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Extracorporeal Endotoxin Removal by Polymyxin B Immobilized Fiber Cartridge: Designing and Antiendotoxin Efficacy in the Clinical Application

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Abstract: We have developed an extracorporeal hemoadsorption cartridge, the PMX cartridge, to eliminate endotoxin from peripheral blood circulation. As an adsorbent, a polymyxin B covalently immobilized fiber (PMX-F) was developed. After the optimization of the condition of immobilization, fixed polymyxin B maintained its ability to adsorb endotoxin and its bactericidal activity. PMX-F could detoxify many kinds of endotoxin in vitro. Fixed polymyxin B was estimated to interact with the lipid A portion of endotoxin. Utilization of fibrous adsorbents enabled us to design the PMX cartridge with a large surface area and low blood pressure drop in the blood flow compartment and to apply it safely to the direct hemoperfusion procedure. In Japan, the PMX cartridge is now being clinically applied as one of the therapeutical interventions for sepsis, septic shock, and septic multiple organ failure. In multicenter clinical studies, the blood endotoxin level has been significantly decreased. Accompanied with elimination of endotoxin, hemodynamic abnormalities such as low blood pressure and low systemic vascular resistance were significantly improved. In more recent multicenter studies, the average number of failed organs; severity of illness score, such as Goris score; and vasopressor dosage were significantly decreased. The PMX cartridge is expected to be effective in the intervention for the treatment of septic shock. Endotoxin may be one of the therapeutical targets for the treatment of sepsis. **Key Words:** Hemoadsorption—Endotoxin removal—Sepsis.

Sepsis and septic shock continue to be life-threatening complications and major causes of deaths in medical and surgical ICUs. During the last decade, the progress of research on the pathogenesis and pathophysiology of sepsis, septic shock, and subsequent septic multiple organ dysfunction syndrome (MODS) has clarified some mechanisms of diseases (1–4). Many kinds of mediators such as cytokines, nitric oxide (NO), reactive oxygen species, and lysosomal enzymes are liberated by the activation of leukocytes, macrophages, and endothelial cells. They are estimated to play a substantial role in the pathophysiology of septic shock and MODS.

Endotoxins, which consist of lipopolysaccharide (LPS), are outer membrane components of gram-negative bacteria and are theorized to be an important pathogeneic substance to trigger the production of a variety of mediators inducing systemic inflammation. In patients with septic shock, the detectable levels of endotoxin in peripheral blood circulation are correlated with positive blood cultures, lactic acidemia, low systemic vascular resistance, and a depressed ventricular ejection fraction. Recently, many different antiendotoxin strategies have been investigated. For example, the antiendotoxin monoclonal antibodies, HA-1A (5–7) and E-5 (8–9), have been developed. Unfortunately, they could not demonstrate a beneficial effect in their clinical trials. The failure of the antiendotoxin monoclonal antibody trials raised the controversy about endotoxin as the therapeutic target. Interest in antiendotoxin therapies persists, the goal being the neutralization of the deleterious effect of endotoxin. The development of novel therapeutic reagents such as rec-BPI (bactericidal/permeability increasing protein) (10,11), rec-ENP (endotoxin neutralizing protein) (12,13), CAP-18 (LPS-binding/antimicrobial protein) (14), and E-

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5531 (specific lipid A analogue) (15) are on-going. On the other hand, approaches to eliminate endotoxin from solution have been suggested since 1970. In the 1970s, Nolan et al. (16) reported endotoxin adsorption by cholestyramine, ion exchange resin, and activated charcoal. Since then, new adsorbents such as polymyxin B (PMX-B) immobilized Sepharose 4B (17–19), histamine immobilized Sepharose 4B (20), PMX-B immobilized polystyrene based fiber (PMX-F) (21), polyethylenimine immobilized macroporous hydrophilic cellulose beads (22), anion exchange resin (23), and PMX-B immobilized acrylic beads (24) have been developed.

From the viewpoint of extracorporeal endotoxin removal, various forms of apheresis techniques have been suggested. Activated charcoal bead hemoperfusion was investigated experimentally in the endotoxin shock of dogs (25). The measurement of blood endotoxin levels demonstrated that the majority of circulating endotoxins could be eliminated from the blood. Activated charcoal was also applied clinically to treat endotoxic shock neonates and demonstrated the efficacy of this therapy (26). Whole blood exchange (27,28), plasmapheresis (29), and direct hemoperfusion using PMX-F have also been applied clinically. Plasma adsorption using PMX-B attached Sepharose 4B (30) and direct hemoperfusion with PMX-B fixed acrylic beads (24) have been evaluated in animal experiments. Recently, a microsphere based detoxification system was proposed (31). Separated plasma is processed in the secondary circuit which includes the microsphere adsorbent. A high blood flow rate and microspheres in the second circuit enable highly efficient protein-bound or hydrophobic substance removal.

Since 1983, we have been developing the PMX endotoxin removal cartridge (Fig. 1) containing PMX-F and applicable in direct hemoperfusion (Fig. 2). Clinical trials were conducted in 6 university hospitals during the time period from 1989 to 1991, demonstrating the safety and effectiveness of the PMX for the treatment of septic shock. Since 1994, the PMX cartridge has been commercially available, and it now is approved as a therapeutic device by the health insurance system in Japan.

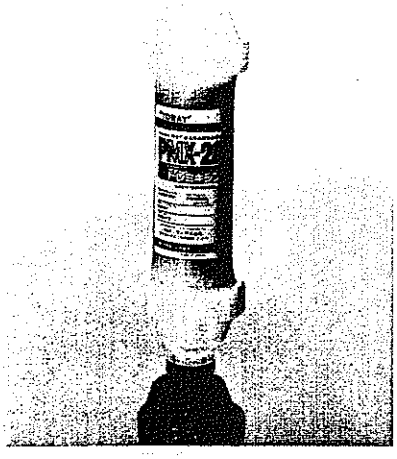
Endotoxin neutralizing reagents have been developed. However, they have not succeeded in multicenter clinical trials. In patients with endotoxemia, their detoxification systems are already depressed (32), thus allowing the overflow of endotoxin into the blood circulation. Therefore, the immune complexes formed with injected reagents and endotoxin cannot be sufficiently processed in the body. Thus, this may result in worsening of diseases. The fate of circulating immune complexes is fully understood. We think it is rational to eliminate circulating endotoxin from the blood circulation rather than neutralize it with reagents in the body.

In this article we describe the designing of the polymyxin B immobilized fiber cartridge and its clinical effectiveness in the treatment of sepsis.

DESIGNING OF POLYMYXIN B IMMOBILIZED FIBER CARTRIDGE TO ADSORB ENDOTOXIN

Polymyxin B antibiotics as ligand for selective adsorbent

Polymyxin B is an antibiotic agent that has a strong bactericidal activity to gram-negative bacte-



Measurements (mm)	length	225
	diameter	49
sterilization		Steam autoclaving
filled fluid		Physiological saline
Priming volume (ml)		135

FIG. 1. The photograph shows the endotoxin adsorption cartridge (PMX) used for clinical application.

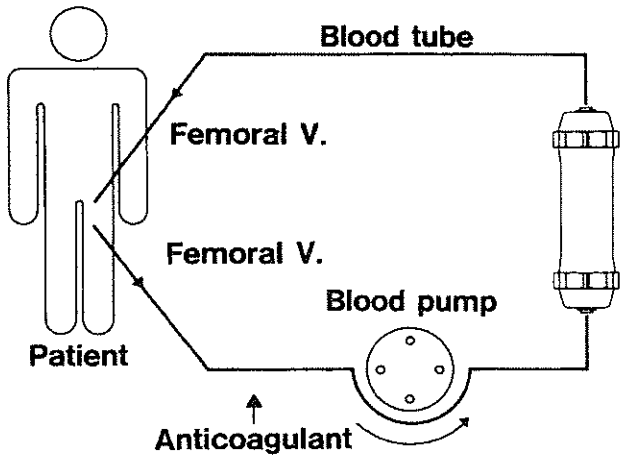


FIG. 2. The circuit diagram for extracorporeal endotoxin removal by the PMX cartridge is shown.

ria, particularly to the *Pseudomanas* species. The mechanism of action is attributed to the electrostatic interaction of cationic antibiotic molecules with acidic phospholipids and the LPS of the outer membrane of gram-negative bacteria, which results in the alteration of the permeability of the outer membrane. Besides this activity, PMX-B has been reported to neutralize endotoxin toxicity, including lethal toxicity (33), the Schwartzman reaction (34), certain blood coagulation defects (35), limulus gelation activity (36), and hemodynamic effects (37). The protective effects of PMX-B on endotoxin induced shock animals have also been reported (38–40).

Electron microscopic studies have demonstrated the structural changes of LPS exposed to PMX-B, from its typical ribbon-like structure to only short sections or completely disaggregated material (41). These changes could be derived from the dissociation of the micellar structure of LPS by the detergent-like activity of PMX-B (42). In 1973, J. Bader and M. Teuber reported the binding of PMX-B to the O-antigenic LPS of *Salmonella typhimurium* due to electrostatic and possibly hydrophobic interactions and pointed to the negatively charged ketodeoxyoctonate-lipid A region of LPS as the binding site (43). This result was also supported by Morrison and Jacobs in 1976 (44). They demonstrated the direct interaction of PMX-B with the lipid A 2-keto-3 deoxyoctulosonate (KDO) region of the LPS molecule and suggested the stoichiometric relationship of the binding of PMX-B to LPS with 1 PMX-B molecule binding to 1 LPS monomer unit.

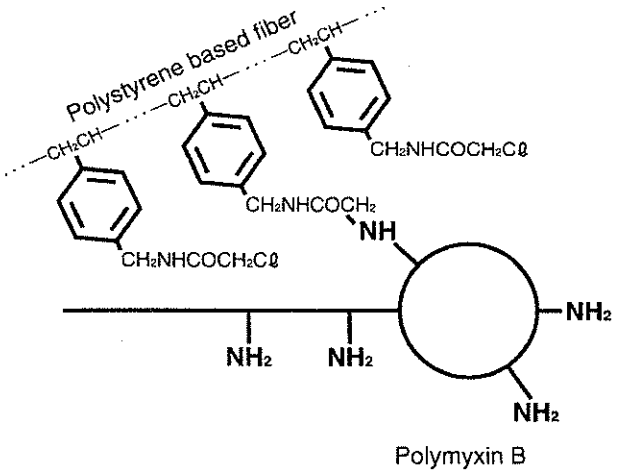
These previous studies clarified the binding of PMX-B to LPS and the resulting detoxification characteristics, the binding being highly related to the mechanism of antimicrobicidal activity of PMX-B. However, due to its nephrotoxicity and neurotoxicity, PMX-B cannot be intravenously injected in clinical usage. These facts have strongly suggested the use of PMX-B as the first candidate ligand to adsorb endotoxin selectively. Thus, PMX-B was attached to an insoluble carrier as a ligand to bind endotoxin.

Immobilization of PL-B (45)

PMX-B was immobilized covalently on the surface of a polystyrene-derived fibrous carrier (Fig. 3). As the carrier we applied fibrous material, which was composed of polypropylene reinforced conjugated fibers. Polystyrene was chemically modified, and the α-chloroacetoamide methyl group was introduced as the functional group to fix PMX-B. PMX-B has 5 primary amino groups originating from α,γ-diaminobutyric acid within the molecule. Covalent bonding was performed by making use of these primary amino groups and an active chlorine atom in the functional groups of the carrier fiber. As shown in Fig. 4, the endotoxin detoxification capacity of PMX-F changes depending on the residual number of primary amino groups in the fixed PMX-B. This means that PMX-F can acquire the best endotoxin detoxification capacity in the case of 1 to 2 points binding to the carrier fiber. This relationship was also observed in an in vivo animal experiment. The survival rate of endotoxin challenged dogs was higher when fixed PMX-B had a large number of primary amino groups (Fig. 5). Because primary amino groups are positively charged, they seem to play a major role in ionic binding to LPS.

Antiendotoxin efficacy of PMX-F in vitro (45)

PMX-F effectively adsorbed synthetic lipid A by the limulus amebocyte lysate (LAL) assay, but the carrier fiber was not effective (Fig. 6). The binding characteristics to lipid A were kept, even in fixed PMX-B. The lipid A portion of endotoxin is the least variable region of active LPS. It does not change from species to species or strain to strain. As is



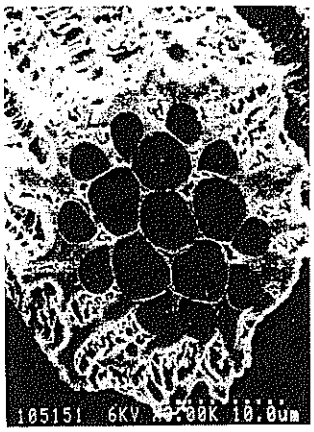


FIG. 3. The schematic diagram of polymyxin B immobilized fiber (PMX-F) accompanies the cross sectional view of PMX-F.

shown in Fig. 7, PMX-F could adsorb many kinds of LPS that have highly changeable O-side chains and chemotypes. These results indicate that fixed PMX-B could bind to the lipid A portion of LPS. Besides the detoxification of the limulus activity of LPS, adsorption of LPS from serum solution by PMX-F proved the neutralization of pyrogenic activity by a rabbit pyrogenic test.

Microbicidal activity of PMX-F

A previous study reported on growth inhibition of *Escherichia coli* and *Pseudomonas aeruginosa* by PMX-B covalently attached to agarose beads (46). They demonstrated that fixed PMX-B can inhibit the growth and respiration of gram-negative bacteria by interacting with the outer surface of these cells. They also proposed that perturbation of the outer membrane structure by PMX-B-agarose indirectly affected the selective permeability of the inner membrane. PMX-F was added to a phosphate buffer solution containing *P. aeruginosa,* the solution given 9 h of continuous stirring, and the changes of bacterial cell counts were traced. The bacterial cell counts were sharply decreased from 10^8 to 10^2 in as few as 4 h (Fig. 8). This demonstrated that PMX-F has microbicidal activity. The previous study and our data clearly demonstrated that immobilized PMX-B could well maintain the original characteristics of PMX-B as antimicrobial reagent.

Designing of PMX cartridge

Knitted fabric of PMX-F was rolled up and embedded into the cartridge case as a structural component of the adsorbents. The PMX cartridge was autoclave-sterilized, and the cartridge was filled with physiological saline. Application of a thin fibrous carrier enabled us to obtain a hemoadsorption cartridge with a large surface area and a low pressure drop in the blood flow compartment. A direct hemoperfusion procedure could be practiced steadily.

Removal of cytokine inducing substances by PMX

Recently, Jaber et al. (47,48) evaluated the in vitro efficacy of the PMX cartridge in a model using 10% human plasma challenged with endotoxin, gram-negative bacteria, or gram-positive bacteria. Cytokine production by peripheral blood mononuclear cells (PBMC) incubated with plasma before and after hemoperfusion was used as the index of removal.

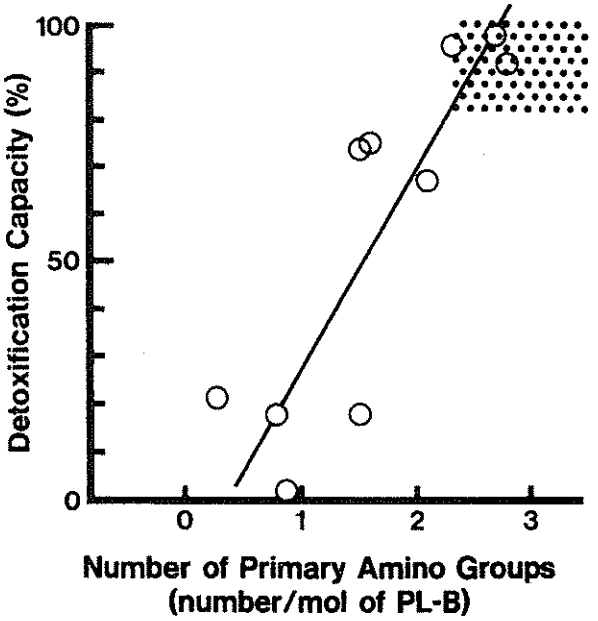


FIG. 4. The graph shows the relationship between the residual number of primary amino groups in fixed polymyxin B and detoxification capacity. PMX-F was added to a bovine serum solution containing *E. coli* LPS with continuous stirring for 2 h, and the concentration was measured by LAL assay. PMX-F had the best endotoxin detoxification capacity when it had 1 to 2 points binding to the carrier fiber.

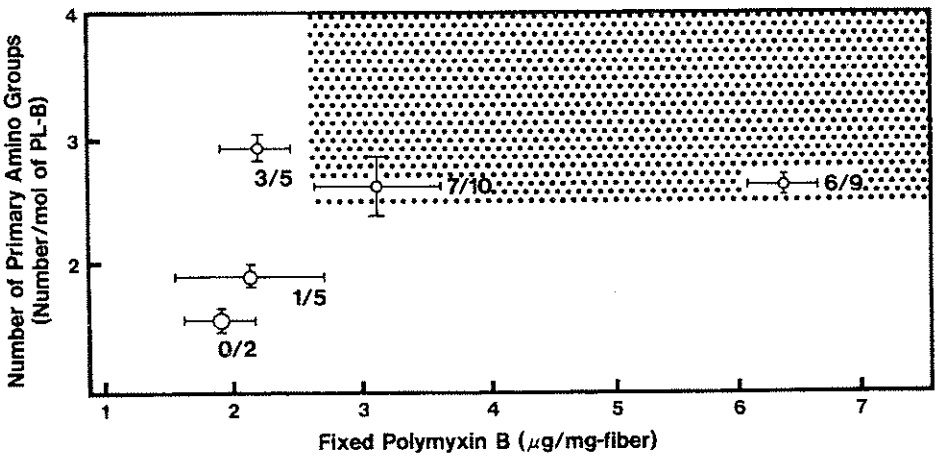


FIG. 5. The graphic shows the relationship between the characteristics of fixed polymyxin B and the survival rate of endotoxin challenged dogs. The number of primary amino groups in fixed polymyxin B more affected the survival rate of dogs than the quantity of attached polymyxin B.

After 2 h of hemoperfusion through PMX, tumor necrosis factor α (TNF α) production was significantly decreased in the 3 settings. The results of hemoperfusion by 10% human plasma challenged with *Staphylococcus aureus* demonstrated the removal of cytokine inducing substances (CISs) such as peptidoglycans and lipoteichoic acids. They indicated that the removal by the PMX cartridge might have been partly due to the stoichiometric binding of the CISs to the PMX cartridge. The hydrophobic nature and large surface area of PMX-F might explain the binding of the CISs to PMX-F. It was thus suggested that the PMX cartridge can be applied not only to gram-negative sepsis, but also to gram-positive sepsis.

ANIMAL EXPERIMENTS

Efficacy of PMX treatment in an endotoxin-challenged canine model

Direct hemoperfusion with the PMX cartridge, a carrier fiber cartridge without fixed polymyxin B, and a polymer coated activated charcoal bead cartridge was performed on LPS challenged (0.75 mg/kg body weight) dogs for 2 h at a blood flow rate of 80 ml/min (49). Blood chemistry, blood cell counts, and hemodynamics were monitored. The 7 day survival rates were 73% (11/15), 0% (0/13), and 20% (1/5), respectively. The group treated with PMX showed quick recovery from severe hypotension. Blood levels of lactic acid were increased to 6.4 times higher than normal in the charcoal group, 3.7 times in the carrier fiber group, and 3.2 times in the PMX group, respectively. The PMX cartridge was effective in decreasing the severity of hypotensive shock and in improving the survival rate in this canine model.

In 1993, Sato et al. (50) evaluated the efficacy of treatment by extracorporeal perfusion on endotoxin infused canine septic shock. Dogs were treated by 3 different techniques: no treatment (sham group), treatment with a PMX cartridge (PMX group), and treatment with plasma perfusion over anion exchange resin (resin group). In the PMX group, the neutrophil phagocytic function evaluated by measuring the opsonic index was higher than in the sham group. The level of CH_{50} in the PMX group was significantly higher than the CH_{50} levels in either the resin or sham groups at 6 h. Blood endotoxin levels in the PMX group were significantly lower with a significant suppression of TNF production. From these results, they hypothesized that hemoperfusion

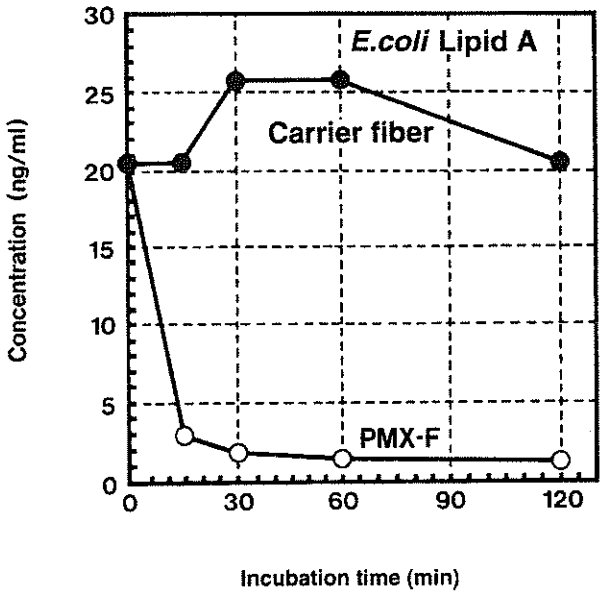


FIG. 6. Shown are changes of lipid A concentration by PMX-F. PMX-F was added to bovine serum solution containing *E. coli* type synthetic lipid A with continuous stirring. The lipid A level was measured by LAL assay.

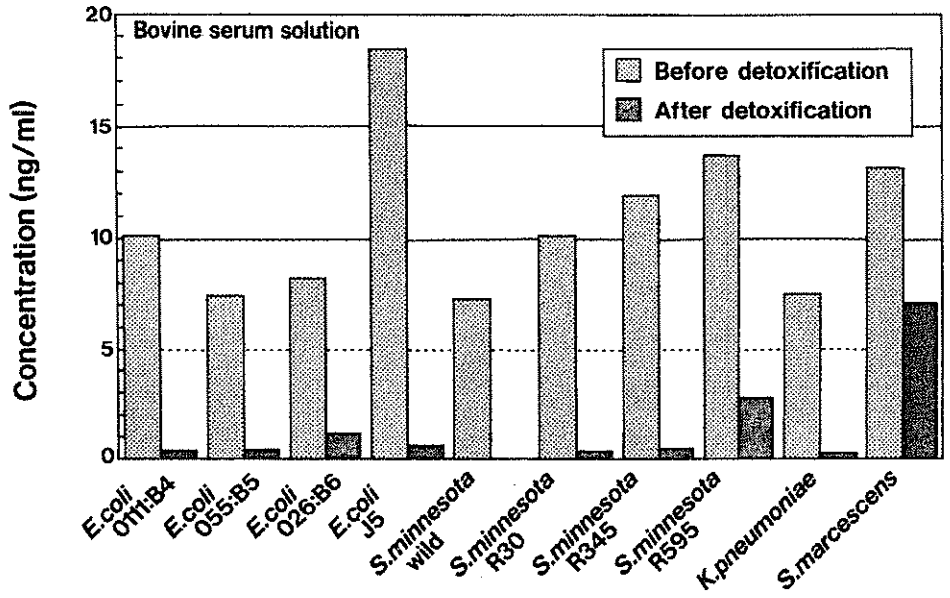


FIG. 7. Shown are the detoxification characteristics of PMX-F against endotoxin extracted from different species or strains of gram-negative bacteria.

over PMX could prevent attenuation of phagocytic function because of the detoxification of endotoxin. As a result, the 24 h survival rates of the sham, PMX, and resin groups were 0%, 80%, and 40%, respectively. They concluded that hemoperfusion over the PMX cartridge detoxified the circulating endotoxin and improved the systemic derangement caused by endotoxic shock.

Efficacy of PMX in a live bacteria challenged septic canine model

Escherichia coli challenged septic dogs were treated with hemoperfusion over the PMX column and concomitant administration of gentamicin as the antibiotic (51). In control experiments, a cartridge without PMX-F adsorbent was used. The method of perfusion was the same as that of the LPS challenged canine experiment. Plasma glucose and lactate levels were measured during the treatment to estimate the metabolic changes over 6 h after bacterial challenge. All dogs in both groups had transient hyperglycemia lasting for 2 h. In the PMX treated group, the glucose level recovered to the pretreatment value at 4 h after hemoperfusion. On the other hand, the plasma glucose level in the control group continuously increased to the hyperglycemic level. The plasma lactate level also recovered after hemoperfusion in the PMX group. However, lactic acidosis persisted in the

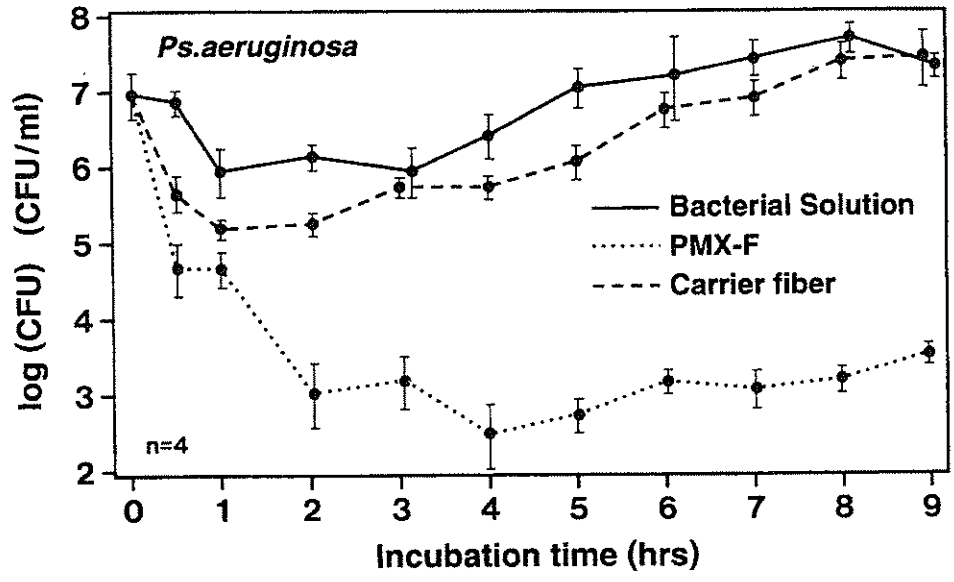


FIG. 8. The graph shows the bactericidal activity of PMX-F against *P. aeruginosa.*

control group. All dogs in the control group died within 18 h of bacterial challenge. In contrast, 2 of the 5 dogs in the PMX group survived, and the other 3 dogs were alive for more than 3 days. These results indicated that the PMX cartridge treatment was effective in improving the survival rate of septic dogs.

CLINICAL EFFECTIVENESS OF PMX FOR THE TREATMENT OF SEPSIS, SEPTIC SHOCK, AND SEPTIC MOF

Patient profile

A multicenter clinical trial was undertaken in 6 domestic university hospitals, mainly in ICU settings from 1989 to 1991 (52–54). Forty-two patients (male, 33; female, 9) were enrolled. PMX cartridge treatment was performed in patients with confirmed infection showing an infection focus or those with positive results for the blood endotoxin concentration who had not responded appropriately to various conventional therapies. Neoplasms were the most frequent underlying diseases (Table 1). Abdominal infections were detected in 27 cases (64.3%), chest infections in 6 cases (14.3%), and other infection focuses in the remaining 9 cases (21.4%). Infections with gram-negative or gram-positive bacteria were seen in twenty-five (59.5%) and 8 patients (19.0%), respectively. In 9 patients (21.4%), bacteria was not detected. Of 42 patients, 38 patients (90.4%) had septic multiple organ failure (MOF). At the initiation of PMX cartridge treatment, 33 patients (78.6%) were receiving vasopressors to maintain blood pressure and 36 (85.7%) were under mechanical ventilation through endotracheal intubation. A total of 61 PMX cartridge treatments were performed in these 42 patients. The frequency of PMX cartridge treatments was determined by the patients' conditions. Thirty patients (71.4%) received 1 PMX treatment, 9 patients (21.4%) received 2, 2 patients (4.7%) received 3, and 1 patient (2.3%) received 7.

TABLE 1. *Underlying diseases of patients receiving PMX treatments*

Mean age: 61.4 years (18–84 years) Sex: males:females (33:9)		
Underlying diseases	Neoplasm	22 patients
	Collagen disease	3
	Diabetes mellitus	2
	Liver cirrhosis	2
	Circulatory disease	11
	Respiratory disease	4
	Subarachnoid hemorrhage	4
	Postoperative	19
	Trauma	9

Extracorporeal hemoperfusion was safely carried out for 2 h at a blood flow rate of 100 ml/min with anticoagulant infusion of heparin or nafamostat mesilate (a protease inhibitor).

A side effect of PMX cartridge treatment was a slight decrease in the platelet counts due to blood cell and adsorbent interaction occurring during direct hemoperfusion. However, neither bleeding tendencies nor exacerbation of hemorrhage was observed.

Endotoxin level

Endotoxin was detected in 37 of 42 patients (88.1%). The average endotoxin level before treatment was 85.0 ± 27.2 pg/ml (50/61 PMX cartridge treatment cases). After extracorporeal hemoperfusion for 2 h, the concentration was significantly decreased to 57.2 ± 28.4 pg/ml.

Hemodynamic improvements

A previous study on endotoxin injection into normal human volunteers showed marked changes in hemodynamic parameters such as blood pressure, the cardiac index, and the systemic vascular resistance (SVR) (55). In this PMX cartridge clinical trial, improvement of hemodynamic abnormalities was clearly recognized. In patients whose systolic arterial blood pressures were below 90 mm Hg even with vasopressor infusion (7/61 cases), blood pressure significantly increased from 75.0 ± 3 mm Hg to 88.0 ± 4.8 mm Hg. In patients whose systolic blood pressure was maintained over 90 mm Hg with the infusion of vasopressors (28/61 cases), blood pressure significantly increased from 125 ± 4.7 mm Hg to 133 ± 4.8 mm Hg. In low SVR cases of 900 dyne-s-cm^{-5} or less, the mean SVR was significantly increased from the pretreatment value of 647 ± 36.5 (18/61 cases) to the posttreatment value of 729 ± 51.4. With the improvement of hemodynamics, antihypotensive drug (usually dopamine, dobutamine, or noradrenalin) infusion was discontinued in 8 patients (24.2%), and the dosage was reduced in 6 patients (18.2%).

Survival rate

As a result, 22 of the 42 patients (52%) had survived at Day 14 after PMX cartridge treatment. In this multicenter clinical trial, the survival rate was compared retrospectively between 37 patients who received PMX cartridge treatment and 34 patients who received conventional therapy without the PMX cartridge (control group). Patients in both groups were endotoxemic and received full intensive care managements. The original severity of illness score was much higher in the PMX cartridge treat-

ment group as shown in higher numbers of failed organs (56) and higher sepsis severity score (57) (Table 2). Despite higher severity of illness in the PMX cartridge treated group, the survival rate of the PMX cartridge treatment group was higher than that of the control group.

Recent multicenter studies

Recently, results of 2 other multicenter studies were presented (58). As a phase IIIa study, 99 patients were enrolled. LAL assay showed a significant reduction of endotoxin levels. The decrease of endotoxin levels as measured by an endotoxin specific assay showed a correlation with the clinical outcome and the changes in parameters such as blood pressure, body temperature, and an oxygenation index. In a phase IIIb study, 86 patients were enrolled. Eighty-one of them were MOF patients, and 77 patients were in shock. The mean APACHE II score was 24.2 ± 1.0. The number of failed organs was improved from 4.6 ± 0.2 before the PMX cartridge treatment to 2.4 ± 0.3 after 14 days, and the Goris score was decreased from 6.1 ± 0.3 to 3.0 ± 0.4. The mean dose of dopamine decreased from 8.8 μg/kg/min to 6.8 μg/kg/min. The survival rate was 51.1% at 14 days and 39.8% at 28 days. Endotoxin adsorption therapy with the PMX cartridge improved the Goris score and the number of failed organs after 14 days. Despite the very high APACHE II scores, the survival rate was shown to be around 50% to 40%.

CONCLUSION

As one of the antiendotoxic strategies for the treatment of sepsis, an endotoxin removal cartridge composed of polymyxin B immobilized fiber, the PMX cartridge, was developed. The PMX cartridge has been safely applied clinically in Japan since 1989 without the occurrence of any critical adverse effects. Improvement of hemodynamic abnormalities is one of the beneficial features of PMX cartridge treatment. Though a control study has not been tried yet, endotoxin adsorption therapy seems to be effective in improving the morbidity and mortality of septic patients. Our study, as well as others, strongly suggested that blood endotoxin could be one of the therapeutical targets for the treatment of sepsis.

TABLE 2. *Comparison of the survival rates of 37 patients who received PMX treatments and 34 patients who received conventional therapy without PMX*

	PMX treatment (M ± SE)	Conventional (M ± SE)	p value
Failed organs	3.8 ± 0.3	3.1 ± 0.2	<0.05
Septic severity score	46.2 ± 3.2	39.1 ± 2.7	<0.05
Survival rate (%)	54 (20/37)	38 (13/34)	<0.05

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