Cytokine Adsorptive Property of Various Adsorbents in Immunoadsorption Columns and a Newly Developed Adsorbent: An in vitro Study

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\textbf{Key Words}

Cytokine \cdot Adsorption experiments \cdot Adsorbents, cytokine adsorptive properties \cdot Immunoadsorption \cdot Plasma adsorption \cdot Direct hemoperfusion \cdot Systemic inflammatory response syndrome \cdot Sepsis \cdot In vitro study

\textbf{Abstract}

\textbf{Background/Aims:} Cytokines play important roles in the pathophysiology of systemic inflammatory response syndrome (SIRS) and sepsis. Therefore, some effective measures to remove cytokines from the bloodstream could be effective in the treatment of SIRS and sepsis. The aim of this study was to evaluate the cytokine adsorptive property of various adsorbents for the purpose of the development of new selective cytokine adsorption columns. \textbf{Methods:} The cytokine adsorptive property of adsorbent in a CF-X column, which consists of cellulose beads cross-linked with hexamethylene-di-isocyanate, was compared with those of various adsorbents in currently available immunoadsorption columns, such as Immusorba TR\textsuperscript{®}, Immusorba PH\textsuperscript{®}, Selesorb\textsuperscript{®}, and Lixelle\textsuperscript{®}, in vitro batchwise test using patients’ plasma. A newly developed adsorbent, MPCF-X, which was modified by coating the surface of the adsorbent in CF-X with 2-methacryloyloxyethyl phosphorylcholine (MPC), was also tested for its cytokine adsorptive property. \textbf{Results:} The adsorbent in CF-X showed a significantly higher adsorption rate for TNF-\textalpha, interleukin (IL)-6 and IL-10 compared with other adsorbents (p < 0.05). Adsorbent in Lixelle\textsuperscript{®} showed good affinity to TNF-\textalpha and IL-8. Especially, the adsorbent in CF-X almost completely removed TNF-\textalpha, whereas it also had considerable affinity to normal IgG. MPCF-X showed decreased affinity to IgG with considerable adsorptive properties to cytokines. \textbf{Conclusion:} Selective cytokine adsorption columns could be developed with improvement of currently available adsorbents. Such a new selective cytokine adsorption column could be clinically applied for the treatment of SIRS/sepsis.

\textbf{Introduction}

Sepsis is defined as systemic inflammatory response syndrome (SIRS) caused by infection in these years [1]. It is widely accepted that excessive cytokine production from macrophages and monocytes plays an important
role in the pathophysiology of sepsis [2, 3]. SIRS is a state in which excessive inflammatory cytokines are present in blood flow, that is, hypercytokinemia. If SIRS becomes more serious or prolonged, circulating neutrophils and various mediator networks are activated, which leads to the development of organ dysfunction. Especially, proinflammatory cytokines such as TNF-α, interleukin (IL)-1β, IL-6 and IL-8 are considered to be closely related to the pathophysiology of sepsis and resulting organ dysfunction. In the last decade, so-called anti-cytokine immunotherapies using monoclonal antibodies, soluble receptors, and receptor antagonists against TNF-α and IL-1β were aggressively studied for the treatment of sepsis [4, 5]. Although these approaches showed satisfactory results in experiments on animals, results of clinical studies have been disappointing. Thus, the effective therapy against hypercytokinemia has not yet been established clinically [6].

Methods to remove cytokines from the bloodstream by means of blood purification have been under study mainly focusing on continuous renal replacement therapies (CRRT) such as continuous hemofiltration (CHF) or continuous hemodiafiltration (CHDF) [7]. However, the effects of these therapies are still controversial [8, 9]. Recent reports have indicated that adsorption to the hemofilter membrane, rather than convection or diffusion, plays an important role in the action mechanism of cytokine removal by CHF or CHDF [10, 11].

On the other hand, adsorbents that selectively adsorb various pathologic substances have been developed recently, and selective adsorption of pathologic substances by means of plasma adsorption or direct hemoperfusion (DHP), so-called immunoadsorption, has been widely applied in clinical settings [12, 13]. If an adsorption column that can selectively remove cytokines could be developed, it would be a new modality for the treatment of SIRS and sepsis. The purpose of the present study is to evaluate the cytokine adsorptive properties of various adsorbents in immunoadsorption columns that have been in clinical use and those still under development, using plasma of patients in vitro, in order to develop new cytokine adsorption columns. In the preliminary experiment, adsorbent in CF-X which is developed for the treatment of immunologic diseases [14] has been revealed to have strong affinity to various cytokines. Therefore, the cytokine adsorptive property of CF-X was compared in vitro with other adsorbents in currently available immunoadsorbent columns. Furthermore, we developed a new adsorbent by modifying adsorbent of CF-X, and its adsorptive property was also evaluated.

### Materials and Methods

**Patients' Plasma**

Forty-milliliter blood samples for adsorption experiments were collected from 3 patients with septic shock admitted to our ICU on days 1 and 2, after obtaining their informed consent. One patient was suffering from septic shock caused by acute obstructive suppurative cholangitis, and the other 2 patients developed septic shock due to panperitonitis caused by perforation of the digestive tract. All of them were male and ages ranged from 71 to 83 years. Two of them died and 1 survived. The blood samples collected from

### Table 1. Adsorbents tested

<table>
<thead>
<tr>
<th>Name of columns</th>
<th>Manufacturer</th>
<th>Adsorbent carrier material</th>
<th>ligand</th>
<th>Main adsorbable substances</th>
</tr>
</thead>
<tbody>
<tr>
<td>CF-X</td>
<td>Ube Industries Ichihara, Japan</td>
<td>Cellulose beads</td>
<td>Hexamethylene-di-isocyanate</td>
<td>Anti-DNA antibody, pemphigus antibody</td>
</tr>
<tr>
<td>Immusorba TR®</td>
<td>Asahi Medical Tokyo, Japan</td>
<td>Polyvinyl alcohol gel</td>
<td>Tryptophan</td>
<td>Anti-acetylcholine receptor antibody, immune complex</td>
</tr>
<tr>
<td>Immusorba PH®</td>
<td>Asahi Medical Tokyo, Japan</td>
<td>Polyvinyl alcohol gel</td>
<td>Phenylalanine</td>
<td>Rheumatoid factor, immune complex</td>
</tr>
<tr>
<td>Selesorb®</td>
<td>Kaneka Osaka, Japan</td>
<td>Cellulose beads</td>
<td>Dextran sulfate</td>
<td>Anti-DNA antibody, anti-cardiolipin antibody</td>
</tr>
<tr>
<td>Lixelle®</td>
<td>Kaneka Osaka, Japan</td>
<td>Cellulose beads</td>
<td>Hexadecyl alkyl chain</td>
<td>β2-Microglobulin</td>
</tr>
</tbody>
</table>
these patients were centrifuged immediately to collect plasma samples. The plasma samples were stored at −70℃ until the use in adsorption experiments. The present study was conducted with the approval of the ethical committee.

**Adsorbents**

Table 1 shows various adsorbents used in this experiment. These adsorbents have the property of selectively adsorbing target pathologic substances by means of electrostatic bonding or hydrophobic bonding, rather than biologic bonding such as antigen-antibody interaction.

CF-X is an immunoadsorption column under clinical trial and the column contains an adsorbent in which cellulose beads are cross-linked with hexamethylene-di-isocyanate. The adsorbent in CF-X has been proven to selectively adsorb antibodies related to systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and pemphigus vulgaris [14]. Among other adsorbents tested, the adsorbent in Immusorba TR® is targeted to adsorb anti-acetylcholine receptor antibody and immune complexes, and is used for the treatment of myasthenia gravis and Guillain-Barré syndrome [15]. The adsorbent in Immusorba PH® adsorbs rheumatoid factor and immune complexes applying to the treatment of SLE and RA [15]. The adsorbent in Selesorb® can remove anti-DNA antibodies efficiently and is used for the treatment of SLE [16]. Adsorbent in Lixelle® can adsorb β₂-microglobulin by DHP and is used for the treatment of dialysis-related amyloidosis [17].

**A Newly Developed Adsorbent**

The adsorbent in CF-X has a strong affinity to various pathologic antibodies and immune complexes. However, as it also shows a strong affinity to immunoglobulin G (IgG), it has the disadvantage of adsorbing and removing normal IgG as well [17]. Therefore, we developed a new adsorbent, MPCF-X, by coating the surface of CF-X with 2-methacryloyloxyethyl phosphocholine (MPC) copolymer [18], a coating material used to improve the biocompatibility in various artificial devices, and studied its cytokine adsorptive property (fig. 1). Two types of adsorbents, MPCF-XM20 and MPCF-XN20, were developed according to the difference in density of MPC coating.

**In vitro Batchwise Adsorption Test**

Pooled patient’s plasma samples were mixed and divided into two to conduct two series of experiments on adsorptive property. In the experiment on adsorptive property, 4.5 ml of patient’s plasma were added to 1.5 ml of adsorbent (volumetric ratio v/v = 3/1), and then the sample was stirred and incubated at 37℃ for 60 min. Immediately afterwards, the sample was centrifuged at 1,000 rpm for 2 min to collect its supernatant. Each of the experiments on adsorptive property was conducted in duplicate.

The total protein (TP), albumin (Alb), IgG, Na, and cytokine in plasma were measured both before and after adsorption (pre- and post-adsorption). Cytokines TNF-α, IL-1β, IL-6, and IL-10 were measured using a Medgenix EASIA kit (Biosource Europe SA, Fleurus, Belgium), and IL-8 was measured using an IL-8 ELISA kit.
Cytokine Adsorptive Property of Various Adsorbents

The corrected adsorption rate ($R_c$) of cytokines was calculated by using Na concentrations in the following formula:

$$R_c [\%] = \left(1 - \frac{C (\text{post})}{C (\text{Na-pre})}/\frac{C (\text{pre})}{C (\text{Na-post})}\right) \times 100$$

Statistics
Data are presented as mean $\pm$ SD. Comparison of corrected adsorption rate among various adsorbents was performed by one-way analysis of variance with post-hoc test using Fisher's PLSD. $p < 0.05$ is considered significant.

Results
The levels of TP, Alb, IgG, Na, TNF, IL-1$\beta$, IL-6, IL-8, and IL-10 in plasma were as shown in Table 2. Because the level of IL-1$\beta$ in plasma was below the minimum detectable concentration (2 pg/ml), it was ruled out of the study.

Figure 2 shows the corrected adsorption rate of each adsorbent. There were significant differences in adsorptive property among the adsorbents ($p < 0.05$). The adsorption rate of TNF-$\alpha$ with the adsorbent in CF-X was 100% and it was significantly higher than other adsorbents ($p < 0.05$). The adsorption rate of IL-6 was 98.9% with the adsorbent in CF-X and was 72.0% with the adsorbent in Lixelle®. The adsorbent in CF-X showed significantly higher adsorption rate than other adsorbents ($p < 0.05$). Adsorption rate of IL-8 was highest with the adsorbent in Lixelle® and was more than 70% in all other adsorbents. IL-10 was well adsorbed with the adsorbent in CF-X or Selesorb®, adsorption rates were 88.7 and 83.7%, respectively. These two adsorbents showed significantly higher adsorption rates than other three adsor-

Table 2. Plasma concentrations of total protein (TP), albumin (Alb), immunoglobulin G (IgG), Na, and cytokines

<table>
<thead>
<tr>
<th>Experimental plasma</th>
<th>TP g/dl</th>
<th>Alb mg/dl</th>
<th>IgG mg/dl</th>
<th>Na mEq/l</th>
<th>TNF-α pg/ml</th>
<th>IL-1$\beta$ pg/ml</th>
<th>IL-6 pg/ml</th>
<th>IL-8 pg/ml</th>
<th>IL-10 pg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. 1</td>
<td>5.2</td>
<td>2,780</td>
<td>1,240</td>
<td>142</td>
<td>36.4</td>
<td>1.1</td>
<td>1,562</td>
<td>207</td>
<td>41</td>
</tr>
<tr>
<td>No. 2</td>
<td>4.2</td>
<td>2,080</td>
<td>1,080</td>
<td>143</td>
<td>58</td>
<td>1.7</td>
<td>6,025</td>
<td>299</td>
<td>28</td>
</tr>
</tbody>
</table>

TNF = Tumor necrosis factor; IL = interleukin.
bents (p < 0.05). In conclusion, the adsorbent in CF-X showed most remarkable adsorptive property for various cytokines and the adsorbent in Lixelle® followed.

Figure 3 shows the adsorptive property of newly developed adsorbent, MPCF-X. The adsorption rate of IgG was as high as 75.5% with the adsorbent in CF-X, while the rate decreased to 23.0 and 0% in MPCF-XM20 and MPCF-XN20, respectively. The adsorption rates of cytokines such as IL-6, IL-8, and IL-10 with MPCF-XM20 or MPCF-XN20 slightly decreased compared to the adsorbent in CF-X. However, it became apparent that they could adsorb TNF-α almost completely.

**Discussion**

Since Bellomo et al. [19] reported that cytokines could be removed by means of continuous veno-venous hemofiltration, attempts to treat sepsis by CRRT have been made energetically [7–10]. Considering that the molecular weights of cytokines are relatively high ranging from 8,000 of IL-8 to 45,000 or 55,000 of a trimer of TNF-α, cytokines are considered to be removed mainly due to the principle of convection. However, the efficiency of removing cytokines by CRRT is lower than the natural clearance in vivo and not high enough to decrease the blood levels, whereby its clinical efficacy has been controversial [8, 9]. We have reported that CHDF using a polymethylmethacrylate (PMMA) membrane hemofilter can remove various humoral mediators including cytokines by means of the principle of adsorption and that it is effective in treating septic multiple organ failure and severe acute pancreatitis [11, 20]. This property is characteristic only in PMMA membrane, and was not observed with other membrane material, such as ethylene vinyl alcohol, polyacrylonitrile, or polysulfone. De Vriese et al. [10] also reported that the removal of cytokines by CHF using AN-69 hemofilter was mainly due to adsorption to a hemofilter membrane.

Recently, the hemodynamic effect of high-volume hemofiltration in which 6 l/h of filtrate was removed to enhance removal of cytokines and other mediators has been reported [21]. However, high blood flow rate (300 ml/h) is necessary to obtain a large volume of filtrate in high-volume hemofiltration. This is impractical, even at risk for hemodynamically unstable patients, such as septic shock. Meanwhile, it has been reported that the adsorptive resin was capable of significantly removing middle molecular weight substances including various cytokines [22]. Ronco et al. [23] developed and reported the continuous plasma filtration adsorption, a combination of plasma filtration and adsorption by resin. Thus, sorbent adsorption of cytokines has been paid attention to recently [24–26].

If a column that can selectively adsorb and remove cytokines in blood by means of DHP could be developed, it can be a new treatment technique for SIRS and sepsis. Recently, we discovered that adsorbent in CF-X, an immunoadsorbent column which has a strong affinity to various antibodies and has been under clinical trial for the treatment of SLE, RA and pemphigus vulgaris, had strong affinity to various cytokines. Therefore, in the

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**Fig. 3.** Adsorptive properties of newly developed cytokine adsorbents, MPCF-XM20 and MPCF-XN20. TNF = Tumor necrosis factor; IL = interleukin; Alb = albumin; IgG = immunoglobulin G.
The present study, we examined the cytokine adsorptive properties of various adsorbents in vitro using adsorbents of immunoadsorption columns currently available and under development, for the purpose of the development of a new cytokine adsorption column. As a result, it became apparent that adsorbents in Lixelle® and CF-X adsorb various cytokines efficiently.

Lixelle® is an adsorption column that has already been in use in the form of DHP for the treatment of dialysis-related amyloidosis in dialysis patients [16]. Recently, Tschida et al. [27] reported that DHP using Lixelle® in 5 SIRS patients decreased the blood levels of IL-1Ra, IL-6, and IL-8 after a 4-hour treatment session by 44.1, 22.1, and 41.4%, respectively, resulting in recovery from shock. They concluded that Lixelle® could be applied to the treatment of hypercytokinemia. A larger-scale controlled study will be necessary to examine whether the removal of cytokines by Lixelle® could improve outcome of SIRS or sepsis in the near future.

The adsorbent in CF-X was found to have a stronger affinity to TNF-α and IL-6 than that in Lixelle®. That is, CF-X may also be able to be applied as a cytokine adsorption column. However, there are problems with the use of CF-X for the treatment of sepsis, because it was designed for plasma adsorption, not for DHP and has a strong adsorptive property for normal IgG [17]. To solve these problems, we developed a new adsorbent MPCF-X by coating adsorbent in CF-X with MPC, aiming at decreasing the adsorption rate of IgG with the cytokine adsorptive property of CF-X maintained and developing a new adsorbent that can be used for DHP. As a result, it was possible to decrease the adsorption rate of IgG with the adsorptive property for various cytokines maintained.

In the future, if further improvements are made to adsorbent in CF-X, there is the possibility that it will be applied to clinical settings as a selective cytokine adsorption column.

On the other hand, there is a limitation to the removal of cytokines by adsorption columns due to saturation of adsorption sites. Under such circumstances, one of strategies to be launched will be to use a cytokine adsorption column in the acute phase where the cytokine level is very high to reduce the blood concentration to some extent, and to perform PMMA-CHDF or HVHF afterwards. Recently, we have introduced a rapid measurement system of the blood IL-6 level using chemiluminescent enzyme immunoassay, with which we can get a result within 30 min [11]. We are now performing PMMA-CHDF as a cytokine modulator for hypercytokinemia caused by SIRS or sepsis while keeping track of IL-6 blood levels in real time [11, 21]. If a cytokine adsorption column becomes clinically applicable, it would be performed at optimal timing with this system.

In conclusion, the present study proved that it is possible to develop a selective cytokine adsorption column by making improvements to immunoadsorbents that have already been in clinical use or those under development. Treatment for hypercytokinemia with cytokine adsorption columns would be a new therapeutic modality for SIRS and sepsis.

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References