Preparation of a superantigen-adsorbing device and its superantigen removal efficacies in vitro and in vivo

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Objective: A new superantigen-adsorbing device (SAAD) was developed, and its characteristics and efficacy in septic animals were evaluated.

Methods: The SAAD was prepared by stepwise chemical modification of a polystyrene-based composite fiber reinforced with polypropylene. Adsorption affinities for several factors and the biological effect of superantigen (SAg) removal were measured in vitro. Also, superantigen-infused rabbits were treated with SAAD, and the efficacy was evaluated in vivo.

Results: When the SAAD was evaluated for its ability to adsorb SAg in human plasma (1 ng/mL each), the adsorption rates were 74%, 76% and 85% for staphylococcal enterotoxins A, B and C, respectively, and 80% and 72% for toxic shock syndrome toxin-1 (TSST-1) and streptococcal pyrogenic exotoxin A, respectively. In addition, the SAAD showed some affinity towards other molecules, such as streptococcal pyrogenic exotoxin B, β2-microglobulin, and vancomycin. Residual activities in whole blood samples containing TSST-1 (1 ng/mL) after incubation with the SAAD were 125 pg/mL for tumor necrosis factor alpha (TNF-α) production, and 359 pg/mL for interleukin-8 (IL-8) production (the initial activities: 194 pg/mL for TNF-α production, and 1029 pg/mL for IL-8 production). When TSST-1/lipopolysaccharide (LPS)-infused rabbits were subjected to extracorporeal blood purification with a SAAD column, 50% of the animals survived for a 14-day period after the infusion. In contrast, all control animals died within 3 days after the infusion.

Conclusion: These results indicate that the SAg-adsorbing device may be useful in treating SAg-related diseases.


INTRODUCTION

Gram-positive sepsis is one of the serious problems seen in severe bacterial infections. Several bacterial products, such as staphylococcal enterotoxins A-E (SEA-SEE), toxic shock syndrome toxin-1 (TSST-1), streptococcal pyrogenic exotoxins A and C (SPEA and SPEC), streptococcal mitogenic exotoxins, and Yersinia-derived mitogen, have recently been shown to have superantigenic activities and are now generally recognized as superantigens (SAgs).

There is accumulating evidence that SAgs are pathogenic substances causing systemic inflammation, sepsis, septic shock, and subsequent multiple organ failure. In fact, SAgs have been shown to be present in the peripheral blood of patients with toxic shock syndrome (TSS) and streptococcal toxic shock syndrome (STSS). Our recent studies have shown that SAgs bind to the major histocompatibility complex (MHC) class II; this event is linked to activation of T cells and induction of pro-inflammatory cytokine production. Furthermore, similar to the case reported with a SAg-related VB, specific T-cell expansion was observed in patients with TSS, neonatal TSS-like exanthematous disease (NTED), and methicillin-resistant Staphylococcus aureus (MRSA)-associated glomerulonephritis.

Considering that SAgs are present in circulating blood and play a major role in the pathogenesis of sepsis during the course of bacterial infections, one of the most direct and promising methods to prevent the development of sepsis would be removal of systemically circulating SAgs from the body fluid by extracorporeal circulation. Although various polymers, including ion-exchange resins and polymer-coated activated charcoal, have been examined, none of these polymers exhibited suitable characteristics, especially in terms of SAg adsorption specificity and bloodflow resistance, for practical use as adsorbents in clinical settings.

In the present study, we have developed a new SAg-adsorbing device (SAAD) that can be used in extracorporeal circulation in clinical settings. The device was prepared by chemical modification of a polystyrene-based composite fiber reinforced with polypropylene. This paper describes the preparation of the device and its effectiveness in adsorbing SAgs in vitro and in vivo.
MATERIALS AND METHODS

Chemicals, biological preparations, and animals

The purified SAgs (SEA, SEB, SEC, TSST-1, and SPEA) and SPEB were purchased from Toxin Technology (Sarasota, FL, USA); staphylococcal protein A (Protein A) was obtained from EY Laboratory (San Mateo, CA, USA); anti-ProteinA chicken polyclonal antibody was obtained from BioGenesis (Poole, UK): *Pseudomonas* exotoxin A (ExA) and anti-ExA antibody were obtained from LIST Biology Laboratories (Campbell, CA, USA); lipoteichoic acid (LTA) (derived from *S. aureus*) was obtained from Sigma (St Louis, IL, USA); endotoxin (lipopolysaccharide (LPS), peptidoglycan (derived from *Micrococcus luteus*) (PepG) and reagent kits for LPS-specific limulus assay and silkworm larval plasma (SLP) reagent assay were obtained from Wako Pure Chemicals (Tokyo, Japan); the purified recombinant cytokines, human interleukin (IL)-6, human IL-8, human macrophage chemoattractant protein 1 (MCP-1) and human tumor necrosis factor alpha (TNF-α), were obtained from Genzyme Tecline (Minneapolis, MN, USA); and human plasminogen activator inhibitors of the active form (PAI-la) and latent form (PAI-11) were obtained from Molecular Innovation (Southfield, MI, USA).

Polyclonal antibodies against SPEB, LTA, PAI-1a, and PAI-11 were prepared by immunization of rabbits with the respective purified antigens.

Vancomycin (VCM) and cefaclor (CCL) were obtained from Shionogi Pharmaceuticals (Osaka, Japan), arbekacin (ABK) and penicillin G (PCG) were obtained from Meiji Seika (Tokyo, Japan), clindafluycin (CLDM) was obtained from Pharmacia and Upjohn (Tokyo, Japan), and miconazole (MCZ) was obtained from Mochida Pharmaceuticals (Tokyo, Japan).

With the approval of the Institutional Review Board of Toray Pioneering Research Laboratory, blood samples were collected from healthy volunteers. Heparin was added to each blood sample to give a concentration of 5 unit/mL immediately after collection. Heparinized blood was taken in aliquots of 50 mL and centrifuged at 2000 g (3000 rev/min) to separate plasma samples.

Male New Zealand white rabbits, 13–15 weeks old, weighing 2.5–3.0 kg, were obtained from a local supplier (Shimizu Laboratory Supplies, Kyoto, Japan).

Preparation of superantigen adsorbent

A polystyrene-based composite fiber reinforced with polypropylene was prepared by a method described elsewhere. In the first step of the modification, composite fiber (100 g) was treated with *N*-methylol-α-chloroacetamide (182 g) in a mixture of sulfuric acid (1300 g) and nitrobenzene (1300 g) at 15°C for 2 h in the presence of paraformaldehyde (2.6 g). The treated fiber was washed once with nitrobenzene and five times with methanol. The fiber was dried at room temperature. In the second step, the fiber was treated with tetraethylpentamethylenediamine (47 g, Katayama Chemical, Osaka, Japan) and triethylamine (77 g, Katayama Chemical) in dimethylformamide (DMF, 3000 mL) for 3 h with mechanical stirring at 30°C. The resulting fiber was thoroughly washed with DMF. In the third step, the fiber was reacted with 4-chlorophenylisocyanate (72 g, Aldrich, Milwaukee, WI, USA) in DMF (3000 mL) at 30°C for 1 h with stirring. The reacted fiber was thoroughly washed with DMF and then with pyrogen-free distilled water. The urea-bond fiber thus obtained was sterilized in pyrogen-free saline by irradiation with gamma rays (2.5 Mrad), and then kept at 4°C until use.

Quantification of the functional group

The number of functional groups (4-chlorophenylurea groups) bound to the fiber was quantitated by spectrophotometric determination of 4-chloroaniline released from the fiber on hydrolysis. Hydrolysis of the SAAD (0.25 g) was carried out in 6 M hydrochloric acid (4 mL) for 20 h at 110°C. For quantitation of the released 4-chloroaniline, 1 mL of the supernatant of the reaction mixture was mixed with 0.5 M hydrochloric acid (1 mL). To the mixture, sodium nitrite (0.04 mg), ammonium sulfamate solution (3 mg/mL, 1 mL) and N-1-naphthyl-ethylenediamine dihydrochloride solution (0.7 mg/mL, 1 mL) were added, in that order. The reaction mixture was allowed to stand at room temperature for 30 min, and then read spectrophotometrically at 545 nm.

Measurement of superantigens and other bacterial products

SAgs were measured by the ELISA method previously reported. Briefly, samples were diluted two-fold with Tris-HCl buffer (pH 8.0) containing 0.25% bovine serum albumin (BSA), 0.05% Tween 20, 10 μg/mL mouse IgG, and 10 μg/mL rabbit IgG. The diluted samples were placed in wells of an anti-SAg polyclonal antibody immobilized ELISA plate (Nunc, Roskilde, Denmark). The plate was incubated for 1 h at 25°C, and each well was then treated with horseradish peroxidase (HRP)-labeled monoclonal antibody to the given SAg. The peroxidase activity was visualized by the reaction with 3,3',5,5'-tetramethylbenzidine (0.2 mg/mL) in 0.1 M citric acid, pH 3.5, in the presence of hydrogen peroxide (0.9%) at room temperature for 30 min. The reaction was terminated with 0.5 M sulfuric acid, and the reaction mixture was read spectrophotometrically at 450 nm. Standard solutions were prepared by serial dilution of the SAg original solution in phosphate-buffered saline (PBS) containing 0.25% BSA and 0.05% Tween-20. Each of the polyclonal antibodies was confirmed to be specific to the given SAg and to exhibit no significant
cross-reactivity with any of the other SAGs, fetal bovine serum, or healthy human plasma.

The bacterial products SPEB, ProteinA, ExA and LTA were also quantified by ELISA in a manner similar to that described above for quantitation of SAGs, except that unlabeled polyclonal and HRP-labeled polyclonal antibodies against these bacterial products were used as the capture and detecting antibodies, respectively. Endotoxin (LPS) and PepG were measured by the LPS-specific limulus assay and SLP reagent assay, respectively, using the respective purified toxin preparations as the standards.

Measurement of cytokines

Concentrations of the cytokines IL-6, IL-8 and MCP-1 were measured by ELISA, using purified recombinant cytokines as the standards.

In vitro batch adsorption of superantigens in fetal calf serum

In order to minimize the contribution of nonspecific adsorption, the SAAD (0.06 g) was pretreated with BSA (0.5 mg/mL) in PBS (pH 7.4, 2 mL) for 1 h at room temperature. The BSA-pretreated SAAD (0.06 g) was incubated with SEA, SEB, SEC, TSST-1 or SPEA (1 ng/mL) in fetal calf serum (FCS, 1 mL) at 37°C for 2 h with gentle rotary mixing. The supernatant of the resulting incubation mixture was analyzed for the residual concentration of the superantigen by ELISA.

In vitro adsorption of non-superantigenic bacterial products, cytokines and antimicrobials

The rates of adsorption on the SAAD of various non-superantigenic bacterial products, cytokines and antibiotics were examined in a similar manner to that described above for adsorption of SAGs. The initial concentrations were: SPEB, 1 ng/mL; ProteinA, 10 ng/mL; ExA, 10 ng/mL; LPS, 1 ng/mL; LTA, 100 ng/mL; PepG, 10 ng/mL; TNF-α, 10 ng/mL; IL-6, 10 ng/mL; IL-8, 10 ng/mL; MCP-1, 10 ng/mL; PAI-1a, 10 ng/mL; PAI-11, 10 ng/mL; VCM, 50 μg/mL; ABK, 10 μg/mL; PCG, 10 μg/mL; CLDM, 20 μg/mL; CCL, 20 μg/mL; MCZ, 10 μg/mL. The initial concentrations of the antimicrobials were selected according to the maximum plasma concentrations in patients seen in clinical settings.

In vitro adsorption of human plasma proteins

Human plasma was incubated in the presence of the SAAD at 37°C for 2 h. After removal of the SAAD, the plasma was analyzed for the residual levels of total protein (TP), albumin (Alb), immunoglobulins G, A and M (IgG, IgA, IgM), high-density and low-density lipoprotein (HDL and LDL), complement factors 3, 4 and 5 (C3, C4, C5), β₂-microglobulin (β2MG), bilirubin (Bil) and myoglobin (Mgb) by the conventional methods.

Biological effect of superantigen removal (cytokine induction assay)

TSST-1-challenged plasma was prepared by the addition of TSST-1 to human plasma at a concentration of 1 ng/mL. TSST-1-challenged plasma was incubated at 37°C for 2 h in the presence of the SAAD (0.065 g/mL, 2 cm²/mL). In a control experiment, TSST-1-challenged plasma was incubated in a similar manner, but in the absence of the SAAD. A portion of the incubated plasma was examined by ELISA for the TSST-1 concentration, and another portion of the plasma was examined for cytokine production-inducing activities.

For determining the cytokine production-inducing activities, the plasma was added to an equal volume of 2.5-fold-diluted human whole blood with RPMI-1169. The mixture was incubated at 37°C for 12 h, and centrifuged at 2000 g (3000 rev/min). The concentrations of TNF-α and IL-8 in the resulting supernatant were determined by ELISA.

Animal experiments

The protocol for the animal experiment was reviewed and approved by the Institutional Animal Care and Use Committee of Toray Pioneering Research Laboratories. In total, 16 rabbits were used: eight were treated with a column containing 6.6 g of SAAD fiber (SAAD column), and eight were treated with an empty column with the same priming volume (control column). Rabbits were anesthetized with sodium pentobarbital by intravenous administration at 30 mg/kg. Anesthesia was maintained by giving an additional 15 mg/kg dose of sodium pentobarbital 2 h after the first injection. The animals were individually subjected to direct hemoperfusion by pumping out blood from the right femoral artery through a cannulated tube, passing it into a SAAD or control column, and returning it into the right femoral vein of the rabbit. Using an electronically controlled infusion pump (TERUMO Corp., Tokyo, Japan), saline containing heparin (100 unit/mL) was infused continuously at a flow rate of 3 mL/h to prevent coagulation.

Animals were allowed to be relaxed during the initial 15-min period after the initiation of the extracorporeal blood circulation. TSST-1 was then infused at a flow rate of 0.05 μg/kg per hour for 1 h. After a 1-h interval from the completion of TSST-1 infusion, LPS was infused from downstream of the hemoperfusion circulation at a flow rate of 0.27 μg/kg per hour for 1 h, to induce shock. Direct hemoperfusion at a flow rate of 10 mL/min was performed from 15 min before the infusion of TSST-1 until 3.5 h after this. After the completion of hemoperfusion, all of the blood in the circuit was returned to the rabbit, the cannulated tubes were removed, and the incisions were closed under sterile conditions. The animals were then allowed to recover from anesthesia, and observed for 3 h after the completion of hemoperfusion and daily thereafter for 14
days. Survival rates in a 14-day period after the treatment were evaluated. After 14 days, the animals were killed with a lethal dose of sodium pentobarbital.

Blood samples were collected from the inlet and outlet of the column in the hemoperfusion circuit. Plasma was separated from each blood sample for the determination of TSST-1 concentrations by ELISA. Blood cell counts, blood gas and blood pH were also measured. Blood pressure was measured with a manometer connected to a tube cannulated to the left femoral artery.

Statistical analysis

The data regarding in vitro clearance of superantigens, cytokine production and TSST-1 concentrations in the blood of TSST-1-infused rabbits were analyzed by Student's t-test. TSST-1 concentrations in the blood samples obtained from the inlet and outlet of the SAAD column were compared by paired t-test, and the survival rates were analyzed by the Kaplan–Meier method.

RESULTS

Preparation of superantigen-adsorbing device (SAAD)

As shown in Figure 1, chemical modification of a polystyrene-based composite fiber reinforced with polypropylene was carried out in the following three steps: (1) α-chloroacetoamidomethylation of the fiber on phenyl rings of the polystyrene component; (2) introduction of polyamine spacer molecules into the acetoamidomethyl moieties by the reaction with tetraethylenepentamine; and (3) modification of the terminal amino groups of the attached spacer molecules with 4-chlorophenylisocyanate to form 4-chlorophenylurea groups.

Under the conditions employed in the present study, 0.8 mmol of 4-chlorophenylurea groups was introduced for each gram of the fiber. No significant changes were noted in the TSST-1-adsorbing abilities of the resulting fiber after gamma ray irradiation at 2.5 Mrad or 6 months of storage at 4°C (data not shown).

Adsorption characteristics of the SAAD

The SAg-adsorbing ability of the SAAD was first examined in vitro by incubating the SAAD (0.065 g, total surface area 2 cm²) with various SAgS (1 ng/mL) in FCS (1 mL) at 37°C for 2 h. The adsorption of TSST-1 occurred in a time-dependent manner, and reached a plateau at 2 h (data not shown). Thus, the adsorption rates determined in 2-h incubations were used as the index in the evaluation of selectivity of SAg adsorption by the SAAD. The resulting adsorption rates were sufficiently high: 74%, 76% and 75% for SEA, SEB and SEC, respectively, and 80% and 72% for TSST-1 and SPEA, respectively (Figure 2A).

Similar levels of adsorption were observed in incubations with non-superantigenic bacterial products such as SPEB (85%) and LTA (75%) (Figure 2B). In general, however, the SAAD exhibited rather poor affinities for non-superantigenic bacterial products, as shown by the adsorption rates of 49%, 10%, 50% and 5% for ProteinA, ExA, LPS and PepG, respectively (Figure 2B).

Rather high adsorption rates were noted in incubations with cytokines under similar conditions: the resulting rates were 90% and 89% for IL-8 and MCP-1, respectively (Figure 2C). In addition, the SAAD showed some affinities for antibiotics; the adsorption rates were 67%, 68%, 50% and 83% for PCG, CCL, MCZ and VCM, respectively (Figure 2D).

When heparinized human plasma samples were incubated in the presence or absence of the SAAD (0.065 g/mL), relatively high adsorption was noted only for P2MG, Bil, and Mgb (84%, 75%, and 80%, respectively) (Figure 2E). In contrast, no significant adsorption was observed with TP, Alb, IgG, IgA, IgM, HDL, LDL, C3, C4, and C5 (less than 5%).

Superantigen-removal efficacy of the SAAD

The SAg-removal efficacy of the SAAD was also examined by assessing the ability of TSST-1 to induce
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Figure 2. Abilities of the superantigen-adsorbing device (SAAD) to adsorb superantigens and other related materials. (A) Superantigens at 1 ng/mL (SEA, staphylococcal enterotoxin A; SEB, staphylococcal enterotoxin B; SEC, staphylococcal enterotoxin C; TSST-1, toxic shock syndrome toxin-1; SPEA, streptococcal pyrogenic exotoxin A). (B) Non-superantigenic bacterial products at 1 ng/mL (SPEB, streptococcal pyrogenic exotoxin B; LPS, lipopolysaccharide), 10 ng/mL (ProteinA, staphylococcal protein A; ExA, Pseudomonas exotoxin A; PepG, peptidoglycan) or 100 ng/mL (LTA, lipoteichoic acid). (C) Cytokines at 10 ng/mL (TNF-α, human tumor necrosis factor alpha; IL-6, human interleukin 6; IL-8, human interleukin 8; MCP-1, human monocyte chemotactic protein 1; PAI-1a and PAI-11, human plasminogen activator inhibitor active form and latent form). (D) Antibiotics tested at 10 μg/mL (ABK, arbekacin; PCG, penicillin G; MCZ, miconazole), at 20 μg/mL (CLDM, clindamycin; CCL, cephaloridin) or at 50 μg/mL (VCM, vancomycin) (all of the above materials were tested in fetal calf serum). (E) Human plasma proteins (TP, total protein; IgG and IgM, immunoglobulins A, G and M; C3, C4 and C5, complement factors 3, 4, and 5; β2MG, β2-microglobulin; Tbil, total bilirubin; Mgb, myoglobin; Alb, albumin; HDL, high-density lipoprotein; LDL, low-density lipoprotein).

cytokine production by human peripheral blood cells in an in vitro system. In this experiment, TSST-1 was added to human plasma at 1000 pg/mL. The plasma was incubated with or without the SAAD, and the residual SAg activity in the plasma was evaluated by determining TNF-α and IL-8 production by human blood cells (Figure 3).

Incubation of TSST-1-containing plasma with the SAAD resulted in a dramatic decrease in the concentration of TSST-1 to 93.8 pg/mL (Figure 3A). No significant decrease in TSST-1 concentration was noted in incubations without the SAAD. On stimulation with the SAAD-treated plasma, human blood cells produced TNF-α and IL-8 to levels of 125 and 359 pg/mL, respectively (Figure 3B,C). In contrast, stimulation with the SAAD-untreated plasma resulted in production of TNF-α and IL-8 to levels of 194 and 1029 pg/mL, respectively (Figure 3B,C). Thus, values for the efficacy of the SAAD in removing TSST-1 from human plasma under the above conditions were calculated to be 36% and 65% in terms of reductions in the induction of TNF-α and IL-8 production, respectively, by human blood cells. Statistical

Figure 3. Toxic shock syndrome toxin-1 (TSST-1) removal efficacy of the superantigen-adsorbing device (SAAD), and the residual TSST-1 activity in inducing cytokine production by human blood cells. (A) Residual TSST-1 concentration after incubation of TSST-1-containing plasma (1 ng/mL) with the SAAD (0.065 g/mL). (B) Tumor necrosis factor alpha (TNF-α) production by human blood cells stimulated with SAAD-treated or untreated TSST-1-containing plasma. (C) Interleukin-8 (IL-8) production by human blood cells stimulated with SAAD-treated or untreated TSST-1-containing plasma. Error bars represent ±SD. *Significantly different from the control by the Student t-test (P<0.001).
analysis indicated that these cytokine production-
inducing activities were significantly lower in the
SAAD-treated plasma than in SAAD-untreated plasma
(P<0.001).

Therapeutic blood purification in a superantigen-
mediated shock model
To evaluate the SAg-removal efficacy, SAAD was
examined in a SAg-mediated shock model using an
experimental extracorporeal blood purification system.
The SAg-mediated shock hypotension, which may cause
death, was induced by intravenous administration of
TSST-1 and LPS into rabbits.

When a SAAD-containing column was used in the
extracorporeal circulation system, 50% of the animals
survived for 14 days after the shock induction by LPS.
In contrast, no survivals were observed even 3 days after
the LPS challenge when the extracorporeal blood
purification was performed using a blank column in
place of the SAAD column (Figure 4A). No deaths were
noted when animals were treated with either TSST-1
alone or LPS alone during the subsequent 14-day
period (data not shown).

The circulating blood was monitored for TSST-1
concentration at both the inlet and the outlet of the
SAAD column at a time point of 60 min after the
initiation of the TSST-1 infusion, and a statistically
significant decrease (inlet, 135 pg/mL±41 pg/mL; out-
let, 124 pg/mL±37 pg/mL; paired t-test, P=0.03) was
detected (Figure 4B). TSST-1 concentration in the
circulating blood decreased with time, and TSST-1 was
no longer detected even at the inlet of the SAAD
column at a time point of 120 min after the initiation
of TSST-1 infusion (in other words, 60 min after
the completion of the TSST-1 infusion). When a blank
column was used in place of the SAAD column, no
significant changes in TSST-1 concentration were
observed during the whole monitoring period of 195 min
after the initiation of the TSST-1 infusion.

Gross observation of the general condition of the
animals revealed hypotension, acidosis, leucopenia and
hypoxemia in all animals after the challenge with LPS,
regardless of whether the extracorporeal circulation was
passed through a SAAD column or a blank column.
Thus, no significant differences were detected in the
incidence of these symptoms between the two groups
(data not shown).

DISCUSSION
In the present study, a new SAAD has been developed
as a material with potential use in an extracorporeal
circulation system for treatment of septic patients.

The basic design of the SAg adsorbent was based
on our previous findings that a polystyrene-based com-
posite fiber exhibited high biocompatibility and low
bloodflow resistance.1 For selective trapping of a given
SAg, an antibody to the given SAg or MHC class II
molecule with high selectivity for certain SAgS would be
a most promising ligand. However, these protein mole-
cules are generally unstable under the conditions usually
employed in autoclave or gamma ray sterilization pro-
cedures. Thus, these molecules were discounted, and
a random screening was carried out with over 100
chemical moieties with different structures, using in vitro
adsorption rates of TSST-1 as the index.

As a result of the screening, 4-chlorophenylurea
groups were selected, and were introduced via a poly-
amine spacer molecule by chemical modification (Figure
1).

To examine the adsorption selectivity, the adsorption
rates were determined by using five different SAgS. In
this connection, cytokine production is considered to be
an important event in the pathogenesis of SAg-related
infectious diseases,19 and also some antibiotics are

Figure 4. Efficacy of the superantigen-adsorbing device (SAAD) in TSST-1/LPS-infused rabbits. (A) Survival curves for rabbits treated
with the SAAD column (n=8) or blank column (n=8). The survival rate in the SAAD column-treated group was significantly higher
than that in the blank column-treated group (by the Kaplan-Meier method and log-rank test, P<0.01). (B) TSST-1 concentrations in
the circulating blood. In the rabbits treated with the SAAD column, the TSST-1 concentration in the circulating blood was significantly
lower at the outlet than at the inlet at a time point of 60 min after the initiation of TSST-1 infusion (by paired t-test, P=0.03).
routinely used in the treatment of patients with bacterial infections. Thus, the abilities of the SAAD to adsorb some cytokines and antibiotics were also examined.

The results revealed that the selectivity of the adsorption by the SAAD was rather broad (Figure 2). Since the five SAGs exhibit no apparent homology in their amino acid sequences, and the SAAD also exhibited high affinities for a wide variety of structurally unrelated non-superantigenic molecules, it is unlikely that the amino acid sequence of SAGs is a major factor in determining their affinities. Thus, we speculate that the urea bond of the 4-chlorophenylurea groups plays a critical role in trapping these molecules by interacting with them, probably through hydrogen bonding, although the detailed adsorption mechanism currently remains to be clarified.

With respect to the affinities of the SAAD toward SAGs, the adsorption rates of over 70% in 2-h incubations were relatively high, compared with those reported with other polymer adsorbents. Furthermore, the observation that the SAAD is resistant to sterilization and is stable for at least 6 months suggests that the SAAD is suitable for practical use for medical devices.

SAG-removal efficacy was evaluated biologically, using the cytokine induction assay. Since, as shown above, the SAAD exhibited some ability to adsorb cytokines, the SAAD was removed before the incubation with blood cells, and the supernatant was examined for the residual activity of TSST-1. Although there were some apparent discrepancies between the removal rate of TSST-1 and the rates of reductions in TNF-α and IL-8 production (Figure 3), the results at least indicated that the SAAD would be effective in reducing the systemic inflammation by trapping circulating SAGs.

The possible application of the SAAD as a blood purification device to treat SAG-associated disorders was examined in an animal model developed in rabbits by treating them with infusions of TSST-1 and LPS. An improvement in survival rate and a decrease in TSST-1 suppression of TSST-1-LPS synergism. Also, this SAg removal by the SAAD. In fact, the dose of 0.05 μg/kg per hour used in our model was selected to obtain blood concentrations around the mean TSST-1 concentration (440 pg/mL) reported to be seen in the circulation of TSS patients;

Despite the remarkable increase in survival, no significant differences were observed in the incidences of the septic symptoms, such as hypotension, acidosis, leucopenia, and hypoxemia. These results suggested that the removal of the TSST-1 by the SAAD did not affect the development of acute shock, but rather the development of more serious toxic events, such as organ failures, that were linked to death in the later stages of sepsis.

Protection against superantigen toxicity in vivo may be achieved by using neutralizing peptides. Continuous use of such antigenic peptides, however, may result in immunosuppression and anaphylactic shock. Thus, repeated or continuous use of these peptides, which are frequently required in the treatment of prolonged infectious diseases, is not considered to be appropriate in clinical practice. In the case of extracorporeal circulation, the chance of such problems arising would be negligible.

In conclusion, the SAAD with 4-chlorophenylurea as the functional group appears to exhibit rather selective and high affinity towards various SAGs, and low affinities towards most of the major protein components of plasma. The markedly improved survival rate observed in the SAAD-treated animals strongly suggests the usefulness of the SAAD for removing SAGs in clinical settings, although further studies are needed to fully characterize the adsorption properties.

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