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Removal of an endotoxin fragment (lipid A) with an endotoxin-retentive filter

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ABSTRACT

Endotoxin-retentive filters are used to purify the dialysate used for hemodialysis, but endotoxin fragments whose molecular weight is below the molecular weight cut-off point of the filter may pass through the filter. The objective of the present study was to determine whether lipid A, an endotoxin fragment, is sufficiently removed by an endotoxin-retentive filter. A filtration test for lipid A was carried out using an endotoxin-retentive filter. The filtrate was tested for the presence of lipid A by a biochemical analysis: the *Limulus* lysate test and a bioassay: the lymphocyte stimulation test. The endotoxin-retentive filter removed lipid A to below the limit of detection by the *Limulus* lysate test. However, the filtrate stimulated lymphocytes, suggesting that a small amount of lipid A whose concentration was below the limit of detection by the *Limulus* lysate test had passed through the filter. In conclusion, the endotoxin-retentive filter was capable of removing lipid A to a concentration below the limit of detection by the *Limulus* lysate test but the filtrate still stimulated lymphocytes.

Keywords: Endotoxin-retentive filter; Endotoxin fragment; Lipid A; *Limulus* lysate test; Lymphocyte stimulation

1. Introduction

Hemodiafiltration (HDF) enhances the potential of dialysis membranes by effectively using both diffusion and convection as driving forces to achieve the larger amount of blood purification over a wide spectrum of molecular weights [1]. On-line HDF, in which the dialysate is used as the replacement fluid, has been favored because it ensures an unlimited amount of replacement fluid at low cost and has been demonstrated to efficiently remove β2-microglobulin and larger solutes [2–4]. However, because a large volume of dialysate flows directly into the bloodstream during on-line HDF, ultrapure dialysate that fulfills the quality requirements of commercially available infusion solutions should be used in on-line HDF [5].

The endotoxin concentration of ultrapure dialysate must be below 1 EU/L, which is the limit of detection by the *Limulus* lysate tests in Japan [6]. Ultrapure dialysate is prepared in many dialysis centers by periodical chemical cleaning of the tube in which dialysate flows and the use of an endotoxin-retentive filter. Endotoxin-retentive filters are usually placed after the water purifying process, after the dialysate making process, and in front of the dialyzer. Endotoxin-retentive filters can remove large amounts of endotoxins, but endotoxin

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fragments whose molecular weight is below the molecular weight cut-off point of the filter may pass through it.

The objective of the present study was to determine whether lipid A, an endotoxin fragment, is sufficiently removed by an endotoxin-retentive filter. To this end, a filtration test for lipid A was carried out using an endotoxin-retentive filter. The filtrate was evaluated by a biochemical analysis: the *Limulus* lysate test and a bioassay: the lymphocyte stimulation test.

2. Materials and methods

2.1. Sensitivity of the Limulus lysate test as a means of measuring endotoxins and lipid A

An endotoxin standard (CSE-L, Seikagaku Corporation, Tokyo, Japan) was diluted in a vial with endotoxin-free distilled water to prepare a standard solution (0.06 ng/vial). The standard solution was used to prepare 1 mL volume of endotoxin solution having concentrations ranging from 1.0 to 10 μg/L by diluting with ultrapure water (made by Gradient-A10, Nihon Millipore, Tokyo, Japan). Chemically synthesized lipid A [7,8] (Peptide Institute, Inc., Osaka, Japan) was diluted with 560 μL of DMSO in a vial to prepare a standard solution having a concentration of 0.1 mg/vial. The standard solution was used to prepare 1 mL volume of endotoxin solution having concentrations ranging from 1.0 to 10 μg/L by diluting with the ultrapure water.

Endotoxins are bacterial lipopolysaccharides, and their fatty acids and sugar structures vary from species to species. The molecular weight of endotoxin molecules is approximately 8,000, and they form dimers or micelles in aqueous solution. The molecular weight of the lipid A used in this study is 1,798. We compared the endotoxin activity of the endotoxin standard and lipid A on the basis of their molar concentrations.

Endotoxin activity in each sample was measured in endotoxin units (EU/L) by a turbidimetric kinetic assay [9]. *Limulus* amebocyte lysate (Limulus ES-II, Wako Pure Chemical) was used in this assay. The turbidity of the sample reacted with the lysate was measured by a Toxinometer (ET-201, Wako Pure Chemical).

2.2. Lymphocyte stimulation by lipid A solutions of different concentrations

Fresh porcine blood obtained from a slaughterhouse in Tokyo (Tokyo Shibaura Zoki, Tokyo, Japan) on the morning of the day of the experiments was carefully transferred in a cool box to our laboratory. Lymphocytes were isolated from the whole porcine blood by a density gradient centrifugation method [10–12] using lymphoprep™ (Axis-S hield, Oslo, Norway). The concentrations of the isolated lymphocytes were adjusted to 2.5 cells/mL by suspending in Hanks' balanced salt solution (without calcium, magnesium, or phenol red, Cambrex Bio Science, Walkersville, MD, USA) with 10 v/v% of porcine plasma obtained from the same blood. After adding 80 μL of lymphocyte solution, 20 μL of sample solution (lipid A solution at concentrations ranging from 3,000 to 0.03 EU/L) and 20 μL of WST-8 solution (Dojindo Laboratories, Kumamoto, Japan) to each well of a 96-well endotoxin-free microplate, the solution was incubated for 1 h at 37°C in a humid CO_2 (5%) incubator, and the absorbance of the sample solution in each well was then measured at 450 nm (reference wavelength: 655 nm) with a microplate reader (model 680, BioRad). WST-8, $C_{20}H_{13}N_6NaO_{11}S_2$, which is reduced by dehydrogenase in cells and produces a water-soluble formazan dye [13,14], was used to detect dehydrogenase activity in the cells. A sample prepared by adding 20 μL of 5 μg/mL concanavalin A (Con A) solution instead of lipid A solution was used as a positive control, and a sample prepared by adding 20 μL of ultrapure water instead of lipid A solution was used as a negative control.

2.3. Removal of lipid A by the endotoxin-retentive filter as measured by the Limulus lysate test and the lymphocyte stimulation test

The endotoxin-retentive filter we used in the resent study was a micro-particle cut filter (EF-01, Nikkiso, Tokyo, Japan), which is the most commonly used type of endotoxin-retentive filter in Japan. The membrane material is a polyester polymer alloy (PEPA), and the area of the membrane is 1.2 m^2. The endotoxin-retentive filter was connected to the circulation circuit composed of silicone tubes, and the circuit connected to the filter was then chemically cleaned by circulating 0.4% sodium hypochlorite solution through it for 48 h, after which the circuit was thoroughly rinsed with ultrapure water. The endotoxin concentration of a sample of the solution in the circuit was confirmed to be below the limit of detection by the *Limulus* lysate test (<0.6 EU/L), and the lipid A filtration test was carried out by adding lipid A to the circulating water at concentrations ranging from 10 to 100 EU/L. The lipid A solution was filtered from the shell-side to the lumen-side of the filter for 4 h at a flow rate of 30 L/h (500 mL/min). During the course of filtration, the filtrate was continuously returned to the tank (filtrate recycle-dead end filtration), and the solution was

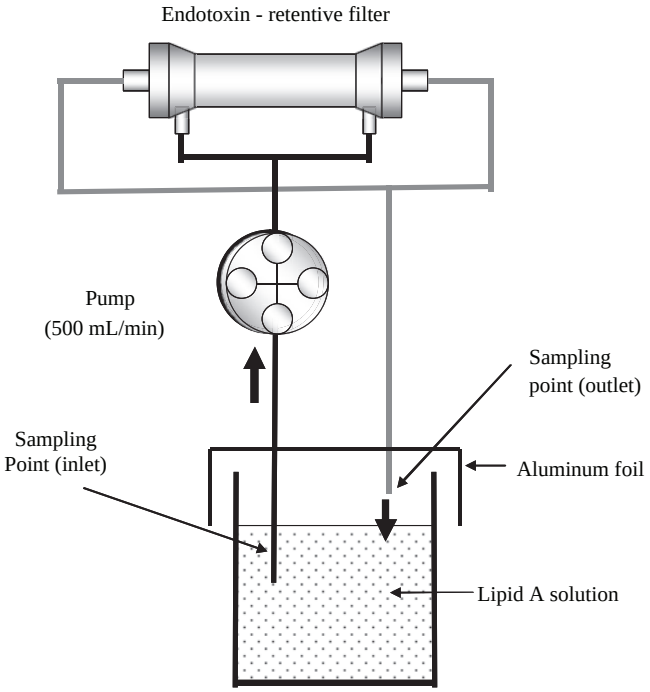

Fig. 1. Experimental apparatus for evaluation of the endotoxin-retentive filter. Filtration tests for lipid A were carried out by adding lipid A to the circulating water to concentrations ranging from 10 to 100 EU/L. The endotoxin-retentive filter was connected to a circulation circuit composed of silicone tubes. The lipid A solution was filtered from the shell-side to the lumen-side of the filter for 4 h at a flow rate of 30 L/h.

sampled from the inlet of the filter (sampled from the tank) and outlet of the filter at 10, 30, 60, 90, 120, 180, and 240 min after the start of filtration (Fig. 1).

The endotoxin activity of each sample was measured in endotoxin units (EU/L) by using *Limulus* amebocyte lysate. Endotoxin activity was determined from the turbidity of the sample mixed with *Limulus* amebocyte lysate by preparation of a standard curve for each measurement. In the standard curve, endotoxin activity was well correlated with the turbidity if the endotoxin activity was over 0.6 EU/L, indicating that the limit of detection was 0.6 EU/L. Therefore all the values of endotoxin activity that were below 0.6 were treated as 0.6. The sample was also evaluated by using a lymphocyte stimulation test to confirm whether the filtrate contained a small amount of lipid A.

2.4. Statistical analysis

All data are reported as the mean ± SD. The statistical significance of differences in paired samples was determined by using paired Student's *t*-test.

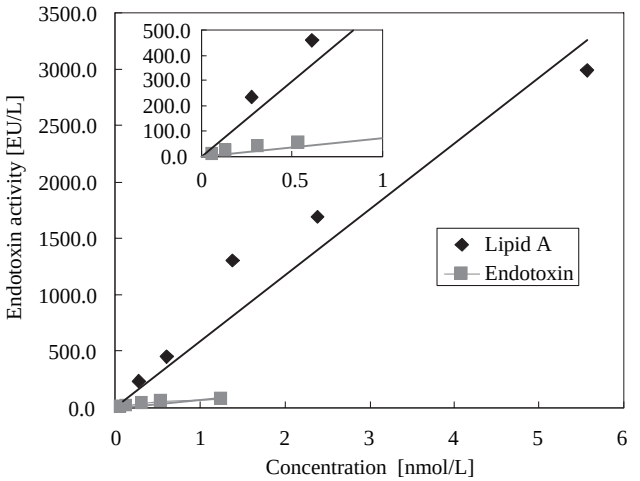

Fig. 2. Sensitivity of the *Limulus* lysate test as a means of measuring endotoxin and lipid A. Endotoxin activity measured by the *Limulus* lysate test at the same molar concentrations was higher for lipid A than for the endotoxin standard. This finding indicated that the activity of lipid A measured by the *Limulus* lysate test was greater than that of the endotoxin standard.

A probability value (*p*-value) of less than 0.05 was considered statistically significant.

3. Results

3.1 Sensitivity of the Limulus lysate test as a means of measuring endotoxin and lipid A

The sensitivity of the *Limulus* lysate test for lipid A was assessed by comparing it to its sensitivity for measuring an endotoxin standard. Endotoxin activity measured by the *Limulus* lysate test for the same molar concentrations of both substances was higher for lipid A than that for the endotoxin standard (Fig. 2), indicating that the activity of lipid A was greater than that of the endotoxin standard.

3.2. Lymphocyte stimulation by lipid A solutions of different concentrations

To determine whether the lymphocyte stimulation test can be used to detect low concentrations of lipid A, we tested the effect of different concentrations of lipid A on lymphocytes, especially of concentrations below the limit of detection by the *Limulus* lysate test. The dehydrogenase activity of the lymphocytes to which lower concentrations of lipid A solution were added was significantly higher than in the absence of lipid A (negative control), indicating that the small amount of lipid A in the solutions is capable of stimulating lymphocytes. Even when the concentration of

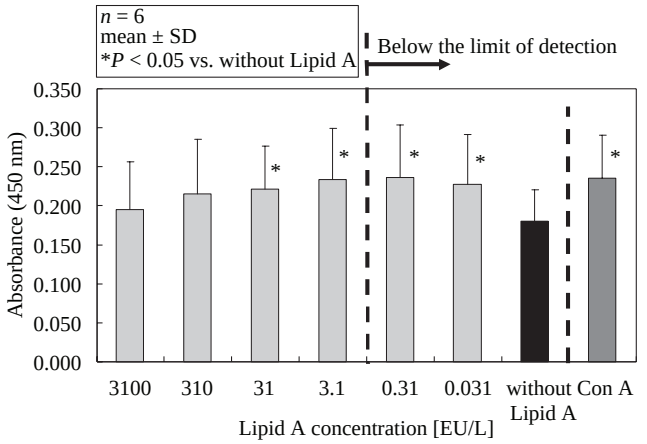

Fig. 3. Lymphocyte stimulation with lipid A solutions of different concentrations. Even when the concentration of lipid A was 1/100 of the limit of detection by the *Limulus* lysate test, the dehydrogenase activity of the lymphocytes after addition of the lipid A solution was significantly higher than in the absence of lipid A (negative control), indicating that a small amount of lipid A below the level of detection by the *Limulus* lysate test can stimulate lymphocytes

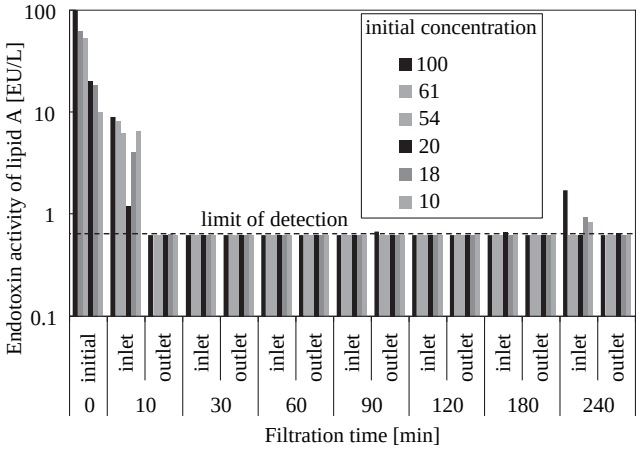

Fig. 4. Removal of lipid A by the endotoxin-retentive filter. Irrespective of the initial concentration of lipid A it was removed until the endotoxin activity of the filtrate was below the limit of detection at every time point measured. The lipid A concentration of the solution at the filter inlet also decreased to almost below the limit of detection after 30 min of filtration, indicating that the filter had removed lipid A by adsorption.

lipid A was 1/100 of the limit of detection by the *Limulus* lysate test, lipid A significantly stimulated the lymphocytes (Fig. 3).

3.3. Removal of lipid A by the endotoxin-retentive filter

The endotoxin concentration of the filtrate was measured by the *Limulus* lysate test. Irrespective of the initial concentration of lipid A, it was removed until the endotoxin activity of the filtrate was below the limit of detection at every time point measured (Fig. 4). The lipid A concentration of the solution at the filter inlet also decreased to almost below the limit of detection after 30 min of filtration, indicating that the filter had removed lipid A by adsorption.

Although lipid A was removed by the endotoxin-retentive filter to a concentration below the limit of detection by the *Limulus* lysate test, it was still unknown whether the small amount of lipid A had passed through the filter. A sample of the solution in the tank (inlet of the filter) and a sample of the filtrate (outlet of the filter) were evaluated by the lymphocyte stimulation test. The solution in the tank and filtrate both significantly increased the dehydrogenase activity in the lymphocytes in comparison to a solution containing no lipid A (Fig. 5), even though the concentration of the filtrate was below the limit of detection by the *Limulus* lysate test. These results suggest that a small amount of lipid A had passed through the filter.

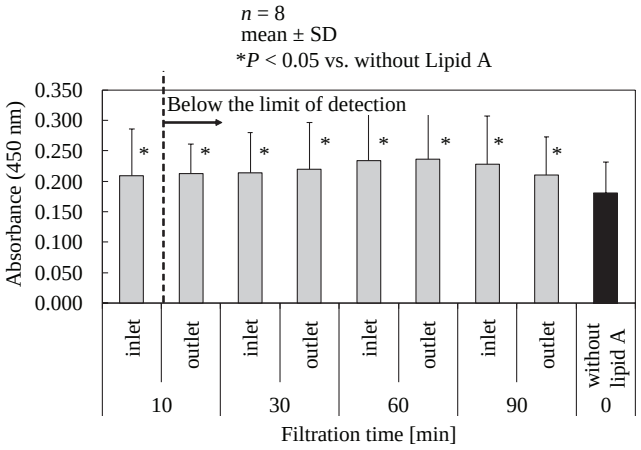

Fig. 5. Stimulation of lymphocytes by the filtrate of the endotoxin-retentive filter. All filtrates whose lipid A concentration was below the limit of detection by the *Limulus* lysate test significantly increased lymphocyte dehydrogenase activity in comparison with a solution that did not contain lipid A, indicating that the lymphocytes had been stimulated. The sample of solution from the tank also stimulated the lymphocytes. These results indicate that the small amount of lipid A that had passed through the filter stimulated the lymphocytes

4. Discussion

To determine whether a lymphocyte stimulation test can be used to detect a small amount of lipid A, a wide range of lipid A concentrations was tested for

their ability to stimulate lymphocytes. The results showed that especially at the lower concentrations, including below the limit of detection by the *Limulus* lysate test, lipid A can be detected by the increase in dehydrogenase activity of the lymphocytes in vitro. Their activity was measured with WST-8, which was developed to measure the dehydrogenase activity of cells as an indicator of cell viability [13,14]. Many types of dehydrogenase are present in cells, and become activated in response to various stimuli. For example, acetaldehyde dehydrogenase acts in the process of metabolism of the mitochondria and converts NAD^+ into NADH [15]. The activation of dehydrogenase in the cells detected by WST-8 was occurred via many activation pathways, so that this test can only detect whether the stimuli had existed or not. Therefore, this test is inappropriate for quantitative analysis, but had much higher sensitivity than the *Limulus* lysate test. In this study, we performed this bioassay with porcine lymphocytes as well as the *Limulus* lysate test to detect the small amount of lipid A that may pass through the filter. Our results also indicates that it will be necessary to increase the sensitivity of the *Limulus* lysate test or develop another method of analysis to detect the small amount of endotoxin fragments which may pass through the filter.

A filtration test for lipid A was carried out using the endotoxin-retentive filter. Firstly, the filtrate and solution in the tank were subjected to the *Limulus* lysate test. Lipid A is an endotoxin fragment whose molecular weight is below the molecular weight cut-off point of the filter, and thus it can pass through the filter if the size exclusion is the only mechanism to determine the solute retention of the filter. However, lipid A can be removed by the endotoxin-retentive filter to a concentration below the limit of detection. The endotoxin concentration of the solution in the tank also decreased to below the limit of detection. Therefore, lipid A was concluded to have been removed by the endotoxin-retentive filter mainly by adsorption.

Since endotoxins are likely to form micelles in the dialysate, almost all the endotoxins are large enough to be removed by the filter. Only a small fraction of endotoxins in the dialysate considered to be endotoxin fragments like lipid A. Therefore, little attention has been given to the small amount of endotoxin fragments in clinical situations. However, the results of the lymphocyte stimulation test indicated that the filtrate of the endotoxin-retentive filter contained a small amount of lipid A if the inlet solution contains endotoxin fragments. Therefore, if dialysate is contaminated by endotoxin fragments, the fragments possibly pass through the filter though the amount is small. Since the dialysate flows directly into the bloodstream during on-line HDF, purifying the dialysate to an endotoxin concentration at least below 0.01 EU/L (1/100 of the limit of detection by the *Limulus* lysate test) would be desirable to ensure safety. Because a single passage through the endotoxin-retentive filter decreased the lipid A level to 1/100 of the inlet concentration, practical solution to reduce the possibility of flowing the endotoxin fragments into bloodstream in on-line HDF is that the endotoxin-retentive filter is used to purify a dialysate whose endotoxin concentration has already been reduced to below the limit of detection. It is recommended that the use of two endotoxin-retentive filters connected in series in front of dialyzer.

Many previous clinical studies have shown that using ultrapure dialysate in hemodialysis has provided many clinical benefits, including a decrease in proinflammatory cytokine production [16], delays in the development of carpal tunnel syndrome and amyloidosis [17,18], a decrease in plasma pentosidine, a marker of carbonyl stress [19,20], an increase in hematocrit, and reduction in the dose of recombinant human erythropoietin [21,22]. The molecular weight cut-off point of the highly permeable dialysis membrane used in the previous study was equivalent to that of the endotoxin-retentive filter. Therefore, if the dialysate had been contaminated by a small amount of endotoxin fragments, endotoxin fragments would have flowed into the blood through the dialysis membrane. These results indicate that ultrapure dialysate should not only be used in on-line HDF but in hemodialysis as well.

4. Conclusions

A endotoxin-retentive filters removed lipid A to a concentration below the limit of detection by the *Limulus* lysate test, but the filtrate still stimulated lymphocytes, suggesting that a small amount of lipid A passed through the filter. It will therefore be necessary to increase the sensitivity of the *Limulus* lysate test or develop another method of analysis to detect the small amount of endotoxin fragments that may pass through the filter.

References

[1] C. Ronco and D. Cruz, Adv. Chronic Kidney Dis., 14 (2007) 231–243.
[2] W. Lornoy, I. Becaus, J.M. Billiouw, L. Sierens and P. van Malderen, Am. J. Nephrol., 18 (1998) 105–108.
[3] W. Lornoy, I. Becaus, J.M. Billiouw, L. Sierens, P. van Malderen and P. D'Haenens, Nephrol. Dial. Transpl., 15 Suppl. 1 (2000) 49–54.
[4] V. Wizemann, C. Lotz, F. Techert and S. Uthoff, Nephrol. Dial. Transpl., (15 Suppl. 1) (2000) 43–48.
[5] I. Ledebo, Adv. Ren. Replace. Ther., 6 (1999) 195–208.

[6] I. Masakane, Ther. Apher. Dial., 10 (2006) 348–354.
[7] M. Imoto, S. Kusumoto, T. Shiba, E.T. Rietschel, C. Galanos and O. Lüderitz, Tetrahedron Lett., 26 (1985) 907–908.
[8] M. Imoto, H. Yoshimura, T. Shimamoto, N. Sakaguchi, S. Kusumoto and T. Shiba, Bull. Chem. Soc. Jpn., 60 (1987) 2205–2214.
[9] H Oishi, Y. Hatayama, H. Shiraishi, K. Yanagisawa and Y. Sakata, J. Pharm. Soc. Jpn. (Yakugaku Zasshi), 105 (1985) 300–303 (Japanese).
[10] A. Boyum, Scand. J. Clin. Lab. Invest. Suppl., 97 (1968) 7.
[11] R. Harris and E.O. Ukaejiofo, Lancet, 2 (1969) 327.
[12] A. Ting and P.J. Morris, Vox Sang., 20, (1971) 561–563.
[13] M. Ishiyama, Y. Miyazono, K. Sasamoto, Y. Ohkura and K. Ueno, Talanta, 44 (1997) 1299–1305.
[14] H. Tominaga, M. Ishiyama, F. Ohseto, K. Sasamoto, T. Hamamoto, K. Suzuki and M. Watanabe, Anal. Commun., 36 (1999) 47–50.
[15] M. Rigoulet, H. Aguilaniu, N. Avéret, O. Bunoust, N. Camougrand, X. Grandier-Vazeille, C. Larsson, I.L. Pahlman, S. Manon and L. Gustafsson, Mol. Cell. Biochem., 256–257 (2004) 73–81.
[16] R. Bambauer, J. Walther, S. Meyer, S. Ost, M. Schauer, W.K. Jung, H. Göhl and J. Vienken, Artif. Organs, 18 (1994) 188–192.
[17] M. Baz, C. Durand, A. Ragon, K. Jaber, D. Andrieu, T. Merzouk, R. Purgus, M. Olmer, J.P. Reynier and Y. Berland, Int. J. Artif. Organs, 14 (1991) 681–685.
[18] S. Schwalbe, M. Holzhauer, J. Schaeffer, M. Galanski, K.M. Koch and J. Floege, Kidney Int., 52 (1997) 1077–1083.
[19] Y. Izuhara, T. Miyata, K. Saito, N. Ishikawa, T. Kakuta, M. Nangaku, H. Yoshida, A. Saito, K. Kurokawa and C. van Ypersele de Strihou, Am. J. Kidney Dis., 43 (2004) 1024–1029.
[20] R. Furuya, H. Kumagai, M. Takahashi, K. Sano and A. Hishida, Blood Purif., 23 (2005) 311–316.
[21] H. Schiffl, S.M. Lang and A. Bergner, Nephron, 83 (1999) 278–279.
[22] P.Y. Hsu, C.L. Lin, C.C. Yu, C.C. Chien, T.G. Hsiau, T.H. Sun, L.M. Huang and C.W. Yang, J. Nephrol., 17 (2004) 693–700.