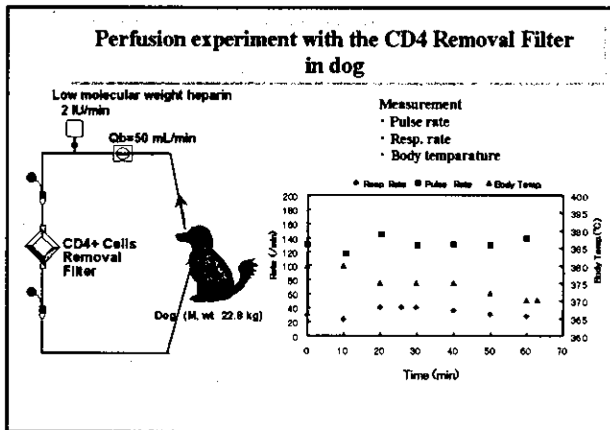


部材の安全性試験

安全性試験	被験物	試験手順・基準	結果
部材溶出物試験	濾材／容器	吸着型血液浄化器基準	合格
VIRUS試験	抗ヒトCD4抗体液	GMP／GLP	6項目全て合格
皮膚感作性試験	濾材	医療用具のガイドライン/ISO10993	皮膚感作を認めず：合格
細胞毒性試験	血清添加培地による濾材抽出液		弱毒性以下：合格
発熱性試験部材	濾材 溶媒(PBS) 抗ヒトCD4抗体液 ブロッキング剤 充填液 滅菌後充填液		左記部材で試験を実施し発熱性を認めず：合格

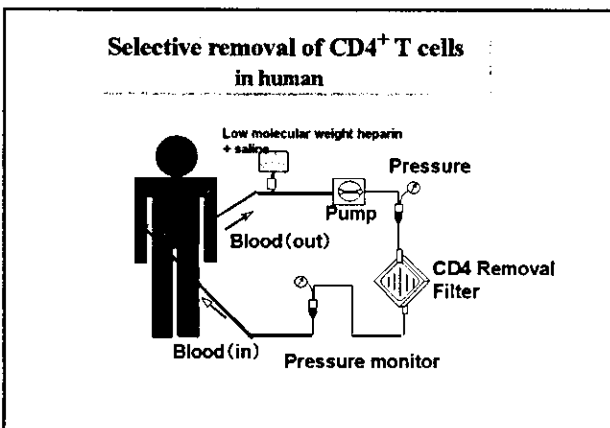
抗体の安全性試験

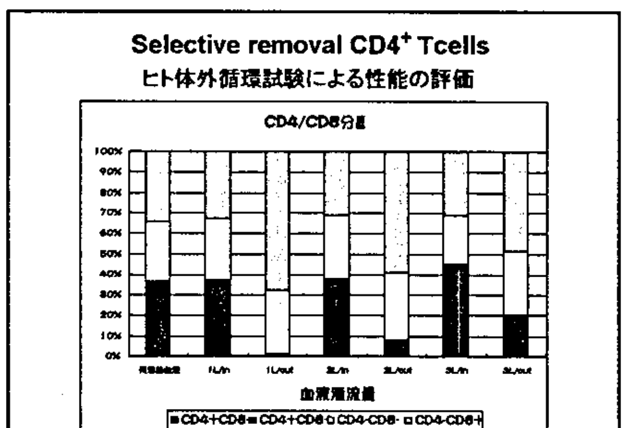
ウイルス否定試験項目：「未加工/未精製バルクにおけるウイルス試験」の試験項目であるin vitro試験を含む、医薬審329号の「ウイルス検出及び同定の為に推奨される試験」全ての項目を実施(下記表)

厚生労働省要求試験	実試験項目	評価項目	評価方法	評価基準	結果	判定
レトロウィルス試験	電子顕微鏡観察試験	電子顕微鏡によるウィルス様粒子の観察	ウィルス感染細胞をコントロールとした電顕による細胞観察	ウィルス様粒子の有無	検出せず	合格
in vitro試験	in vitro試験	ヒト・サル・ウス3種の細胞にてウィルス否定試験	細胞変性・凝集作用	細胞の形態	正常形態	合格
in vivo試験	in vivo試験	細胞変性・血球凝集等の臨床兆候の無いウィルス検出	マウス・モルモット・卵に接種し、ウィルス有無	生死/臨床症状の赤血球凝集	異常無し	合格
抗体産生試験	MAP試験	マウス由来の16種類のウィルス否定試験	マウス由来16種類ウィルスの有無	ウィルス検出有無	検出せず	合格
―	BSAウィルス混入試験	牛由来9種のウィルス否定試験	細胞変性・血球吸着・免疫蛍光測定	ウィルス検出有無	検出せず	合格

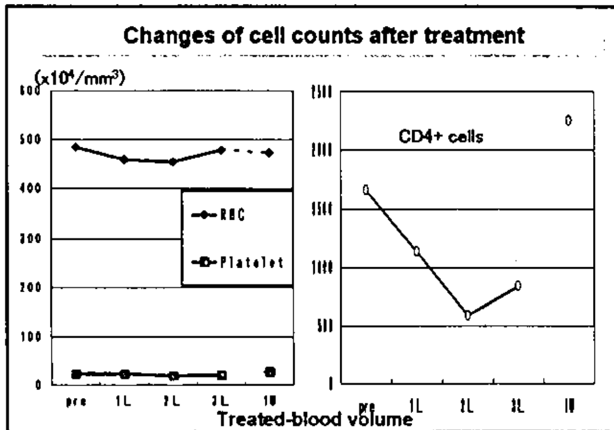
製品の安全性試験

安全性試験	被験物	試験手順・基準	結果
生物学的試験 皮内反応試験 急性毒性試験 溶血性試験 発熱性試験	生食による抽出液 37℃×72hr 抽出	医療用具のガイドライン/ISO10993	合格
流出異物数試験	充填液	吸着型血液浄化器基準	合格
耐圧漏洩試験	容器		合格
無菌試験	実スケール品		合格





Changes of cell counts after treatment



発熱性物質試験

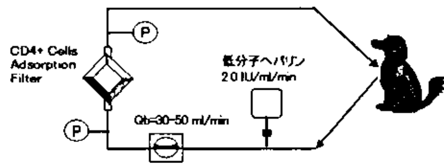
- 目的: CD4-0312の濾材に発熱性物質が含まれていないことを検証する
- 方法: ウサギを用いた発熱性物質試験(第十四改正日局)
- 抽出: ウサギ血漿抽出(37℃X15分)
- 結果:

検体	体温上昇(℃) 基準:0.6℃以下			3羽合計 (℃) 基準:1.4℃以下	判定
	n=1	n=2	n=3		
血漿のみ	0.24	0.45	0.33	1.02	陰性
CD4-0312	0.10	0.05	0.08	0.23	陰性
CD4-01	1.27	1.59	1.42	4.28	陽性

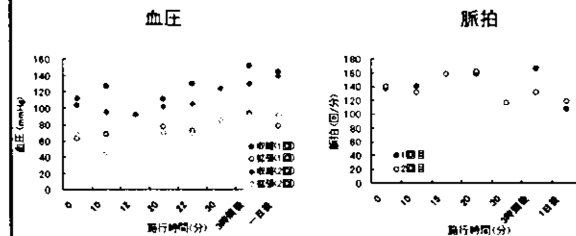
- 結論:対策品CD4-0312は発熱のリスクが十分低減されている

イヌ体外循環複数回施行での安全性確認

	ヒト(参考)	イヌ 1回目	イヌ 2回目
体重	60kg	22.4kg	21.8kg
体液量(a)	4.6L	1.9L	1.8L
血液処理量(b)	3.0L	1.0L	1.2L
(b)/(a)	0.65	0.53	0.66

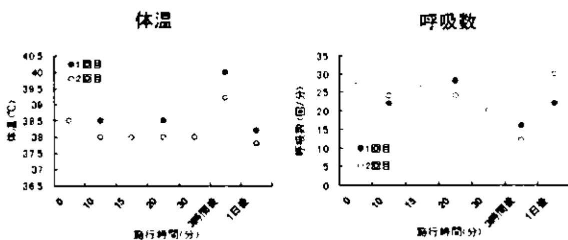


測定結果



- 施行中の緩やかな血圧上昇は生食フィードによる体液増加、また終了後の血圧上昇は生食返血による体液増加に起因するものと考えられる.

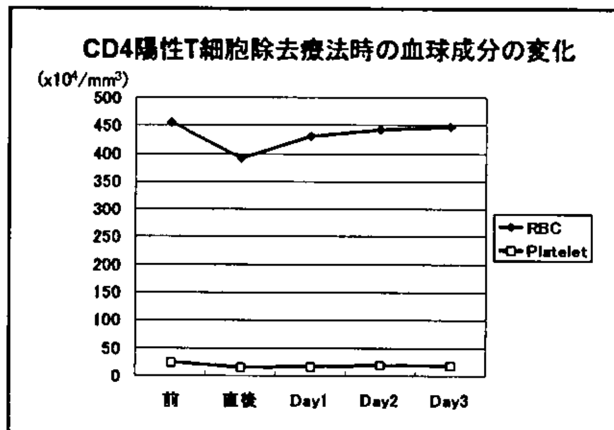
測定結果

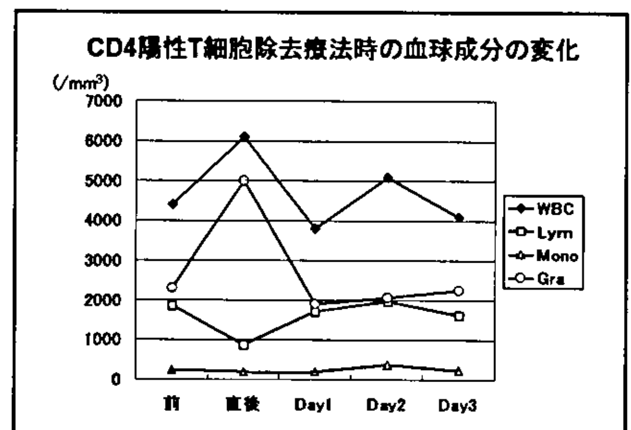


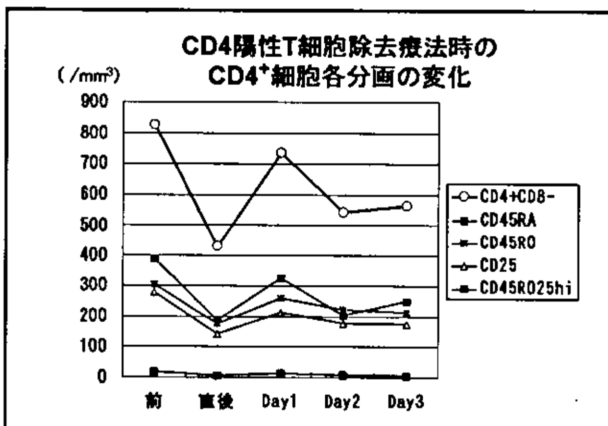
- 施行終了3時間後の体温上昇は麻酔下での呼吸数低下により放熱できないことに因るものと考えられる.
- 発赤等のアレルギー反応は認められなかった.

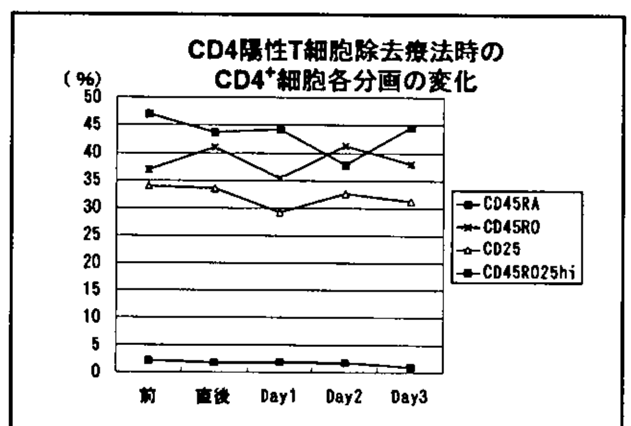
改良型CD4+T細胞吸着器による健常人の体外循環試験

- 低分子ヘパリンを抗凝固剤とし, 流速40-50ml/分で3Lを処理.
- 施行時の血圧低下, 発熱などの有害事象なし.
- 施行前後で血液・生化学検査では白血球以外の異常変動は認められなかった.









結 論

- 免疫性神経疾患の治療目的に選択的CD4+T細胞吸着器を開発し，その臨床応用を行った．
- 健常人の体外循環試験で，in vitroと結果と同様の性能を確認した．
- エンドトキシンによる有害事象を確認し，治療器の改良を行い，安全性を確認した．

II. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Onodera H, Ninomiya K, Yoshida M, Matsuo H, Shibuya N.	Development of a device for selective removal of CD4+ T cells.	Ther Apher Dial	7	329-333	2003
Nakane S, Matsuo H, Goto H, Yoshinaga-Matsumoto M, Ohtsuru I, Ichinose K, Onodera H, Yoshida M, Shibuya N.	Cytapheresis with a filter for selective removal of CD4+ T cells in experimental autoimmune encephalomyelitis	Multiple Sclerosis	9	579-584	2003
Matsuo H, Goto H, Kambara C, Fukudome T, Mizota T, Onodera H, Yoshida M, Shibuya N.	Selective adsorption of human CD4+ T cells.	Ther Apher Dial	8	194-196	2004

III. 研究成果の刊行物・別刷

Therapeutic Apheresis and Dialysis
7(3):329–333, Blackwell Publishing, Inc.
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Development of a Device for Selective Removal of CD4+ T Cells

*Hirokazu Onodera, *Kasumi Ninomiya, *Makoto Yoshida, †Hidenori Matsuo, and †Noritoshi Shibuya

**Research and Development Laboratory 2, Asahi Medical Co., Oita, †Department of Neurology, Kawatana National Hospital, Kawatana, Japan*

Abstract: To control antigen (Ag)-specific immune cells is important in the treatment of autoimmune diseases. In particular, controlling the immune response of autoimmune T cells is effective in the treatment of these diseases. The development of a device that can remove CD4+ T cells specifically by extracorporeal circulation is now in progress, with the aim to deplete autoimmune T cells. We developed a removal material made of polypropylene non-woven fabrics with anti human CD4 monoclonal antibody immobilized on the surface. Using a column packed with the removal material, we succeeded in removing CD4 + T cells specifically from peripheral whole blood by direct perfusion. Moreover, CD4+ T cells can be specifically removed even from blood with lower surface antigen density by in vitro activation. **Key words:** Autoimmune disease—CD4+—Monoclonal antibodies—Non-woven fabrics—Polypropylene—Selective cell removal.

CD4+ T cells are deemed to play a major role in the development of autoimmune diseases. It is known that CD4+ T cells function to manage the immune response system. It has already been proved that autoimmune diseases appear as a result of the entry of CD4 membrane antigen-positive T lymphocytes (helper T cells) into the tissue in response to autoantigens, where they destroy the body's own tissue. With such autoimmune diseases, abnormality in cellular immunocompetence appears as deteriorated suppressor functions, an increase in the ratio of CD4+ and CD8+, an increase in activated T cell counts, and a reduction in the counts of natural killer cells.

With this in mind, immunomodulation of CD4+ T cells is considered an effective means of therapy for autoimmune disease. In particular, immunomodulation ex vivo by means of extracorporeal circulation acts directly on CD4+ T cells and removes the cells with less therapeutic strain on patients. Thus, this immunomodulation has attracted significant attention as a prospective therapy. The development of a device that can remove CD4+ T cells specifically by extracorporeal circulation, is now in progress with a view towards depleting autoimmune T cells (1). We have developed a removal material made of polypropylene non-woven fabrics with anti human CD4 monoclonal antibody immobilized on the surface (2). We report here on the development of a new device for selective removal of CD4+ T cells.

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Address correspondence and reprint requests to Dr Hirokazu Onodera, Research and Development Laboratory 2, Asahi Medical Co. Ltd., 2111-2 Oaza-Sato, Oita-shi, Oita 870-0396, Japan. Email: onodera.hc@om.asahi-kasei.co.jp

MATERIALS AND METHODS

Induction of active group into polypropylene non-woven fabrics

For the preparation of activated solution, sulfuric acid (15 mL), nitrobenzene (20 mL), paraformaldehyde (0.098 g), and 2-hydroxymethyliodoacetamide (HMIAA) (1.59 g) were added to a glass beaker and magnetically stirred for 20 min. Polypropylene non-woven fabric (0.8 g) was immersed with the activated solution at room temperature for 24 h. (Fig. 1) After the reaction, the activated non-woven fabrics were washed with ethanol and water.

Preparation of immobilized monoclonal antibody removal material

For the preparation of removal material, the activated non-woven fabrics were immersed with antihu-

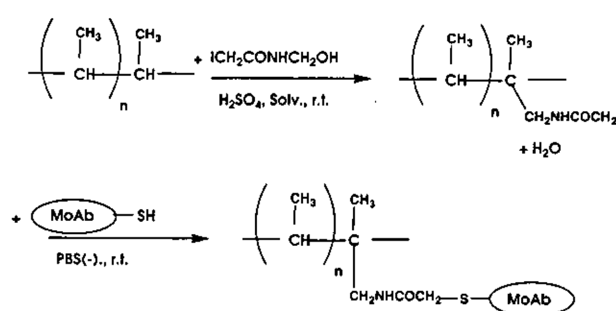

FIG. 1. MoAb activation.

man CD4 monoclonal antibodies (mouse IgG1, clone: NU-T; Nichirei Corporation, Japan) in phosphate-buffered saline (PBS) solution at room temperature and allowed to react to the antibodies by covalent bonding. The remaining activated group was blocked with Tween 20 (Tokyo Kasei Co., Tokyo, Japan) (Fig. 1). After the reaction, the removal material was washed with PBS.

Preparation of the mini-scale column

The immobilized monoclonal antibody removal material (18 mg) was packed in layers in a 6.8 mm diameter column (priming volume, 1 mL). The sterilization-protecting agent, 1% chitosan/PBS solution, was added into the mini-scale column and it was irradiated with 25 kGy by the use of cobalt 60 γ-ray irradiation equipment.

In vitro whole blood evaluation of the mini-scale column

Phosphate-buffered saline solution (10 mL) was passed through the mini-scale column at the flow rate of 1 mL/min for the priming. The priming solution was expelled with air. A 5 mL sample of blood from a healthy donor and acid citrate dextrose (ACD)-A (blood : ACD-A = 9:1), was passed through the mini-scale column at the flow rate of 1 mL/min using a syringe pump. The blood was then counted before and after the column treatment. White blood cells were stained with Turcq liquid and counted using a microscope to obtain the white blood cells recovery ratio. The thrombocyte count was counted using the automatic blood cell counter (Sysmex Inc., Tokyo, Japan) to obtain the thrombocyte recovery ratio. In addition, analysis of flow cytometry using fluorescent antibodies was conducted, and lymphocyte subsets were analyzed by two-color analysis of CD4 and CD8 (EPICS ELITE; Beckmann Coulter, Tokyo, Japan) to obtain the CD4+ T cell removal ratio and CD4– cells recovery ratio.

Activated blood evaluation of mini-scale column

The activated solution was prepared using 10% fetal calf serum, phorbol myristate acetate (PMA), and ionomycin with RPMI1640. The activated solution above was added to fresh blood with heparin (1000 IU/L) from healthy donors and was incubated in a CO_2 incubator at 37°C for 4 h. After the incubation, cold PBS solution was added as a suspended solution to obtain an activated treatment sample. Similar procedures with the in vitro mini-scale column blood evaluation were performed to obtain the CD4+ T cells removal ratio and the CD8+ T cells recovery ratio.

Preparation of the clinical investigation scale column

We cut the immobilized monoclonal antibody removal material into 96.5 mm squares, and the material (9 g) was layered in a 60 mL column to make a clinical investigation scale column. The column was filled with a 1% chitosan/PBS solution, the sterilization-protecting agent, and it was irradiated with 25 kGy using cobalt 60 γ-ray irradiation equipment.

In vitro clinical investigation scale column evaluation

Blood was taken from a number of healthy donors, and ACD-A (blood : ACD-A = 8 : 1) was added as an anticoagulant. Physiologic saline (2000 mL) was passed through the clinical investigation scale column at a flow rate of 50 mL/min for the priming. Using a blood pump, 1500 mL of blood was circulated at the flow rate of 30 mL/min, and 1200 mL of blood was circulated at a flow rate of 50 mL/min. A sample of blood was taken before and after the column treatment. This was analyzed using the same method as with the mini scale: the CD4+ T cell recovery ratio and the CD8+ T cell recovery ratio were calculated.

RESULTS

Mini-scale blood evaluation

We performed a removal test of CD4+ T cells using 5 mL of blood in a mini-scale column ($N = 50$), which was made from a removal material using polypropylene non-woven fabric as a carrier. The CD4+ T cell removal ratio was 90.5% ± 13.3% (mean ± SD, $N = 50$), and the CD4– T cell recovery ratio was 88.1% ± 13.5% (mean ± SD, $N = 50$). This result indicated that CD4+ T cells can be removed selectively. (Fig. 2)

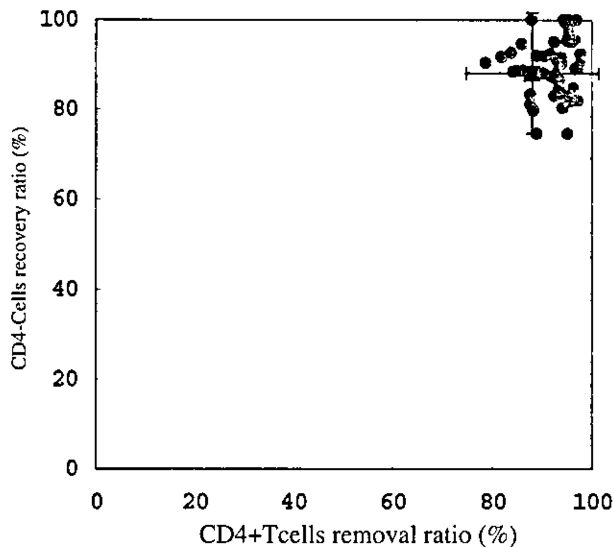

FIG. 2. In vitro cell removal test ($N = 50$). Flow rate, 1 mL/min, human blood (ACD-A), 5 mL; ■, average.

Discussion about the effect of CD4+ T cell removal in activated cells

When we activated leukocytes from the fresh blood of healthy donors using PMA/ionomycin, the down-regulation of CD4 antigens was confirmed. (Fig. 3). After the treatment, the CD4+ T cell removal ratio was 91.9% ± 2.7% ($N = 4$, mean ± SD) and the CD8+ T cell recovery ratio was 99.7% ± 31.1%. These percentages were almost equivalent to the percentages calculated concurrently from non-activated blood; the CD4+ T cell removal ratio was 95.9% ± 4.3% ($N = 4$, mean ± SD.), and the CD8+ T cell recovery ratio was 67.9% ± 14.8%. Thus, it indicated that CD4+ T cells could be removed selectively. (Fig. 4)

The recovery ratio of activated blood constituents were as follows: leukocytes, 48.1% ± 9.6% ($N = 4$, mean ± SD); erythrocytes, 98.3 ± 1.7%; and thrombocytes, 91.8% ± 30.74% ($N = 4$, mean ± SD). The leukocyte recovery ratios were as follows: lymphocytes, 51.2% ± 12.9% ($N = 4$, mean ± SD); granulocytes, 21.9% ± 63.2%; and monocytes, 53.6% ± 45.2% ($N = 4$, mean ± SD). The lymphocyte subsets were as follows: CD4+ T cell removal ratio, 95.3 ± 3.3% ($N = 4$, mean ± SD); and CD8+ T cell recovery ratio, 83.8 ± 11.55% ($N = 4$, mean ± SD), resulting in the selective removal of CD4+ T cells.

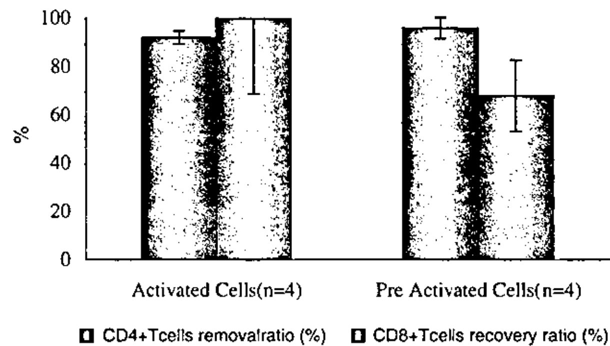

FIG. 4. Effect of CD4+ T cells removal in activated cells. Blood: Healthy donor blood, Heparin (1000 IU/L); Treated blood volume: 5mL. Cell activation reagents: phorbol myristate acetate (PMA)/Ionomycin. Flow rate, Qb = 1mL/min.

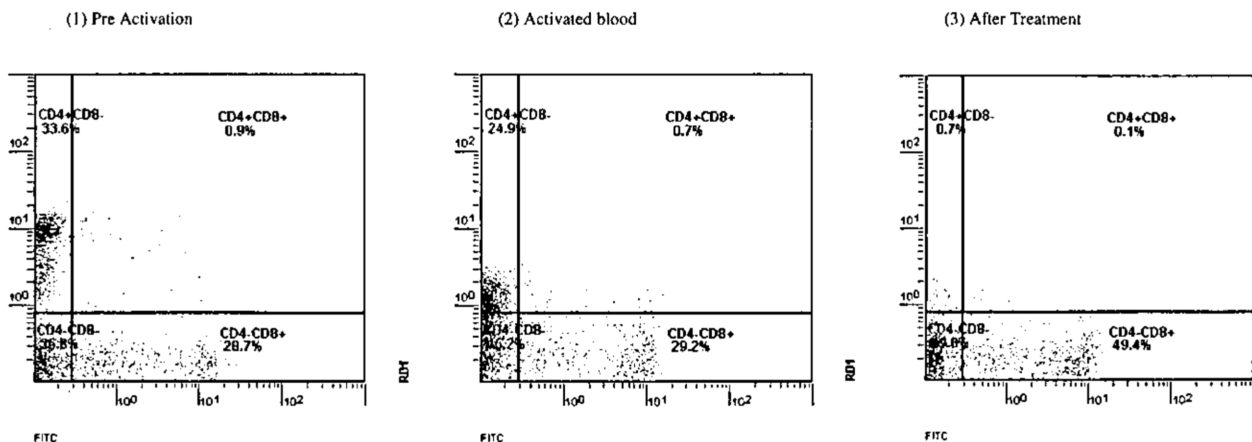

FIG. 3. Flow cytometry anlysis of phorbol myristate acetate (PMA)/Ionomycin Activated Cells. Blood: Healthy donor blood, Heparin (1000 IU/L); (1) Pre Activation, (2) PMA/Ionomyasin Activated Blood, (3) After Treatment, CD4-FITC, CD8-PE labelled.

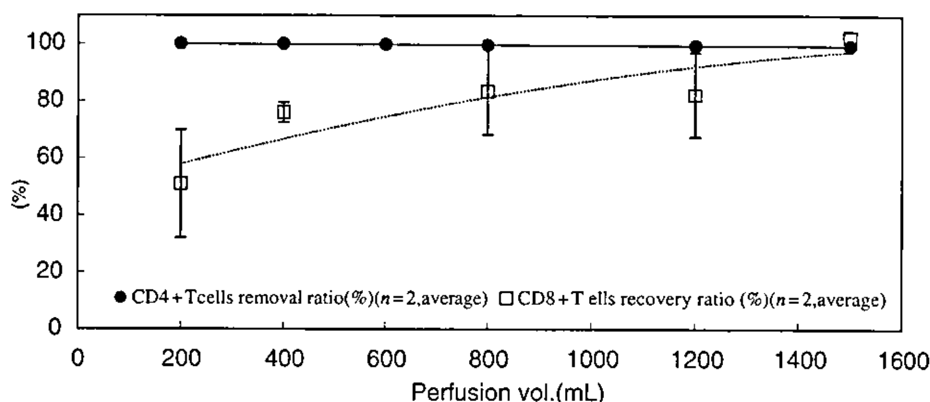

FIG. 5. In vitro clinical investigation scale evaluation ($N = 2$). Blood from healthy donor (Blood: ACD-A=8:1). Blood flow rates were 30 mL/min and 50 mL/min.

Evaluation of removal of CD4+ T cells using a clinical investigation scale column

As we treated the blood taken from healthy donors using the clinical investigation scale column at a flow rate of 30–50 mL/min, the CD4+ T cell removal ratio for the treatment of 1200 mL of blood was 99.4% ± 2.7% ($N = 2$, mean ± SD), and the CD8+ T cell recovery ratio was 82.2% ± 14.6%, indicating that selective removal was possible. The average recovery ratio for 1200 mL of blood was as follows: leukocytes recovery ratio, 58.0%; erythrocytes, 99.9%; and thrombocytes, 87.7%. In addition, treatment at the flow rate of 30 mL/min treated up to 1500 mL of blood. The CD4+ T cell removal ratio was 99.3%, and the CD8+ T cell recovery ratio was 99.9% after the treatment of 1500 mL (Fig. 5).

DISCUSSION

We performed the study to immobilize the antibodies on the material surface through covalent bonding using polypropylene non-woven fabrics as a carrier for the immobilized monoclonal antibody removal material. The fact that CD4+ T cells can be selectively and effectively removed was confirmed on the basis of in vitro blood evaluation using the blood taken from healthy donors. Through this study, we concluded that it is possible to use polypropylene as a carrier material. Polypropylene is considered to demonstrate selectivity for CD4+ T cells approximately equivalent to that of polystyrene (3). Because polypropylene has a high formability for non-woven fabrics, polypropylene non-woven fabrics with various qualities are now being developed. As physical properties of non-woven fabrics are important in performing specific cell adsorption and non-specific adsorption control, we concluded that it is useful to adopt polypropylene non-woven fabrics as a carrier for the development of medical device.

In addition, when we activated leukocytes in vitro by PMA/ionomycin, the depletion of down-regulated CD4+ T cells was confirmed by surface observation using flow cytometry analysis. At first, we were concerned that the representation of the reduction of CD4 antigen could be associated with a decrease in the capacity of the removal device to eliminate the CD4 antigen. However, it was later confirmed that the CD4+ T cell removal ratio could be maintained at a high level, just as in the removal of blood from healthy donors. Therefore, CD4+ T cells can be selectively removed even if the CD4 antigen representation density is decreased due to activation of the cells in autoimmune diseases. It also indicates the possibility that the proportion of CD4 and CD8 can be effectively controlled.

We made a clinical investigation scale column that is 500 times the volume of the mini-scale column to perform the study of in vitro perfusion using 1200 mL of blood taken from healthy donors. The selective removal of CD4+ T cells from up to 1500 mL of blood was possible without decreasing its removal competence. As for CD8+ T cells recovery ratio, non-specific adsorption was recognized in the beginning, but the percentage increased to more than 80% during the latter half of the treatment and an upward trend was confirmed. Regarding the clinical investigation scale column, a high recovery ratio of both erythrocytes and thrombocytes was obtained, with ratios similar to those obtained when using the mini-scale. Moreover, the result of this study indicates the possibility of effective treatment of a large amount of blood by perfusing the blood at the practicable flow rate of 30–50 mL/min through extracorporeal circulation. From this examination, we found that the amount of blood treated can be

increased by increasing the removal material. Thus, the possibility of using the column as a medical device was confirmed.

CONCLUSION

We made selective removal material for CD4+ T cells using polypropylene as a carrier. This removal material can selectively remove CD4+ T cells in vitro. In addition, CD4+ T cells were selectively removed even from blood with a lower CD4 antigen density, which was activated in vitro. We made a clinical investigation scale column to treat 1500 mL of blood and found that the column could remove CD4+ T cells selectively.

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3. Shibuya N, Ichinose K, Oturu Matsuo H et al. Newly designed column for selective removal CD4 T cells. *Jpn J Artif Organs* 1996;25:165–8.

Multiple Sclerosis 2003; **9**: 579–584
www.multiplesclerosisjournal.com

Cytapheresis with a filter for selective removal of CD4$^+$ T cells in experimental autoimmune encephalomyelitis

Shunya Nakane[1], Hidenori Matsuo[,1,2], Hirofumi Goto[1,2], Megumi Yoshinaga-Matsumoto[3], Izumi Ohtsuru[1], Katsuhiro Ichinose[1], Hirokazu Onodera[4], Makoto Yoshida[4] and Noritoshi Shibuya[1]*
[1]Department of Neurology, [2]Division of Clinical Research and [3]Department of Surgery, Kawatana National Hospital, Nagasaki, Japan; [4]Research and Development Laboratory, Asahi Medical Co., Ltd., Oita, Japan

Experimental autoimmune encephalomyelitis (EAE) is a major animal model of human multiple sclerosis (MS). CD4$^+$ T cells are thought to play a pivotal role in the pathogenesis of EAE and MS. In order to investigate the depletion of CD4$^+$ T cells from the systemic circulation as an effective strategy for the treatment of MS, we performed extracorporeal CD4$^+$ T cell adsorption, using a filter to which anti-CD4$^+$ antibody is immobilized as a ligand, in adoptively transferred EAE. Rats treated with CD4$^+$ T cell removal filter (CD4RF) exhibited milder clinical signs of EAE and earlier recovery than those receiving sham treatment. Moreover, the thymic cells from EAE rats treated with CD4RF exhibited a suppressed proliferative response and IFN-γ production to myelin basic protein. These results suggest that depletion of CD4$^+$ T cells from the systemic circulation by extracorporeal treatment is a potentially useful strategy for treatment of acute phase and relapsing MS.
Multiple Sclerosis *(2003)* **9**, 579–584

Key words: *autoimmunity; CD4; cytapheresis; experimental autoimmune encephalomyelitis (EAE); immunotherapy; T cells*

Introduction

T cells specific for self-antigens (Ags) normally exist in the peripheral blood, and, upon activation, may become involved in autoimmune pathogenesis by targeting tissue Ags. Multiple sclerosis (MS) is a chronic relapsing inflammatory demyelinating disease of the central nervous system (CNS), generally considered to result from Ag-sensitized T cells reactivity to myelin Ags in the CNS.[1–3] In experimental autoimmune encephalomyelitis (EAE), an animal model of MS, CNS demyelination and inflammation are induced by immunization with myelin proteins.[2] EAE can be induced in many susceptible strains of rodents and primates by immunizing with whole brain homogenate or purified neural Ags, such as myelin basic protein (MBP), proteolipid protein and myelin oligodendrocyte protein.[4,5] Adoptive transfer of T cells reactive to these neural Ags is sufficient to induce the disease.[6–8] Attention has focused on CD4$^+$ T cells because susceptibility to MS is associated with the MHC class II gene.[9–11] Studies have indicated that MS and EAE are mediated by Th1 type CD4$^+$ T cells specific for various myelin proteins, which migrate from the peripheral blood into the CNS. Therefore, CD4$^+$ T cells play a key role in initiating and accelerating CNS inflammation and demyelination in both MS and EAE. Elimination or inhibition of CD4$^+$ T cells prevents the adoptive transfer of EAE in mice, and this subset predominates in the early lesions of EAE/MS.[12–16] Inactivation or blocking of CD4$^+$ T cells before or shortly after disease induction was also shown to prevent disease onset and/or disease progression in EAE.[17,18] In fact, anti-CD4$^+$ monoclonal antibodies inhibit the development of EAE in rodents,[19] and uncontrolled human clinical trials indicate that anti-CD4$^+$ antibody therapy may have beneficial effects on several autoimmune diseases.[20–23] In patients with MS, extensive depletion of CD4$^+$ T cells also made minor but significant improvements in relapse rates and reduced levels of magnetic resonance imaging (MRI) activity.[24,25]

On the other hand, previous studies of murine monoclonal antibodies against various cell surface Ags in humans have revealed several major limitations to this strategy. These antibodies often do not significantly deplete the function of the target cells,[20,23,24] and predicting the extent of the defect in the immune defence resulting from antibody administration is difficult. Murine antibodies also provoke a host anti-mouse response that may limit the effectiveness of therapy, especially with repeated treatment.[20,24] Tp minimize these problems, we designed a filter to adsorb CD4$^+$ T cells *ex vivo*. Anti-CD4$^+$ monoclonal antibody was immobilized on an activated substance. This filter removes CD4$^+$ T cells selectively from the circulation by direct perfusion of whole blood.

**Correspondence: Hidenori Matsuo, MD, Kawatana National Hospital, Kawatana, Higashisonogi-gun, Nagasaki 859-3615, Japan.
E-mail: hidenori@kawatana.hosp.go.jp*
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Previous studies on lymphocytapheresis in chronic progressive MS demonstrated that the total number of lymphocytes collected ranged from 3.7×10^{10} to 8.8×10^{10},[26] or the mean number of lymphocytes removed per pheresis ranged from 3.0×10^{9} to 3.7×10^{9}.[27] At most, these numbers represent 0.5% of total lymphocytes in the human body. This raised the question of whether or not such a small, unselective and transient depletion of lymphocytes can be effective for the treatment of autoimmune diseases. Our strategy of selective removal of $CD4^+$ T cells from the circulation, including pathogenic autoreactive T cells, must also address this question. We previously reported that a preliminary trial of *ex vivo* treatment with the filter in active EAE rats resulted in a modest fall in the percentages of circulating $CD4^+$ lymphocytes, but failed to alter the course of EAE.[28] Since the adsorption rate of the filter was nearly 90%, $CD4^+$ T cells in the circulation, once depleted, must be supplied rapidly from the lymphoid organ. This causes the difficulty in estimating the degree of $CD4^+$ T cell depletion after cytapheresis. Furthermore, there was no specific surface marker for the pathogenic $CD4^+$ T cells to evaluate the changes after treatment.

In order to address these questions, we investigated the alterations in the clinical course of adoptive transfer EAE following an attempt to remove $CD4^+$ T cells from the systemic circulation. In adoptive transfer EAE, activated CNS Ag-specific $CD4^+$ T cells are increased in the circulation for a few days after cell transfer, resembling the clonal expansion of specific T cells in the peripheral blood of patients with MS. Therefore, we chose to assess anti-MBP responses of T cells that migrated into the organ instead of measuring $CD4^+$ T cells in the circulation.

Materials and methods

Filters

The $CD4^+$ T cell removal filter (CD4RF) consists of polystyrene nonwoven fabric on which purified mouse anti-rat $CD4^+$ IgG was immobilized using hydroxymethyliodeacetoamide, and blocked with bovine serum albumin.[29] A control filter (CF) without anti-$CD4^+$ monoclonal antibody was used for sham experiments.

Animals

Lewis rats were purchased from The Charles River Co. (Tokyo, Japan). All rats were housed under barrier conditions at Kawatana National Hospital. Animal experiments were conducted under the guidelines of the Animal Care and Use Committee of Nagasaki University, and the Institutional Review Board had approved all procedures.

Production of Ag-specific CD4 T cell lines and induction of adoptive transfer EAE

MBP-specific T cell lines were isolated from the draining lymph nodes of Lewis rats immunized in the hind footpads (0.2 mL total) with 50 μg whole guinea pig MBP (Sigma Chemical Co., St Louis, MO, USA) in complete Freund's adjuvant (CFA) containing 100 μg /rat *Mycobacterium tuberculosis*. To establish a T cell line that induces severe adoptive EAE, some rats received subcutaneous injections of *Bordetella pertussis* organisms (PTX, 1×10^{10}/0.5 mL) at the time of immunization.[30] The lymph node cells were cultured with MBP (10 μg/mL) in stimulation medium containing antibiotics, 1 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol (ME) and 1% fresh syngeneic serum for four days. The responding lymphoblasts were isolated on a Ficoll density gradient and expanded further in growth medium containing 10% foetal bovine serum, antibiotics, L-glutamine and 2-ME as above, and 5% supernatant of 24-hour rat splenocyte cultures with concanavalin A (ConA) (rat growth factor). The line cells were propagated with syngeneic mitomycin C (MMC)-treated thymocyte, and MBP, and subsequent rat growth factor.[7]

Adoptive transfer EAE was induced in naive Lewis recipients (female, age 12 weeks) by injection of 2×10^{6}–3×10^{6} MBP-specific, $CD4^+$ activated T cells. In brief, T cells (3×10^{5}/mL, after at least five cycles of restimulation) were restimulated with MBP (10 μg/mL) and MMC-treated thymocytes (2×10^{7}/mL as APC). Seventy-two hours later, activated T cell blasts were separated by Ficoll density gradients, and 2×10^{6}–3×10^{6} blasts were injected intravenously into each rat.

Clinical evaluation of EAE

All rats were weighed and examined daily for neurologic signs as previously described according to the following criteria: Grade 0, no disease; Grade 1, decreased tail tone or slightly clumsy gait; Grade 2, tail atony and/or moderately clumsy gait; Grade 3, paraplegia; Grade 4, paraplegia with forearm weakness; Grade 5, moribund state or death. Data are plotted as the mean clinical score.

Treatment with the filter

Treatment with CD4RF or CF was performed only once within one hour after the adoptive transfer. Under ether anaesthesia, using a pump, a total 30 mL of blood (1 mL/min) was drawn from the tail artery, passed through the filter, and then returned into the tail vein using 27 G needles. Low molecular weight heparin (125 IU/rat) was given intravenously as an anticoagulant.

Flow cytometry

Flow cytometry determination of lymphocyte subsets was performed using EPICS Elite (Coulter). Red blood cells were lysed with ammonium chloride (NH_4Cl), and lymphocytes were stained with anti-rat $CD4^+$ (W3/25; Cedarlane Laboratoroies Ltd., Ontario, Canada) and anti-rat $CD8^+$ (MRC OX-8; Cedarlane Laboratoroies Ltd., Ontario, Canada) antibodies conjugated to fluorescein isothiocyanate or phycoerythrin, respectively.

In vitro proliferation assay of the thymic cell suspension

To estimate the number of MBP-specific T cells that migrated into tissue from the circulation, on day 6 post-transfer, thymic glands were removed from three rats in each group treated with either CD4RF or CF. The thymuses were placed in Hanks' solution and forced through mesh

screens to yield the thymic cell suspension. This cell suspension was tested for proliferative responses to MBP and mitogen (Con A). In order to measure the MBP-specific T cell proliferation, the thymic cells were washed and plated into flat-bottomed microwells at 1×10^5 cells/well, and then incubated with MBP (10 μg/mL) or Con A (2 μg/mL). Quadruplicate cultures with 200 μL stimulation medium were set up, with and without Ag. After 72 hours of culture at 37°C in 5% CO_2, cultures were pulsed with the thymidine analogue, 5-bromo-2′-deoxyuridine (BrdU) for two hours and proliferative responses were quantified with the Cell Proliferation ELISA kit (Boehringer Mannheim, Germany). The resultant colour development is proportional to the concentration BrdU in the DNA synthesizing cells in each microwell. Absorbances were measured with an optical densitometer.

Measurement of cytokine by ELISA

One hundred microlitres of supernatant were removed from each well for cytokine ELISA after 24 hours of above thymic cell cultures. Production of IFN-γ was evaluated with an ELISA kits (TFB, Tokyo, Japan), following the manufacturer's protocol. Cytokine production was calculated from a standard curve of the corresponding concentration of recombinant rat IFN-γ. The amount of cytokine secreted was expressed in pg/mL.

Statistical analysis

The two-way repeated measures ANCOVA (Student–Newman–Keuls method; $P < 0.05$) was applied to evaluate differences in the clinical status of each of the groups of the adoptive EAE rats. For data that were not normally distributed, the Mann–Whitney *U*-test was employed.

Results

We established two MBP-specific T cell lines. Preliminary experiments revealed that Line 1 was able to induce EAE by adoptive transfer of 3×10^6 cells in 10 of 10 naive Lewis rats with the maximum clinical score of 2.0 (mean 1.3 ± 0.35) on day 6 post-transfer. Line 2 was established from Lewis rats that received *Bordetella pertussis* toxin injection at the time of immunization, induced more severe EAE than Line 1. Adoptive transfer of 2×10^6 lead to 100% occurrence of EAE with a maximum clinical score 4.0 (mean 2.6 ± 0.70, $n = 10$) on day 6. Both MBP-specific T cell lines consisted of more than 95% of $CD4^+$ T cells. The first clinical signs, caudally to cranially progressing paralysis, became apparent on day 4 or 5 post-transfer in all animals. Maximal symptoms developed by day 7 and resolved fully by day 13. The neurological deficit was accompanied by weight loss.

Effects of CD4 T cell removal column on adoptive transfer EAE

We followed the chronological changes of clinical scores of the mild and severe adoptive transfer EAE rats after treatment of extracorporeal circulation with experimental and control filters (Figures 1 and 2, respectively).

In the mild model (eight rats in total), all four rats treated with CF showed the mild disease course of EAE. The first clinical signs were apparent on day five, and by day eight, symptoms reached their maximum (grade 2). Full recovery was achieved by day nine. Rats treated with CD4RF were less affected than the control; only three of four rats developed the illness and the first clinical sign appeared on day five in one rat and on day six in two others. The maximum severity of the disease was grade 1 in all rats affected. Full recovery occurred by day nine.

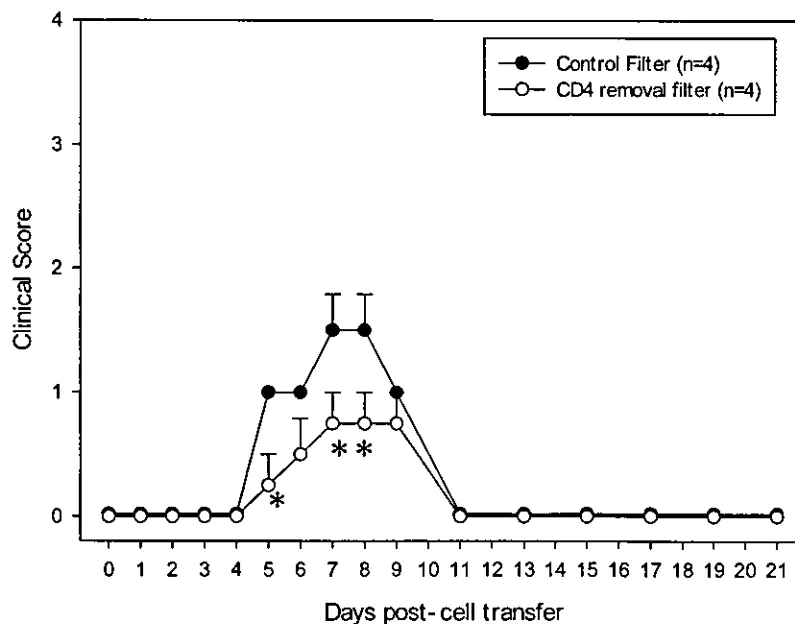

Figure 1 *Effect of cytapheresis with CD4RF on adoptive transfer EAE. Cytapheresis on the day of transfer of Line 1 marginally suppresses clinical signs of EAE. Mean ± SD of four rats per group are plotted. Rats are scored for clinical disease according to the scale specified in Materials and methods. * There are statistical differences between clinical scores at days five, seven and eight (P < 0.05).*

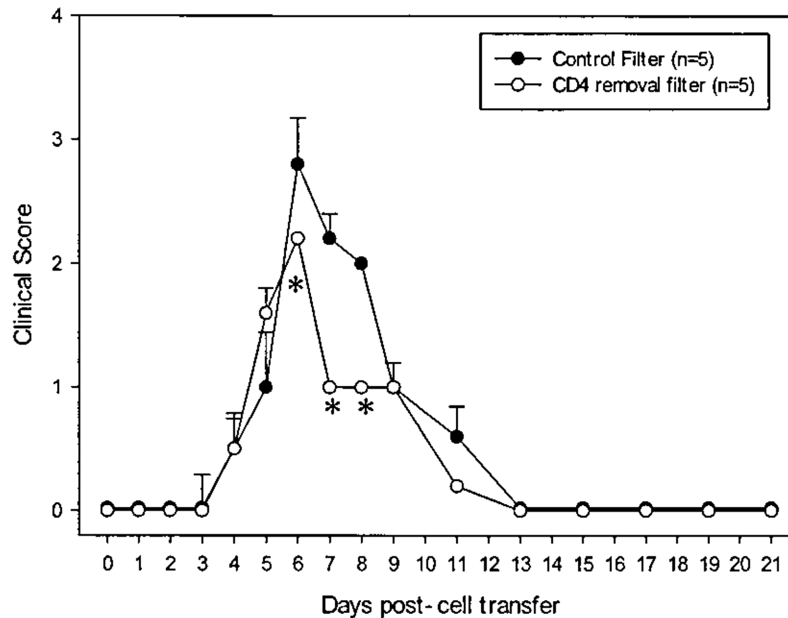

Figure 2 *Treatment of the severe model of adoptive transfer EAE by cytapheresis with CD4RF. On days six, seven and eight, the clinical signs in the group treated with CD4 removal filter are significantly milder than those treated with the control filter (* The two-way repeated measures ANOVA;* $P < 0.05$). Mean±SD of five rats per group are given.

There were statistical differences between clinical scores in CD4RF- versus CF-treated rats at day five, seven and eight ($P < 0.05$, respectively) (Figure 1).

In the severe model (10 rats total), EAE was induced in all 10 rats treated with either CF or CD4RF. Rats treated with CF exhibited the typical disease course of adoptive transfer EAE. The first clinical signs became apparent on day four in all five rats, and by day six all animals developed paraplegia but recovered fully by day 13. The rats treated with CD4RF were less affected than the control group. Only two rats developed paraplegia by day six. On days six–eight, the mean clinical scores of rats treated with CD4RF were significantly milder than those of controls ($P < 0.05$, respectively). These results indicated that treatment of the CD4RF promoted recovery from the illness (Figure 2).

The MBP-specific T-cell response in the thymus

Naparstek *et al.* reported that activated anti-MBP T cells accumulated in the thymus as well as the brain after adoptive transfer.[31,32] On day four post-transfer, there was a highly significant accumulation of anti-MBP T cells in the brain and thymus, which lasted for nearly a week. Therefore, we decided to investigate anti-MBP responses of T cells that migrated into the thymus instead of the brain. Thymic cells from the rats treated with the CD4RF exhibited a markedly reduced proliferation to MBP in contrast to those treated with CF. Whereas the proliferative responses of thymic cells to Con A were almost the same in both groups (Figure 3).

We also measured IFN-γ production of thymic cells when cultured with MBP. As shown in Figure 4, MBP-stimulated IFN-γ production was suppressed to a greater extent in the group treated with CD4RF than in the group treated with CF.

Discussion

We demonstrated that transient depletion of $CD4^+$ T cell from the systemic circulation by cytapheresis with the CD4RF was able to alter the course of adoptive transfer EAE. Furthermore, this may be associated with a decrease in the number of $CD4^+$ T cells migrating from the circulation into the target organ. Although we could not

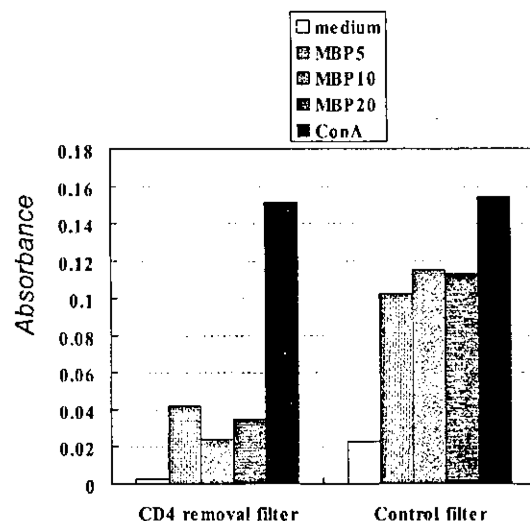

Figure 3 *Proliferative response of thymic cells taken on day six post-transfer. The results shown are from pooled thymic cells of three rats per group. The two groups are represented on the x axis for the animals receiving cytapheresis with CD4RF and CF. MBP was used at a concentration 5, 10 or 20 μg/mL. ConA was used as a positive control at a concentration 2.0 μg/mL. All results are in quadruplicate with SDs of < 10%. Data are representative of at least two experiments.*

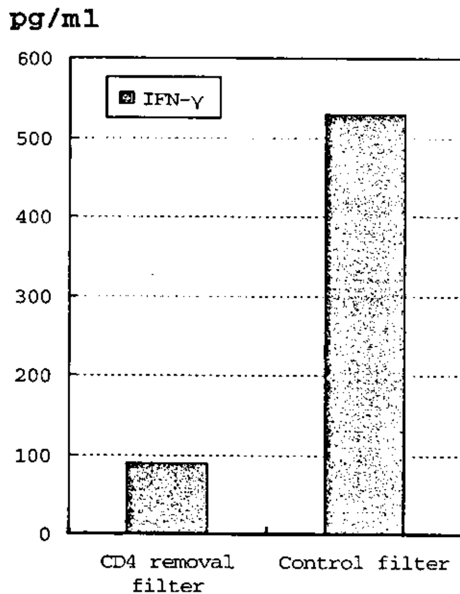

Figure 4 IFN-γ production of thymic cells taken on day six post-transfer. The results shown are from culture supernatant of the pooled thymic cells used in the experiments described in Figure 3. All results are in triplicate with SDs of <5%. Data are representative of at least two experiments.

measure the MBP-specific T-cell response from the CNS, we demonstrated that *ex vivo* adsorption of CD4$^+$ T cells, including MBP-specific CD4$^+$ T cells, caused depletion of the MBP-specific T cell response in the thymus.

Our data suggest that cytapheresis of particular T cells may play a role in the treatment of T cell dependent autoimmune disorders. Previous studies failed to confirm the clinical effect of lymphocytapheresis in chronic progressive MS,[26,27] in which lymphocytapheresis was done to achieve an immunosuppressive state. Major differences between our strategy and previous lymphocytapheresis are not only to remove CD4$^+$ T cells selectively, but also to apply this treatment during the acute phase of the disease, when pathogenic CD4$^+$ T cells clonally expand in the peripheral blood. More specifically, our strategy is to reduce pathogenic T cells in the circulation rather than to achieve an immunosuppressed state. In adoptive transfer EAE, clinical severity depends on the dose of MBP-specific T cells.[7] In the present study, treatment with CD4RF just after cell transfer should reduce the circulating CD4$^+$ T cells, including pathogenic MBP-specific CD4$^+$ T cells that transiently increased in the circulation. Nevertheless, most rats developed EAE. This may be because a portion of pathogenic MBP-specific CD4$^+$ T cells adhered to the endothelium in the CNS immediately after the cell transfer. Perhaps the cytapheresis removed remaining MBP-specific CD4$^+$ T cells with other nonpathogenic CD4$^+$ T cells, which led to a milder clinical course of EAE.

In human MS, clonal expansion of autoimmune T cells has been reported.[33,34] If we can remove these pathogenic T cells from the circulation, further disease progression may be prevented. Along with corticosteroids for suppression of the autoimmune response and inflammation, cytapheresis with CD4RF may benefit patients with acute phase of MS.

Cytokines also play an important role in the initiation and maintenance of the inflammatory reaction in MS/EAE.[35,36] MHC class II-restricted CD4$^+$ T cells, producing IFN-γ, IL-2, lymphotoxin and TNF-α, have been defined as Th1 cells and have been clearly shown to be pathogenic EAE. In the present study, we focused on IFN-γ because it has been related in the pathogenesis of the disease. Our data indicating reduced IFN-γ production in thymic cells culture from CD4RF treated rats compared with those of the CF-treated group is consistent with disability scores on day six post-cell transfer.

In conclusion, these current results indicate that, by selective removal CD4$^+$ T cells, it may be possible to attenuate the CD4$^+$ T cell immune responses in EAE/MS. The technology used in the CD4RF may be applicable to adsorb other target cells, such as lymphocytes with other surface markers or other immune cells, by selecting the appropriate antibody as a ligand. Recently, several reports suggested that myelin-specific CD8$^+$ T cells could function as effector cells in the pathogenesis of EAE/MS.[37-39] Such CD8$^+$ T cells might be another target of this strategy. Apparently, the CD4$^+$ cells populations that the filter adsorbed should include nonpathogenic or even disease-protective T cells. By targeting other surface molecules, such as markers for activated T cells, adhesion molecules or particular T cell receptors, we may remove the pathogenic cells more efficiently. The paradigm set in this study for autoimmune demyelination may also apply in a broad range of chronic inflammatory autoimmune disease, and may prove useful and potential in the future design of rational treatments.

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Selective Adsorption of Human CD4$^+$ T Cells

Hidenori Matsuo,[1,2] Hirofumi Goto,[1,2] Chiaki Kambara,[1,2] Takayasu Fukudome,[1,2] Takamitsu Mizota,[2] Hirokazu Onodera,[3] Makoto Yoshida,[3] and Noritoshi Shibuya[2]

[1]*Division of Clinical Research and* [2]*Department of Neurology, Kawatana National Hospital, Nagasaki and* [3]*Research and Development Laboratory 2, Asahi Medical Co. Ltd, Oita, Japan*

Abstract: The pathogenesis of most autoimmune diseases directly involves CD4$^+$ helper T cells. To remove CD4$^+$ T cells selectively from the circulation, we designed a new column in which an anti-CD4 monoclonal antibody was immobilized on the activated substance. Nearly 90% of CD4$^+$ T cells were selectively adsorbed from whole blood with a single passage through the column in vitro, resulting in depletion of the antigen-specific T cell responses. We conclude that this new column would be potentially useful for treatment of T cell-mediated autoimmune diseases.
Key Words: Cytapheresis, CD4 helper T cell, Immunotherapy.

Antigen (Ag)-specific CD4$^+$ T cells play a central role in the pathogenesis of most of autoimmune diseases (1,2). Monoclonal antibodies to the CD4 Ag have proven effective in the control of experimental autoimmune diseases (3), and uncontrolled clinical trials indicate that anti-CD4 antibody therapy is beneficial in various human autoimmune diseases (4–12). However, previous studies of murine monoclonal antibodies against various cell surface Ags in humans have revealed several major limitations of this strategy. Murine monoclonal antibodies often do not significantly deplete the function of the target cells (4,6,7,12), and it is often difficult to know how many cells can be affected after administration the antibodies. Murine antibodies also provoke a host antimouse response which may limit the effectiveness of therapy, especially with repeated treatment (5,6,12). To minimize these problems we designed a column to adsorb CD4$^+$ T cells ex vivo, using anti-CD4 monoclonal antibody. Reported here are the property of the column and the effects on the human peripheral lymphocytes.

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Address correspondence and reprint requests to Dr Hidenori Matsuo, Division of Clinical Research, Kawatana National Hospital, Kawatana, Higashisonogi-gun, Nagasaki 859-3615, Japan. Email: hidenori@kawatana.hosp.go.jp
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MATERIALS AND METHODS

The column to adsorb CD4$^+$ T cells consists of polystyrene non-woven-fabric on which an anti-CD4 monoclonal antibody was fixed (CD4 removal column). The monoclonal antibody, purified antihuman CD4 mouse IgG ak 2 (clone BL 4), was immobilized on the carrier using hydroxymethyliodeacetoamide, and blocked with bovine serum albumin. By not detecting particular fluorescent intensity in the filtrate when fixing antibodies conjugated with fluorescein isothiocyanate on the carrier, we confirmed that the antibodies were not released from the column after extensive washing. The column, 1 mL in volume, can treat 10–15 mL whole blood at the flow rate of 1 mL/min. A column without anti-CD4 monoclonal antibody was used for sham experiments.

We performed a flow cytometric determination of lymphocyte subsets in whole blood before and after the above treatment, using EPICS Elite (Coulter). Red blood cells were lysed with ammonium chloride (NH_4Cl), and lymphocytes were stained with anti-CD4, CD8, CD3, and CD20 antibodies conjugated to fluorescein isothiocyanate or phycoerythrin.

To assess functional activity of the lymphocytes from untreated or treated blood with the CD4- column, we measured the proliferation to mitogens and purified protein derivatives of tuberculin (PPD). First, PPD-specific CD4$^+$ T cell lines were raised from the peripheral blood mononuclear cells (PBMCs), which maintained by restimulation with PPD and autologous mitomycin (MMC)-treated PBMCs and

TABLE 1. *Adsorption rate (%)† of each cell population when whole blood was treated with the control column or the CD4-column*

Cell populations	Control column mean percentage ± SD ($N=4$)	CD4-column mean % ± SD ($N=5$)	*P*-value*
RBC	3.1 ± 1.4	2.1 ± 2.2	0.45
Platelets	19.5 ± 14.1	25.3 ± 15.8	0.58
WBC	23.0 ± 7.6	18.0 ± 4.7	0.26
Granulocytes	22.6 ± 8.1	7.0 ± 3.0	0.02
Monocytes	39.4 ± 10.0	47.0 ± 9.1	0.27
Lymphocytes	31.9 ± 10.0	36.5 ± 9.8	0.51
CD3+ CD20-	29.9 ± 10.8	53.4 ± 6.1	<0.01
CD3-CD20+	41.0 ± 9.5	9.8 ± 6.1	<0.01
CD4+ CD8-	28.5 ± 10.3	88.2 ± 4.9	<0.01
CD4-CD8+	30.4 ± 11.5	10.0 ± 6.0	0.01

†, Absorption rate (%) = 100 × (cell number of pretreatment – cell number of post-treatment)/(cell number of pretreatment); *, Student's *t*-test or Welch's *t*-test was used where appropriate.

expansion with recombinant human IL-2. The autologous whole blood(10 mL) supplemented with the PPD-specific $CD4^+$ T cells(1×10^6) were treated with the column, then PBMCs were separated on a Ficoll density gradient from both untreated and treated blood, and 2×10^5 cells/well were cultured in 200 μL medium. 1×10^5 autologous MMC-treated PBMCs were added as additional Ag presenting cells because non-woven fabric filters mechanically adsorb a portion of monocytes. After 72 h of culture at 37°C in 5% CO_2, ^{3}H-thymidine was added to each well for the following 18 h, and cells were harvested on filters for scintillation counting.

RESULTS

A single pass of human whole blood through the column reduced a great number of $CD4^+$ T cells. $CD4^+$ $CD8^-$ cells decreased from 27.4 to 1.3% (mean, $N=7$), whereas $CD8^+$ $CD4^-$ cells did not. The CD4-column selectively adsorbed significantly many more $CD4^+$ T cells than the control. Granulocytes and B cells were less adsorbed by the CD4-column. Red blood cell counts remained unchanged (Table 1).

PBMCs from the untreated blood responded well to PPD (stimulation index; SI = 42.6), which was markedly suppressed after treatment with the CD4-column (SI = 7.1) but not with the control column (SI = 30.2). Lymphoproliferation to phytohemagglutinin or concanavalin A was well conserved. PBMCs which passed through the column did not proliferate spontaneously without PPD or mitogens (Fig. 1).

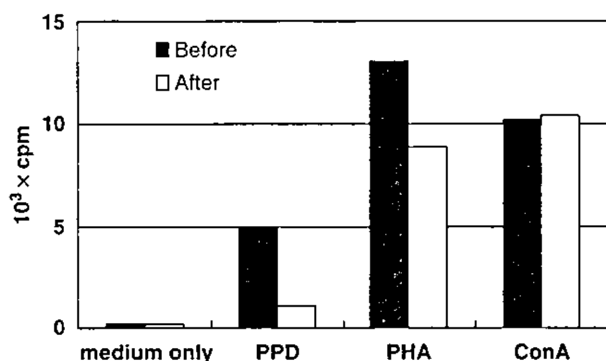

FIG. 1. Proliferative responses of peripheral blood mononuclear cells from the blood treated with the CD4-column or control column. PPD, purified protein derivatives of tuberculin; PHA, phytohemaggulutin; ConA, concanavalin A. The blood (10 mL) contains the autologous PPD-specific $CD4^+$ T cell clones(1×10^6) before treatment.

DISCUSSION

Although lymphocytapheresis has been used to treat autoimmune diseases with some benefit, unselective removal of lymphocytes from the peripheral blood does not appear to be a reasonable therapeutic modality (13). We demonstrated that the CD4-column efficiently adsorbed $CD4^+$ T cells directly from whole blood. In vitro, Ag-specific $CD4^+$ T cells were also adsorbed with the column, resulting in abrogation of the Ag- specific T cell responses. These results suggest that the column is potentially useful in treatment of T cell-mediated autoimmune diseases. However, like lymphocytapheresis (13), a single treatment with the column can remove target cells only from the circulation and may not be sufficient to suppress disease activity. Repeated treatment would overcome this problem. In human autoimmune diseases, clonal expansion of autoimmune T cells has been reported (14,15). If we can remove these pathogenic T cells from the circulation, further disease progression may be prevented. Along with corticosteroids for suppression of the autoimmune response and inflammation, cytapheresis with the CD4-column may benefit patients with acute