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Original

Development of a Small-sized Leukapheresis Column for the Treatment of Inflammatory Diseases

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Cytapheresis methods have been extended to remove cells such as leukocytes components, and are used for autoimmune disease treatment. However, the large blood volumes in conventional columns may restrict clinical use. We developed a small cytapheresis column. We evaluated its leukocyte adsorption property and inhibitory effect on gonarthritis. The priming volume was reduced to approximately one-third that of conventional columns. Leukocytes adsorbed to a mini-column for rabbits were counted following a 1-hour circulation immediately and 24 hours after the elicitation of inflammation. The diameter of joint swelling was measured at 24 hours and 48 hours. The adsorption property was found to differ; the number of removed leukocytes in the conventional column was largerimmediately after the elicitation, but was smaller at 24 hours, compared to the developed column. Thus, the developed column has a higher adsorption rate. For the inhibitory effect on gonarthritis, swelling decreased in the developed column group before and after the 24-hour circulation, compared with the sham column. Thus, the developed and conventional columns have equal inhibitory effects. These results show that the developed column improved selectivity and removal efficiency for granulocytes and monocytes and showed performance comparable to the conventional column.

Key Words: leukapheresis column, extracorporeal circulation, small-sized priming volume, gonarthritis

Introduction

In the 1910s, the dialyzer[1] was developed by Abel, and since the 1960s, when Kiil et al developed a laminated flat-membrane[2] dialyzer made of cuprophane, dialyzers have become widely known. In the 1970s, efficient and disposable hollow fiber membrane[3] dialyzers made of synthetic macromolecules were developed, and the clinical use of dialysis treatment spread throughout the world. In addition to the principle of dialysis, new techniques and devices related to filtration or adsorption were developed, and these therapies gained adoption under the overall concept of blood purification therapy[4]. Japan in particular has played a leading role in furthering this therapy. In blood purification therapy, disease-causing agents (such as urotoxins, antibodies and inflammatory cytokines) or cells (such as lymphocytes, granulocytes or viruses) are removed from the blood by extracorporeal circulation, as therapy for organs that are difficult to treat, or for intractable diseases such as immunodeficiency, with the aim of improving the patient's condition. At present, there are therapies that use various "columns" (blood purification devices) including 1) dialyzers, 2) combinations of plasma separators and plasma component separators (plasma exchange)[5], 3) adsor-

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ption-based blood purifiers[6] and 4) cytapheresis-based purifiers (cytapheresis therapy)[7].

Cytapheresis therapy has attracted particular attention in recent years. A "column" that removes cells such as granulocytes, monocytes and lymphocytes[8)9)] has been developed for cytapheresis therapy, and this therapy has come to be widely used in blood purification therapy, particularly in autoimmune inflammatory diseases (mainly diseases such as ulcerative colitis, Crohn's disease and rheumatoid arthritis[10)]). Two types of column are in clinical use: a type using an adsorption method[11)], filled with acetyl cellulose beads, which employs the complement activation on the surface of the beads to remove mainly granulocytes and monocytes, and a type using a filtration method[12)13)], with layers of rolled sheets of nonwoven fabric to remove white blood cells in general. However, the columns in both these types tend to be large, in the former type because a large surface area is required to adsorb white blood cells, and in the latter type to reduce clogging when large numbers of white blood cells are removed. Because of this, conventional "columns" have a large capacity for blood, 130 mL or more, which can lead to restrictions[14)15)] on clinical use such as hindrances to the use of the device in children or the elderly due to concerns about problems such as decreases in blood pressure during external diafiltration. Therefore, the present research aimed to develop a new smaller cytapheresis column that could also be used safely for cytapheresis therapy in children or the elderly, with a performance equivalent to that of conventional cytapheresis products, but with about 1/3 of the blood capacity (50 mL). Specifically, we attempted to make a small-sized column that could remove activated granulocytes or monocytes selectively with high efficiency, by using ultrafine fibers for the adsorbent to increase the adsorbent surface area, and developing a new adsorbent made of ultra-low-density unwoven fabric with reduced clogging of air gaps. We assessed the ability of the new adsorbent to adsorb white blood cells *in vitro*, and then conducted extracorporeal circulation in a model of adjuvant arthritis, to assess the effect of the developed column at suppressing gonarthritis *in vivo*[16)]. The results suggested that the developed column had a therapeutic effect equivalent to conventional columns, despite its small size, and that it had different characteristics, showing a large amount of adsorption only when inflammation was present. We report on these results below.

Materials and Methods

1. Basic design of the new adsorbent

To decrease the column size, we reconsidered the structure used for adsorbing cells employed in existing cell adsorption columns. Firstly, to increase the surface area of the adsorbent, we decided to use fabric with narrow fiber diameters, rather than beads. Next, to stabilize adsorption of white blood cells during extracorporeal circulation, it was necessary to stabilize and decrease the bulk density of the adsorbent when in contact with the blood, and so we designed a new unwoven fabric structure with a 3-dimensional combination of fibers for adsorbing white blood cells and fibers for stabilizing the frame (Fig. 1). As the structure of this adsorbent was extremely bulky and became deformed easily, we employed a low-density 3-layered structure with a scrim sandwiched between 2 sheets of nonwoven fabric, to suppress stretching in horizontal directions. The adsorbent with this 3-layered structure can be expected to reduce to a minimum the removal of lymphocytes, which have an immune memory function, during leukapheresis, and also increase the efficiency of the selective removal of cells such as granulocytes or monocytes, which are activated by events such as inflammation in the body, thus improving the selectivity of leukapheresis. We have confirmed in earlier research that fibers of 10 μm or less actively adsorb granulocytes and monocytes. Given the structural restrictions of the adsorbent, we decided to use fibers with a diameter of 4 μm. To improve the adsorption selectivity, we chose a bulky structure with a lower density adsorbent. We expected this structure to have a mechanism whereby cells such as nearly-spherical lymphocytes with no phagocytic ability could pass through, and cells with protrusions and high phagocytic ability such as granulocytes and monocytes

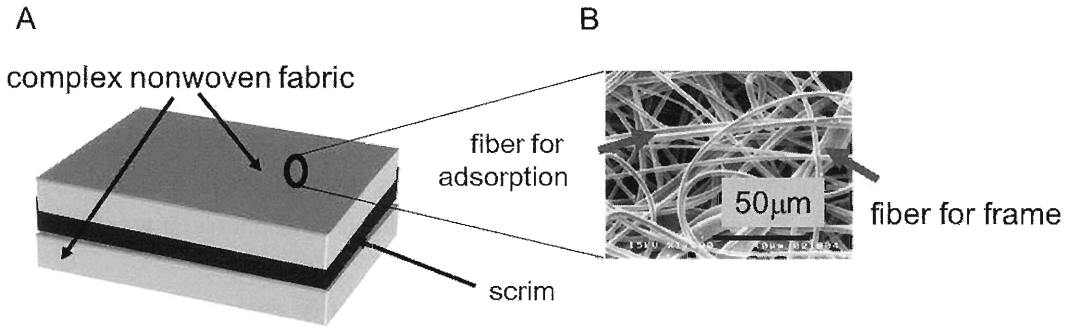
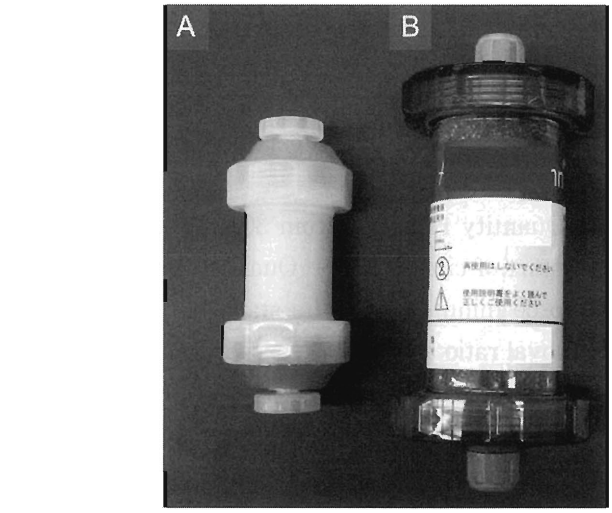


Fig. 1 A schematic view and a photograph of the absorbent in the developed column
A: Three-layered structure of complex nonwoven fabric with an ultra-low bulk density.
B: Scanning Electron Microscope (SEM) photograph of the adsorbent. Diameter of fiber for adsorption is 4 μm.



priming volume	45 mL	130 mL
surface area	1.41 m^2	0.44 m^2

Fig. 2 Appearance of the developed column
A: Developed column.
B: Conventional column.
Comparison of priming volume and surface area between developed column and conventional column. The developed column has an ample surface area (developed column: 1.41 m^2, conventional column: 0.44 m^2), despite being of a smaller size and containing around 1/3 the volume of blood (developed column blood volume: 45 mL, conventional column blood volume: 130 mL).

would be treated as foreign objects by the fibers and enclosed and thus adsorbed, so that they could not pass through.

In addition, as the column is intended mainly for use in inflammatory diseases, we employed a special macromolecular material to avoid causing stimulation of the white blood cells passing through the adsorbent. The external appearance of the small-sized column with a blood capacity of 45 mL we created to meet these requirements is shown in Fig. 2.

2. Assessment of adsorbency performance

1) *In vitro* tests

Sheets of 1 cm in diameter were punched out from the obtained adsorbent, to create a mini-column containing 3 sheets (with a diameter of 1 cm and a length of 0.51 cm). To investigate the adsorbent's ability to remove stimulated white blood cells, lipopolysaccharide (LPS) 10 ng/mL was added to heparinized fresh human blood, and the mixture was left at rest for 30 minutes at 37 ℃, and then sent into the mini-column over 60 minutes at a rate of 0.57 mL/min. The blood that had passed through the mini-column was sampled at 2-minute intervals, and compared with blood without additives. In addition, the adsorbent of a conventional column (Adacolumn®) was assessed in the same way, for the case where white blood cells were stimulated. The granulocyte counts (in particular the neutrophil count), lymphocyte count and monocyte count of the sampled blood were measured using an automated multi-item blood cell analyzer (XT-1800i, Sysmex Corporation), and the average leukapheresis ratio was calculated.

2) *In vivo* tests

Extracorporeal circulation was conducted between the auricular artery and vein in a rabbit model of arthritis induced by egg white albumin sensitization[17], using a 1/20 mini-column because of

the difference in the body weight of rabbits and humans (n = 10). Three standards of columns were used for assessment: a sham column (simply a vinyl tube), a conventional column (Adacolumn®) and the developed column, and these columns were created with a blood capacity of 4.1 mL, 8.1 mL and 1.9 mL, respectively.

A water-in-oil emulsion was made by dissolving ovalbumin (OVA) (made by Sigma) at a concentration of 4 mg/0.5 mL in sterilized normal saline solution and then mixing it in a 1:1 ratio with Freund's complete adjuvant (made by Gibco). This emulsion, with a final OVA concentration of 4 mg/mL, was used as a sensitization antigen, and injected into the skin on the backs of Japanese White household rabbits (Kitayama Labes), and 14 days later, sensitization was performed again using the same method. OVA dissolved in a normal saline solution at a concentration of 5 mg/mL was used for an antigen challenge evoking gonarthritis, by administering 1 mL into the right knee joint cavity at 5 days after the second sensitization. An equivalent quantity of normal saline solution was administered to the left knee joint cavity as a control. Extracorporeal circulations were performed twice, with the speed of blood circulation was set at 2 mL/min, for 1 hour per time; at 10 minutes after arthritis was evoked, blood was circulated for the first time, from the left auricular artery to the right auricular vein, and at 24 hours later, arterial and venous lines were again secured, with the left and right reversed, and blood was circulated for the second time. At 30 minutes and 60 minutes after the start of circulation, 0.5 mL samples of the blood on each side of the column were taken, and the blood cell counts of the samples were measured using an automated multi-item blood cell analyzer (XT-1800i, Sysmex Corporation), and the removal ratio of each type of blood cell was calculated. The removal ratios for blood cells (white blood cells, granulocytes*1, lymphocytes, monocytes, red blood cells and platelets) were calculated as follows.

Blood cell removal ratio (%) {(Number of blood cells before passing through column − Number of blood cells after passing through column)/Number of blood cells in column entrance} × 100

*1The number of granulocytes is the sum of neutrophils, eosinophils and basophils.

Total quantities of removed blood cells (white blood cells, granulocytes*1, lymphocytes, monocytes, red blood cells and platelets) were calculated as follows.

Total quantity removed (cells) = Quantity removed during the 30 minutes from the start of circulation*2 + Quantity removed from 30 to 60 minutes after the start of circulation*3

*1The number of granulocytes is the sum of neutrophils, eosinophils and basophils.

*2Quantity removed during the 30 minutes from the start of circulation = Quantity processed during the 30 minutes from the start of circulation*4 × Removal ratio at 30 minutes after the start of circulation

*3Quantity removed from 30 to 60 minutes after the start of circulation = Quantity processed from 30 to 60 minutes after the start of circulation*5 × Removal ratio at 60 minutes after the start of circulation

*4Quantity processed during the 30 minutes from the start of circulation = (Concentration of blood cells before circulation + Concentration of blood cells in the column entrance at 30 minutes after the start of circulation)/2 × 2 mL/min (quantity circulated) × 30 min

*5Quantity processed from 30 to 60 minutes after the start of circulation = (Concentration of blood cells in the column entrance at 30 minutes after the start of circulation + Concentration of blood cells in the column entrance at 60 minutes after the start of circulation)/2 × 2 mL/min (quantity circulated) × 30 min

Swelling of the knee joint was calculated by measuring the diameter of each knee joint before inflammation was evoked, after the first extracorporeal circulation, and before and after the second extracorporeal circulation and at 48 hours after inflammation was evoked, and then finding the difference between the diameter of the knee joint to which OVA was administered and the diameter of the knee joint to which normal saline solution was

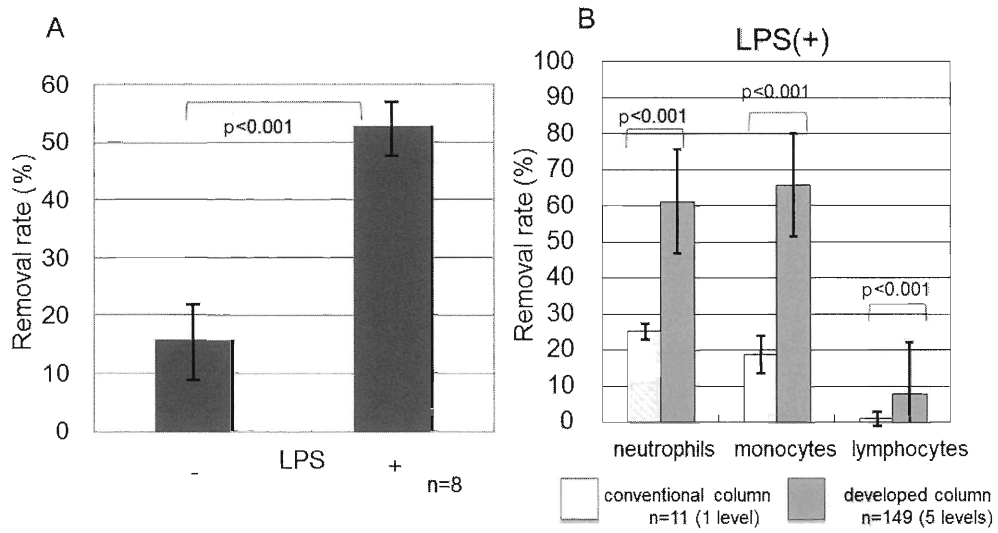


Fig. 3 An evaluation of the leukocyte adsorption characteristics using human fresh blood
Blood is passed through the column once. Lipopolysaccharide (LPS) 10 ng/mL was added to heparinized fresh human blood, and the mixture was left at rest for 30 minutes at 37 ℃, and then sent into the mini-column over 60 minutes at a rate of 0.57 mL/minute. The blood that had passed through the mini-column was sampled at 2-minute intervals, and compared with blood without additives.
A: Leukocyte adsorption ratio for fresh human blood using the developed column adsorbent, with and without the addition of LPS.
B: Comparison of the leukocyte adsorption ratios of the adsorbents in the developed column and the conventional column, for fresh human blood with LPS added.
The statistical analysis was performed using Student's t test.

administered.

The statistical analysis was performed using Student's t test when dispersion of the difference of mean value was equal, or by Aspin-Welch's t test when dispersion of the difference of mean value was not equal. Dispersion of the difference of mean value was performed using the F test.

The experimental methods used for both human and animal experiments followed the Institutional Review Board (IRB) rules, which Toray Corporation follows, in general.

Results

1) *In vitro* tests

The results of *in vitro* adsorption tests are shown in Fig. 3. In the developed column, when blood stimulated with LPS was used, the removal ratio for neutrophils was significantly higher ($p < 0.001$), over 50% (Fig. 3A). When blood with LPS added was used, the leukocyte removal ratios (mean ± SD) for the developed column (n = 149) were as follows: neutrophils 61.2 ± 12.7%, monocytes 65.7 ± 11.9%, lymphocytes 7.8 ± 3.3%, and those for the conventional column (n = 11) were as follows: neutrophils 25.3 ± 2.2%, monocytes 18.8 ± 5.8%, lymphocytes 1.0 ± 2.0%, and thus removal ratios for the developed column were significantly higher (Fig. 3B).

2) *In vivo* tests

For both the conventional column and the developed column, the removal rate for lymphocytes was low. During circulation directly after inflammation was evoked, the conventional column removed more white blood cells than the developed column, but at 24 hours after inflammation was evoked, the quantity of cells removed by the conventional column increased 1.5-fold, while the quantity of cells removed by the developed column increased at least 5-fold, and thus at this time it was the developed column that removed more cells (Fig. 4). Assessment of the effect at reducing arthritis (Fig. 5) showed a significant decrease in swelling in the developed column group, compared with the sham column group in which adsorption was not performed (blood volume 4.08 mL), with the amount of swelling at 24 hours after inflammation was evoked

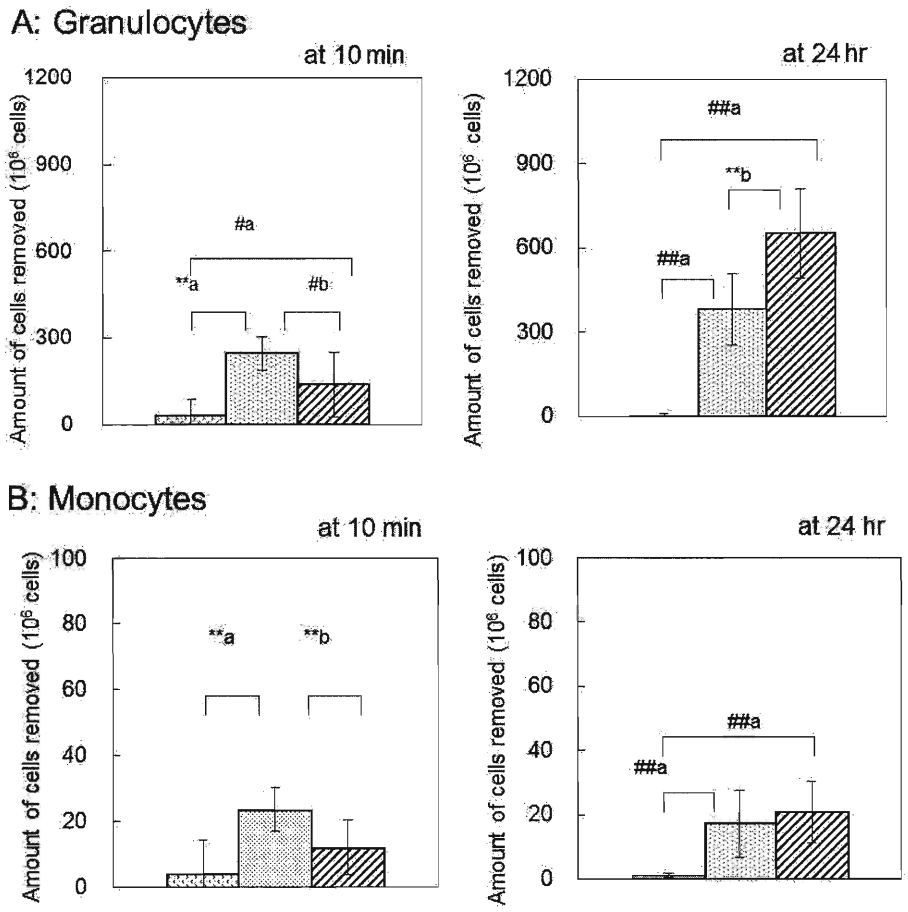


Fig. 4 Effect of treatment of gonarthritis

Comparison of the number of cells removed by extracorporeal circulation at 10 min and at 24 hr after inflammation was evoked.

Extracorporeal circulations for which the speed of blood circulation was set at 2 mL/min, for 1 hour per time. were performed. The conventional column removed more white blood cells than the developed column at 10 min, but at 24 hr after inflammation was evoked, the developed column removed more white blood cells than the conventional column.

A: Granulocyte removed.

B: Monocyte removed.

▨ sham colum, ▩ conventional column, ▨ developed column.

Sham: n=9, conventional, developed: n=10.

**: $p<0.01$ (Student's t test) a: sham vs conventional, sham vs developed, b: conventional vs developed.

#: $p<0.05$, ##: $p<0.01$ (Aspin-Welch's t test) a: sham vs conventional, sham vs developed, b: conventional vs developed.

being as follows before circulation: sham column group 5.1 ± 1.1 mm, developed column group 3.7 ± 1.1 mm, $p=0.012$ and after circulation: sham column group 5.2 ± 0.5 mm, developed column group 4.0 ± 1.1 mm, $p=0.005$. In the conventional column group, the diameter of the swollen joint after circulation at 24 hours after inflammation was evoked was 4.1 ± 1.1 mm, and thus no significant difference between the conventional column and the developed column was found. Measurements for the swelling of the knee joint at 48 hours after inflammation was evoked were 2.7 mm in the developed column group and 3.7 mm in the conventional column group, compared to 6.8 mm in the sham column group, and thus the developed column group and the conventional column group showed a marked decreasing trend.

Discussion

1. Discussion of the smaller size of the column

In the *in vitro* adsorbency tests, assessment of the

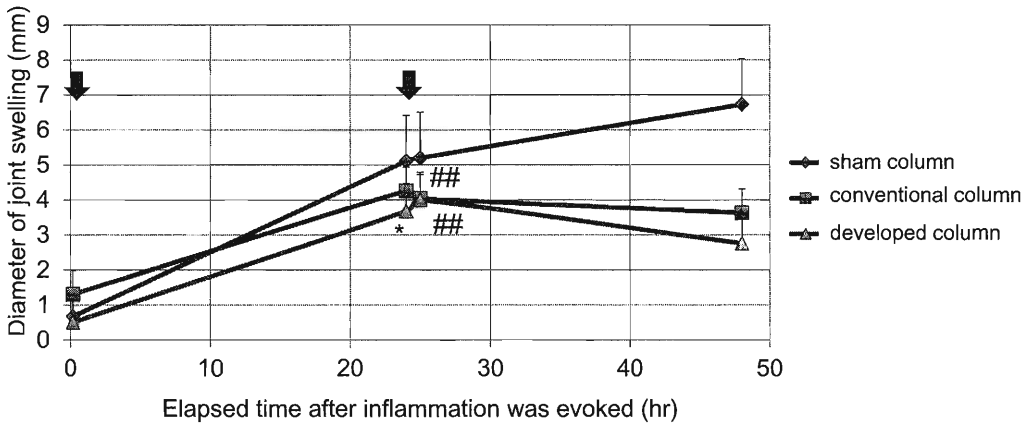


Fig. 5 Comparison of the arthritis-reducing effect of the developed column and conventional column vs sham column. Swelling of the knee joint was calculated by measuring the diameter of each knee joint before inflammation was evoked, after the first extracorporeal circulation, and before and after the second extracorporeal circulation and at 48 hr after inflammation was evoked, and then finding the difference between the diameter of the knee joint to which OVA was administered and the diameter of the knee joint to which normal saline solution was administered.
⬇: Extracorporeal circulations.
10 min and 24 hr after inflammation was evoked: sham: n = 9, conventional and developed: n = 10.
48 hr after inflammation was evoked: sham: n = 2, conventional and developed: n = 3.
*: $p < 0.05$ (Student's t test) sham vs developed (before 2nd extracorporeal circulation).
##: $p < 0.01$ (Aspin-Welch's t test) sham vs conventional, sham vs developed (after 2nd extracorporeal circulation).

white blood cell removal ratio for fresh human blood stimulated with LPS showed that the developed column had a significantly higher removal rate than the conventional column, and so it is considered that the developed column has adsorbency performance that is not inferior to that of the conventional column, despite its smaller size. As the conventional column uses large acetyl cellulose beads with a diameter of 2 mm[11], it is conceivable that a smaller column with equivalent adsorbency properties could be created by decreasing the diameter of the beads and increasing their number, thus increasing the adsorbent surface area. However, decreasing the diameter of the beads would make the route along which the blood flows longer and narrower, making accumulation of coagulative cells such as red blood cells and platelets more likely, and thus increasing the chance of clogging, hindering the process of designing the column. The use of an adsorbent employing a filtration method, with layers of paper-thin rolled sheets of nonwoven fabric to remove white blood cells in general, is one possible approach, but it is considered likely that if attempts were made to implement this design on a smaller scale, the blood filtration area would decrease in a similar way, simply leading to a decrease in cell removal properties, and therefore this design is not feasible. Even if improvements were made by increasing the affinity of the adsorbent for white blood cells, eventually the risk of clogging by coagulative cells such as red blood cells or platelets would increase due to the decrease surface area of the adsorbent, and therefore, it is considered that the design is not feasible. The developed column has an ample surface area (developed column: 1.41 m^2, conventional column: 0.44 m^2), despite being of a smaller size and containing around 1/3 the volume of blood (developed column blood volume: 45 mL, conventional column blood volume: 130 mL). We consider that it was possible to decrease the size by employing a new design using a nonwoven fabric structure that maintained the bulkiness of the structure, but with a lower-density adsorbent, thus creating a new structure where adsorption of white blood cells was

possible even on the inside of the nonwoven fabric structure. The small-sized column described in this paper can be safely used for children and elderly people without losing functional capability[13)14)].

2. Discussion of adsorbency properties

During *in vitro* adsorbency tests, the developed column significantly improved the efficiency of adsorption of white blood cells when stimulation with LPS was performed, and therefore, it is considered that it has a high affinity for activated white blood cells. In addition, it was considered that during *in vivo* tests in household rabbits, the reason why the developed column removed fewer white blood cells than the conventional column at first but removed more later was that at 10 minutes after inflammation was evoked, there were few activated white blood cells, and thus the developed column removed a low quantity of white blood cells, while at 24 hours after inflammation was evoked, as the diameter of the swollen joint increased and it was likely that arthritis had progressed, the number of white blood cells activated due to the inflammation had increased and the quantity of white blood cells removed by the developed column increased rapidly as a result. It is considered that using a complement activation structure[18)] and giving an oxidative stress which is caused by hydroxyl groups of sugar chains[19)], for the conventional column it gave adsorbency properties that are independent of differences in the degree of inflammation. In summary, this suggests that the column developed for the present research is efficient at removing activated white blood cells even *in vivo*.

In addition, in extracorporeal adsorption at 24 hours after inflammation was evoked, the quantities of the white blood cells removed by extracorporeal circulation were 7.1 ± 1.8 (10^8 cells) in the developed column and 4.7 ± 1.4 (10^8 cells) in the conventional column. This suggests that despite the small size of the developed column, its white blood cell removal performance is good in clinical use, and that if a 20-fold larger column for humans were used, it could be expected to remove white blood cells at a rate of the order of 10^{10} cells per hour of extracorporeal circulation. From the above, it is considered that a large difference in adsorbency was observed in the developed column compared to the conventional column, in that the developed column removes white blood cells in quantities directly proportional to the degree of inflammation.

From these results, even though the developed column is of a smaller size, it has an ample surface area (developed column: 1.14 m^2, conventional column: 0.44 m^2), increasing its adsorbency for white blood cells, and it is considered that for this reason, an anti-inflammatory effect equivalent to that of the conventional column was achieved.

Conclusion

It was shown that, even though the developed column uses about 1/3 the volume of blood used in the conventional column, it has improved selectivity and removal performance for granulocytes and monocytes, and has an ability to relieve inflammation equivalent to that of a conventional column.

Masaaki Shimagaki is an employee of Toray Industries, Inc. (currently Toray Medical Co., Ltd.), and receives an allowance from this company. He conducted this research as a student at the Graduate School of Medicine, Tokyo Women's Medical University, from 2009 to 2013.

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炎症性疾患に対する白血球除去療法のための小型カラム開発

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細胞除去療法において，除去対象を顆粒球，単球，リンパ球などの白血球，すなわち細胞にまで拡張した方法が開発され，特に自己免疫性の炎症性疾患（主に潰瘍性大腸炎，クローン病，関節リウマチなど）に対して広く使われている．従来のカラムは血液容量が大きいため，体外循環時の血圧低下などの懸念から小児や高齢者への適用の困難性などの臨床使用上の制約となっている場合があったため，小型カラムを開発した．吸着体には白血球の吸着表面積拡大のため直径 4 μm 程度の極細繊維を採用し，血液循環によって空間がつぶれることなく高い空隙率を保持できる構造とした．吸着体の白血球吸着特性の評価を行ない，さらに炎症抑制効果を確認するため，*in vivo* 試験で，アジュバント関節炎モデルでの膝関節炎抑制効果を評価した．血液容量は，従来品に比べ 1/3 程度の 50 mL とし，体重比を元に家兎用サイズのミニカラムを作成して炎症惹起直後および 24 時間後に各 1 時間循環して吸着量を測定し，炎症惹起後 24・48 時間後に関節腫脹径を測定した（n＝10）．吸着特性は異なり，惹起直後の循環では従来カラム（アダカラム）は開発カラムに比べ白血球除去量は多く，24 時間後には逆転した．*In vitro* 試験で，開発カラムでは従来カラムに比べ LPS 刺激した白血球の吸着率が高く，*in vivo* でも体内の炎症部位で活性化した白血球を効率的に除去できていると考えられる．また，膝関節炎抑制効果の確認では，吸着体の入っていないシャムカラム群（血液容量 4.08 ml）と比較して，開発カラム群では惹起 24 時間目の循環前（シャムカラム群：5.1 ± 1.1 mm，開発カラム群：3.7 ± 1.1 mm，p＝0.012）及び循環後（シャムカラム群：5.2 ± 0.5 mm，開発カラム群：4.0 ± 1.1 mm，p＝0.005）で腫脹が有意に軽減した．従来カラム群では同条件下で惹起 24 時間目の循環後に関節腫脹径が 4.1 ± 1.1 mm であり，開発カラムが従来カラムと同等の性能を示した．以上のように，本研究で開発したカラムは，顆粒球や単球の選択性や除去性を向上させ，かつ血液容量を従来品の 1/3 程度の 50 mL 以下にし，従来品と同等の性能が得られた．