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Short Communication

Specific Removal of Monocytes from Peripheral Blood of Septic Patients by Polymyxin B-immobilized Filter Column

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Lipopolysaccharide (LPS) is one of the major causes of septic shock. The polymyxin B-immobilized filter column (PMX) was developed for the adsorption of endotoxin by direct hemoperfusion and has been used for the treatment of LPS-induced septic shock. In this study, we demonstrated that PMX also specifically bound monocytes from the peripheral blood leukocytes of septic patients by mean of an analysis of bound cells using immunocytochemical and electron microscopic techniques. The specific removal of monocytes from septic patients may produce beneficial effects by reducing the interaction between monocytes and functionally associated cells including vascular endothelial cells.

Key words: septic shock, polymixin B-immobilized column, monocyte, adsorptive removal

Sepsis and septic shock, often associated with multiple organ failure, still remain important causes of morbidity and mortality in intensive care units. Many types of therapeutic trials for the treatment of septic shock have failed however, recent phase III studies using recombinant activated protein C demonstrated the effectiveness of this therapy [1, 2]. Lipopolysaccharide (LPS), one of the major causes of septic shock, together with LPS binding protein, binds to CD14 on the surfaces of monocytes/macrophages, leading to the activation of the signaling molecule complex of Toll-like receptor-4 (TLR-4) and MD2. Polymyxin B can bind LPS and neutralize its biological activity therefore, the polymyxin B-immobilized filter (PMX) column was developed for the adsorption of endotoxin by hemoperfusion [3]. There is now increasing evidence supporting the usefulness of this treatment, showing improvement of the survival rate in LPS-induced circulatory disorders and systemic inflammatory response syndrome. Moreover, the effectiveness of hemoperfusion with this column for treating septic shock beyond LPS endotoxemia [4] prompted us to investigate additional mechanisms. Since it is well known that different populations of leukocytes are activated during septic shock and change their adhesive phenotype, we hypothesized that some population of leukocytes may be adsorbed in the column and removed from the blood circulation after treatment. To examine this hypothesis, we investigated the cellular components in the PMX columns after direct hemofiltration in 4 septic patients.

The original diseases of the 4 patients were ileus of sigmoid colon (case 1; 69 ys male), embolism of superior mesenteric artery (case 2; 76 ys male), pye-

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lonephritis (case 3; 67 ys female) and liver abscess (case 4; 58 ys female). The patients were admitted to the university hospital or educational training hospitals in Okayama city from 2003 to 2004. The systolic and diastolic blood pressures before and after the treatment were 87.5 ± 20.2 and 48.3 ± 7.7 mmHg vs. 105.8 ± 29.2 and 56.3 ± 15.3 mmHg, respectively (mean $\pm$ SEM). The investigations were carried out with the approval of the ethical review committee of Okayama University Medical School. Written informed consent was obtained from the families of all patients.

The used PMX columns were washed with 500 ml of saline in the ICU. Then, the columns were cooled on ice and moved into a cold room at 4℃. The columns were successively washed with 500 ml of ice-cold saline at a flow rate of 16 ml/min 3 times, and the cells in each 500 ml fraction were analyzed. The columns were then opened and the cells attached to the filters were collected by shaking the filter in ice-cold saline. The collected cells from the fiber were smeared on the slide glass and subjected to May-Grunwald-Gimsa or immunocytochemical staining with PE-conjugated anti-CDl4 and FITC-conjugated anti-CD68 antibodies. For scanning electron microscopic and transmission electron microscopic examinations, the PMX and the collected cells from the PMX column were fixed with 1% glutaraldehyde in 0.1 M phosphate buffer. FACS analysis was performed on the collected cells as well as peripheral blood mononuclear cells as described previously [5].

We found huge amounts of cellular components in the hemoperfused PMX columns from 4 patients (Fig. 1A). Fig. 1C shows the nuclear staining of leukocytes on the filter by hematoxylin when the filter was fixed immediately after opening the column. Fig. lA is the corresponding picture made by scanning electron microscopy in which the cells include leukocytes and erythrocytes. About 70 to 80% of the total cells were recovered from the filter by gentle shaking in ice-cold saline (Figs. 1B and D). Fig. 2B clearly shows by May-Grunwald-Gimsa staining that the recovered leukocytes have the typical nuclear shape of monocytes with slight basophilic cytoplasm. Immunocytochemical staining confirmed that almost all leukocytes were immunoreactive for both CDl4 and CD68 (Figs. 2C–F). The transmission electron microscopy also revealed that most of the cells recovered from the PMX filter have monocyte features polymorphic nuclei, few specific granules and many vacuoles (Fig. 3). Apoptotic changes such as nuclear condensation were not observed. These findings were common to four cases. Fig. 4 summarizes the leukocyte populations in the washings and in the PMX columns from four patients. Even in the washing fraction more than 75% cells were monocytes, although the cell numbers in the washings were less than 1% of those attached to the filter. The CD68 localization on the plasma membrane as well as within the cytoplasm (Fig. 2E) and the ultrastructural appearance of monocytes with irregularly shaped processes (Fig. 3) and numerous phagocytic vesicles strongly suggested that the monocytes trapped in the column had been activated through the pathological processes of septic shock. These results as a whole indicated that the PMX column specifically adsorbed monocytes (98.5% purity) among leukocytes from the peripheral blood of septic patients.

Analysis of the expression of adhesion molecules in monocytes from the peripheral blood as well as those recovered from PMX columns revealed that the expression levels of all adhesion molecules examined in patients were lower than those in normal volunteers (Fig. 5). The comparison of the expression levels of each adhesion molecule among monocytes from pre-treatment PBMC and those from PMX columns showed that the expression levels of CD11b in monocytes from PMX columns were lower than those in pre- and post-treatment PBMC. Also, CD62L expression in monocytes from PMX columns was lower than that in post-treatment PBMC. CD62L and CD11b, an α chain of the Mac-1 molecule, were considered to be involved in the cellular interaction therefore, the reduced levels of CD11b and 62L in monocytes in PMX columns may represent the shedding of these molecules after the stimulation of monocytes. Although the detailed phenotype features of bound monocytes are not known at present, it is quite likely that specific populations of monocytes show an increased affinity to PMX. It is not clear what enabled the monocytes with low levels of CD11b and CD62L to bind selectively to PMX. However, the removal of particular populations of monocytes from blood may provide a new strategy for the treatment of septic shock. Further research is necessary to confirm the causative relationship between the removal of specific

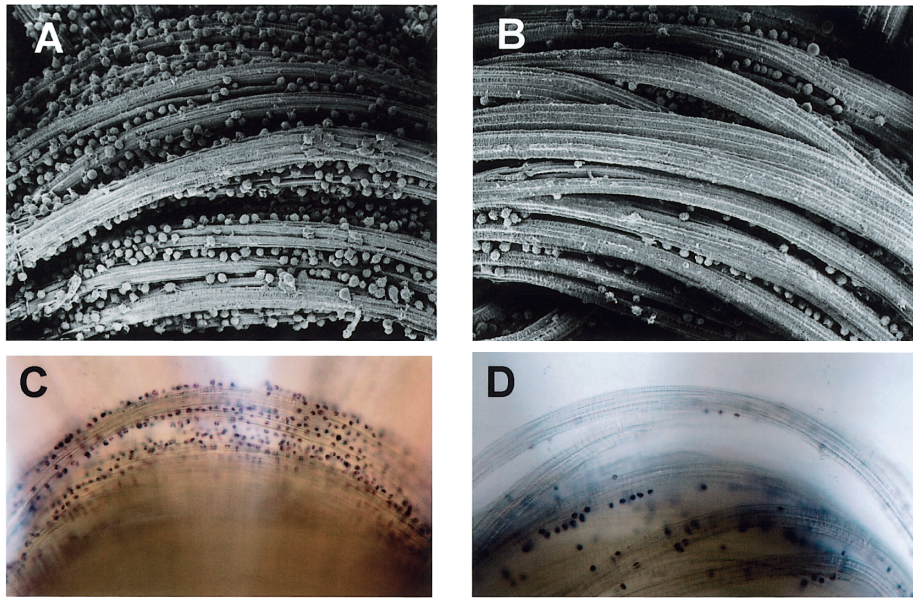

Fig. 1 Analysis of cellular components in the PMX columns. Scanning electron micrographs of the filter (**A**, **B**) and light micrographs of the hematoxylin-stained filter (**C**, **D**) show the cellular components in the PMX column before (**A**, **C**) and after the cells were collected (**B**, **D**). Numerous leukocytes were trapped on the filter, and a major portion of these cells were easily collected from the filter.

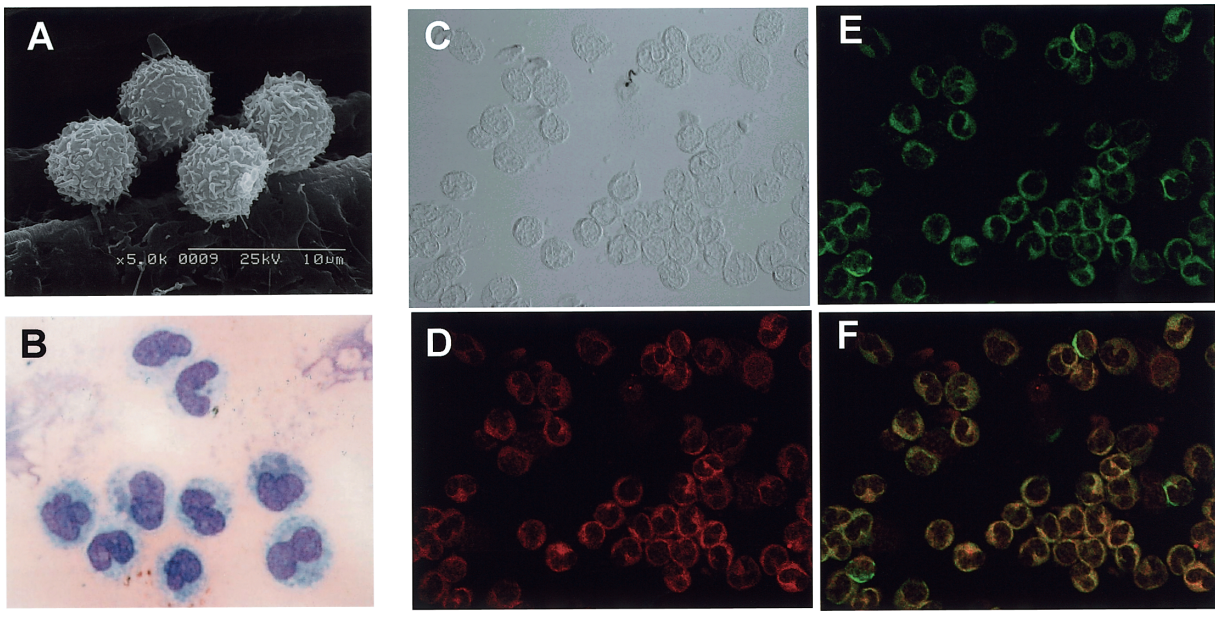

Fig. 2 Identification of leukocyte population. (**A**) Image of scanning electron microscopy of leukocytes on PMX filter. Smeared leukocytes from the column stained with May-Grunwald-Giemsa presented the typical monocyte appearance (**B**). Smeared samples (phase contrast image (**C**)) were immunostained with PE-conjugated anti-CDl4 (**D**) and FITC-conjugated anti-CD68 (**E**). Merged view (**F**). The specimen was from the patient identified as case 2.

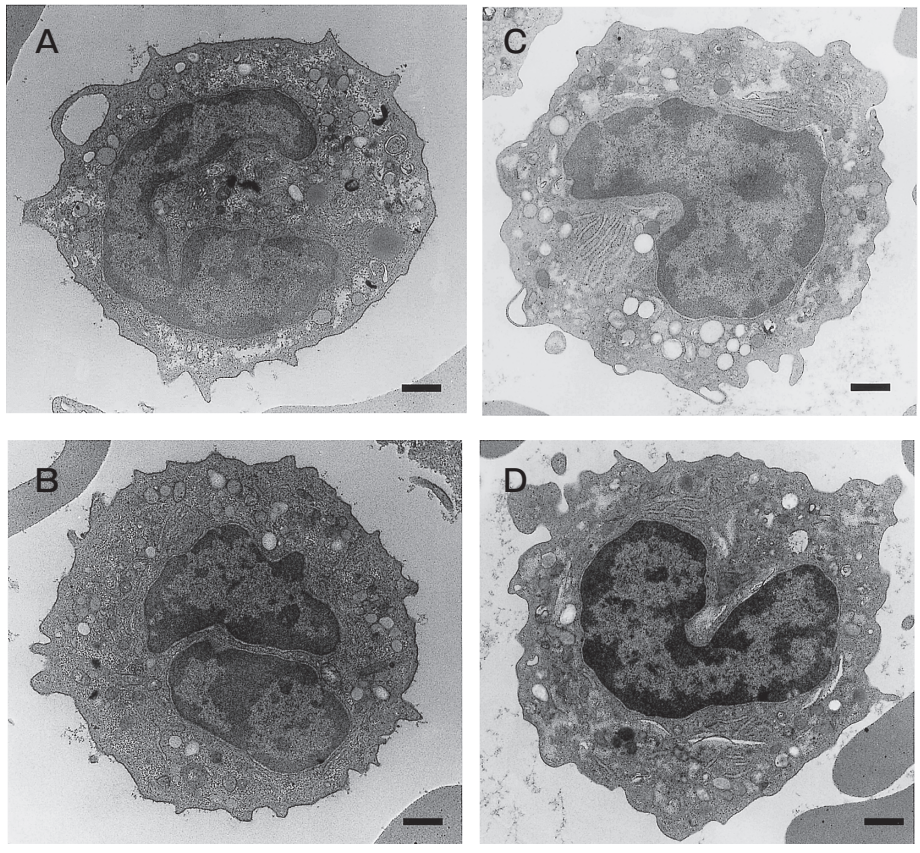

Fig. 3 Transmission electron micrograph of monocytes detached from the filter. The recovered leukocytes were fixed for transmission electron micrography. As shown in Fig. 2, most of the cells were monocytes. Four representative 4 cells are shown. There were vacuoles observed in the cytosol however, apoptotic changes such as condensation of nuclei were not present. The specimen was from the patient identified as case 2. Scale bars equal 1 μm.

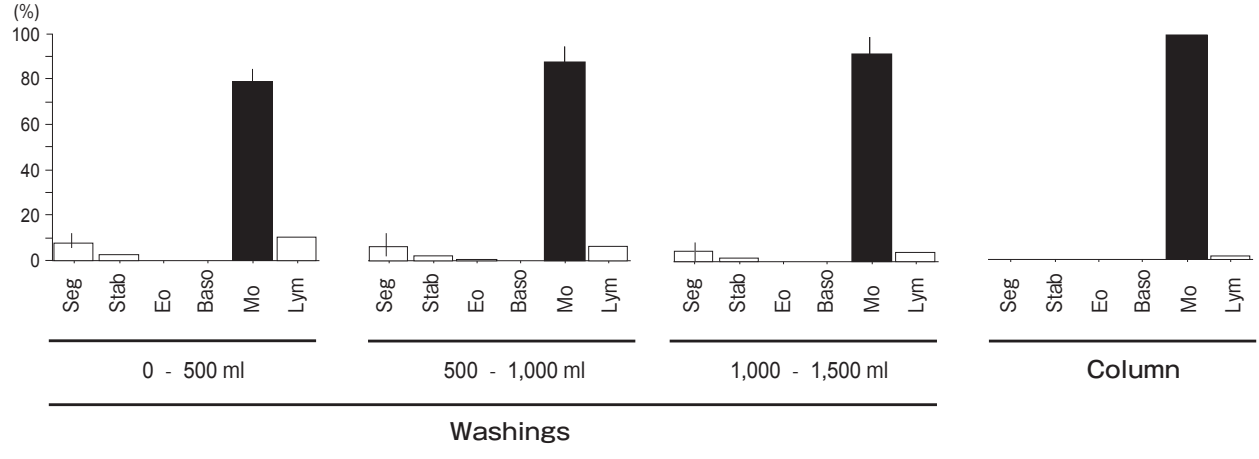

Fig. 4 Classification of leukocyte populations from washings and polymixin B-immobilized column. Smeared samples from each 500 ml washing and polymixin B-immobilized filter were used for the classification of leukocytes by May-Grunwald-Giemsa staining. The results are expressed as percentages of total cells (means $\pm$ SEM of 4 patients). Seg, segmented neutrophil; Stab, stab neutrophil; Eo, eosinophil; Baso, basophil; Mo, monocytes; Lym, lymphocyte.

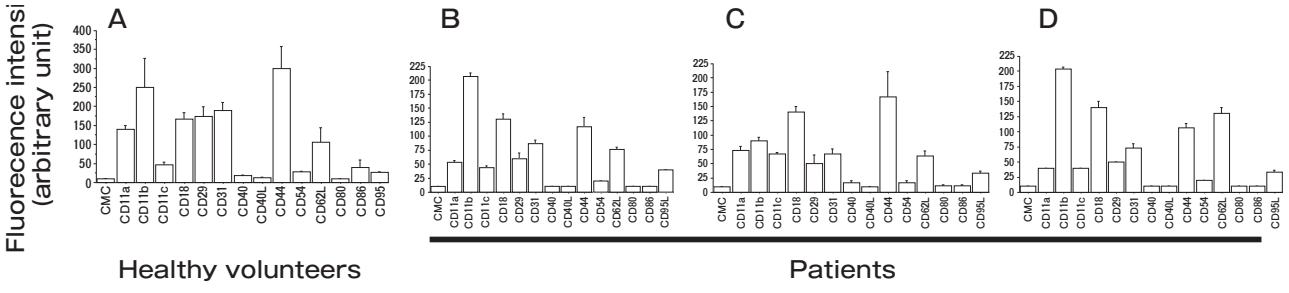

Fig. 5 FACS analysis of adhesion molecule expression on monocytes from healthy volunteers and patients. The peripheral blood was collected from 4 healthy volunteers (**A**) and patients before (**B**) and after (**D**) PMX treatment. PBMC was prepared from the peripheral blood. A diverse range of adhesion molecules on CD14-positive monocytes was analyzed by FACS. In **C**, the data on monocytes collected from PMX columns are shown. The data are the means ± SEM of 4 individuals.

monocytes and the beneficial effects of PMX treatment in septic shock.

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