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CLINICAL STUDY

Inhibitory Factors of Serum Opsonic Activity in Serum from Patients with Chronic Renal Failure

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ABSTRACT

We investigated the mechanism of impaired serum opsonic activity in patients suffering from chronic renal failure (CRF). Prehemodialysis pooled serum from CRF patients was fractionated using Sephadex G-50 gel chromatography, and the effects of the obtained fractions on serum opsonic activity were examined using luminol-dependent chemiluminescence responses. Number 25–30 fractions in the prehemodialysis serum contained factors that may inhibit serum opsonic activity. The presumed molecular weight of these fractions is between 300 and 2800 daltons. These findings indicate that the inhibitory factors of serum opsonic activity may belong to middle molecules.

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INTRODUCTION

Infection is a common complication and the major cause of death among chronic renal failure (CRF) patients (1). Several investigators have reported impaired granulocyte functions in uremia (2–12), but there are few reports on impaired serum opsonic activity (8).

Serum opsonic activity is one of the humoral components of the host immune system, and it plays an important role in the host defense against infections by promoting phagocytosis.

When neutrophils phagocytize opsonized microorganisms, enzyme systems on the cell membrane are activated and microbicidal oxidants are produced. These microbicidal oxidants are electronically excited molecules which emit light or chemiluminescence (CL), relaxing to a stable ground state (13). CL measurements have therefore been used in order to evaluate the functions of neutrophils and serum opsonic activities (14,15).

Babb et al. (16) suggested that compounds with a molecular weight range of 500–5000 Da (middle molecules, MMs) accumulate in uremia and may exert toxic effects such as peripheral neuropathy. After the presentation of this MM hypothesis, several authors (2,3,17–21) reported relationships between MMs and the symptoms of uremia, such as neuropathy and impaired granulocyte functions. No investigations into the effect of MMs on serum opsonic activity have been reported, as far as we know. We previously found that the fraction of prehemodialysis (HD) serum below 5 kDa contain inhibitory factors of serum opsonic activity. In present study, we examined in more detail which fractions of pre-HD serum contain the inhibitory factors of serum opsonic activity.

MATERIALS AND METHODS

Neutrophils

Neutrophils were separated from one healthy adult volunteer by Histopaque (Sigma, USA) density gradient centrifugation according to the method described previously (22), and resuspended to 3×10^6 cells/mL in HBSS.

Sera

Pre-HD pooled serum (HeS) was obtained by pooling sera from 869 CRF patients receiving hemodialysis, and healthy pooled serum (NoS) was obtained by pooling sera from 892 healthy subjects. Serum concentrations of creatinine and blood urea nitrogen were 11.9 mg/dL and 84 mg/dL in HeS, and 0.8 mg/dL and 14 mg/dL in NoS, respectively.

Fractionation of Sera

The serum fraction below or above 10 kDa was obtained by ultrafiltrating HeS or NoS with ULTRACENT-10 (TOSOH, Japan) (MW cutoff: 10 kDa). The ultrafiltrated solution containing HeS fraction below 10 kDa was lyophilized, and the lyophilized material was resolved in distilled water so that the final concentration of the material was 10 times the concentration in the ultrafiltrated solution. The concentrated solution containing HeS fraction below 10 kDa was applied onto a Sephadex G-50 (Pharmacia Fine Chemicals, Sweden) column (0.5×55 cm) in a volume of 2.0 mL, and each fraction was eluted with HBSS containing 0.002% ICl as a preservative. Next, 1.5-mL fractions were collected, and the absorption of each fraction was measured at 280 nm using a spectrophotometer, and the

effects of the collected fractions (number 23–40 fractions) on serum opsonic activity were studied. The column was standardized with products of known molecular weight: carbonic anhydrase (MW 29,000, Sigma), cytochrome c (MW 12,500, Sigma), aprotinin (MW 6500, Sigma), vitamin B_{12} (MW 1355, Wako Pure Chemical Industries, Japan), glutathion reduced (MW 300, Sigma).

Opsonized Zymosan (OZ)

In all experiments, opsonized particles were washed twice with HBSS after incubation (to exclude the effects of scavengers on CL responses) and resuspended in a concentration of 1 mg/mL in HBSS.

In the comparison of HeS and NoS opsonic activities, zymosan A (Sigma) was suspended in HBSS in a concentration of 1 mg/mL, and opsonization was then performed by adding HeS or NoS to this suspension at a rate of 40:6 (volume of zymosan suspension: volume of serum) and incubating at 37°C for 30 min. Each serum opsonic activity was evaluated by each zymosan-activated CL response.

In all the following opsonization, the rate of the volume of zymosan suspension and added NoS and each serum fraction or HBSS was 40:6:4.

In the examination of the effect of HeS fraction below or above 10 kDa on serum opsonic activity, opsonization was performed by adding NoS and HeS fraction below or above 10 kDa to the zymosan suspension. Each zymosan-activated CL response was compared with the control study. The control was measured by CL response upon stimulation with zymosan opsonized by adding NoS and HBSS to the zymosan suspension.

In the examination of the effect of NoS fraction below or above 10 kDa on serum opsonic activity, opsonization was performed by adding NoS and NoS fraction below or above 10 kDa to the zymosan suspension. Each zymosan-activated CL response was compared with the control study as described above.

In the examination of the effects of the fractions obtained by gel chromatography on serum opsonic activity, opsonization was performed by adding NoS and each isolated fraction to the zymosan suspension. Each zymosan-activated CL response was compared with the control study, whose CL response was set at 100%. The control was measured by CL response upon stimulation with zymosan opsonized by adding NoS and HBSS containing 0.002% ICl to the zymosan suspension. In this experiment, the effects of number 23–40 fractions (the presumed molecular weight of these fractions was below about 5 kDa) on serum opsonic activity were studied (Fig. 1).

Luminol-Dependent CL Response

CL responses were measured using a Lumi Box U-800 II (Maikurotekku Nition, Japan). In the measurement of CL response with this instrument, following addition of prepared luminol (Sigma) solution (50 μL, 2×10^{-3} M) and OZ (100 μL) in each well of a 96-well microplate (Greiner Japan, Japan), the neutrophil suspension (50 μL, 3×10^6 cells/mL) was added to each well (23). Immediately after the addition of neutrophil suspension, the microplate was placed in the imaging box and the CL responses were automatically measured according to preselected measurement conditions. The peak value in the response curve was defined as the maximal chemiluminescence intensity (MCI), and the reaction time to reach the MCI was defined as the peak time (PT), using analyzing software developed in our laboratory. The MCI and PT were expressed as counts and seconds, respectively.

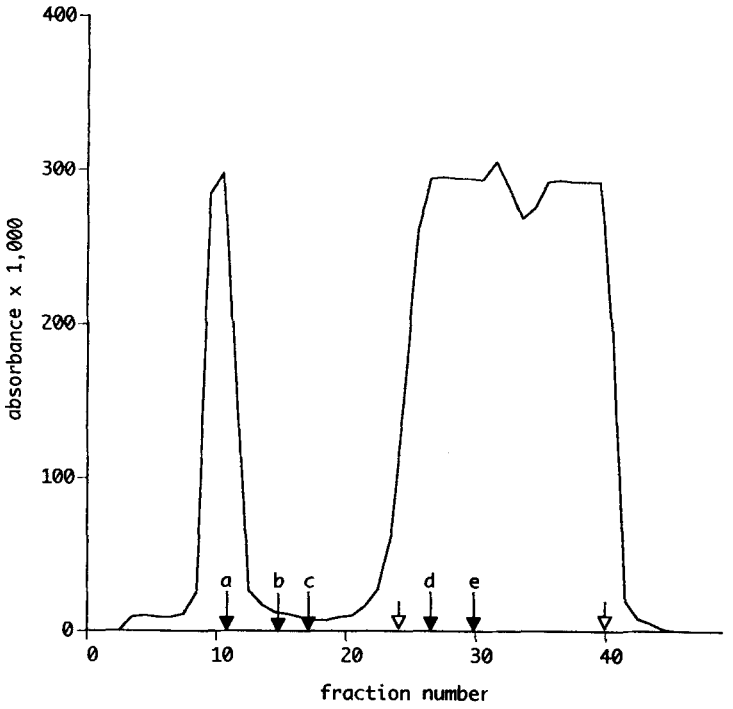

Figure 1. The chromatographic profile obtained by Sephadex G-50 gel chromatography of the concentrated solution containing HeS fraction below 10 kDa. a: carbonic anhydrase (MW 29,000), b: cytochrome c (MW 12,500), c: aprotinin (MW 6500), d: vitamin B_{12} (MW 1355), e: glutathion reduced (MW 300). The fractions eluted at the range indicated by downward-pointing arrows (numbers 23–40) were studied.

Statistical Analysis

All results are presented as means ± standard deviation (*SD*). The statistical significance of differences was calculated using Student's *t* test or the Mann–Whitney *U* test. A probability < 0.05 was regarded as significant.

RESULTS

Comparison Between HeS and NoS Opsonic Activity

As shown in Table 1, A, the MCI was significantly lower ($p < 0.01$) and the PT delayed ($p < 0.01$) in HeS than in NoS.

Effect of HeS Fraction Below or Above 10 kDa on Serum Opsonic Activity

The effect of the fraction below or above 10 kDa is shown in Table 1, B. This experiment was performed in order to investigate the possibility that there may be factors in HeS that

Table 1

CL Responses Upon Stimulation with Each OZ

	Fraction Added in Opsonization	MCI	PT
A.	NoS	172944 ± 15038	892 ± 50
	HeS	116340 ± 12613*	1359 ± 106*
B.	HBSS (control)	148993 ± 12785	897 ± 42
	HeS fraction below 10 kDa	134685 ± 15203†	1034 ± 64†
	HeS fraction above 10 kDa	143680 ± 16175	794 ± 45†
C.	HBSS (control)	118558 ± 6797	1329 ± 35
	NoS fraction below 10 kDa	124665 ± 7553‡	1195 ± 77‡
	NoS fraction above 10 kDa	134894 ± 8509‡	807 ± 26‡

Note. Data are expressed as counts for MCI and seconds for PT. A: The comparison between HeS and NoS opsonic activity. B: The comparison between the control study and CL response upon stimulation with zymosan opsonized by adding NoS and HeS fraction below or above 10 kDa. C: The comparison between the control study and CL response upon stimulation with zymosan opsonized by adding NoS and NoS fraction below or above 10 kDa.
*$p < 0.01$ NoS vs. HeS; †$p < 0.01$ control vs. HeS below or above 10 kDa; ‡$p < 0.01$ control vs. NoS below or above 10 kDa.

inhibit serum opsonic activity, because HeS opsonic activity was significantly reduced compared with NoS opsonic activity. The MCI and PT of the CL response in the group added HeS fraction below 10 kDa were significantly lower ($p < 0.01$) and delayed ($p < 0.01$), respectively, compared with that of the control study.

Effect of NoS Fraction Below or Above 10 kDa on Serum Opsonic Activity

As shown in Table 1, C, neither the fraction below nor that above 10 kDa had any inhibitory effects on serum opsonic activity.

Effects of the Fractions Obtained by Gel Chromatography on Serum Opsonic Activity

As shown in Figure 2, the MCI and PT of the CL response in the group added the isolated number 25–30 fractions were significantly lower ($p < 0.01$) and delayed ($p < 0.01$), compared with that of the control study.

DISCUSSION

Patients with renal failure have an increased susceptibility to bacterial infections (1). This susceptibility appears to be caused by impaired phagocytosis or serum opsonic activity (2–12). The present study was performed to investigate the possible role of MMs in impaired serum opsonic activity.

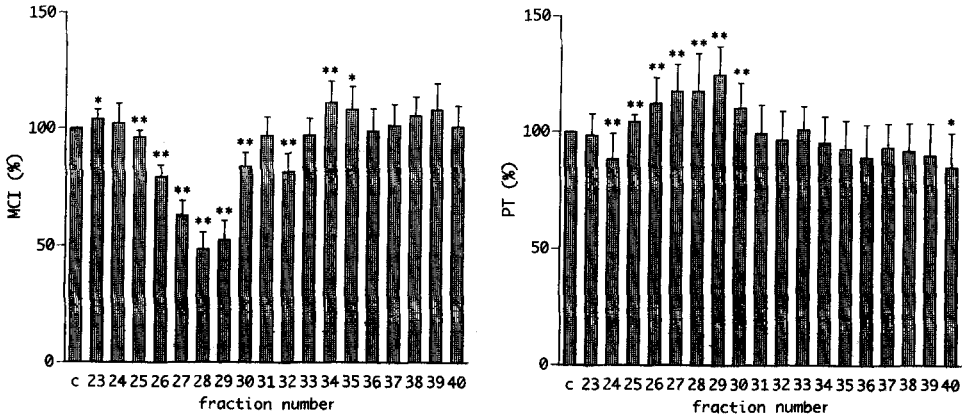

Figure 2. Effects of the fractions (numbers 23–40) obtained by gel chromatography on serum opsonic activity. Left panel: the effect on MCI. Right panel: the effect on PT. The results are expressed as the means ± *SD* of a ratio (%) of the control study, whose CL response was set at 100%. $^{*}p < 0.05$; $^{**}p < 0.01$ c (control) vs. the fraction number.

Our data showed that HeS opsonic activity was significantly less than that of NoS (Table 1, A), and we also confirmed that HeS fraction below 10 kDa has an inhibitory effect on serum opsonic activity (Table 1, B). Therefore we propose that the inhibition of serum opsonic activity observed in CRF patients may be in part due to inhibitory factors of serum opsonic activity.

The middle-molecule hypothesis proposed by Babb et al. (16) suggested that the syndrome of uremia is characterized by the retention of a variety of MMs whose molecular weights are about 300–2000 Da. Asaba et al. (24) also reported that after successful renal transplantation, uremic symptoms subside and the uremic MMs disappear rapidly from serum while being excreted in the urine; they suggested that middle molecules may exert toxic effects in uremia. Toxic effects of MMs have been shown on oxidative metabolism and phagocytosis of neutrophils (3,11), and granulocyte iodination capacity (2).

In the present study, we observed that HeS fraction below 10 kDa may have inhibitory factors of serum opsonic activity. We therefore investigated the effects of the fractions obtained by Sephadex G-50 gel chromatography of the solution containing HeS fraction below 10 kDa on serum opsonic activity, in order to determine which parts of HeS contain the inhibitory factors.

In this examination, when the number 25–30 fractions of HeS were added to the zymosan suspension with NoS in opsonization, the MCI and PT of the CL responses were significantly lower and delayed compared with that of the control study (Fig. 2), and when the CL response of the control study was set at 100%, the MCI decreased from 48.4% (No. 28 fraction) to 96.2% (No. 25 fraction), and the PT delayed from 104.3% (No. 25 fraction) to 124.7% (No. 29 fraction).

In this experiment, opsonized zymosan particles were washed with HBSS twice to exclude the effect of scavengers in serum on CL responses. Accordingly, the inhibitory effect of these fractions on serum opsonic activity may be due, not to a light quenching effect by scavengers, but to actions that these fractions exerted in opsonization, although these mechanisms are unknown. The presumed molecular weight of these inhibitory factors is 300–2800 Da, and that of the number 28 and 29 fractions, which showed marked

inhibitory effects, is 300–1355 Da. Accordingly, these factors, which inhibit serum opsonic activity, may be middle-molecular substances.

In summary, our results showed that serum opsonic activity in CRF patients was significantly reduced, compared with that of healthy subjects. We also found that MMs in uremic serum inhibited serum opsonic activity. It may thus be concluded that the combined effects of MMs on serum opsonic activity (a humoral component) and oxidative metabolism of phagocytes (a cellular component) cause the increased susceptibility to infections of patients with CRF.

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