**TABLE 1.** *Laboratory data*

| | Chemistry | Immunology |
|---|---|---|
| Urinalysis | | |
| pH 6.0 | ALP 209 IU/L | IgG 3228 mg/dL |
| Protein (+) | GOT 20 IU/L | IgA 905 mg/dL |
| Sugar (–) | GPT 8 IU/L | IgM 2537 mg/dL |
| Sediment | LDH 398 IU/L | ANA (–) |
| RBC 5–9/HPF | GTP 24 IU/L | RF 33230 IU/mL |
| WBC 1–4/HPF | CHE 189 IU/L | RAPA 40960 X |
| hyaline Cast (+) | CPK 111 IU/L | CH50 24.7 |
| Granular Cast (+) | TBIL 0.8 mg/dL | |
| | TP 8.9 g/dL | |
| Peripheral blood | | |
| WBC 25800/mm$^3$ | ALB 3.1 g/dL | CIC(C1q) 1.9 μg/mL |
| Neut 74.0% | UA 6.5 mg/dL | MPO-ANCA 86 EU |
| Lymph 18.4% | BUN 20 mg/dL | C-ANCA <10 EU |
| Mono 13.1% | CREA 1.03 mg/dL | BGA (R.A) |
| Eos 0.1% | Na 138 mEq/L | PH 7.394 |
| Baso 1.1% | Cl 104 mEq/L | PCO2 37.8 torr |
| RBC 260 × 10$^4$/mm$^3$ | K 4.29 mEq/L | PO2 89.1 torr |
| Hb 9.4 g/dL | Ca 9.0 mg/dL | HCO3 23.1 mmol/L |
| Ht 28.8% | AMY 99 IU/L | BE –1.1 mmol/L |
| PLT47.9 × 10$^4$/mm$^3$ | GLU 100 mg/dL | $SaO_2$ 96.8% |
| HbA1C 5.5% | TCHO 151 mg/dL | KL-6 1390 U/mL |
| ESR 129 mm/ h | TG 65 mg/dL | |
| CRP 315.8 mg/dL | | |
| 24 h Ccr 57.0 mL/min | | |
| Stool occult blood (–) | | |

ALB, albumin; ALP, alkaline phosphatase; AMY, amylase; ANA, antinuclear antibody; Baso, basophil; BE, base excess; BGA (R.A.), blood gas analysis (room air); BUN, blood urea nitrogen; C-ANCA, cytoplasmic (proteinase-3)-antineutrophil cytoplasmic antibody; Ccr, creatinine clearance; CHE, cholinesterase; CIC (C1q), circulating immune complex (c1q method); CPK, creatine phosphorkinase; CREA, serum creatinine; CRP, c-reactive protein; Eos, eosinophil; ESR, erythrocyte sedimentation rate; GLU, glucose; GOT, glutamic oxaloacetic transaminase; GPT, glutamic pyruvic transaminase; Hb, hemoglobin; HbA1c, hemoglobin A1c; HCO3, bicatbonate; HPF, high power field; Ht, hematocrit; IgG, immunoglobulin G; IgA, Immunoglobulin A; IgM, immunoglobulin M; LDN, lactate dehydrogenase; Lympho, lymphocyte; mono, monocyte; MPO-ANCA, myeloperoxidese-antineutrophil cytoplasmic antibody; Neu, neutrophil; PLT, platelet; RBC, red blood cell count; RF, rheumatoid factor; $SaO_2$ saturation $O_2$; TBIL, total bilirubine; TCHO, total cholesterol; TG, triglyceride; TP, total protein; UA, uric acid; WBC, white blood cell count.

talized at the Surgery Division of the Chiba Social Insurance Hospital on 5 December 2001, however, the cause was still not understood, in spite of examinations. Additionally C-reactive protein (CRP) was revealed at an extremely high level (136 mg/dL). Therefore, the Rheumatology Division of the hospital was consulted, and the service was changed to this division on 12 December.

He was emaciated (his height 160 cm, body weight 48.7 kg), slightly anemic, with a body temperature of 39.3°C. His pulse was irregular due to atrial fibrillation, which had been managed since 67 years of age (pulse rate 90 beats/min.). Velcro rales were audible posteriorly at his right lung base. Lymph node swelling and hepatosplenomegaly were not recognized. Cutaneous nodules were recognized at the bilateral extensor side of the elbows (right, 7 × 8 mm, left, 12 × 15 mm). He also complained of numbness in the bilateral fingers.

Blood tests showed active inflammatory status according to the high levels of erythrocyte sedimentation rate (ESR) and extremely high level of CRP (Table 1). Other tests showed hypergammapathy and extremely high levels of rheumatoid factor (RAPA 40960 X). Additionally hypocomplementemia was exhibited (CH50 24.7) and LDH was elevated (398 IU/mL). Urinalysis showed slight proteinuria, hyaline cast and granular cast. Although blood gas examination was within normal range, a marker of interstitial pneumonitis increased (KL-6, 1390 U/mL).

Examination findings are summarized in Table 2. A chest X-ray showed ground-glass shadow in the right medial field and reticulogranular shadow over the bilateral lower lung field. The joint destructions were at the stage III of Steinbrocker's criteria (3) according to a hand X-ray which showed erosions and subluxation.

**TABLE 2.** *Examination findings*

| Examination | Findings |
|---|---|
| Chest X-ray | CTR 47.7%, ground-glass shadow in right medial field and reticulogranular shadow over the bilateral lower lung field |
| Hand X-ray | Erosion visible at the joints of bilateral second to fifth PIP, bilateral fifth MP and carpal bones. Subluxation visible at right fifth MP joint. |
| ECG | Atrial fibrillation |
| Abdominal echo | Hemangioma in S5, left renal cyst |
| UCG | Mild TR. wall motion good, pericardial effusion (–) bacterial vegetation (–) |
| Abdominal CT | Normal study |
| Thoracic CT | Emphysematous change in bilateral upper lung field reticulogranular shadow over the bilateral lower lung field |
| Gastric fiber | Superficial gastritis |
| Colon fiber | Small polyp and small superficial ulceration in transverse colon, small scar in descending colon |
| Ophthalmology | No abnormal findings |

CT, computed tomography; CTR, cardiothracic ratio; ECG, electrocardiography; MP, metacarpophalangeal; PIP, proximal interphalangeal; TR, tricuspid valve regurgitation; UCG, ultrasound cardiography.

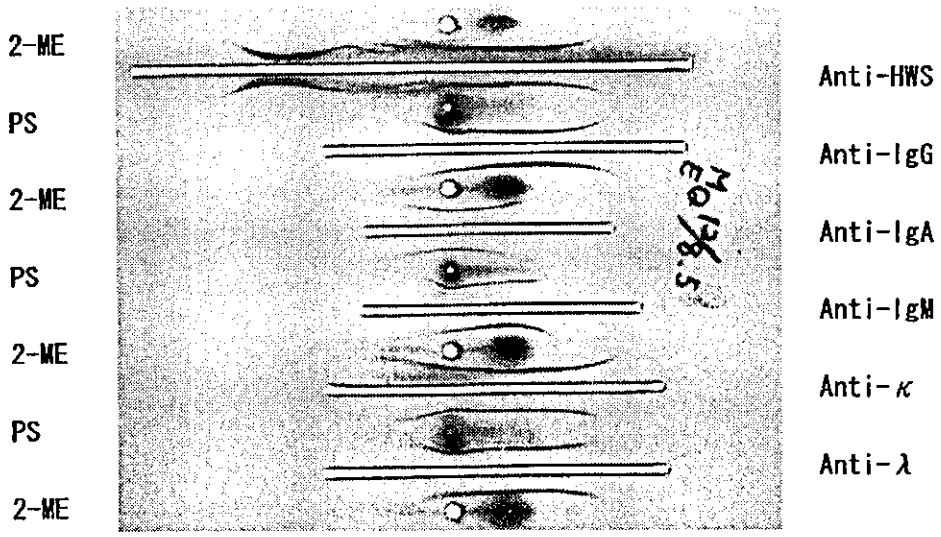


FIG. 1. Immunoelectrophoresis shows IgM type M protein (K-chain) existed in the patients serum. PS, patients sample; 2-ME: 2-mercaptoethanol treated patients sample.

Based upon these findings (such as high levels of rheumatoid factor, hypocomplementemia, polyneuropathy, rheumatoid nodules, and interstitial shadow in chest X-ray), he satisfied the criteria of the Japanese Research Committee for RA with vasculitis, so called malignant rheumatoid arthritis. Prednisolone (30 mg/day) was prescribed and a survey conducted for infectious diseases. It was, however, not significant. One week after changing the service, MPO-ANCA proved to be positive (86 EU) and cyclophosphamide (50 mg/day) was initiated orally. Additionally, IgM K-chain M-protein was revealed (Fig. 1). The differentiation between auto-immune and hematologic diseases was required for further drug prescriptions.

Therefore, DFPP was initiated from 2 weeks after changing the service. Kuraray KM-8800 (Kuraray Medical Inc., Okayama, Japan) was used as the monitor of DFPP. Plasmaflo OP-05 (Asahi Medical Co. Ltd., Tokyo, Japan) was used as the first membrane and Kuraray Evaflux 4, a plasma filter (Kuraray Medical Inc.) was used as the second membrane. Bilateral antecubital veins were used as the blood access, and as an anticoagulant, heparin sodium was used with bolus shot 2000 U and continuous infusion 1000 U/h. QB and QP were set at 60 mL/min and 30% of QB, respectively. The partial discard method was used and the filtration fraction was set at 0.8. As the replacement fluid 500 mL of 5% albumin was used. The treated plasma volume was 2000 mL on each session and the treatment was performed weekly. As a result of bone marrow examination, there was no evidence of hematologic diseases. Additionally, hypergammapathy was improved and CRP and MPO-ANCA decreased to the normal level after 3 sessions while the symptoms were also much improved and cutaneous nodules disappeared (Fig. 2). Prednisolone was tapered and he was discharged. It was suggested that the case presented here was quite rare, having an extremely high level of CRP which was successfully managed by utilizing DFPP.

## DISCUSSION

The present case was quite a rare RA case with vasculitis, having benign M-proteinemia, positive MPO-ANCA and an extremely high level of rheumatoid factor and C-reactive protein.

MPO-ANCA is a significant marker of systemic vasculitis (4,5) such as microscopic polyangiitis, rapidly progressive glomerlonephritis and Churg–Strauss syndrome. It has been reported that 12–36% of RA patients are MPO-ANCA positive and some of these patients rapidly develop progressive glomerlonephritis (6,7). Therefore, a high dose of prescription steroids or intravenous administration of cyclophosphamide had been considered as the treatment for the present case initially. However, IgM K-chain M-protein was revealed and the differentiation between auto-immune and hematologic diseases was required for further drug prescriptions. Based upon the severity of blood testing and the interference to the diagnosis and the management, blood purification was considered as the proper treatment until the differentiation was made. As a result, CRP, MPO-ANCA decreased to the normal level after the initiation of DFPP. It was considered that DFPP enhance the effectiveness of steroids and cyclophosphamide

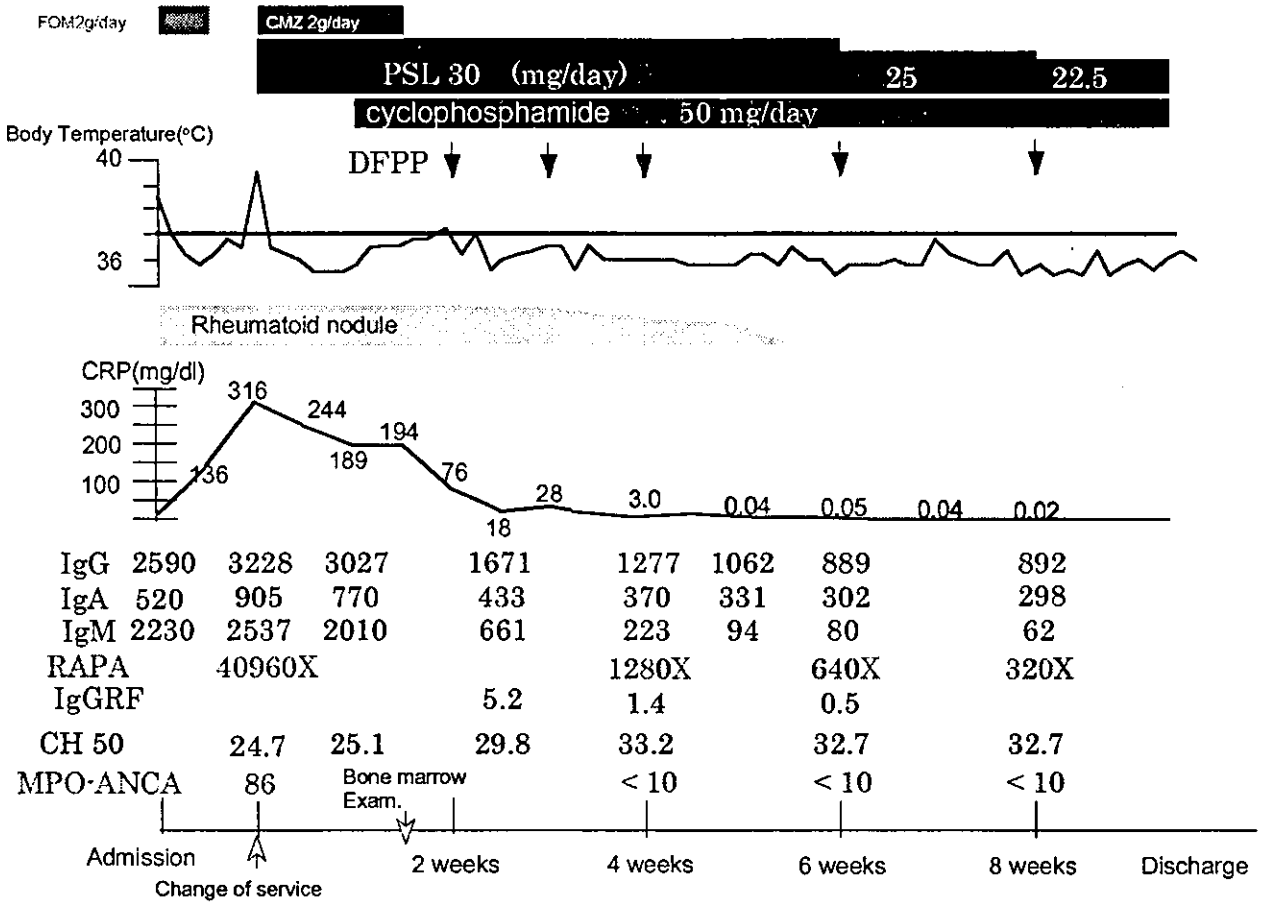


**FIG. 2.** Course of treatment.

which had been prescribed before the initiation of DFPP, through the removal of MPO-ANCA, rheumatoid factor and immunoglobulin, etc.

C-reactive protein is one of the acute-phase proteins. The serum CRP concentration increases in response to infectious disease, non-infectious inflammatory disease, neoplasm, tissue damage, and necrosis, etc. Therefore, it has been used as an available marker for monitoring disease activity including rheumatoid arthritis. The present case had an extremely high level of C-reactive protein and it might indicate the severity of the patient status. However, there are some reports describing that high titre of rheumatoid factor and/or hypergammapathy interfere with the assay of CRP (8–10). Since immunoglobulin and rheumatoid factor can be removed by DFPP, there is a possibility that the normalization of CRP was achieved through the improvement of hypergammapathy and reduction of rheumatoid factor by DFPP in the present case. We investigated whether the rise in CRP was true or false, however, we could not get the evidence. Additionally, there is a report demonstrating the correlation between hyperlipidemia and CRP (11), however, such metabolic disorder was not recognized in the present case.

Several humoral factors such as MPO-ANCA, rheumatoid factor, and hypergammapathy were considered to be related to the status of the present case having RA with vasculitis. The cause of RA is still unknown, however, humoral factors such as immunoglobulin, rheumatoid factors, cytokine, chemokine, proteolytic enzyme, superoxide are considered to be related to developing RA. As a method of apheresis for the treatment of RA, DFPP can manage such humoral immunological factors. It is suggested that DFPP was effective for the management of RA with vasculitis having high levels of humoral pathological factors like the present case.

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# Filtration Leukocytapheresis Therapy in the Treatment of Rheumatoid Arthritis Patients Resistant To or Failed with Methotrexate

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**Abstract:** Filtration leukocytapheresis (LCP) is a treatment for abnormal autoimmune states, which removes responsible leukocytes from the peripheral blood. To examine the efficacy of LCP therapy in the treatment of rheumatoid arthritis (RA), nine patients were selected, who were either resistant to methotrexate, or failed with methotrexate due to drug ineffectiveness or adverse side effects. For these patients, LCP therapy was performed once a week for five weeks. After five LCP treatments, the patients were observed for 12 weeks, to test the efficacy of the treatment. The definition of improvement given by the American College of Rheumatology (ACR core set) was used for efficacy evaluation of LCP therapy. As the result, 77.8% of the patients showed an ACR 20% response and 44.4% of the patients showed an ACR 50% response. With improvement of joint symptoms, IL-6 was significantly decreased at 8 weeks and 12 weeks after the treatment. The expression of adhesion molecules CD11a, CD11b, and CD18 on granulocytes decreased directly after the LCP treatment. No adverse side effect was monitored during the study period. These results indicates that LCP treatment is a useful treatment for RA patients who were resistant to methotrexate, or failed with methotrexate due to ineffectiveness or side effects of the drug. **Key Words:** ACR core set, Leukocytapheresis, Leukocyte, Methotrexate, Rheumatoid arthritis.

Rheumatoid arthritis (RA) is a chronic inflammatory arthropathy with destructive synovitis and immunological processes within the synovial tissue. Synovitis of this disease is characterized by extensive inflammation involving leukocytes and proliferation of the synovial tissue of the joints. Cytokines such as IL-1 and TNF-α are produced by this synovial tissue. T lymphocytes and macrophages are involved in the inflammatory process. Although etiology of RA is unknown, many factors, such as viral infection and genetic factors, are thought to be involved. The chronic inflammatory process of RA often leads to destruction and deformation of the joints, and a worsening of the quality of life. The purposes of the treatment of RA are to control the inflammatory process and to stop the destruction of the joints. Drug treatment is one of the important treatments of RA, in addition to education of the patients, and physical therapy. Nonsteroidal anti-inflammatory agents, disease-modifying antirheumatic drugs, corticosteroids, and immuno-suppressive agents are often used in the treatment of RA. Adverse side effects of these agents are, however, great obstacles in treatment. Apheresis therapy is also one useful treatment for RA, as an adjunct to drug therapy. It is sometimes used in rheumatoid vasculitis and some cases of refractory RA.

Leukocytes are an important factor in the inflammatory process of RA. To control this, cytapheresis has been used as a treatment for autoimmune related diseases in several fields of rheumatology, gastroenterology, and neurology. Two major methods of extracorporeal leukocyte removal therapy have been performed in clinical fields. These include the centrifugal method and the adsorptive method with fiber or beads. In the United States of America and Europe, the centrifugal method is the main method of leukocyte removal. The adsorptive method became the main stream in Japan, however, because of ease of operation and cell removal efficiency.

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What we can expect from removing leukocytes from autoimmune diseases, such as RA, is removal of the granulocytes involved in inflammation, suppression of cytokine production, and removal of T cells in antibody production and inflammation (1). In order to remove leukocytes from the circulation, thoracic duct drainage and centrifugal methods have been used in the past (2,3). In Japan, a leukocyte removal filter has been developed and modified. In a clinical setting, a leukocyte removal filter, which was originally made for blood transfusion tubing, was developed for treatment of systemic lupus erythematosus in 1985 (4). After this experience, the leukocyte removal filter technique was modified and used in autoimmune diseases, such as ulcerative colitis and RA. In the present study, we selected RA patients who were resistant to methotrexate treatment, or who could not continue using methotrexate due to its side effects. Filtration leukocytapheresis (LCP) therapy was performed and the efficacy of the therapy evaluated.

## PATIENTS AND METHODS

### Patient selection

For the present study, patients had to satisfy the criteria of the American College of Rheumatology for RA, and have had RA for at least 6 months. Patients had to be between the ages of 20 and 75, to be resistant to two or more kinds of drug treatment, including methotrexate, for more than three months, or be unable to use methotrexate due to its side effects.

Also the patient had to have six or more tender joints, three or more swollen joints, C-reactive protein (CRP) more than 1.0 mg/dL or erythrocyte sedimentation rate (ESR) more than 30 mm/h, and the dosage of methylprednisolone had to be less than 15 mg/day. No change of the medication was allowed one month prior to entering the study and during the study period.

The study population consisted of 9 patients with RA (2 men, 7 women; mean age 51.9 years). Three of the nine patients were currently on methotrexate. The rest of the patients had experienced use of methotrexate in the past. These patients profiles are shown in Table 1.

### Leukocytapheresis

Leukocytapheresis, an extracorporeal circulation therapy utilizing a fiber filter, was performed once a week for 5 weeks. A total amount of 3 L of blood was filtrated with a leukocyte removal column equipped with a fiber filter (CS-100 Cellsorba, Asahi Medical Co. Ltd, Tokyo, Japan) at a blood flow rate of 50 mL/min for 60 min. Nafamostat mesilate (Futhan, Torii Pharmaceutical Co. Ltd, Tokyo, Japan) was used as an anticoagulant at a dose of 50 mg/h. Study of this treatment was approved in Juntendo University Hospital ethics committee, and informed written consent was obtained from each patient.

**TABLE 1.** *Patient profile*

| | | | Steinbrocker | | Medication | | |
|---|---|---|---|---|---|---|---|
| Patient | Age | Sex | Stage | Class | Current or past methotrexate (mg/week) | Other medication in use | Past medication stopped due to no effect or side-effect |
| 1 | 64 | M | IV | 3 | 6 mg/week stopped due to abnornal liver function | prednisolone(5 mg/d), auranofin, NASID | |
| 2 | 56 | F | III | 3 | 6 mg/week in use | NSAID | auranofin, bucillamine, salazosulfapyridine |
| 3 | 53 | F | IV | 3 | 8 mg/week in use | prednisolone(10 mg/d), salazosulfapyridine, NSAID | |
| 4 | 57 | F | IV | 3 | 6 mg/week stopped due to abnornal liver function | prednisolone(6 mg/d), azathioprine, penicillamine | |
| 5 | 55 | F | III | 3 | 4 mg/week in use | prednisolone(8 mg/d), bucillamine, NSAID | |
| 6 | 62 | F | IV | 4 | 6 mg/week stopped due to no effect | prednisolone(15 mg/d), cyclosporine, NSAID | auranofin, bucillamine, salazosulfapyridine, sodium aurothiomalate, actarit, bucillamine |
| 7 | 54 | M | III | 2 | 6 mg/week stopped due to hair loss and skin rash | prednisolone(10 mg/d), NSAID | |
| 8 | 37 | F | IV | 4 | 6 mg/week stopped due to no effect | bucillamine, NSAID | |
| 9 | 31 | F | IV | 3 | 7.5 mg/week stopped due to no effect | prednisolone(6 mg/d), bucillamine, actarit, NSAID | |
| Average | 51.9 | | 3.7 | 3.0 | | | |

The CS-100 filter is composed of two different kinds of unwoven fabric made of extra fine polyethylene terephthalate fibers that are rolled up to form two concentric layers and then are packed into a column housing. The inner main filter is composed of approximately 11 g of unwoven fabric made of fibers with an average diameter of 2.6 μm. This is surrounded by the outer prefilter, which is made of fibers with large average diameters of between 10 and 40 μm. While blood introduced into the column flows through the outer prefilter into the inner main filter, leukocytes are entrapped by the filter matrix and are gradually removed from the blood flow. According to Yamaji et al. when this treatment was applied to healthy donors, one LCP session treating 3 L of whole blood removed approximately $6.6 \times 10^9$ leukocytes in total, including approximately $3.5 \times 10^9$ neutrophils, $2.5 \times 10^9$ lymphocytes, $2.3 \times 10^8$ monocytes, and $3.7 \times 10^8$ eosinophils (5). We have reported the efficiency of cell removal and reported that LCP performed on RA patients removes $1.0–1.6 \times 10^{10}$ leukocytes, $3.2–3.5 \times 10^9$ lymphocytes, and $5.63 \times 10^{11}$ platelets (6,7). Another institution reported that LCP in patients with RA removed $1.2 \times 10^{10}$ leukocytes, $9.7 \times 10^9$ neutrophils, and $1.9 \times 10^9$ lymphocytes (8).

**Clinical evaluation**

Clinical response to the treatment was evaluated based on the American College of Rheumatology (ACR) core set of disease activity measures (9): swollen joint count; tender joint count; patient assessment of pain by 100-point visual analog scale (VAS); patient assessment of global severity by 100-point VAS; physician assessment of global severity by 100-point VAS; modified Health Assessment Questionnaire (MHAQ) (10); and CRP. The efficiency of the treatment was evaluated based on the ACR definition of improvement in RA (11), which requires at least 20% improvement in joint tenderness and joint swelling counts, as well as improvement in 3 of 5 other measures (patient assessment of pain, patient assessment of global severity, physician assessment of global severity, MHAQ, and CRP; ACR 20% response). Improvement of at least 50% in the variables was also assessed (ACR 50% response).

**Study period**

Each patient was evaluated for treatment response before the each LCP session. After the five LCP sessions, each patient was examined at 1 week, 2 weeks, 4 weeks, 8 weeks, and 12 weeks after the last treatment period. Side effects from the treatment were also monitored during the study period.

**Laboratory examination**

Laboratory examinations were performed during the treatment period and after the treatment. Complete blood cell counts with leukocyte differential, blood chemistry, immunological parameters (IgG, IgA, IgM, C3, C4, CH50, rheumatoid factor [RF], monoclonal RF [mRF]), and CD4/CD8 ratio were measured. Cytokine levels IL-1β, IL-2, IL-10, and IF-γ were measured using the quantitative sandwich enzyme-linked immunosorbent assay (ELISA) from commercially available kits (BioSource Europe, Nivelles, Belgium). IL-6 level was measured by chemiluminescent immunoassay (Fujirebio Co. Ltd, Tokyo, Japan). TNF-α level was measured by using ELISA from commercially available kits (Japan Immuoresearch Laboratories Co. Ltd, Gunma, Japan). The detection thresholds for those cytokines were IF-γ, 0.1 IU/mL; IL-1β, 10 pg/mL; IL-2, 0.8 U/mL; IL-6, 4 pg/mL; IL-10, 2 pg/mL; and TNF-α, 5 pg/mL. Matrix metalloproteinase-3 (MMP-3) was measured by enzyme immunoassay (Daiichi Fine Chemical Co. Ltd, Toyama, Japan). Granulocytes were isolated and marked with monoclonal antibody CD11a, CD11b, and CD18 (Becton Dickinson), and flow cytometry study was conducted. Lymphocytes were isolated and marked with monoclonal antibody HLA-DR, CD4, and CD8 (Becton Dickinson) and two-color flow cytometry study was performed. Also, lymphocytes were isolated and marked with monoclonal antibody CD3 (Becton Dickinson), CD19 (Coulter Immunology, Tokyo, Japan) and two-color flow cytometry study was conducted. All of the blood samples were taken directly from the vein. The study protocol is shown in Table 2.

**Statistical analysis**

Changes in parameters were analyzed using the Wilcoxon signed rank test. A $P$-value of $<0.05$ was considered to be significant. Values are expressed as mean ± SE.

## RESULT

**ACR core set**

During the five LCP therapy periods, patient 5 satisfied the ACR 20% response from the third treatment, and patient 1 satisfied the criteria from the fourth treatment. One week after the scheduled five treatments, patients 3, 6, and 7 satisfied the ACR 20% response. Four weeks after the therapy patients 2 and 4 satisfied the ACR 20% response. During the study, three patients dropped out. From four weeks after the therapy, the patients 8 and 9 dropped out of the study because no improvement in symptoms was

**TABLE 2.** *Protocol of the study*

| | Treatment period | | | | | Post treatment period | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Week | 1 | 2 | 3 | 4 | 5 | 1 | 2 | 4 | 8 | 12 |
| LCP treatment | #1 | #2 | #3 | #4 | #5 | — | — | — | — | — |
| CBC | x | x | x | x | x | x | x | x | x | x |
| Chemistry | x | x | x | x | x | x | x | x | x | x |
| Immunology | x | — | x | — | x | x | x | x | x | x |
| MMP-3 | x | — | x | — | x | x | x | x | x | x |
| Cytokines | x | — | x | — | x | x | x | x | x | x |
| Adhesion molecules | x | — | x | — | x | x | x | x | x | x |
| Lymphocytes study | x | — | x | — | x | x | x | x | x | x |
| Clinical evaluation | x | x | x | x | x | x | x | x | x | x |

LCP, Leukocytapheresis procedure; CBC, complete blood cell count; x, study performed; —, study not performed.

observed, and the patient 7 dropped out because of worsening of the joint pain and required modification of the medication. The remaining patients were monitored until 12 weeks after the therapy. During the 17 weeks of the study period (5 weeks for the LCP treatments and 12 weeks for the follow-up period), the ACR 20% response was 77.8% (7 out of 9 patients). 85.7% of these patients (6 out of 7 patients) showed continuation of the ACR 20% response at 12 weeks after the therapy. During this study period, the ACR 50% response was 44.4% (4 out of 9 patients) (Table 3). Of the patients who remained in the study, no adverse side-effects were observed during the entire study period.

In terms of each ACR core set activity measurements, significant improvement in swollen joint count and tender joint count was observed one week after the therapy ($P < 0.05$) (Table 4). Four weeks after the therapy, swollen joint count, tender joint count, and physician assessment of global severity significantly improved ($P < 0.05$). Eight weeks after the therapy, swollen joint count, tender joint count, patient assessment of pain, patient assessment of global severity, physician assessment of global severity, and CRP significantly improved ($P < 0.05$). Twelve weeks after the therapy, swollen joint count, tender joint count, patient assessment of pain, patient assessment of global severity, and physician assessment of global severity significantly improved ($P < 0.05$).

**Other parameters**

No significant difference was observed in ESR during the study. Eight weeks after the therapy, RF significantly decreased ($P < 0.05$) Table 4. For C3, C4,

**TABLE 3.** *Appearance of LCP treatment effectiveness*

| | Treatment period (week) | | | | | Post treatment period (week) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Week | | | | | | | | | | |
| Patient | 1 | 2 | 3 | 4 | 5 | 1 | 2 | 4 | 8 | 12 |
| ACR20%* | | | | | | | | | | |
| 1 | — | — | — | x | x | x | x | x | x | x |
| 2 | — | — | — | — | — | — | — | x | x | x |
| 3 | — | — | — | — | — | x | x | x | x | x |
| 4 | — | — | — | — | — | — | — | x | x | x |
| 5 | — | — | x | x | — | x | x | x | x | x |
| 6 | — | — | — | — | — | x | x | x | x | x |
| 7 | — | — | — | — | — | x | x | — | drop out | |
| 8 | — | — | — | — | — | — | — | — | drop out | |
| 9 | — | — | — | — | — | — | — | — | drop out | |
| ACR50%† | | | | | | | | | | |
| 1 | — | — | — | — | — | — | — | x | x | x |
| 2 | — | — | — | — | — | — | — | — | — | — |
| 3 | — | — | — | — | — | — | x | x | x | x |
| 4 | — | — | — | — | — | — | — | — | — | — |
| 5 | — | — | — | — | — | x | x | — | x | — |
| 6 | — | — | — | — | — | — | — | — | x | — |
| 7 | — | — | — | — | — | — | — | — | drop out | |
| 8 | — | — | — | — | — | — | — | — | drop out | |
| 9 | — | — | — | — | — | — | — | — | drop out | |

x, satisfaction of the American College of Rheumatology (ACR) definitions of 20% response and 50%; —, ACR core set was not satisfied; drop out, Patients 7, 8, and 9 dropped out of the study as shown; *, Overall ACR core set 20% = 77.8%; †, Overall ACR core set 50% = 44.4%.

**TABLE 4.** *Changes in disease activity measures in RA patients and other parameters*[†]

| | Before treatment ($N = 9$) | 1 week after treatment | $P^a$ | 4 weeks after treatment | $P^a$ | 8 weeks after treatment | $P^a$ ($N = 6$) | 12 weeks after treatment | $P^a$ |
|---|---|---|---|---|---|---|---|---|---|
| Tender joint count (range 0–48)[‡] | 15.2 ± 1.7 | 8.9 ± 2.2 | <0.05 | 7.7 ± 2.0 | <0.05 | 5.5 ± 2.0 | <0.05 | 5.8 ± 1.0 | <0.05 |
| Swollen joint count (range 0–46)[‡] | 6.9 ± 0.7 | 3.6 ± 0.8 | <0.01 | 3.7 ± 1.1 | <0.05 | 2.7 ± 0.9 | <0.05 | 2.7 ± 0.9 | <0.05 |
| Patient assessment of pain, VAS (range 0–100)[‡] | 67.7 ± 7.1 | 56.6 ± 9.3 | NS | 1.0 ± 0.2 | NS | 40.8 ± 9.8 | <0.05 | 38.0 ± 8.9 | <0.05 |
| Patient assessment of global severity, VAS (range 0–100)[‡] | 74.0 ± 6.0 | 58.9 ± 8.6 | NS | 53.7 ± 9.2 | NS | 39.7 ± 10.7 | <0.05 | 36.3 ± 8.2 | <0.05 |
| Physician assessment of global severity, VAS (range 0–100)[‡] | 81.3 ± 3.2 | 54.6 ± 5.7 | NS | 54.6 ± 6.3 | <0.05 | 46.3 ± 8.3 | <0.05 | 41.8 ± 7.7 | <0.05 |
| MHAQ (range 0–3)[‡] | 1.2 ± 0.2 | 1.1 ± 0.2 | NS | 1.0 ± 0.2 | NS | 1.1 ± 0.3 | NS | 1.1 ± 0.3 | NS |
| CRP mg/dL[‡] | 3.8 ± 1.1 | 3.2 ± 0.8 | NS | 3.0 ± 0.8 | NS | 1.7 ± 0.2 | <0.05 | 3.6 ± 2.0 | NS |
| ESR mm/h | 68.8 ± 10.4 | 77.8 ± 9.5 | NS | 72.3 ± 10.4 | NS | 54.5 ± 6.6 | NS | 57.8 ± 9.5 | NS |
| RF IU/mL(normal <20) | 183.6 ± 65.5 | 202.3 ± 78.2 | NS | 241.3 ± 112.0 | NS | 163.5 ± 80.3 | <0.05 | 153.7 ± 84.5 | NS |
| C3 mg/dL (normal 84–151) | 142.6 ± 7.4 | 142.2 ± 6.7 | NS | 141.3 ± 5.1 | NS | 152.7 ± 8.7 | NS | 148.8 ± 9.1 | NS |
| C4 mg/dL (normal 17–40) | 25.6 ± 1.9 | 24.1 ± 2.3 | NS | 23.9 ± 2.1 | NS | 25.2 ± 2.1 | NS | 25.2 ± 1.6 | NS |
| CH50 (normal 25.0–48.0) | 39.6 ± 2.3 | 43.2 ± 3.2 | NS | 42.4 ± 2.8 | NS | 46.2 ± 2.9 | NS | 44.8 ± 3.5 | NS |
| IgG mg/dL (normal 880–1800) | 1569.1 ± 228.8 | 1648.9 ± 244.2 | NS | 1672.6 ± 250.5 | <0.05 | 1338.3 ± 183.6 | NS | 1310.2 ± 204.1 | NS |
| IgA mg/dL (normal 126–517) | 392 ± 79.6 | 429 ± 97.4 | NS | 411.4 ± 68.8 | NS | 397.8 ± 87.1 | NS | 381.2 ± 83.4 | NS |
| IgM mg/dL (normal 52–270) | 150.0 ± 23.1 | 172.1 ± 29.4 | NS | 197.8 ± 47.5 | NS | 132.3 ± 28.4 | NS | 126.2 ± 29.3 | NS |
| IL-6 pg/mL(normal <4.0) | 30.9 ± 7.7 | 20.9 ± 5.4 | NS | 20.9 ± 5.4 | NS | 7.6 ± 1.5 | <0.05 | 9.5 ± 1.9 | <0.05 |
| MMP-3 ng/mL (normal; M 36.9–121, F 17.3–59.7) | 452.1 ± 78.6 | 357.0 ± 69.9 | <0.05 | 361 ± 72.1 | NS | 306.5 ± 49.7 | NS | 308.8 ± 43.9 | NS |
| CD4/CD8 ratio | 2.4 ± 0.5 | 2.4 ± 0.5 | NS | 2.1 ± 0.4 | NS | 2.0 ± 0.5 | NS | 2.4 ± 0.8 | NS |

[†], Values are the means ± SE; NS, not significant; [‡], Activity measures used for assessment of the American College of Rheumatology (ACR) definitions of 20% response and 50% response. See text for details on ACR core set; [a], $P$ from Wilcoxon signed rank test.

and CH50, no significant difference was observed. There was significant improvement in IgG four weeks after the therapy, but no significant change was observed in the rest of the period. IgA and IgM showed no significant changes. Changes in mRF levels were observed, but only patient 6 had elevated mRF (normal; < 4.2 IU/mL) with 7.2 IU/mL at the beginning of the therapy, and it decreased to 3.8 IU/mL at 12 weeks after the therapy. The cytokine levels of IF-γ, IL-1β, IL-2, IL-6, IL-10, and TNF-α were measured, but only IL-6 level was above the detection threshold level. IL-6 levels significantly decreased at 8 and 12 weeks after the therapy ($P < 0.05$) Table 4. The IL-6 level of patient 2 was 68.4 pg/mL at the beginning of the study and it decreased to 13.4 pg/mL at 12 weeks after the therapy. The IL-6 level of patient 6 was 70.9 pg/mL at the beginning and it decreased to 7.6 pg/mL at 8 weeks after the therapy. MMP-3 involved in the cartilage and connective tissue destructive process decreased significantly one week after the therapy ($P < 0.05$) Table 4. The MMP-3 level of patient 3 was 800 ng/mL which decreased to 381 ng/mL at 12 weeks after the therapy. The MMP-3 level of patient 4 was 659 ng/mL which decreased to 156 ng/mL at 12 weeks after the therapy. In peripheral blood, total numbers of leukocytes, granulocytes, and lymphocytes were decreased significantly directly after the each LCP treatment, but no significant difference was observed after the therapy period when it was compared to the data before the treatment (Table 5). CD4/CD8 ratio was unchanged during the entire study period. In order to examine the lymphocyte activation, surface markers for HLA-DR+CD4+ and HLA-DR+CD8+ were analyzed by using two-colored flow cytometry. There was no HLA-DR+CD4+ (%) significant difference in HLA-DR+CD4+ (%). HLA-DR+CD8+ (%) decreased significantly at the third LCP treatment when it was compared before and after the treatment, and it increased significantly at four weeks after the therapy ($P < 0.05$) (Tables 5 and 6). For changes in T and B lymphocytes, CD3 and CD19 surface markers were analyzed. CD3-CD19+ (%) decreased significantly at each LCP treatment before and after the session. CD3+CD19- (%) increased significantly at the fifth LCP treatment, but no significant change was observed during the rest of the period. Changes in expression of the adhesion molecules CD11a, CD11b, and CD18 on granulocytes were examined by flow cytometry at first, third, and fifth LCP and after the therapy period. The expression of CD11a tended to decrease directly after the LCP treatment, and it decreased significantly at the third treatment

**TABLE 5.** *Changes in leukocytes during LCP treatment period*†

| | LCP treatment 1 | | | LCP treatment 3 | | | LCP treatment 5 | | |
|---|---|---|---|---|---|---|---|---|---|
| | before | after | P§ | before | after | P§ | before | after | P§ |
| WBC/μl | 8688.9 ± 839.1 | 5811.1 ± 778.8 | <0.05 | 8277.8 ± 731.8 | 5344.4 ± 1161.2 | NS | 8011.1 ± 680.2 | 4766.7 ± 782.4 | <0.05 |
| Granulocyte/μl | 6847.3 ± 794.9 | 4728.1 ± 648.5 | <0.05 | 6390 ± 598.1 | 4129.3 ± 952.1 | NS | 6239.7 ± 636.0 | 3780.2 ± 663.2 | <0.05 |
| Lymphocyte/μl | 1419.8 ± 188.3 | 840.2 ± 123.6 | <0.05 | 1574.0 ± 187.8 | 993.3 ± 221.1 | <0.05 | 1321.9 ± 134.8 | 712.6 ± 117.7 | <0.05 |
| CD19+CD3- (%) | 9.0 ± 1.6 | 5.7 ± 1.4 | <0.05 | 9.6 ± 1.6 | 6.6 ± 1.6 | <0.05 | 9.9 ± 2.2 | 6.3 ± 1.5 | <0.05 |
| CD3+CD19- (%) | 72.0 ± 3.3 | 76.8 ± 3.9 | NS | 72.7 ± 3.6 | 78.5 ± 3.6 | NS | 72.6 ± 3.8 | 79.5 ± 2.4 | <0.05 |
| HLA-DR+CD4+ (%) | 4.2 ± 0.4 | 4.3 ± 0.4 | NS | 4.6 ± 0.4 | 4.3 ± 0.5 | NS | 4.3 ± 0.4 | 4.1 ± 0.6 | NS |
| HLA-DR+CD8+ (%) | 7.3 ± 1.5 | 6.4 ± 1.4 | NS | 8.3 ± 1.7 | 5.3 ± 1.0 | <0.05 | 6.9 ± 1.4 | 5.4 ± 1.6 | NS |

†, Values are the means ± SE; NS, not significant; Lymphocytes study for CD3, CD19, HLA-DR, CD4, CD8 surface markers were carried out as stated in text; §, *P* from Wilcoxon signed rank test.

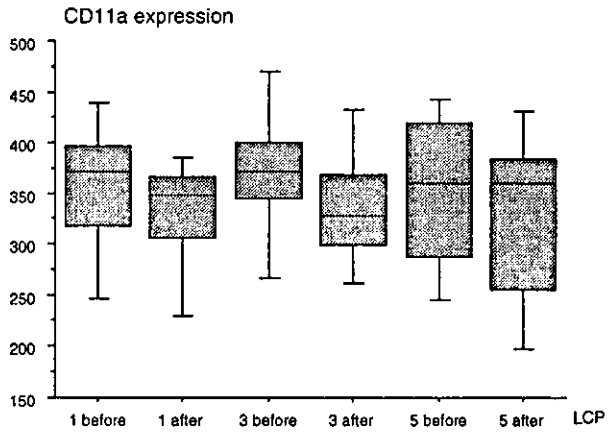


**FIG. 1.** Flow cytomety studies of the granulocyte adhesion molecule CD11a expression before and after LCP treatment. Y axis, arbitrary mean fluorescence units. Mean values and *P* values are shown in Table 7.

($P < 0.05$) (Table 7 and Fig. 1). The expression of CD11b and CD18 decreased significantly at the first, third, and fifth LCP treatment (Table 7 and Figs 2 and 3). No significant changes of these surface markers were noted during the 12 weeks of the observation period (Table 7).

## DISCUSSION

The effectiveness of the LCP therapy has been reported in the past. Ueki et al. (12) reported an ACR 20% response of 64%, and ACR 50% response of 16% when three LCP treatments were performed to 25 RA patients who were resistant to drug therapy. Hidaka et al. (13) also reported an ACR 20% response of 79%, and an ACR 50% response of 25%


**FIG. 2.** Flow cytometry studies of the granulocyte adhesion molecule CD11b expression before and after LCP treatment. Y axis, arbitrary mean fluorescence units. Mean values and *P* values are shown in Table 7.

**TABLE 6.** *Changes in leukocytes after LCP treatment*[†]

| | Weeks after the treatment | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 1 | $P$[‡] | 4 | $P$[‡] | 8 | $P$[‡] | 12 | $P$[‡] |
| WBC/μl | 8800 ± 802.3 | NS | 8588.9 ± 800.3 | NS | 8116.7 ± 747.2 | NS | 7666.7 ± 694.1 | NS |
| Granulocyte/μl | 6932.6 ± 648.9 | NS | 6837.4 ± 733.7 | NS | 6217.5 ± 761.2 | NS | 5780.7 ± 590.0 | NS |
| Lymphocyte/μl | 1281.2 ± 174.7 | NS | 1280.7 ± 156.8 | NS | 1273.2 ± 135.4 | NS | 1358.0 ± 204.6 | NS |
| CD19+CD3-(%) | 9.3 ± 1.7 | NS | 9.3 ± 1.7 | NS | 9.7 ± 2.8 | NS | 11.8 ± 3.3 | NS |
| CD3+CD19-(%) | 71.1 ± 3.6 | NS | 70.7 ± 4.0 | NS | 64.4 ± 4.9 | NS | 63.3 ± 5.5 | NS |
| HLA-DR+CD4+(%) | 4.1 ± 0.5 | NS | 5.0 ± 0.6 | NS | 4.1 ± 0.4 | NS | 3.7 ± 0.5 | NS |
| HLA-DR+CD8+(%) | 7.1 ± 1.4 | NS | 9.3 ± 2.1 | <0.05 | 9.0 ± 3.1 | NS | 9.7 ± 2.9 | NS |

[†], Values are the measn ± SE; NS, not significant; [‡], $P$ from Wilcoxon signed rank test. Two-color flow cytometry study of lymphocytes for CD3, CD19, HLA-DR, CD4, CD8 surface markers were carried out as stated in laboratory examination.

when three LCP treatments were performed to 25 RA patients who were resistant to drug therapy. In the present study, RA patients whose symptoms were not controlled adequately by methotrexate or patients who experienced adverse side-effects from methotrexate were selected as stated before. Three out of the nine selected patients were actually using methotrexate during the entire study period (Table 1). The rest of the patients stopped using methotrexate because of adverse side effects, such as abnormal liver function test and hair loss, or stopped using methotrexate because the drug was not effective. Our study of LCP therapy for these patients revealed an ACR 20% response of 77.8% and an ACR 50% response of 44.4%.

After initiation of the study, the ACR core set activity measures: swollen joint count; tender joint count; patient assessment of pain; patient assessment of global severity, physician assessment of global severity; and CRP; improved significantly except MHAQ (Table 4). The probable reason for no changes in MHAQ is that the Steinblocker stages of the nine patients were relatively high. It may mean that joint deformity was too extensive to show some improvement, or the questionnaire used in MHAQ was not adequate to reflect the improvement (14). The ACR 20% and 50% responses tended to appear after the therapy period. Improvements in symptoms appeared relatively slowly, but the effects were sustained until 12 weeks after the therapy. 85.7% of the patients (6 out of 7 patients) still had improvements in symptoms 12 weeks after the therapy. This phenomenon is one of the characteristics of this therapy. It was reported that treatment effect of the LCP therapy appeared slowly but lasted longer when compared with double filtration plasmapheresis (15).

The filter used in this study is able to remove about $3.5 \times 10^9$ neutrophils, and $2.5 \times 10^9$ lymphocytes when used in healthy volunteers (5). In the present study, leukocytes in peripheral circulation were significantly decreased (Table 5), but no adverse side effect from the therapy was observed. It implies that this amount of temporal reduction in leukocytes is not harmful to the patients. We know that leukocytes play very important roles in inflammatory processes. Yamasaki et al. (16) reported that enzyme activities of leukocytes were modulated by the LCP therapy. It is known that in the situation of inflammation, leukocytes move out from the blood circulation into tissue by using adhesion molecules on leukocytes and cells of the blood vessels (17). Since great numbers of leukocytes including granulocytes are removed by the LCP therapy, we have studied changes in the expression of the adhesion molecules CD11a, CD11b, and CD18. Flow cytometric study revealed that the expression of CD11a tended to decrease directly after the LCP treatment, and it decreased significantly at the third treatment ($P < 0.05$; Table 7

**TABLE 7.** *Changes in expression of the granulocyte adhesion molecules CD11a, CD11b, CD18 by LCP*[†]

| | LCP treatment 1 | | | LCP treatment 3 | | | LCP treatment 4 | | |
|---|---|---|---|---|---|---|---|---|---|
| | before | after | $P$[‡] | before | after | $P$[‡] | before | after | $P$[‡] |
| CD11a | 358.3 ± 23.6 | 331.3 ± 20.0 | NS | 370.5 ± 24.4 | 336.5 ± 20.7 | <0.05 | 351.4 ± 25.4 | 326.8 ± 29.7 | NS |
| CD11b | 1470.6 ± 141.3 | 1223.0 ± 147.3 | <0.01 | 1518.5 ± 163.8 | 1179.9 ± 146.5 | <0.05 | 1333.0 ± 107.4 | 1026.3 ± 106.8 | <0.01 |
| CD18 | 307.5 ± 25.0 | 259.4 ± 27.9 | <0.05 | 305.3 ± 32.1 | 242.9 ± 25.0 | <0.01 | 302.6 ± 20.5 | 234.9 ± 15.8 | <0.01 |

[†], Flow cytometric studies of the granulocyte adhesion molecules CD11a, CD11b,and CD18 expression before and after LCP treatment; NS, not significant; [‡], $P$ from Wilcoxon signed rank test. See Figures 1, 2, and 3 for graphical changes of the adhesion molecules. Values are mean ± SE of the mean arbitrary fluorescence intensity unit.

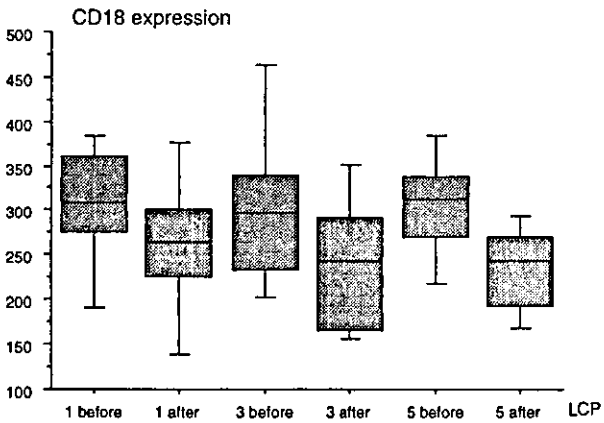


**FIG. 3.** Flow cytometry studies of the granulocyte adhesion molecule CD18 expression before and after LCP treatment. Y axis, arbitrary mean fluorescence units. Mean values and *P* values are shown in Table 7.

and Fig. 1). The expression of CD11b and CD18 decreased significantly at the first, third, and fifth LCP treatment ($P < 0.05$ Table 7, Figs 2 and 3). Stimulated granulocytes are known to produce protease and superoxide which leads to vasculitis (18). Mac-1 (CD11b/CD18) of granulocytes are involved in production of hydrogen peroxide (19). It is reported that unstimulated granulocytes exhibit CD11a/CD18 (LFA-1) dependent attachment to intercellular adhesion molecule-1 (ICAM-1), and chemotactic stimulation enhances the attachment of human granulocytes to ICAM-1 by a MAC-1-dependent process (20). Observed improvements in the joint symptoms of the RA patients may be related to decrease in expression of the adhesion molecules CD11a, CD11b, and CD18, and this phenomenon probably influenced enzyme production from the granulocytes.

Lymphocytes also play important roles in inflammation. Our study revealed a significant decrease in the numbers of lymphocytes when measured directly after the each LCP treatment (Table 6), but the CD4/CD8 ratio showed no significant changes (Table 4). HLA-DR+CD8+ (%) decreased significantly at the third LCP treatment and increased significantly 4 weeks after the therapy, but HLA-DR+CD4+ (%) did not change significantly. In order to study changes in T and B lymphocytes, CD3 and CD19 surface markers were analyzed. CD3-CD19+ (%) decreased significantly at each LCP treatment before and after the LCP session. CD3+CD19- (%) increased significantly at the fifth LCP treatment, but no significant change was observed during the rest of the period (Tables 5 and 6). Our study showed a decrease in numbers of lymphocytes and CD3-CD19+ cells in each LCP treatment. The changes in lymphocytes were temporal, as in the case of the granulocytes, but these changes probably influenced the inflammation of the joints.

In RA, MMP-3, IL-6, and CRP are related in terms of disease activity (21). In our case, MMP-3 levels significantly decreased 1 week after the therapy, and IL-6 levels significantly decreased at 8 and 12 weeks after the therapy. CRP level also significantly decreased 8 weeks after the therapy ($P < 0.05$ Table 4). Also, 8 weeks after the therapy, RF levels decreased significantly. It seems that most of the patients disease activities were under control during this period, 8 weeks after the LCP therapy.

In the present study, ESR levels did not change significantly. Hidaka et al. (13) reported the same result in their study. On the other hand, CRP levels decreased significantly at 8 weeks after the therapy, but it tended to increase at 12 weeks (Table 4). IgG levels increased significantly only at 4 weeks after the therapy. This finding may be related to the worsening symptoms of the three patients who dropped out from the study. IgA and IgM levels showed no significant changes, and Ueki et al. (12) reported similar results, that IgG, IgM, and CH50 did not change significantly with the LCP therapy.

In summary, the LCP therapy for the RA patients selected in this study revealed the ACR 20% response of 77.8% and the ACR 50% response of 44.4%. Three of the nine selected patients were using methotrexate during the entire study period. The rest of the patients stopped using methotrexate because of adverse side effects, such as abnormal liver function test and hair loss, or stopped using methotrexate because the drug was not effective. Our study showed the effectiveness of the LCP therapy for these patients. In Japan, the allowed usage of methotrexate is a maximum dose of 8 mg/week. This dosage is much less than the Unites States and Europe. It has been suggested that the maximum dosage of 8 mg/week is not sufficient in Japan. Clearly this issue needs to be considered in the treatment of RA in Japan (22). However, we often face some patients who have experienced adverse side effects from methotrexate. For these patients, LCP therapy may be one of the alternative treatment options. We have not experienced any side effects in our study, and other studies had a similar result (12,13). It is clear that this treatment is a relatively safe treatment to perform. The effect of the LCP therapy seems to last longer, as 85.7% of the patients still had improved symptoms at 12 weeks after the therapy. It is not clear, however, whether destruction of the joints is really controlled by this therapy. Radiological study of the joints may be necessary to clarify this problem.

Factors such as treatment numbers, timing, and volume of blood of one treatment also need to be studied.

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# Suppression of Collagen-Induced Arthritis by Natural Killer T Cell Activation With OCH, a Sphingosine-Truncated Analog of α-Galactosylceramide

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*Objective.* **OCH, a synthetic analog of α-galactosylceramide with a truncated sphingosine chain, stimulates natural killer T (NKT) cells to produce predominantly Th2 cytokines. Thus, OCH may be a potential agent for the treatment of Th1-mediated autoimmune diseases. This study was designed to evaluate the protective effects of OCH on collagen-induced arthritis (CIA) in mice.**

*Methods.* **Mice were immunized with type II collagen (CII) and injected intraperitoneally twice per week with OCH, before or after the onset of CIA. They were monitored to assess the effect of OCH treatment on the severity of disease. Anti-CII antibodies and cytokine production were measured by enzyme-linked immunosorbent assay. Expression of cytokine genes was determined by quantitative reverse transcriptase–polymerase chain reaction.**

*Results.* **OCH inhibited CIA in wild-type C57BL/6 (B6) mice but not in NKT-deficient mice. OCH suppressed CIA in SJL mice, which are prone to autoimmune diseases and have a deficiency in the number and function of NKT cells which is similar to that in patients with autoimmune diseases, even after disease has already developed. Disease protection conferred by OCH correlated with its ability to selectively induce Th2 cytokine production mediated by NKT cells and to promote collagen-specific Th2 responses. Neutralization of interleukin-4 (IL-4) or IL-10 with monoclonal antibodies abolished disease protection by OCH, indicating a critical role for these cytokines.**

*Conclusion.* **Taken together, our findings suggest that OCH holds possibilities as a therapeutic agent for autoimmune diseases such as rheumatoid arthritis.**

Rheumatoid arthritis (RA) is a common autoimmune disease characterized by persistent inflammation of joints resulting in progressive destruction of cartilage and bone. Although its precise etiology is not clearly understood, cumulative evidence suggests that Th1 cells secreting interferon-γ (IFNγ) and tumor necrosis factor α (TNFα) exacerbate disease, whereas Th2 cells producing interleukin-4 (IL-4) and IL-10 suppress arthritis (1). Studies with animal models have demonstrated that systemic or locally administered IL-4 and IL-10 can effectively protect against arthritis in mice (2–11).

Natural killer T (NKT) cells are a unique subset of T cells that coexpress T cell receptor α/β (TCRα/β) and receptors from the NK lineage. NKT cells express an invariant TCRα chain (encoded by a $V_\alpha14$–$J_\alpha281$ rearrangement in mice and a homologous $V_\alpha24$–$J_\alpha Q$ rearrangement in humans). Unlike conventional T cells that recognize peptides in association with major histocompatibility complex (MHC), NKT cells recognize glycolipid antigens bound to the nonpolymorphic class I MHC–like protein, CD1d. NKT cells have been implicated in a variety of immune responses such as infection and tumor immunity. One striking feature of NKT cells is their capacity to secrete a large amount of cytokines,

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including IL-4 and IFNγ, in response to TCR stimulation (12–14). Recently, a number of reports have indicated that NKT cells play a critical role in the regulation of autoimmune responses. Abnormalities in the numbers and functions of NKT cells have been observed in patients with autoimmune diseases (15–18) as well as in a variety of mouse strains that are genetically predisposed to the development of autoimmune diseases (19–23).

While the natural ligand for NKT cells remains to be determined, α-galactosylceramide (α-GC) (Figure 1A), a derivative of marine sponge, has been shown to bind to CD1d and strongly stimulate NKT cells to produce IFNγ and IL-4, both in humans and in mice (24–26). Previously, we have shown that OCH (Figure 1A), an analog of α-GC with a truncated sphingosine chain, efficiently inhibits induced experimental autoimmune encephalomyelitis (EAE) in C57BL/6 (B6) mice, due to its ability to stimulate NKT cells to selectively produce Th2 cytokines; in contrast, α-GC had little effect on EAE (27,28).

In the present study, we found that OCH inhibits collagen-induced arthritis (CIA), a murine experimental model for RA, in wild-type B6 but not NKT-deficient $J_{\alpha}281$-knockout mice. We also demonstrated that OCH inhibits CIA in SJL mice even after arthritis has already developed. Experiments with anti-IL-4 or anti-IL-10 administration revealed that IL-4 and IL-10 are critical for OCH-mediated suppression of CIA. These results suggest that stimulation of NKT cells with OCH could be an attractive means of intervention in autoimmune diseases such as RA.

## MATERIALS AND METHODS

**Mice.** B6 mice were purchased from Clea Laboratory Animal Corp. (Tokyo, Japan). SJL mice were obtained from Charles River Japan (Yokohama, Japan). $J_{\alpha}281$-knockout mice were kindly provided by Dr. Masaru Taniguchi (Chiba University Graduate School of Medicine, Chiba, Japan). The animals were kept under specific pathogen-free conditions and studied at 7–10 weeks of age.

**Induction of CIA.** Mice were immunized intradermally at the base of the tail with 100 μg of either chicken type II collagen (CII) (for B6 mice) or bovine CII (for SJL mice) (Collagen Research Center, Tokyo, Japan) emulsified with an equal volume of Freund's complete adjuvant (CFA), containing 250 μg of H37RA *Mycobacterium tuberculosis* (Difco, Detroit, MI). The animals were boosted by intradermal injection with the same antigen preparation on day 21. Mice were examined for signs of joint inflammation 3 times per week, and joint involvement was scored as follows: 0 = no change, 1 = focal redness of the limb or swelling and redness of 1 digit, 2 = mild swelling and erythema of the limb or swelling of >2 digits, 3 = marked swelling and erythema of the limb, 4 = maximal

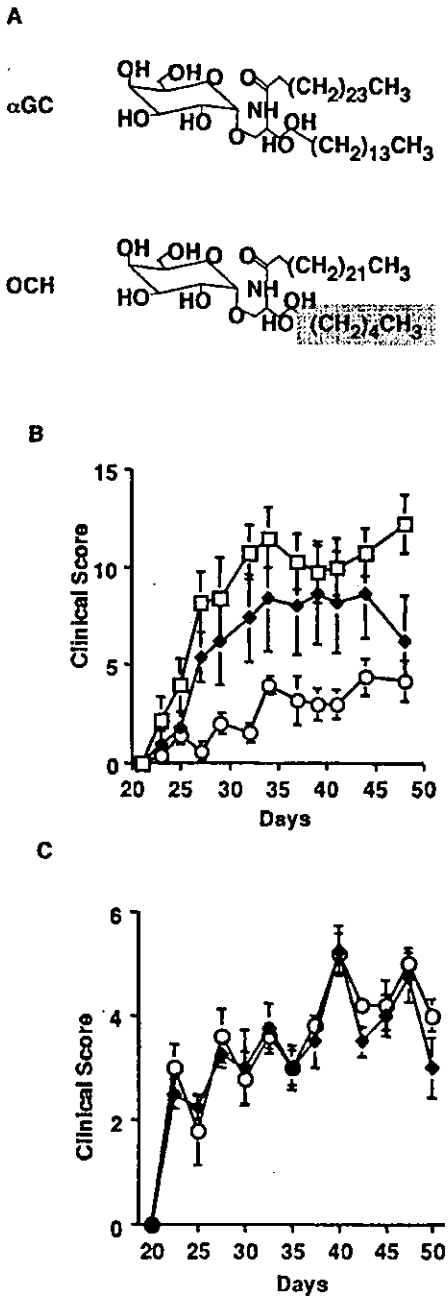


**Figure 1.** **A,** Structure of α-galactosylceramide (α-GC) and OCH, a sphingosine-truncated analog of α-GC. **B** and **C,** Effect of OCH on collagen-induced arthritis (CIA) in C57BL/6 (B6) and $J_{\alpha}281$-knockout mice. **B,** Clinical score of CIA in B6 mice treated with 500 μg/kg of α-GC (◆), OCH (○), or vehicle (□) twice per week starting from day 21. **C,** Clinical score of CIA in $J_{\alpha}281$-knockout mice treated with 500 μg/kg of OCH (○) or vehicle (◆) twice per week starting from day 21. Data shown are from a single experiment representative of 2 identical experiments; values are the mean ± SEM (5 mice per group).

swelling and redness of the limb and later, ankylosis. The average of the macroscopic score was expressed as a cumulative value for all paws, with a maximum possible score of 16 per mouse. The in vivo experiments were performed with 10 mice per group and repeated twice to ensure reproducibility.

**In vivo glycolipids treatment and antibody treatment.** Synthetic glycolipids were used to treat CIA. Starting from the indicated day, mice were injected intraperitoneally twice per week with either OCH or α-GC at a dose of 500 μg/kg. The control mice were injected with vehicle alone (10% DMSO in phosphate buffered saline [PBS]). To neutralize IL-4 or IL-10, anti–IL-4 monoclonal antibody (mAb) (11B11) or anti–IL-10 mAb (JES-2A5) (500 μg per mouse) was injected intraperitoneally 2 hours before glycolipid administration.

**Histopathologic study.** Forepaws were removed from mice killed 50 days after the first immunization of CII, then fixed in buffered formalin, decalcified, embedded in paraffin, sectioned, and stained with hematoxylin and eosin for histopathologic analysis.

**NKT cell preparation.** NK1.1-positive T cells were purified from the liver of 6–8-week-old B6 mice. Liver mononuclear cells were prepared by Percoll density-gradient centrifugation. Prepared cells were then incubated with phycoerythrin-conjugated NK1.1 mAb and fluorescein isothiocyanate–conjugated CD3 mAb (BD PharMingen, San Jose, CA). The stained cells were sorted into $NK1.1^+$,$CD3^+$ cells. The purity of the sorted cells was >95%.

**Quantitative reverse transcriptase–polymerase chain reaction (RT-PCR).** Total RNA was extracted with an RNeasy kit (Qiagen KK, Tokyo, Japan) from purified $NK1.1^+$ T cells. Random hexamer-primed complementary DNA was prepared with the First-Strand cDNA Synthesis Kit (Invitrogen, Carlsbad, CA). For quantitative analysis of cytokines, we used the LightCycler quantitative PCR system (Roche Molecular Biochemicals, Mannheim, Germany) and performed quantitative PCR with a commercial kit (LightCycler-DNA Master SYBR Green I; Roche Molecular Biochemicals). The PCR amplification was repeated 40 times (for 15 seconds at 95°C, 5 seconds at 60°C, and 10 seconds at 72°C). All PCR reactions were normalized by GAPDH expression.

**Enzyme-linked immunosorbent assay (ELISA).** To detect CII-specific IgG1 and IgG2a, chicken CII or bovine CII (5 μg/ml) was coated onto ELISA plates (Sumitomo Bakelite, Tokyo, Japan) at 4°C overnight. After blocking with 3% bovine serum albumin in PBS, serially diluted serum samples were added onto CII-coated wells. The plates were incubated with biotin-labeled anti-IgG1 and anti-IgG2a (Southern Biotechnology, Birmingham, AL) or anti-IgG antibody (CN/Cappel, Aurora, OH) for 1 hour and then incubated with streptavidin–peroxidase. After addition of substrate, the reaction was evaluated. The levels of IL-4, IL-10, and IFNγ in serum were measured by standard sandwich ELISA, using purified and biotinylated mAb pairs and standards (BD PharMingen).

## RESULTS

**Suppression of CIA development by OCH.** In order to determine whether stimulation of NKT cells modulates arthritis, we first examined the effect of α-GC, a prototypic ligand for NKT cells, on the development of CIA. Early studies showed that CIA is restricted to mouse strains bearing the $H\text{-}2^q$, $H\text{-}2^r$, or $H\text{-}2^s$ haplotype (29) and is generally induced in DBA/1 mice. More recently, modified immunization conditions that are sufficient to induce CIA in B6 mice have been developed; this allowed us to study CIA in knockout mice with a B6 background, which eliminated the need to backcross the knockout mice onto a DBA/1 background (30,31). The histologic and immunologic characteristics of the disease induced in B6 mice have been shown to be similar to those in DBA/1 mice, even though B6 mice have a slightly delayed onset and less uniformly severe disease (31). We immunized B6 mice with chicken CII in CFA to elicit CIA as described previously (30) and then injected mice intraperitoneally with either α-GC or vehicle alone twice per week starting from the day of the second immunization. As shown in Figure 1B, α-GC treatment did not improve the arthritis score significantly. We next examined the effect of OCH on CIA. The mean maximum clinical CIA score was profoundly reduced in OCH-treated B6 mice (Figure 1B and Table 1). The incidence and the time of onset of disease were not significantly different between the OCH-treated group and the control group.

**Table 1.** Clinical scores of collagen-induced arthritis in C57BL/6 and $J_\alpha281$-knockout mice*

| Mice, treatment | Incidence, % | Maximum score, mean ± SEM | Days to onset, mean ± SEM |
|---|---|---|---|
| C57BL/6 (wild-type) | | | |
| Vehicle | 100 | 13.0 ± 0.83 | 23.8 ± 0.48 |
| α-GC | 100 | 9.8 ± 2.37 | 24.2 ± 0.48 |
| OCH | 100 | 4.6 ± 0.92† | 24.2 ± 0.48 |
| $J_\alpha281$-knockout | | | |
| Vehicle | 100 | 5.5 ± 0.5 | 24 ± 0.58 |
| OCH | 100 | 5.75 ± 0.25 | 23.5 ± 0.5 |

* C57BL/6 mice or $J_\alpha281$-knockout mice were sensitized with chicken type II collagen for induction of arthritis.
Vehicle or 500 μg/kg of α-galactosylceramide (α-GC) or OCH was injected intraperitoneally twice per week from day 21. Data are from 5 mice per group.
† $P < 0.05$ versus control vehicle, by Mann-Whitney U test.

To investigate the role of $V_\alpha14$ NKT cells in the suppression of CIA by OCH, we examined the ability of OCH to modulate disease in $J_\alpha281$-knockout mice, in which $V_\alpha14$ NKT cells are absent (32). Administration of OCH did not modulate the clinical course of CIA in $J_\alpha281$-knockout mice compared with mice treated with vehicle alone (Figure 1C and Table 1). These results indicate that OCH-mediated suppression of CIA requires NKT cells.

In addition to visual scoring, on day 50 after

disease induction we analyzed the histologic features in the joints of forepaws of wild-type B6 mice treated with OCH or α-GC. As shown in Figure 2, in the control and α-GC–treated groups there was severe arthritis in the joints, associated with massive cell infiltration, cartilage erosion, and bone destruction. These histologic features were significantly less apparent in the group of mice treated with OCH. These results demonstrated that administration of OCH ameliorated CIA, whereas α-GC had little effect on CIA in B6 mice.

**Selective induction by OCH of NKT cell–mediated IL-4 and IL-10 production.** Activation of NKT cells leads to the rapid production of a variety of cytokines, including IL-4, which promotes Th2 differentiation, and IFNγ, which promotes Th1 differentiation. Previously we demonstrated that OCH stimulates NKT cells to produce predominantly IL-4, whereas α-GC stimulates NKT cells to produce both IL-4 and IFNγ (27). IL-10, as well as IL-4, has also been reported to suppress CIA (1) and to be involved in α-GC–mediated inhibition of diabetes in the NOD mouse (33). These data led us to examine whether IL-10 was induced by OCH stimulation. We measured serum levels of IL-10 in addition to IL-4 and IFNγ, 2 hours and 12 hours after intraperitoneal injection of either OCH or α-GC into B6 mice. As shown in Figure 3A, OCH, as well as α-GC, caused an elevation in IL-10 levels. Consistent with previous results, OCH injection induced a rapid rise in IL-4 levels along with a much less marked increase in levels of IFNγ. Injection of α-GC induced the production of IL-4 and IFNγ (Figure 3A). NKT cell–deficient $J_{\alpha}281$-knockout mice did not exhibit a response to either α-GC or OCH (Figure 3A), indicating that the increase in cytokine levels in B6 mice was mediated by NKT cells.

To further clarify the difference in gene expression by NKT cells stimulated with OCH or α-GC, we performed quantitative RT-PCR to detect the levels of expression of major contributors to joint destruction, such as TNFα and receptor activator of NF-κB ligand (RANKL) in stimulated NKT cells in vivo. We sorted $NK1.1^+$ T cells from liver mononuclear cells of B6 mice 1.5 hours after administration of either OCH or α-GC. As shown in Figure 3B, α-GC stimulation induced expression of TNFα and RANKL genes. In contrast, OCH stimulation induced much lower levels of TNFα and RANKL expression.

**Efficient inhibition by OCH of CIA development in SJL mice.** In a screen of laboratory mouse strains, NKT cells in SJL mice, which exhibit a marked propensity to the Th1-mediated autoimmune diseases, were

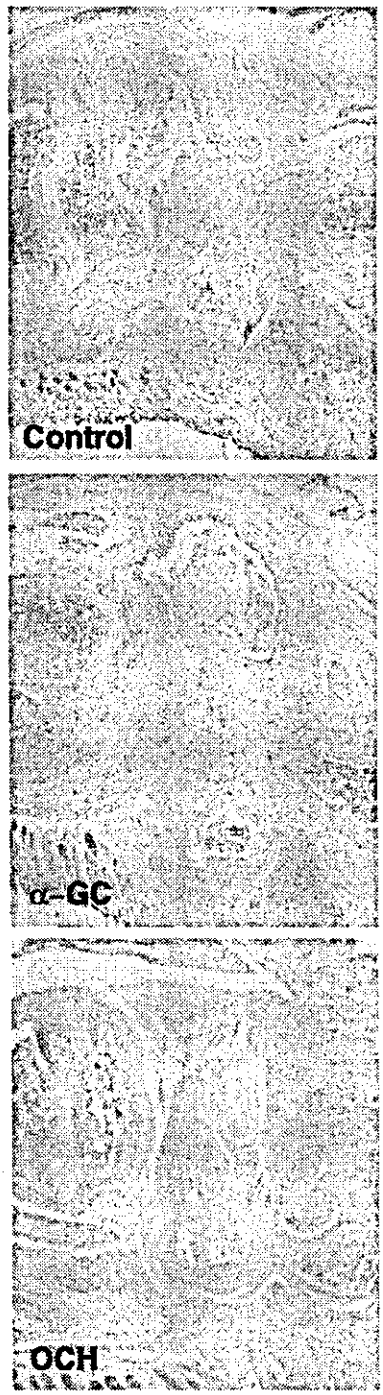


**Figure 2.** Histopathologic assessment of arthritic wrist joints of mice treated with control vehicle, α-galactosylceramide (α-GC), or OCH (hematoxylin and eosin stained; original magnification × 20).

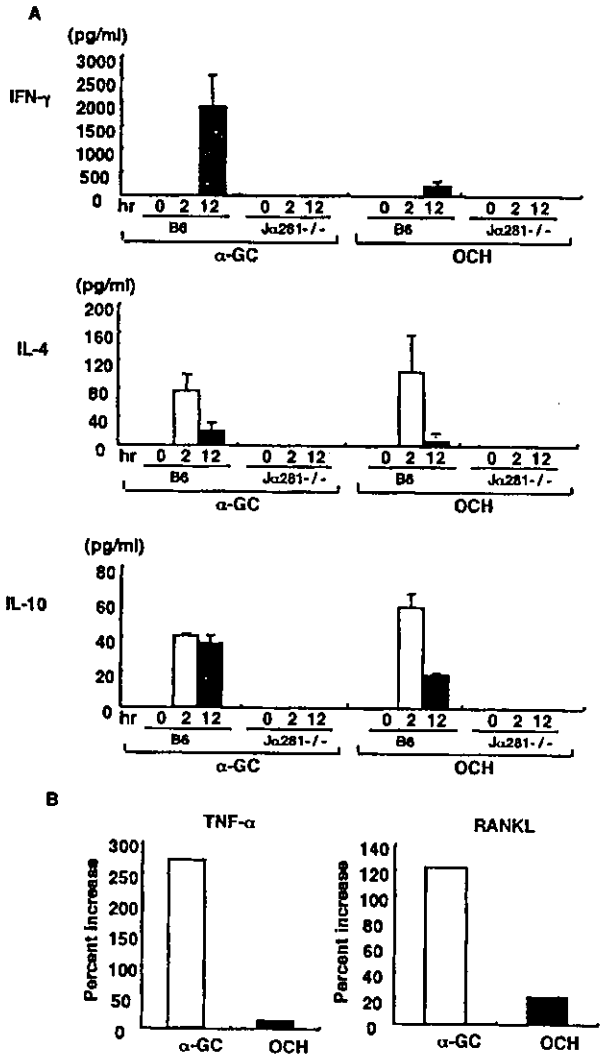


**Figure 3.** A, Change in serum cytokine levels after injection of OCH or α-GC in wild-type B6 and $J_{\alpha}281$-knockout mice. Serum levels of interferon-γ (IFNγ), interleukin-4 (IL-4), and IL-10 in B6 mice or $J_{\alpha}281$-knockout mice 2 hours (open bars) and 12 hours (solid bars) after intraperitoneal injection of OCH or α-GC were measured by enzyme-linked immunosorbent assay. B, Quantitative analysis of levels of tumor necrosis factor α (TNFα) and receptor activator of NF-κB ligand (RANKL) by reverse transcriptase–polymerase chain reaction. Total RNA was isolated from $NK1.1^+$ T cells of OCH- or α-GC–treated mice, and the percent increase was calculated based on the expression of each gene obtained in cells of vehicle-treated mice. Values are the mean and SEM (4 mice per group). See Figure 1 for other definitions.

found to be reduced in number and to have a profound defect in IL-4 secretion (19–23). Furthermore, recent studies of human autoimmune diseases demonstrated that patients with these diseases exhibited a decreased frequency of NKT cells in the periphery (15–18). These data led us to investigate whether OCH could ameliorate CIA in the autoimmune-prone mice with reduced numbers of and functional defects in NKT cells. Administration of OCH in SJL mice resulted in a rapid appearance of IL-4 and IL-10, although the levels of these cytokines were lower than those in B6 mice (data not shown). In contrast, IFNγ was barely detectable in the serum of SJL mice treated with OCH (data not shown), indicating that the cytokine profile induced by OCH stimulation was similar to that seen in B6 mice.

Next, we immunized SJL mice with bovine CII to induce CIA and then treated the mice with either OCH, α-GC, or vehicle alone. As shown in Figure 4A and Table 2, OCH administration efficiently inhibited the clinical course of CIA, whereas α-GC treatment had little effect on CIA in SJL mice. To examine the

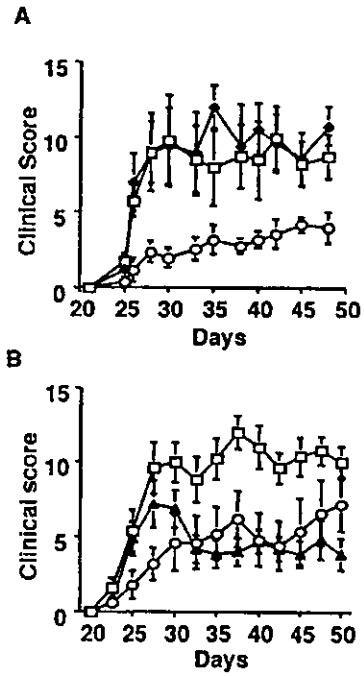


**Figure 4.** Effect of OCH on CIA in SJL mice. A, Clinical score of CIA in mice treated with 500 μg/kg of α-GC (◆), OCH (○), or vehicle (□) twice per week starting from day 21. B, Clinical score of CIA in mice treated with 500 μg/kg of vehicle starting from day 21 (□) or with OCH twice per week starting from day 21(○) or day 28 (▲). Data shown are from a single experiment representative of 2 identical experiments; values are the mean ± SEM (6 mice per group). See Figure 1 for definitions.

Table 2. Clinical scores of collagen-induced arthritis in SJL mice*

| Time of injection, treatment | Incidence, % | Maximum score, mean ± SEM | Days to onset, mean ± SEM |
|---|---|---|---|
| Day 21 after immunization | | | |
| Vehicle | 100 | 11.0 ± 2.35 | 24.0 ± 0.00 |
| α-GC | 100 | 12.4 ± 1.50 | 24.4 ± 0.40 |
| OCH | 100 | 5.2 ± 0.58† | 28.8 ± 2.42 |
| Day 21 or 28 after immunization | | | |
| Vehicle, day 21 | 100 | 12.6 ± 1.03 | 23.4 ± 0.40 |
| OCH, day 21 | 100 | 7.0 ± 2.17† | 26.8 ± 2.84 |
| OCH, day 28 | 100 | 7.8 ± 1.35† | 24.0 ± 0.45 |

* Mice were sensitized with chicken type II collagen for induction of arthritis. Vehicle or 500 μg/kg of α-galactosylceramide (α-GC) or OCH was injected intraperitoneally twice per week. Data are from 6 mice per group.
† $P < 0.05$ versus control vehicle, by Mann-Whitney U test.

potential therapeutic effect of OCH on established CIA, we injected OCH beginning on day 28 after the first immunization, when arthritis had already developed (Figure 4B and Table 2). The severity of arthritis gradually decreased after OCH treatment, and the disease was efficiently suppressed within 1 week. These results suggest that OCH has a therapeutic effect on established CIA in autoimmune-prone mice.

**Promotion of CII-specific Th2 responses by OCH.** OCH has been demonstrated to alter the cytokine profile of autoantigen-specific T cells in vivo (27). We therefore speculated that the OCH-mediated inhibition of arthritis might be due to a modulation of the Th1/Th2 balance, resulting from a Th2 bias of CII-reactive T cells. To explore this possibility, we measured CII-specific immunoglobulin isotype levels 50 days after induction of CIA. It is generally accepted that elevation of antigen-specific IgG2a antibody results from augmentation of a Th1 immune response to the antigen, whereas a higher level of IgG1 antibody would reflect a stronger Th2 response to the antigen. In OCH-treated mice there was a greater reduction in the level of IgG2a antibody specific to CII versus IgG1 specific to CII (Figure 5A). Consequently, the IgG1:IgG2a ratio was elevated in mice treated with OCH (Figure 5B), indicating that the suppression of CIA by OCH is associated with a Th2 bias of CII-reactive T cells.

**Critical role of IL-4 and IL-10 in OCH-mediated suppression of CIA.** To confirm the involvement of IL-4 and IL-10 in the suppression of CIA, we next examined whether the inhibitory effect of OCH was abrogated after neutralization of IL-4 or IL-10 in vivo. Groups of SJL mice were injected with anti–IL-4 or anti–IL-10 mAb 2 hours before OCH or vehicle was administered. OCH-mediated suppression of CIA was partially abolished when anti–IL-4 mAb was injected (Table 3). More remarkably, in the presence of anti–IL-10 mAb, the protective effect of OCH against CIA was no longer evident at all (Table 3). Injection of neutralizing antibody to either IL-10 or IL-4 reversed the beneficial effect of administration of OCH in B6 mice also (data not shown). These results imply that IL-4 and IL-10 are critical in the OCH-mediated suppression of CIA and are consistent with our hypothesis that OCH modulates CIA by stimulating production of Th2 cytokines by NKT cells.

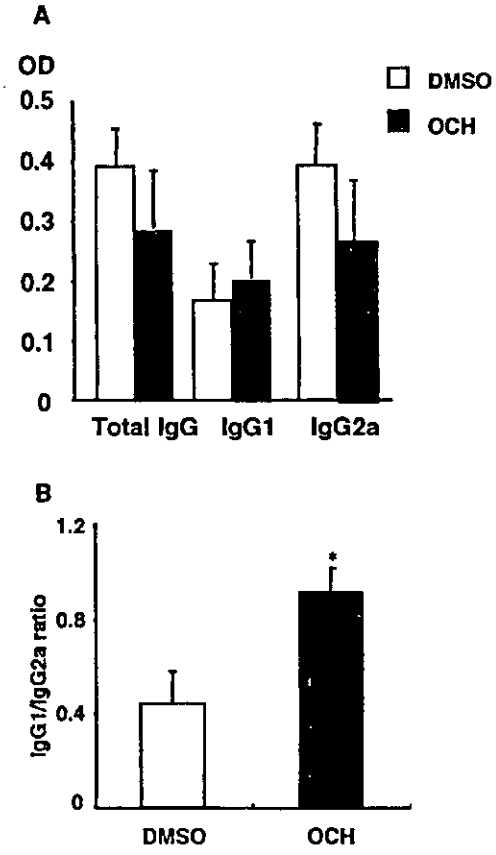


Figure 5. Effect of OCH on type II collagen (CII)–specific responses. Individual serum samples obtained on day 50 after induction of arthritis were analyzed as indicated in Materials and Methods. A, CII-specific antibody responses in OCH- or control vehicle (DMSO)–treated mice. B, IgG1:IgG2a ratio in OCH- or vehicle-treated mice. Values are the mean and SEM (n = 5). * = $P < 0.05$ versus control, by Mann-Whitney U test. OD = optical density.

Table 3. Abrogation of the ability of OCH to suppress collagen-induced arthritis after neutralization of IL-10 or IL-4 in vivo*

| Treatment | Incidence, % | Maximum score, mean ± SEM | Days to onset, mean ± SEM |
|---|---|---|---|
| Control IgG | | | |
| Vehicle | 100 | 12.8 ± 1.85 | 28.4 ± 0.60 |
| OCH | 90 | 5.0 ± 2.00† | 29.0 ± 0.00 |
| Anti–IL-10 mAb | | | |
| Vehicle | 100 | 10.5 ± 1.86 | 29.0 ± 0.00 |
| OCH | 100 | 11.4 ± 1.44 | 28.4 ± 0.60 |
| Anti–IL-4 mAb | | | |
| Vehicle | 100 | 9.75 ± 2.84 | 27.5 ± 0.87 |
| OCH | 100 | 7.0 ± 0.45 | 27.8 ± 0.73 |

* SJL mice were sensitized with bovine type II collagen for induction of arthritis. Vehicle or 500 μg/kg of OCH was injected intraperitoneally twice per week from day 21. Anti–interleukin-10 (anti–IL-10) or anti–IL-4 monoclonal antibody (mAb) (500 μg per mouse) was injected each time vehicle or OCH was administered. Data are from 5 mice per group.
† $P < 0.05$ versus control vehicle, by Mann-Whitney U test.

## DISCUSSION

A number of studies have shown that treatment with Th2-promoting cytokines or with monoclonal antibodies directed against Th1-promoting cytokines can effectively protect against the development of CIA in mice (1–11). Here we have demonstrated that specific activation of NKT cells with their ligand OCH provides an alternative way to shift the balance from a pathogenic Th1 response toward a protective Th2 response and that disease protection is dependent on NKT cells.

We also identified a critical role of the Th2 cytokines IL-4 and IL-10 in the ability of OCH to confer protection against CIA. Recently, local delivery of Th2 cytokines, using hybridomas (8) or dendritic cells (10,11) transfected with either IL-4 or IL-10, was found to be effective in the prevention of arthritis in animal models. In light of the fact that NKT cells are known to rapidly invade and accumulate in inflammatory lesions in a manner similar to inflammatory cells (34), stimulation of NKT cells to selectively induce Th2 cytokines might be a powerful strategy to deliver these cytokines to inflammatory lesions. It has been shown in vivo that neutralizing of IL-10, but not IL-4, increases the severity of CIA in DBA mice (3). However, we did not observe worsening of the clinical course of arthritis by neutralizing IL-4 or IL-10 in SJL mice in this study. Also in B6 mice, the clinical course of arthritis was not worsened when we neutralized IL-4 or IL-10 using the same mAb. Although the precise reason for the discrepancy with results of the earlier study is not clear, it may be because IL-4 and IL-10 levels were not high enough to modulate the severity of the disease in the natural course of arthritis in these strains.

The maximum score of CIA in the $J_\alpha 281$-knockout mouse was relatively low compared with that observed in wild-type B6 mice, suggesting that NKT cells may act as a modifier of the inflammation in the natural course of arthritis. We also observed a lower maximum score of CIA in CD1d-knockout mice (data not shown). Although the CD1d-knockout mice were backcrossed to B6 mice for only 6 generations, this observation further supports the notion that NKT cells increase the inflammation in the natural course of CIA. In contrast, the NOD and SJL strains of mice, which exhibit a marked propensity to the Th1-mediated autoimmune diseases, were found to have reduced numbers of NKT cells. Increasing the number of NKT cells in NOD mice by either transfer or transgenic expression of the invariant TCR $V_\alpha$ chain ($V_\alpha 14$–$J_\alpha 281$) resulted in a decrease in insulitis and diabetes, suggesting that NKT cells play a protective role in the development of diabetes (35,36). In these strains of mice, the defect of NKT cells may contribute to disease susceptibility. Identification of a natural antigen for NKT cells would provide further insight into the precise role of NKT cells in the pathogenesis of autoimmune diseases such as arthritis.

Alpha-galactosylceramide, a prototypic ligand for NKT cells, has been reported to prevent diabetes in NOD mice (22,33,37). Even though we confirmed that α-GC inhibited the development of diabetes in NOD mice (data not shown), we did not observe any inhibitory effect of α-GC on CIA. In a previous study, we demonstrated the inhibitory effect of α-GC on EAE induced in IFNγ-knockout mice but not in wild-type B6 mice (28), suggesting that α-GC is not effective in B6 mice because NKT cell–derived IFNγ would mask the therapeutic effect of the IL-4 simultaneously produced by NKT cells. In fact, the serum levels of IL-4 and IL-10 after administration of OCH or α-GC were similar, whereas IFNγ production was much lower after injection of OCH compared with α-GC injection. This suggests that the balance of Th1/Th2 cytokines produced by NKT cells is important with regard to the protection against Th1-mediated disease conferred by the glycolipid ligand. Thus, OCH is a unique ligand that is beneficial in the treatment of a wide variety of Th1-mediated autoimmune diseases.

Another advantage of using OCH rather than α-GC is the reduced production of factors that are harmful in arthritis, such as TNFα and RANKL. TNFα is one of the major contributors to joint inflammation and destruction (38). It induces the production of other