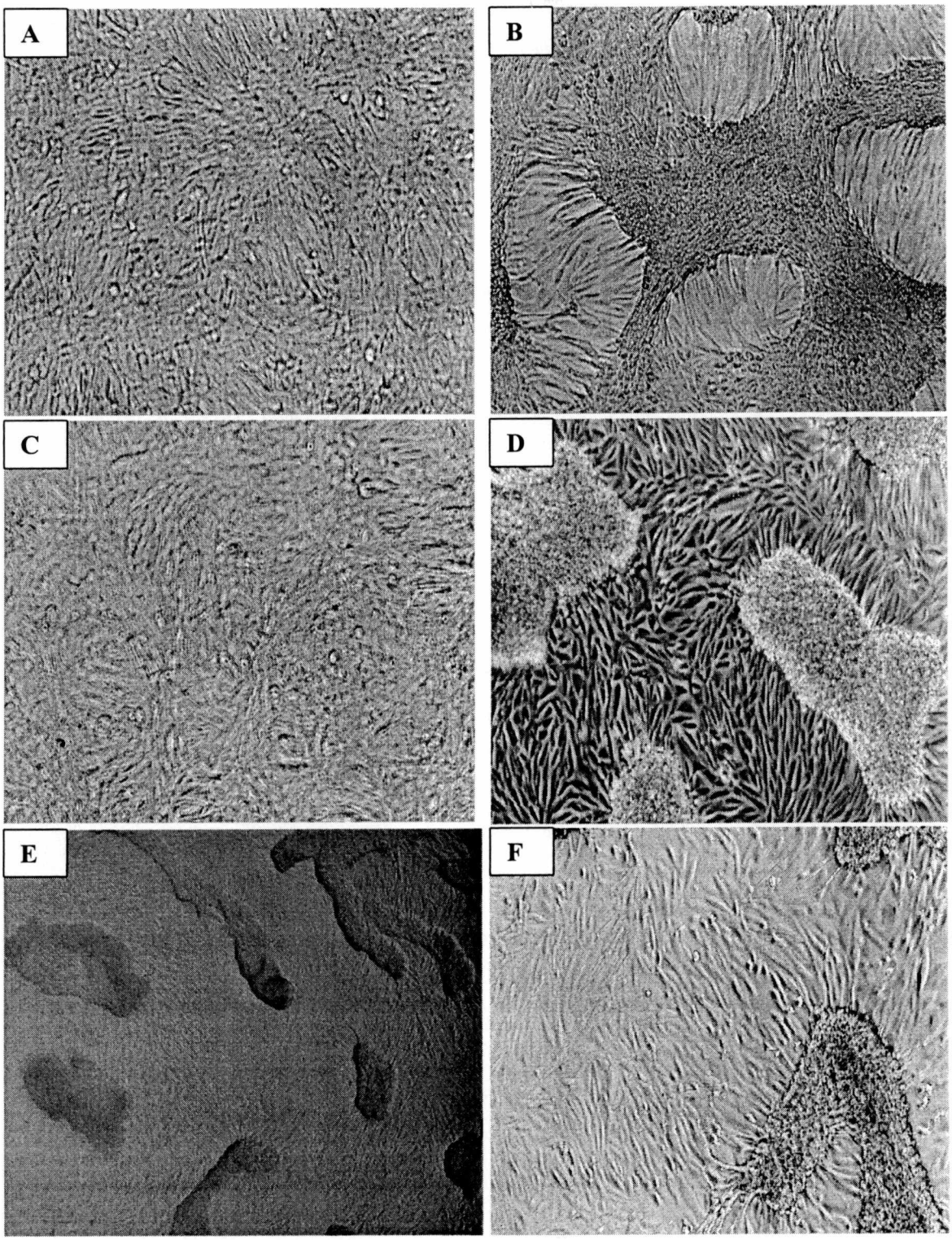


Figure 9: Dot blot of recombinant PMT proteins with anti-PMT rabbit polyclonal serum. Approximately 10 μg of each of the protein samples or bacterial lysates were directly adsorbed onto nitrocellulose membrane using a Convertible Filtration manifold System (Life Technologies, Inc., Gaithersburg, MD) and the membrane was probed with anti-PMT rabbit polyclonal serum produced in this study. The two unlabeled bands are two elution fractions in rPMT purification.

Figure 10: Morphological changes in Vero cell monolayer caused by recombinant PMT.

A. Normal Vero cell monolayer without addition of rPMT; B. Vero cell monolayer 24 hours after addition of rPMT (250 ng/ml); C. Vero cell monolayer 48 hours after addition of heat inactivated rPMT (500 ng/ml): D. Vero cell monolayer 48 hours after addition of rPMT (250 ng/ml); E. Vero cell monolayer 72 hours after addition of rPMT (250 ng/ml)(low magnification); F. Vero cell monolayer 72 hours after addition of rPMT (250 ng/ml).


A
B
C
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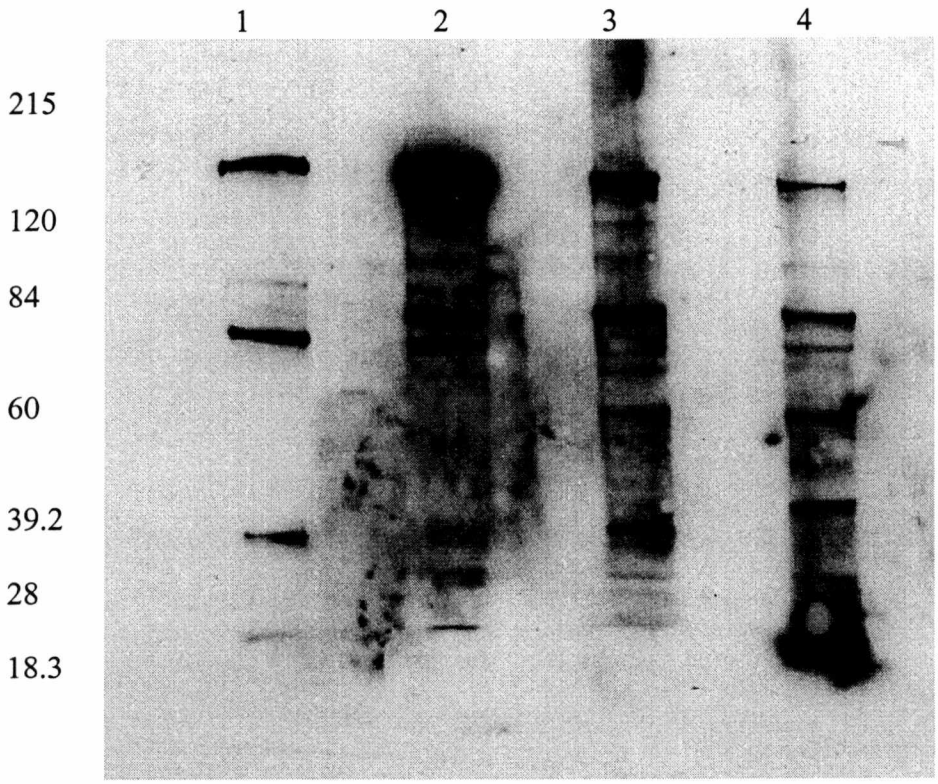

Figure 11: Western Blot Comparison of purified rPMT produced in this study with rPMT produced in another laboratory and with cell lysates containing recombinant and native PMT. The proteins and the cleared lysates were run by SDS-PAGE on an 8% polyacrylamide gel, electro-tranferred on to nitrocellulose membrane and probed with MAb CRL-1965. The membrane was developed with chemiluminescent substrate (SuperSignal West Pico chemiluminescent Substrate, PIERCE). The protein marker used (not shown) was the Blue ranger Prestained Protein Molecular Weight Marker Mix (PIERCE). Lane 1, rPMT 50ng (this study); lane 2, rPMT 50 ng (provided by Dr. Brenda Wilson); lane 3 pmt-TA2 *E. coli* lysate; lane 4, *P.multocida* NCTC 12178 lysate.

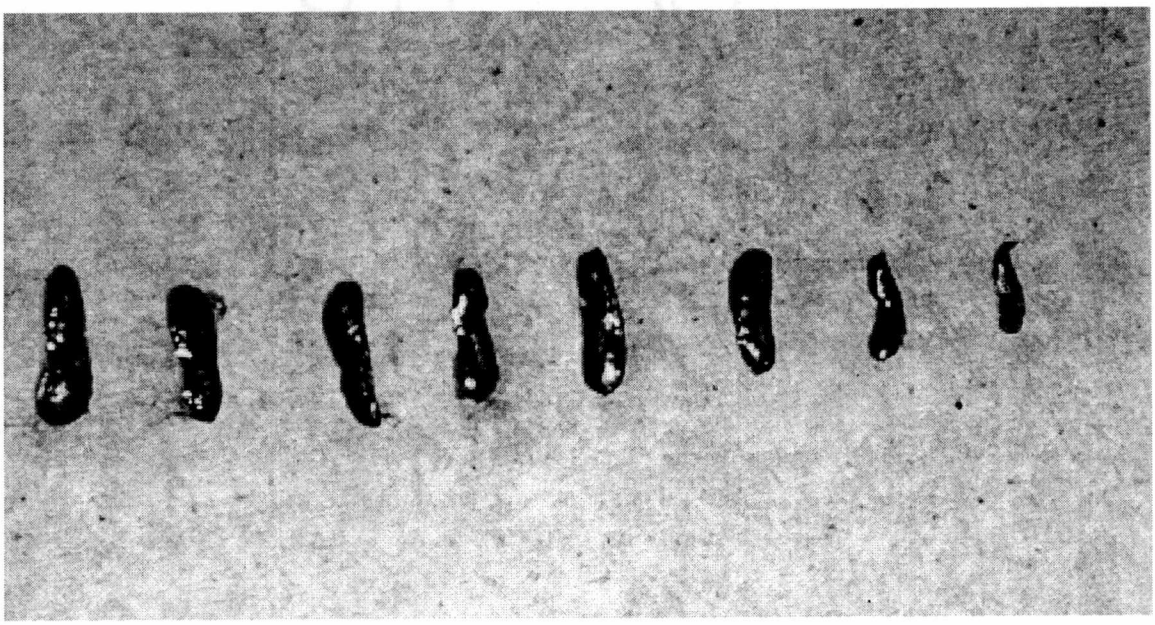

Figure. 12 : Splenic atrophy in mice following intraperitoneal inoculation of different doses of rPMT. The spleens are from the mice which survived the 7-day period after rPMT challenge in the LD 50 study. From left; spleen of control mouse, spleen from mouse given 4 μg, 8 μg, 16 μg, 32 μg, 64 μg, 125 μg and 250 μg of rPMT, respectively.

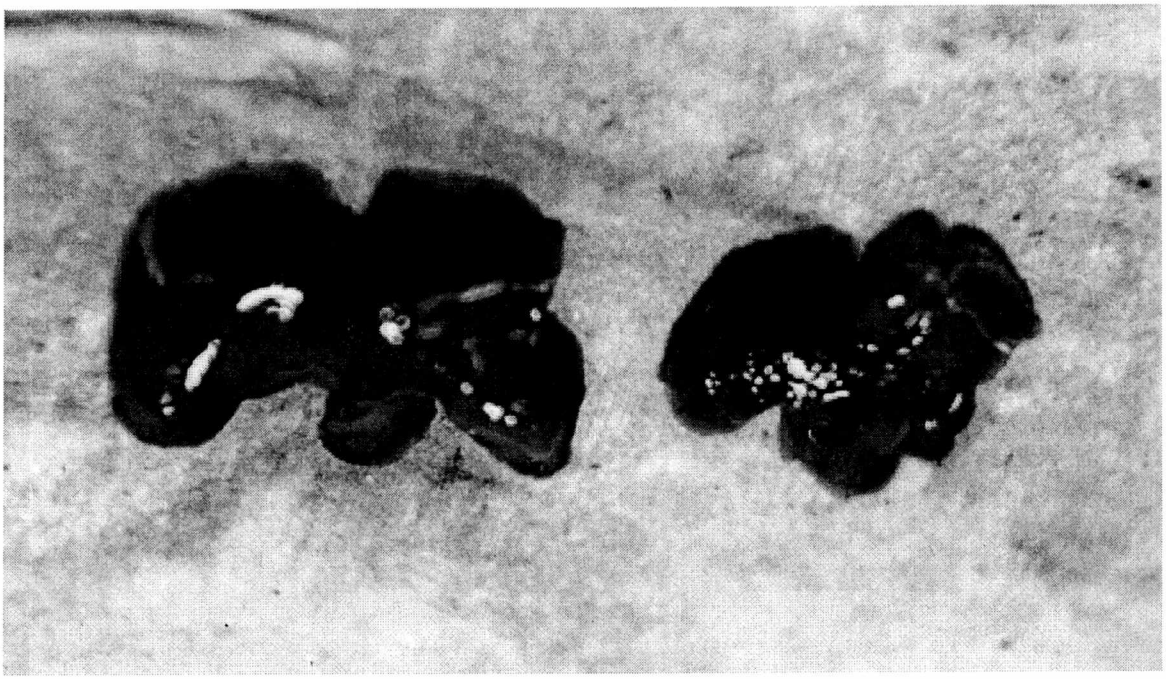

Figure. 13: Liver atrophy following intraperitoneal rPMT exposure. Left, normal liver from unexposed control mouse. Right, liver of the single mouse that survived seven days after intraperitoneal administration of 250 μg of rPMT.

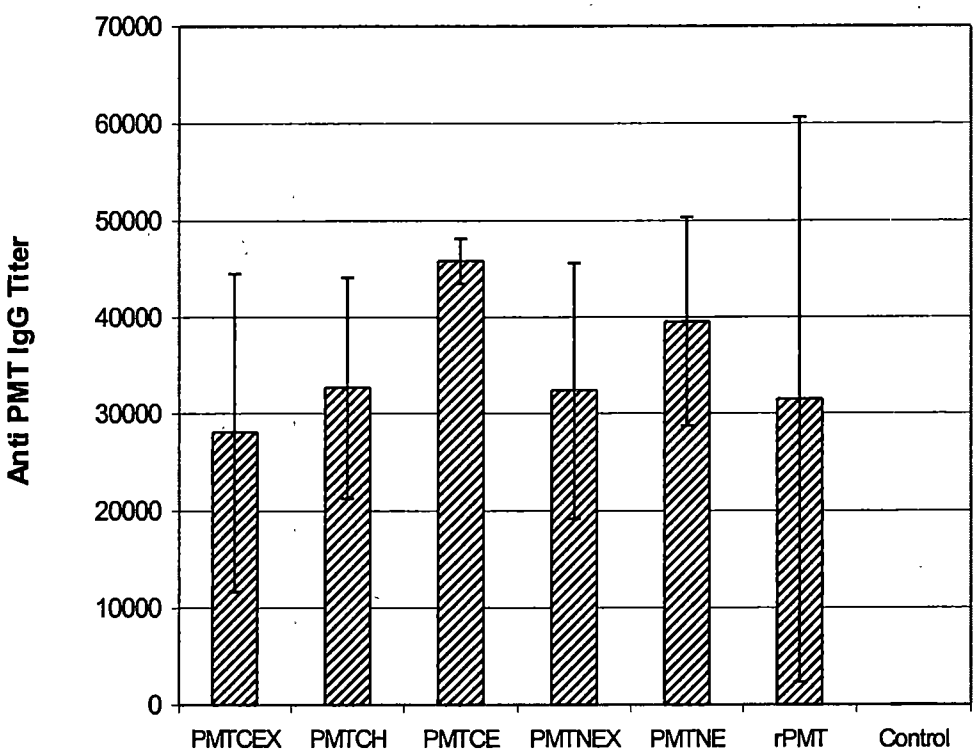

Figure 14. Mean anti-PMT antibody response of individual groups of mice immunized with recombinant PMT proteins. Mice were immunized with three successive injections of antigens on days 1, 14 and 35 as described in the text. A control group received IFA+PBS. The different groups are labeled on the X-axis. Mean anti-PMT IgG ELISA titers +/- SD is reported. The titers were determined as the reciprocal of the highest dilution of serum that gave an absorbance reading three times the conjugate control.

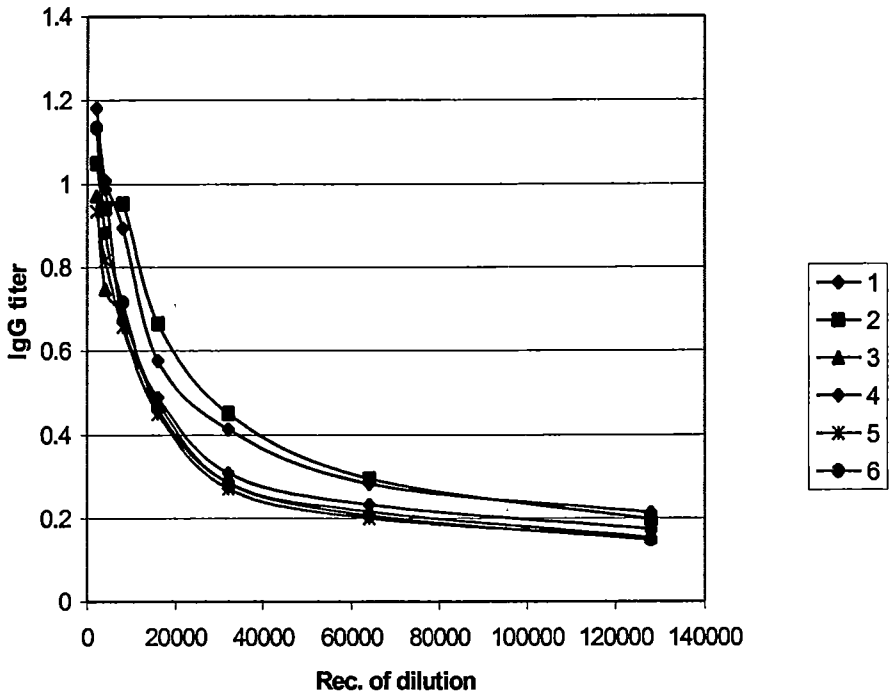

Figure 15: Anti PMT antibody responses of mice immunized with PMT-CE. Mice were immunized with three successive injections of PMT-CE in IFA on days 1, 14 and 35 as described in the text, bled on day45, and the serum evaluated in a direct ELISA to measure anti-PMT IgG responses. Each mouse is represented by a different style bullet in the graph.

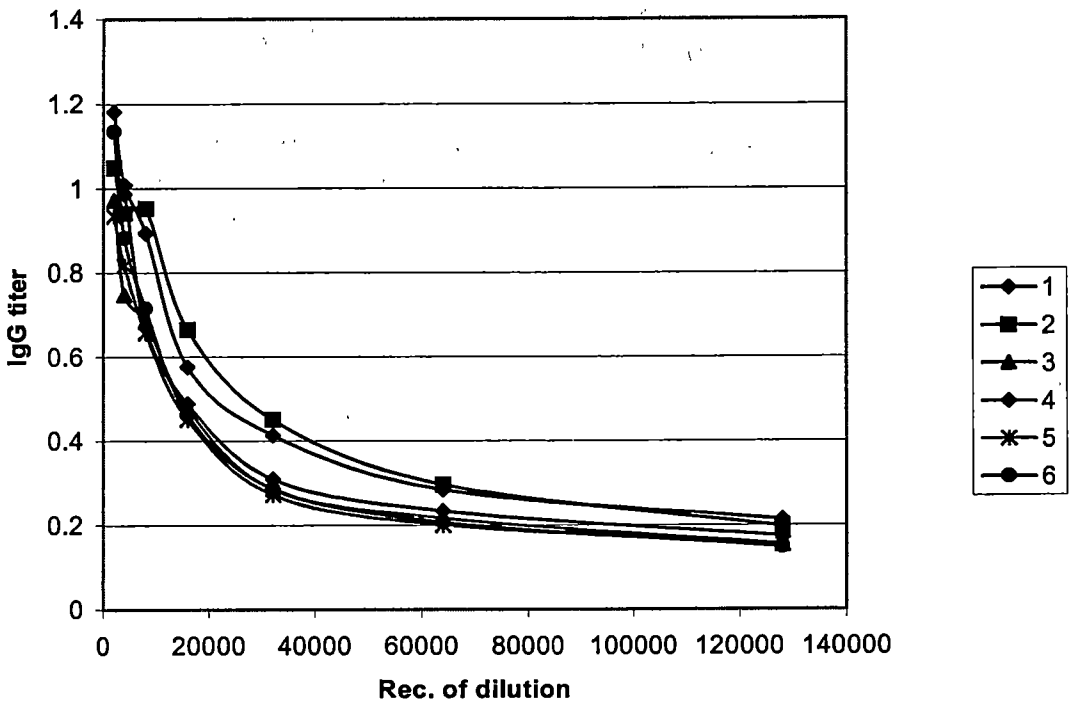

Figure 16: Anti PMT antibody responses of mice immunized with PMT-CH. Mice were immunized with three successive injections of PMT-CH in IFA on days 1, 14 and 35 as described in the text, bled on day45, and the serum evaluated in a direct ELISA to measure anti-PMT IgG responses. Each mouse is represented by a different style bullet in the graph.

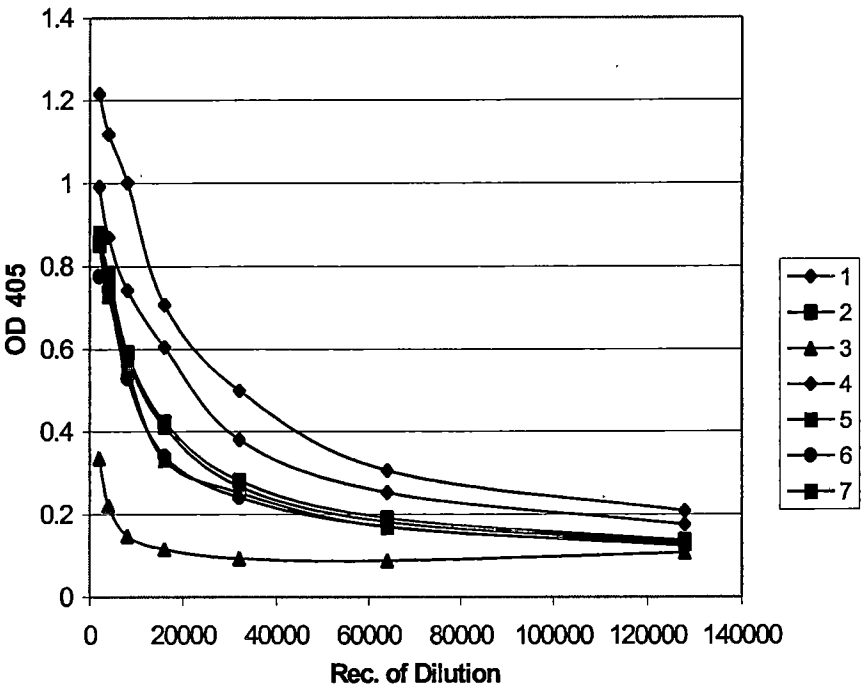

Figure 17: Anti PMT antibody responses of mice immunized with PMT-CEX. Mice were immunized with three successive injections of PMT-CEX in IFA on days 1, 14 and 35 as described in the text, bled on day45, and the serum evaluated in a direct ELISA to measure anti-PMT IgG responses. Each mouse is represented by a different style bullet in the graph.

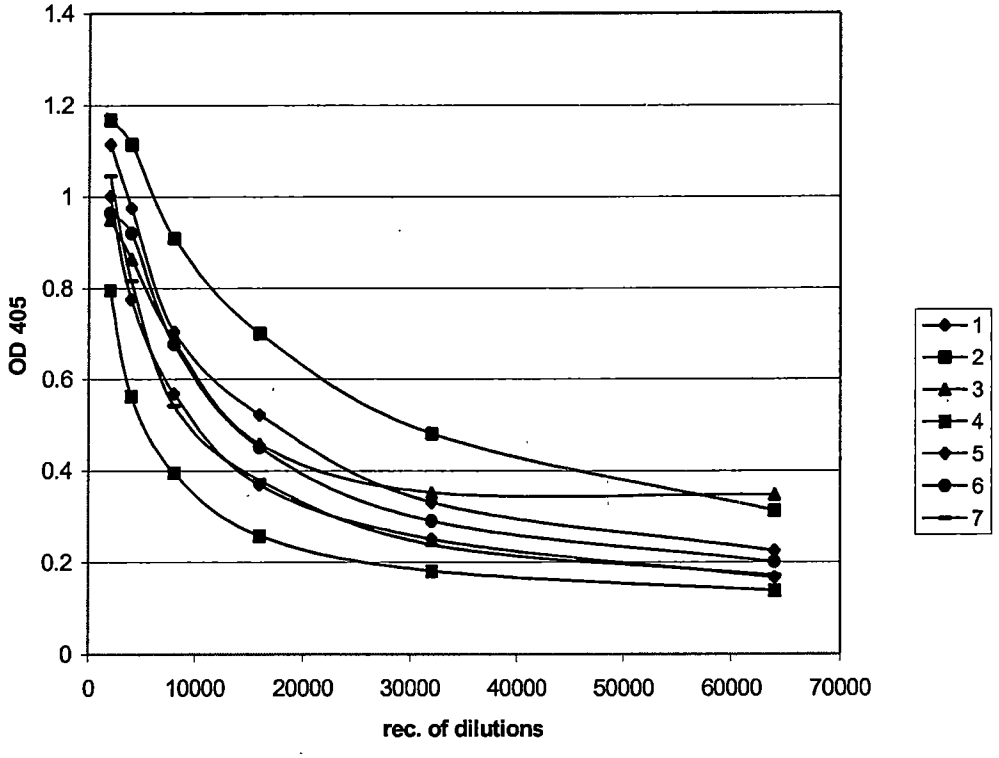

Figure 18: Anti PMT antibody responses of mice immunized with PMT-NEX. Mice were immunized with three successive injections of PMT-NEX in IFA on days 1, 14 and 35 as described in the text, bled on day45, and the serum evaluated in a direct ELISA to measure anti-PMT IgG responses. Each mouse is represented by a different style bullet in the graph.

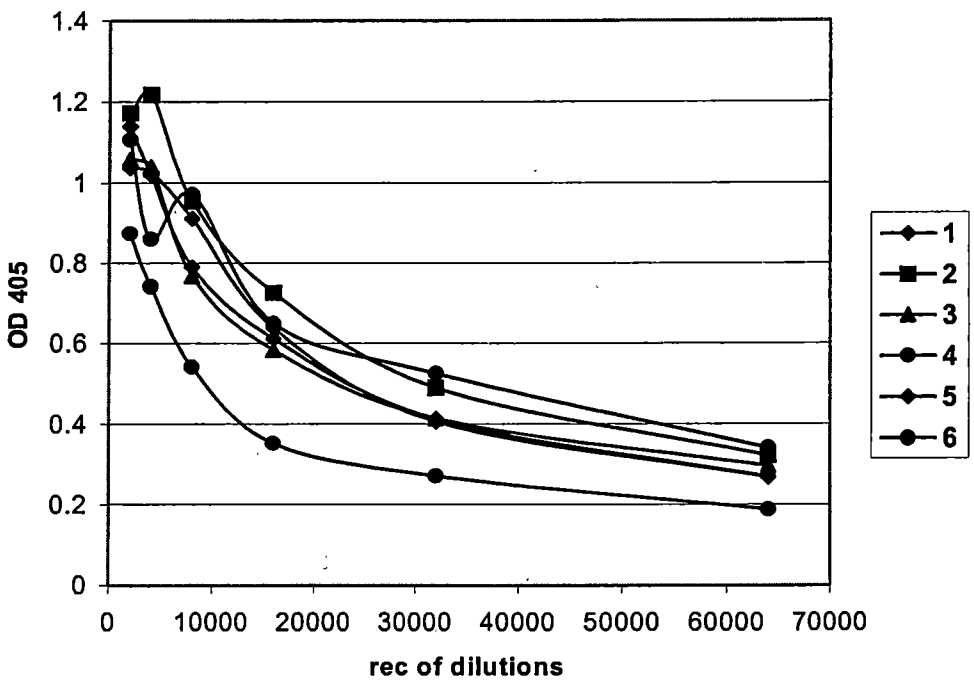

Figure 19: Anti PMT antibody responses of mice immunized with PMT-NE. Mice were immunized with three successive injections of PMT-NE in IFA on days 1, 14 and 35 as described in the text, bled on day45, and the serum evaluated in a direct ELISA to measure anti-PMT IgG responses. Each mouse is represented by a different style bullet in the graph.

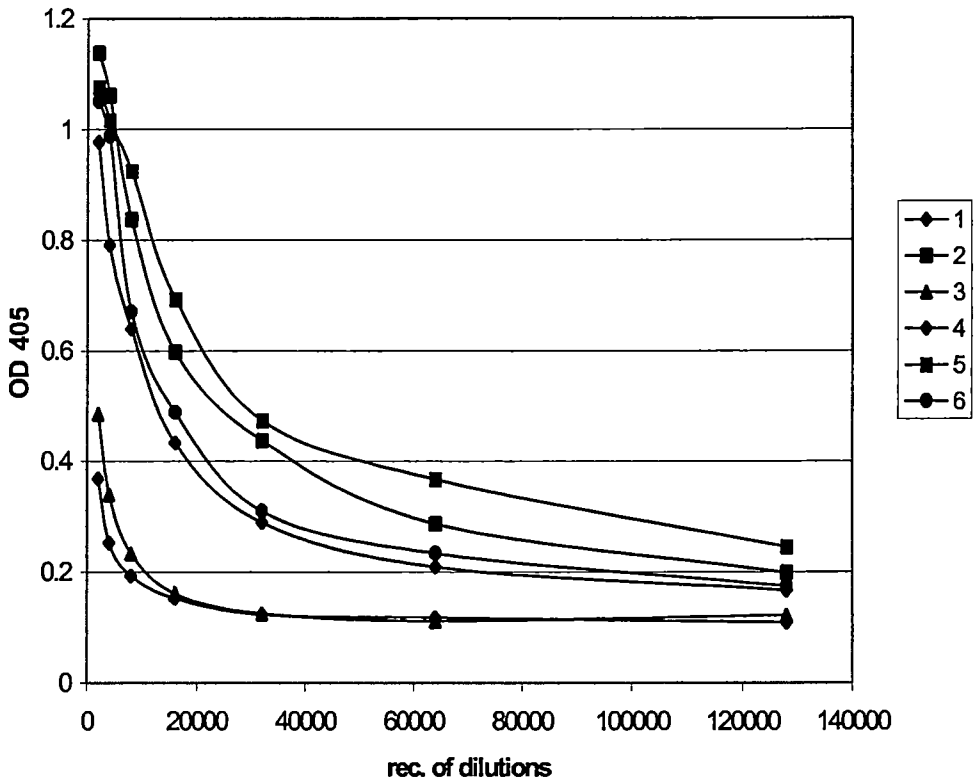

Figure 20: Anti PMT antibody responses of mice immunized with heat inactivated rPMT. Mice were immunized with three successive injections of rPMT (h.i) in IFA on days 1, 14 and 35 as described in the text, bled on day45, and the serum evaluated in a direct ELISA to measure anti-PMT IgG responses. Each mouse is represented by a different style bullet in the graph.

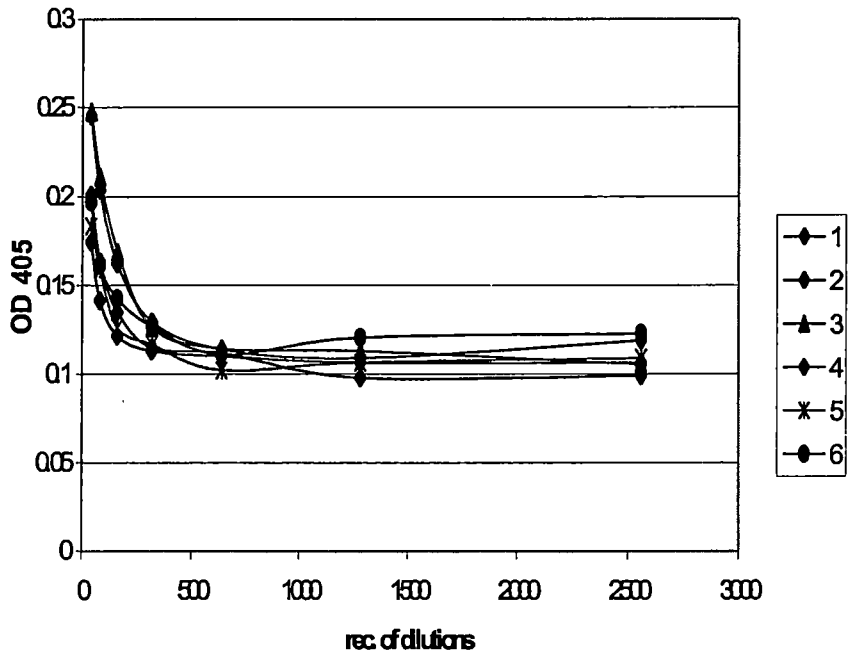

Figure 21: Anti PMT antibody responses of mice immunized with IFA+PBS (control group). Mice were immunized with three successive injections of IFA+PBS in IFA on days 1, 14 and 35 as described in the text, bled on day45, and the serum evaluated in a direct ELISA to measure anti-PMT IgG responses. Each mouse is represented by a different style bullet in the graph.

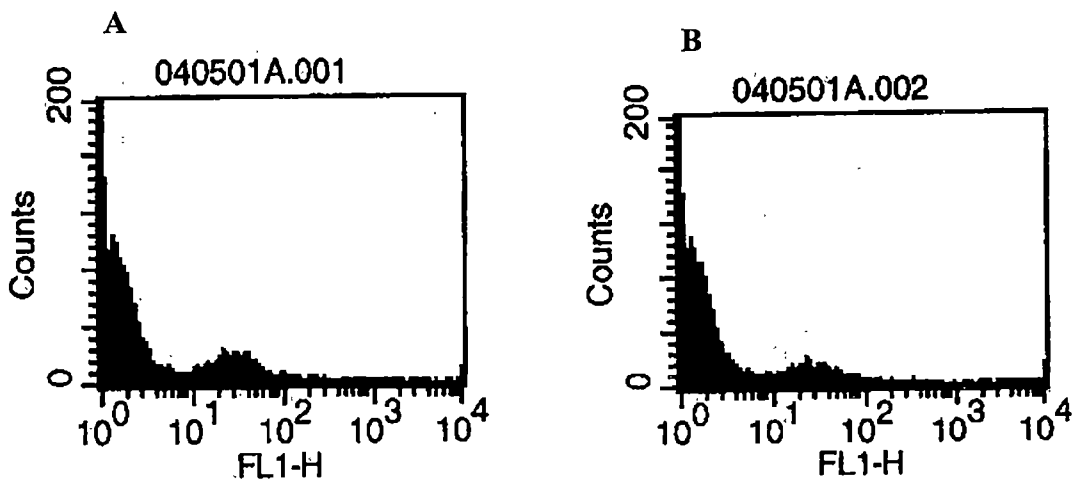

Figure. 22: FACS analysis of Vero cells treated with recombinant PMT proteins. Approximately 10^5 Vero cells were treated with 10 μg of PMT-CE or rPMT proteins in 1 ml blocking buffer (4% non-fat milk in PBS). MAb CRL-1965 was used as the primary antibody and FITC conjugated anti-mouse IgG as secondary antibody. The cells were analyzed by flow cytometry. Mean fluorescence for rPMT was 46.5 (A) and for PMT-CE was 60.4 (B).

VITA

Rajeev Vasukuttan Nair was born in Changanasserry, Kerala State, INDIA on May 3, 1963. He graduated from N.S.S. High School, in Changanasserry, Kerala in March 1978. He had his Pre-degree Courses at St. Berchmans' College, Changanasserry, Kerala. After his Pre-degree, in 1980 he joined the College of Veterinary and Animal Sciences in Kerala Agricultural University at Trissur, Kerala. In March 1986, he graduated with a Bachelor of Veterinary Sciences and Animal Husbandry (BVSc&AH). He worked for the Kerala Government as a Veterinary Surgeon from April 1986 to July 1997. He was an Undergraduate Student at University of Tennessee, Knoxville from August 1998 to July 1999 and received a second Undergraduate degree in Animal Science. He began graduate studies in the Comparative and Experimental Medicine graduate program at The University of Tennessee, Knoxville in August 1999. He completed his graduate studies in August 2001.