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Effect of a novel adsorbent on cytokine responsiveness to uremic plasma

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Background. Middle molecules such as β_2-microglobulin (β_2M) and advanced glycation end products (AGE)–modified proteins contribute to inflammation in uremia. The BetaSorb™ column is a new adsorptive device, which contains copolymeric beads, suitable for removal of β_2M and other middle molecules. We assessed the effect of this column on the bioreactivity of uremic plasma, as measured by cytokine responsiveness.

Methods. Uremic plasma was perfused in vitro through the column (10 mL/min) and samples were collected after 10 to 30 passes. Endotoxin-stimulated tumor necrosis factor-α (TNF-α) and interleukin-10 (IL-10) production by THP-1–derived monocytes was measured following brief exposure to uremic plasma. β_2M levels were measured. The contribution of AGE-modified proteins to the bioreactivity of uremic plasma was explored.

Results. TNF-α and IL-10 production markedly decreased after 30 passes (629 ± 78 vs. 144 ± 62 pg/mL; 207 ± 25 vs. 117 ± 23 pg/mL; $P = 0.04$). The column removed β_2M efficiently with a marked decline in plasma levels by 99% after 30 passes. Neutralization of AGE receptor (RAGE) resulted in a further reduction in the bioreactivity of uremic plasma. This was observed with nonperfused, as well as perfused, uremic plasma, suggesting that AGE-modified proteins were biologically active and still present after perfusion.

Conclusion. The sorbent beads removed uremic solute(s) that prime monocytes to enhanced cytokine production. Removal of β_2M was efficient, and of native and AGE-modified middle molecules likely.

Hemodialysis patients are constantly exposed to a microinflammatory environment. Indeed, exposure to bacterial contaminants from dialysis water systems, poorly biocompatible dialyzer membranes, and bacterial infections induce the release of proinflammatory cytokines, which generate an acute phase reaction. This results in a chronic microinflammatory state, which increases cardiovascular morbidity [1]. Furthermore, retention of middle molecules such as β_2-microglobulin (β_2M) may also independently contribute to this inflammatory state by promoting cytokine generation. Interestingly, human β_2M per se has not been reported to directly affect cytokine generation whereas the modification of β_2M by advanced glycation end products (AGE-β_2M) can result in many biological activities including production of proinflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) [2], matrix expansion, vascular damage, and accelerated atherosclerosis [3]. Therefore, removal of uremic toxins, including native and AGE-modified middle molecules, may prevent or at least reduce the uremia-induced inflammatory responses. Modalities for removal of middle molecules from the blood include diffusive, convective, and adsorptive techniques such as hemoperfusion. The latter approach employs matrices that bind the hydrophobic moieties of middle molecules [4]. Recent advances in these technological approaches have led to the development of a new adsorption column, which contains hyper-cross-linked styrene divinylbenzene copolymer beads (BetaSorb™, RenalTech International, New York, NY, USA) [5–7]. The sorbent beads contained in this column have a pore size distribution of 2 to 20 Å, which is suitable for removing molecules in the weight range of 0.3 to 15.0 kD, and restricting adsorption of larger molecules such as serum albumin [5, 6]. A list of putative plausible uremic toxins removed by this sorbent have been reviewed previously [6]. Ex vivo (abstract; Bosch et al, *J Am Soc Nephrol* 11: 257A, 2000), animal (abstracts; Brady et al, *J Am Soc Nephrol* 11: 560A, 2000; Cowgill et al, *ASAIO J*: 47:61, 2001), and preliminary clinical studies [7] have demonstrated 95% removal of β_2M by this column, with high degree of biocompatibility [7]. By contrast, little

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is known of the effect of this column on inflammatory responses.

The objectives of this study were to assess whether the removal of middle molecules by the sorbent beads contained in the BetaSorb™ column affects the bioreactivity of uremic plasma. We hypothesized that uremic plasma primes monocytes to enhance cytokine responsiveness, and that the sorbent beads reduce this "priming effect." TNF-α and IL-10 production by endotoxin-stimulated THP-1–derived monocytes were used as indicators of cytokine responsiveness following brief exposure to uremic plasma. Plasma β_2M was measured as a surrogate marker of middle molecules. Finally, in separate experiments, the contribution of AGE-modified proteins to the bioreactivity of uremic plasma was explored.

METHODS

Reagents

The human promonocytic THP-1 cell line was obtained from the American Tissue Culture Collection ATCC (Rockville, MD, USA). All culture reagents were purchased from Gibco Life Technologies (Rockville, MD, USA). Trans-retinoic acid, 1,25 dihydroxy cholecalciferol, interferon-γ, purified *Escherichia coli* lipopolysaccharide (LPS) (serotype 055:B5) and glucose were obtained from Sigma Aldrich Chemical (St. Louis, MO, USA). β_2M, TNF-α and IL-10 ELISA kits were purchased from R&D Systems (Minneapolis, MN, USA). Receptor for AGE (RAGE) antibody was purchased from Research Diagnostics, Inc. (Flanders, NJ, USA).

In vitro plasma perfusion experiments

A 30 mL aliquot of plasma was obtained from a dialysis patient before the start of five distinct dialysis sessions and used for in vitro perfusion experiments. In addition, plasma obtained from six healthy volunteers and six dialysis patients was used to prime cells during in vitro static experiments. The Human Investigation Review Committee approved the study, and all participants gave written informed consent.

In vitro plasma perfusion was carried out at 37°C. In brief, an in vitro closed-loop perfusion circuit was created with standard dialysis tubing (Medisystems Corp., Seattle, WA, USA) and a column packed with 10 mL of hypercross-linked copolymer biocompatible beads (BetaSorb™, RenalTech International). Before the start of the in vitro perfusion, the column was rinsed with pyrogen-free isotonic saline. Plasma was then perfused over the column at a flow rate of 10 mL/min using an AK-10 system pump (Gambro Instrumenta AB, Lund, Sweden) and samples were collected after 10, 20, and 30 passes. All plasma samples were frozen at −70°C.

THP-1 cell culture

For these studies, we used the human promonocytic THP-1 cell line, which was originally characterized by Tsuchiya et al [8]. This cell line has distinct human monocytic markers, and during culture, THP-1 cells maintain these monocytic characteristics [8]. Furthermore, THP-1 cells express CD14 and Toll-like receptors [9], two important LPS ligands.

In brief, THP-1 cells were grown in suspension culture at the concentration of 0.25×10^6 cells/mL in RPMI-1640 medium (Sigma Aldrich Chemical) containing 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL fungizone. Cells were maintained in a humidified atmosphere with 5% CO_2 at 37°C. Differentiation to monocytes (0.35×10^6 cells/mL) was induced by incubating cells with transretinoic acid (1 μmol/L), 1,25 dihydroxy cholecalciferol (10^{-7} M), and interferon-γ (10^{-5} g/L) for 3 days [10].

TNF-α and IL-10 production by THP-1–derived monocytes

To examine the effect of the sorbent column on cytokine responsiveness, differentiated THP-1 cells (0.7×10^6 cells/mL) were incubated for 2 hours in a humidified atmosphere with 5% CO_2 and at 37°C with uremic plasma before (pass 0) and after 30 passes (pass 30) over the column. The plasma was then removed, and cells were washed with phosphate-buffered saline (PBS) and stimulated with 10 ng/mL of LPS for 16 hours. Cell supernatants were aspirated, and TNF-α and IL-10 were measured by sandwich enzyme-linked immunosorbent assay (ELISA). Similar experiments were performed to examine the "priming effect" of normal and uremic plasma on cytokine responsiveness.

In separate experiments, we examined the contribution of AGE-modified proteins to cytokine responsiveness. In brief, we preexposed differentiated THP-1 cells to serial dilutions of a neutralizing RAGE monoclonal antibody. After a 3-hour incubation, cells were washed and primed with uremic plasma for 2 hours followed by LPS, as previously described. Cell supernatants were then assayed for TNF-α.

Finally, we evaluated indirectly whether the column also removed AGE-modified proteins by performing similar experiments, using plasma obtained after 30 passes over the column.

Measurement of middle molecules

β_2M was used as a surrogate marker of middle molecules. In brief, plasma β_2M levels were measured by a competitive binding enzyme immunoassay, according to the manufacturer's instructions. Lower limit of detection was 0.2 μg/mL. The average intra- and interassay coefficient of variation for β_2M was <10%.

Statistical analysis

Statistical analyses were performed using the Statistical Package for Social Sciences version 10.0 (SPSS, Chicago, IL). Kruskal-Wallis ANOVA, Mann Whitney U test (paired and unpaired), and Friedman test were employed to test the significance of differences. The Spearman rank test was employed to test for correlation between continuous variables. Results are expressed as mean ± standard error of the mean (SEM). Differences were considered statistically significant at $P < 0.05$.

RESULTS

Effect of the sorbent beads on cytokine responsiveness

We first examined the effect of normal or uremic plasma on cytokine responsiveness to LPS. Compared with normal plasma, exposure to uremic plasma resulted in significantly higher TNF-α production (261.1 ± 21.6 vs. 440.5 ± 93.0 pg/mL; $P = 0.05$). By contrast, IL-10 production was nearly significantly lowered after exposure to uremic plasma compared with normal plasma (131.1 ± 35.2 vs. 85.9 ± 20.1 pg/mL).

We next examined the effect of the sorbent column on this enhanced proinflammatory cytokine release by THP-1–derived monocytes. As shown in Figure 1A, compared with baseline, TNF-α production markedly decreased after 30 passes (629.0 ± 77.7 vs. 144.4 ± 62.2 pg/mL; $P = 0.043$). Similarly, as shown in Figure 1B, IL-10 production also markedly declined after 30 passes (206.8 ± 25.1 vs. 116.9 ± 22.9 pg/mL; $P = 0.043$). Overall, TNF-α and IL-10 production decreased by 78 ± 9% and 47 ± 8%, respectively.

Effect of the sorbent beads on plasma removal of β_2M

Predialysis β_2M plasma levels were markedly elevated at 53.3 ± 1.4 μg/mL. However, after 10, 20, and 30 passes over the 10 mL sorbent column, plasma β_2M levels markedly decreased to 4.7 ± 1.0 μg/mL, 0.8 ± 0.2 μg/mL and 0.2 ± 0.0 μg/mL, respectively ($P = 0.002$ by Friedman test; Fig. 2). After 20 passes, any further decrease in β_2M levels did not reach statistical significance. There was a significant correlation between plasma β_2M levels and TNF-α production levels ($r = 0.760$; $P = 0.011$ by Spearman rank test).

Effect of RAGE antibody on TNF-α responsiveness

We first examined the contribution of AGE-modified proteins to the priming of THP-1–derived monocytes by uremic plasma. As shown in Figure 3, cells preexposed to rising concentrations of RAGE antibody and then primed with uremic plasma had marked reduction in LPS-stimulated TNF-α production ($P = 0.02$ by Friedman test). Indeed, compared with no antibody, LPS-stimulated TNF-α production markedly decreased after exposure to the 1:10 RAGE antibody dilution (553.5 ± 135.9 vs. 326.6 ± 70.8 pg/mL; $P = 0.04$).

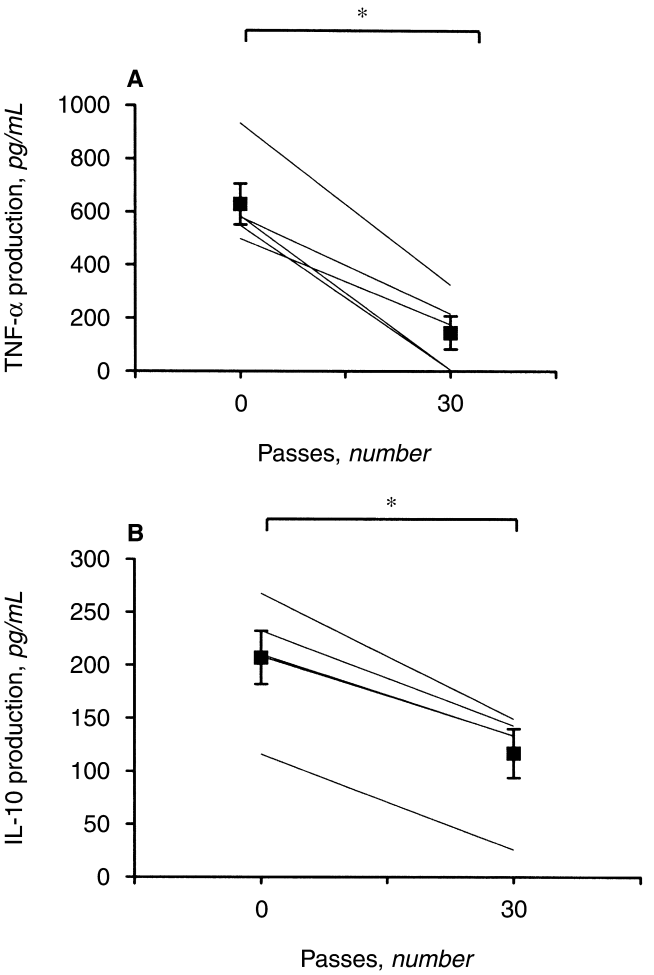

Fig. 1. Effect of the sorbent beads on tumor necrosis factor-α (TNF-α) (*A*) and interleukin-10 (IL-10) (*B*) responsiveness by THP-1–derived monocytes. Differentiated THP-1 cells were primed for 2 hours with uremic plasma, which was obtained before and after 30 passes over the perfusion column. Cells were then washed and stimulated with lipopolysaccharide (LPS) (10 ng/mL) for 16 hours. TNF-α and IL-10 were measured in cell supernatants by sandwich enzyme-linked immunosorbent assay (ELISA). Data are mean ± SEM of five experiments. *$P = 0.04$ vs. pass 0.

We finally examined the effect of uremic plasma obtained after 30 passes over the perfusion column on THP-1 responsiveness after RAGE neutralization. As shown in Figure 4, preexposure of cells to the neutralizing RAGE antibody (at 1:10 dilution) resulted in a further decrease in TNF-α production by 46 ± 15% ($P = 0.068$). These results suggest that despite plasma perfusion, AGE-modified proteins of the middle- and/or large-molecular-weight range were still present in the plasma.

DISCUSSION

In the present study, we explored whether the sorbent beads contained in the BetaSorb™ hemoperfusion col-

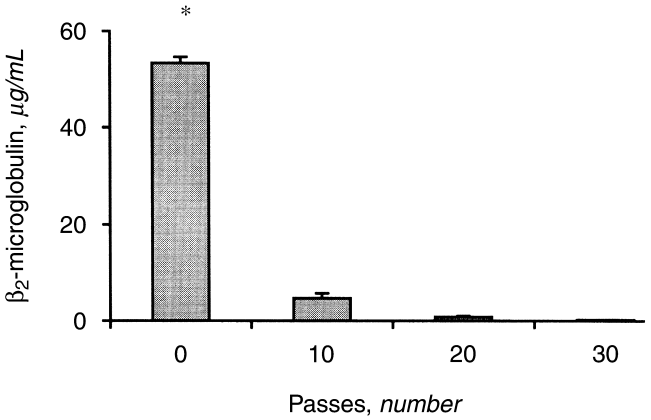

Fig. 2. Effect of the sorbent beads on removal of beta$_2$-microglobulin (β_2M). β_2M level was measured in plasma samples at baseline and after 10, 20, and 30 passes over the copolymeric beads contained in the BetaSorb™ perfusion column by a competitive binding enzyme immunoassay technique. Data are mean ± SEM of five experiments. *P = 0.04 vs. pass 0.

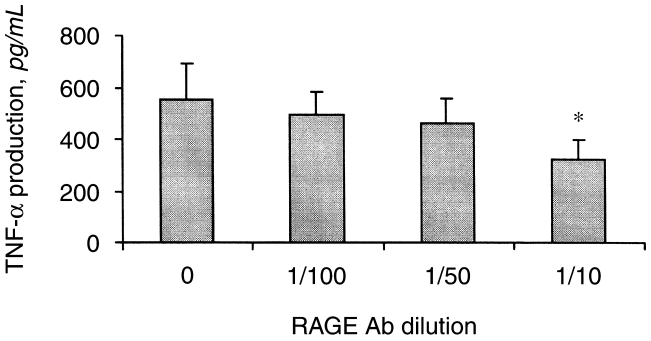

Fig. 3. Effect of receptor for advanced glycation end products (RAGE) antibody on THP-1–derived monocyte priming by uremic plasma. Differentiated THP-1 cells were preexposed to RAGE antibody for 3 hours, then washed and primed with uremic plasma for 2 hours. followed by lipopolysaccharide (LPS) (10 ng/mL) for an additional 16 hours. Tumor necrosis factor-α (TNF-α) was measured in cell supernatants. Data are mean ± SEM of five experiments. P = 0.02 by Friedman test. *P = 0.04 vs. no antibody.

umn modulate cytokine responsiveness by THP-1–derived monocytes. Our results first indicate that compared with normal plasma, uremic plasma enhanced LPS-stimulated TNF-α production, which was, in turn, markedly reduced by the sorbent. Of note, 30-pass TNF-α levels were normalized to those observed with nonuremic plasma. LPS-stimulated IL-10 production was not significantly lowered after priming with uremic plasma compared with normal plasma. However, IL-10 production further decreased after in vitro perfusion over the column. Of note, the concomitant reduction in IL-10 production is not inconsistent with the anti-inflammatory property of this molecule, which is normally produced in response to TNF-α, and acts to diminish transcription and production of TNF-α and other proinflammatory cytokines [11]. The

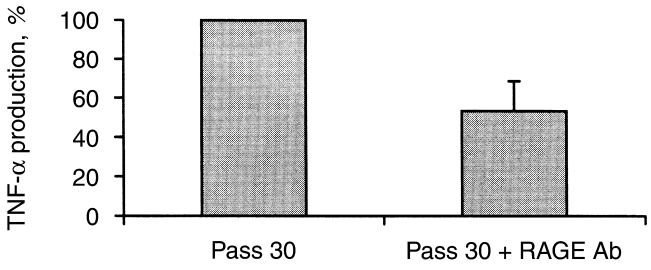

Fig. 4. Effect of receptor for advanced glycation end products (RAGE) antibody on THP-1–derived monocyte priming by perfused uremic plasma (pass 30). Differentiated THP-1 cells were preincubated with or without RAGE antibody (1:10 dilution) for 3 hours, then washed and primed for 2 hours with pass 30 uremic plasma, followed by polysaccharide (LPS) (10 ng/mL) for an additional 16 hours. Tumor necrosis factor-α (TNF-α) was measured in cell supernatants. Data are mean ± SEM of five experiments. P = 0.068 vs. no antibody.

results also indicate that the column removes β_2M efficiently, a surrogate marker of middle molecules.

The enhanced cytokine responsiveness following exposure of monocytes to uremic plasma suggests that uremic retention solute(s) prime(s) cells to enhanced production of proinflammatory cytokines. This may contribute to the chronic microinflammatory state of uremia. The marked decrease in the bioreactivity of uremic plasma following perfusion over the sorbent beads incriminates the removal of toxins in the middle-molecular-weight range. Although the mechanism behind the marked decline in cytokine production by monocytes after in vitro perfusion is elusive, we attempted to examine this question. Compared to AGE-modified β_2M, native β_2M has not been reported to affect cytokine production by monocytes [2]. Consequently, we performed additional experiments using neutralizing RAGE antibodies, where we first observed that AGE receptor blockade markedly reduces monocyte priming by uremic plasma, suggesting a strong involvement of this toxin solute in cytokine production. Because AGE-β_2M levels were not measured in this study, we can only speculate whether the sorbent beads also removed AGE-β_2M, resulting in marked reduction in cytokine generation by monocytes. These data are in agreement with recent findings, which demonstrated that AGE-β_2M prepared in vitro can enhance cytokine production [12]. Of note, patients with chronic kidney disease have high concentrations of AGEs in tissue and plasma, due to impaired elimination. In addition, new findings suggest that AGE-β_2M levels are also elevated among patients with end-stage renal disease [13, 14].

We attempted to examine indirectly whether the column also removes AGE-modified proteins. This was achieved by adding RAGE antibody to plasma samples that had been subjected to 30 passes over the column. We observed that even in this maximally perfused plasma sample, THP-1 cell preincubation with RAGE antibody resulted in a further decline in TNF-α production, sug-

gesting that AGE-modified proteins of middle- or larger-molecular-weight range were still present in the plasma. These speculations require further validation using various AGE-modified middle- and large-molecular-weight proteins, including β_2M, parathyroid hormone and albumin.

CONCLUSION

In summary, these in vitro studies indicate that the sorbent beads remove uremic solute(s) that prime monocytes to enhanced cytokine production. Although this column also removes β_2M efficiently, a surrogate marker of middle molecules, we were unable to directly incriminate this or other middle molecules in this reduced bioreactivity of uremic plasma.

Future in vitro studies will be required to examine the effect of the sorbent beads on the removal of middle- and large-molecular-weight toxins, including AGE-modified proteins, and dissect the signaling pathways leading to "priming" of monocytes. Finally, clinical studies with the BetaSorb™ column will need to be conducted, to examine whether these in vitro findings translate in vivo, into the attenuation of the exuberance of inflammatory responses, reflected by a decrease in systemic markers of inflammation, such as C-reactive protein levels.

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