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ORIGINAL ARTICLE

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Effect of G-1 column (Adacolumn) therapy in rats with adjuvant arthritis on the migration and immunoreactivity of peripheral and splenic leukocytes

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Abstract The G-1 column (Adacolumn), a novel extracorporeal adsorption device, is now available for the treatment of such chronic inflammatory diseases as ulcerative colitis and rheumatoid arthritis. G-1 column treatment sometimes results in a rapid decrease in clinical inflammatory parameters and/or has a delayed beneficial effect on disease activity. In order to identify the scientific basis for such clinical benefits, we studied rats with adjuvant arthritis induced by immunization with *Mycobacterium butyricum* antigen. The potential role of G-1 column treatment on the migratory properties and immunoreactivities of leukocytes was investigated. Treatment of arthritic rats for 60 min with an extracorporeal perfusion through the G-1 column led to the adsorption of a small proportion (20%) of circulating granulocytes and monocytes. However, after G-1 treatment, the migration of radiolabeled blood granulocytes and monocytes to sites of acute dermal inflammatory reactions decreased significantly, in the case of granulocytes, almost by half. The migration of granulocytes to the inflamed hindpaws of severely affected animals was diminished in the G-1 treated group. Granulocytes that have passed through the G-1 column may stay in the bloodstream because of their markedly diminished number of adhesion molecules. A slightly increased accumulation in the liver and a decreased localization in the lung was also observed. These results may be relevant to the rapid clinical anti-inflammatory effect observed in rheumatoid arthritis and possibly also in ulcerative colitis, without any pulmonary complications. In contrast, the adsorption rate by the G-1 column of T lymphocytes was very low, and their migration pattern to sites of dermal inflammatory reactions was not altered after treatment. However, the antigen (*Mycobacterium* purified protein derivative) reactivity of T lymphocytes in blood was almost completely abolished after G-1 column treatment of arthritic rats. This unexpected qualitative effect on T lymphocytes of G-1 treatment warrants further detailed study.

Key words Adjuvant arthritis · Antigen-specific T lymphocyte · Cytapheresis · G-1 column · Leukocyte migration

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Introduction

The G-1 column[1] (Adacolumn), a novel therapeutic apheretic device, is a cylindrical column packed with 2-mm-diameter cellulose diacetate beads. The G-1 column was designed to adsorb adherent populations of leukocytes from the bloodstream. In fact, it has been demonstrated that apheretic therapy with the G-1 column results in marked clinical improvement in patients suffering from various intractable inflammatory diseases, such as ulcerative colitis[2–4] and rheumatoid arthritis[5–14] and even acquired immune deficiency syndrome (AIDS).[15] In animal models, G-1 column treatment decreased the severity of adjuvant arthritis in rats[12–14] and in a rabbit model for Glynn's arthritis.[10] These studies showed that some blood leukocytes were adsorbed onto the column, and chemical modulation of cellular and plasma components of blood also occurred on the G-1 beads in the column. Transient activation of the hypothalamic–pituitary–adrenal axis was also observed.[14] However, these results obtained from animal models so far do not explain the dramatic clinical effects seen in human ulcerative colitis and rheumatoid arthritis.

We hypothesized that the leukocytes that pass through the G-1 column are altered in their capacity to migrate to an inflammatory site and/or have altered immunological

reactivities. To test this hypothesis, we conducted a series of studies in rats with adjuvant arthritis, a model of human chronic arthritis.

Materials and methods

G-1 column

The G-1 beads, that is, 2-mm-diameter surface-treated cellulose diacetate beads, were a gift from Japan Immunoresearch Laboratories (JIMRO, Takasaki, Japan) and were used as follows. One gram of G-1 beads (Lot AS-4-1) in saline was autoclaved for 15 min, packed aseptically into individual disposable plastic syringes (Terumo, Tokyo, Japan), and stored at 4°C until use in G-1 columns for rat experiments. Before use, each column was flushed with 10 ml of saline containing 5 U/ml heparin.

Adjuvant arthritis in rat

For the migration studies, male Lewis rats, 170–230 g each (Harlan-Sprague Dawley, Indianapolis, IN, USA), were immunized by subcutaneous injection into two sites at the base of their tails of 0.5 mg heat-killed, powdered *Mycobacterium butyricum* (Mb; Difco Labs, Detroit, MI, USA) suspended in 0.05 ml mineral oil, referred to as adjuvant. The migration studies were performed at the peak of clinical signs of arthritis, around 14–15 days after immunization either as a donor or a recipient of cells.

In the immunoreactivity studies, 7-week-old female Lewis rats (Charles River Japan, Yokohama, Japan) were immunized with 0.05 mg Mb suspended in 0.05 ml liquid paraffin by injection into the plantar surface of the right hindpaw. In both studies, the arthritic activity was measured by the weight of the hindpaw of arthritic rats.[16]

Isolation and radiolabeling of leukocytes

Granulocytes and monocytes

Granulocytes and monocytes were isolated from peripheral blood and radiolabeled as previously described in detail.[17,18] Briefly, blood was collected from arthritic rats into acid-citrate-dextrose anticoagulant by exchange transfusion using hydroxyethyl starch (Hespan; Dupont Merck, Wilmington, DE, USA). The leukocyte-rich plasma obtained by sedimentation (at 1 *g*) of erythrocytes was layered on discontinuous 63% and 74% autologous plasma (10%)-Percoll (Pharmacia Fine Chemicals, Dorval, Canada) gradients. After centrifugation (350 *g*, 30 min., 22°C), granulocytes (synonym for polymorphonuclear leukocytes and essentially all neutrophils) of >95% purity were harvested from the interface between 63% and 74% Percoll layers, washed with Ca^{2+}/Mg^{2+}-free Tyrodes solution (T^{--}) and labeled with ^{111}In in T^{--} with 15 μCi of ^{111}In-oxine (Amersham, Oakville, Ontario, Canada) at room temperature for 10 min.

Mononuclear cells (MNCs) on top of the 63% Percoll layer were collected, made slightly hypertonic by gradual addition of 9% NaCl, and layered onto a discontinuous hypertonic Percoll gradient (40%/55%/58%). After centrifugation (350 *g*, 30 min), monocytes of >85% purity were harvested from both the 40%/55% and 55%/58% interfaces and radiolabeled with 75 μCi of $Na_2{}^{51}CrO_4$/ml of T^{--} 5% plasma (Amersham) at 37°C for 30 min.

Splenic T lymphocytes

In previous experiments,[19] we observed that the T lymphocytes from spleen and blood had similar migratory properties. Therefore, splenic T lymphocytes (SPTs) were used in the following experiments. SPTs were isolated by mincing the spleen with scissors, washing the minced spleen, and lysing the red cells with NH_4Cl (0.84%, 3 min, 37°C). T lymphocytes were purified by passage through nylon–wool columns, as previously described.[18] SPTs were confirmed >95% viable by trypan blue dye exclusion, and were radiolabeled with 50 μCi of $Na_2{}^{51}CrO_4$/ml of RPMI 1640 with 5% fetal calf serum (FCS) at 37°C for 45 min.

Preparation of G-1 treated labeled leukocytes for migration studies

For granulocyte and monocyte migration studies, ^{111}In-labeled granulocytes and ^{51}Cr-labeled monocytes were added to heparinized (5 U/ml) blood (5×10^6 radiolabeled cells/ml of blood) obtained from an arthritic rat. A 3-ml aliquot of blood containing radiolabeled cells was circulated through a G-1 column in a closed circuit for 60 min at 37°C with a peristaltic pump at 0.16–0.17 ml/min. At the end of the treatment, all remaining blood in the circuit was washed out with heparinized saline (5 U/ml) and collected. Sham treatment of the same amount of blood was carried out in a closed circuit without the G-1 column, and this blood sample was used as the control.

For T-lymphocyte migration studies, ^{51}Cr-labeled SPTs were suspended in heparinized blood [$(1–2) \times 10^7$ cells/ml] and circulated through the G-1 column or sham circuit as described for the granulocyte and monocyte migration studies.

Migration of radiolabeled leukocytes to various inflammatory sites

Granulocytes and monocytes

A 1-ml aliquot of blood containing ^{111}In-labeled granulocytes [$(2–4) \times 10^5$ cpm] and ^{51}Cr-labeled monocytes [$(1–2) \times 10^5$ cpm] was G-1 or sham treated and transfused intravenously into an arthritic rat under halothane anesthesia. Immediately after the transfusion, skin inflammatory reactions were induced on the shaved back of the rat by the intradermal injection of 0.05 ml of RPMI 1640 with 0.5% human serum albumin (HSA, pyrogen-free; Canadian Blood Services) containing one of the following:

1. 1 ng *Escherichia coli* 0111 endotoxin (LPS; List Biologicals, Campbell, CA, USA)
2. 25% zymosan-activated serum (ZAS: containing $C5a_{desArg}$, generated as described previously[18]
3. 10 ng recombinant rat TNF-α (rTNFα; Peprotech, Mississauga, Ontario, Canada)
4. Diluent only

The transfused blood containing labeled granulocytes and monocytes was allowed to circulate for 2 h, and the animals were then euthanized. As was described previously,[16] blood was collected by cardiac puncture into acid-citrate-dextrose anticoagulant, and 12-mm-diameter skin samples were punched out at the injection sites. The spleen and samples of both liver and right lung were also removed and weighed. The hindpaw on both sides, including the talar and metatarsophalangeal joints, were resected. Radioactivity in these specimens was measured with an LKB 1280-2 spectrometer set for ^{111}In and ^{51}Cr dual-label detection. Migration of granulocytes and monocytes into the organs (spleen, liver, and lung) was expressed as cpm/mg tissue weight, and that into joints and skin as cpm/tissue.

Splenic T lymphocytes

A 1-ml aliquot of blood, which contained ^{51}Cr-labeled SPTs [$(1.6–1.8) \times 10^5$ cpm], was treated with the G-1 column or sham treated and injected intravenously into arthritic rats. Immediately after this transfusion, various inflammatory reactions were induced in the skin of the back by injection of one of the following in 0.05 ml of diluent (RPMI-1640 with 0.5% HSA):

1. 100 ng LPS
2. 20 μg heat-killed Mb
3. A mixture of 300 U of rat recombinant interferon (IFN)-γ (a gift from Dr. Peter van der Meide, TNO Primate Center, Rijswik, The Netherlands) and 10 ng of rTNFα
4. 200 ng polyinosine-cytosine (polyI:C; Sigma, St Louis, MO, USA)
5. Diluent only

Labeled SPTs were allowed to circulate for 20 h, and then the animals were euthanized. The accumulation of ^{51}Cr-labeled SPTs was expressed in the same way as in the granulocyte and monocyte migration studies.

Analysis of immunological reactivity after G-1 column treatment

G-1 column treatment of arthritic rats

Arthritic rats were anesthetized by intravenous injection of 30 mg/kg sodium pentobarbital (Dainippon Seiyaku, Osaka, Japan), put in dorsal position, and kept warm. For the extracorporeal perfusion, two 25G butterfly needles with connection tubing were inserted into the right and left jugular veins, and sodium heparin (500 U/kg) was injected. The free end of each piece of tubing was connected to each end of a G-1 column, and perfusion was started at a pump speed of 0.16–0.17 ml/min. The blood perfusion was carried out for 60 min. After perfusion, the remaining blood in the G-1 circuit was washed into the jugular inflow with heparinized (5 U/ml) saline for 5–6 min.

Proliferative response of mononuclear cells to purified protein derivatives

For time-course studies, peripheral blood of five sensitized animals was collected every day from the third to the thirteenth day after immunization. MNCs were separated from peripheral blood by density gradient centrifugation using Nycoprep (NycoMed Pharma, Oslo, Norway). MNCs were suspended in RPMI 1640 supplemented with 2 mM L-glutamine, 20 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 mM 2-mercaptoethanol, and 5% FCS. Collected MNCs (2×10^5/well) were apportioned into 96-well flat-bottomed plates each containing various amounts (0–10 μg/ml) of purified protein derivative (PPD) (Japan BCG, Tokyo, Japan). After incubation at 37°C in a humidified 5% CO_2 incubator for 72 h, wells were pulse-labeled with ^{3}H-thymidine (NEN, Boston, MA, USA) for another 18 h, and then the incorporated ^{3}H-thymidine was measured by radiometry (Topcount; Packard Instrument, Meriden, CT, USA). ^{3}H-thymidine incorporation was expressed as cpm normalized to the T-lymphocyte count in the MNCs, which was considered the sum of the CD4- and CD8-positive cells after flow cytometry (FACSCalibur; Becton Dickinson, Franklin Lakes, NJ, USA). In order to assess the effects of G-1 column treatment on thymidine incorporation by MNCs, the rats were extracorporeally treated with the G-1 column on the fifth day after immunization, and inflow and outflow blood of the G-1 column was collected after a 60-min G-1 column treatment. The remaining cells in the G-1 column were also harvested by washing with ice-cold saline containing 5 U/ml of heparin. The thymidine incorporation by the MNCs was performed as described above.

T-lymphocyte response of arthritic rats to PPD-pulsed antigen-presenting cells

Arthritic rats received an extracorporeal G-1 column treatment as above. The peripheral blood was collected before and after the G-1 column treatment. The adsorbed cells in the column after G-1 column treatment were washed out of the column with ice-cold heparinized saline. The blood and the adsorbed cell suspensions from five animals were pooled, and MNCs were isolated by Nycoprep. From the MNC fraction, T lymphocytes (>95% purity) were purified with the aid of a negative selection column, Rat T-cell Enrichment Column (RTE Column; R&D, Minneapolis MN, USA) according to manufacturer's instructions.

Normal splenic adherent cells were separated from splenocytes of normal Lewis rats by culturing in a plastic flask for 3 h. These were incubated with step-wise concentrations (0–30 μg/ml) of PPD and 50 μg/ml mitomycin C (Sigma) at 37°C for 60 min to serve as PPD-pulsed antigen-presenting cells (APCs). These were detached by scraping,

and washed five times with medium (RPMI 1640 containing 2 mM glutamine, 20 mM HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 mM 2-mercaptoethanol, and 5% FCS), and used as the PPD-APCs.

The purified T lymphocytes (2×10^5 cells/well) and PPD-APCs [$(1–2) \times 10^5$ cells/well, suspended in 100 µl medium] were cocultured in 96-well culture plates for 72 h at 37°C. After 3 days of culture, ^{3}H-thymidine (NEN) was added to each well, and the cultures were incubated for another 18 h at 37°C. Cells were harvested, and then the incorporated ^{3}H-thymidine was measured.

Statistical analysis

Data are presented as means ± SD or SEM. Statistical significance was determined by Student's t test and the Pearson correlation coefficient test. Values of $P < 0.05$ were considered statistically significant.

Results

The effect of closed G-1 circulation on the migration capacity of leukocytes into various inflammatory sites

Granulocytes

After G-1 column treatment (60 min) of the ^{111}In labeled granulocytes, which were added to the peripheral blood of arthritic rats, these cells had a slight but not significant decrease in migration capacity to the arthritic joints when compared with sham treatment (Fig. 1A). The rate of migration of granulocytes into the arthritic joints was quite variable from joint to joint in all of the experimental animals. However, after G-1 treatment, the rate of migration generally decreased with less variation, and it showed an increasing tendency through the course of joint inflammation with a higher correlation coefficient. (G-1; $r = 0.79$, $P < 0.0001$, Sham; $r = 0.51$, $P = 0.03$) (Fig. 1D).

The granulocyte migration to skin inflammatory sites induced by various neutrophil-attracting substances (ZAS, LPS, or TNFα) was significantly decreased, by almost 50%, after G-1 treatment (Fig. 1B). The accumulation of G-1-treated granulocytes in the reticuloendothelial organs such as the spleen and liver increased slightly but not significantly, while localization to the lung tended to decrease (Fig. 1C).

Monocytes

A considerable number of ^{51}Cr-labeled monocytes trafficked to the arthritic paw on day 14 after sensitization. After G-1 column treatment, the monocytes tended to migrate less into the arthritic joints than did sham-treated cells, although this difference was not significant (Fig. 2A). However, migration to sites of acute dermal inflammation induced by various single proinflammatory substances decreased after circulation through the G-1 circuit, being significantly lower for LPS-induced inflammation (Fig. 2B). Accumulation in other tissues showed no significant change after G-1 column treatment (Fig. 2C).

Splenic T lymphocytes

Labeled SPTs migrated into the arthritic paws (Fig. 3A) and to sites of skin inflammatory lesions induced by various proinflammatory stimuli (Fig. 3B). The G-1 treatment had no significant effects on the migration of SPTs into either of these inflammatory sites.

Effects of G-1 column treatment on the immunoreactivity of lymphocytes of adjuvant-arthritic rats

The clinical course of adjuvant arthritis and the antigen reactivity of blood lymphocytes

The time course of joint inflammation and antigen reactivity of circulating lymphocytes of adjuvant-arthritic rats is shown in Fig. 4. The first and maximum peak of responsiveness in blood MNCs to PPD was found on day 5, and the second peak around days 10–12 after sensitization (Fig. 4). The first peak (day 5) was targeted in the following study, because it preceded the dissemination of the immune reaction such as to joint inflammation, and at this stage there was relatively low variance among individual animals.

Effects of G-1 treatment on the antigen reactivity of circulating mononuclear cells

Before G-1 column treatment, the MNCs in the peripheral blood showed high reactivity against PPD (Fig. 5, closed circles). Following G-1 column treatment for 60 min, on the fifth day after adjuvant inoculation, the MNCs in the effluent blood (outlet; Fig. 5, gray column) showed substantially less response to the antigen (PPD). It appeared that the G-1 column preferentially adsorbed the antigen-competent T lymphocytes from circulation (inside; Fig. 5, black column). At the final point of treatment, the lymphocytes in the peripheral blood had significantly less reactivity to PPD, as was shown by the low antigen reactivity at the inlet site of the G-1 column (Fig. 5, white column).

Effects on the antigen reactivity of peripheral T lymphocytes

Blood T lymphocytes after 60 min of G-1 column treatment clearly lost their antigen reactivity to PPD almost completely (Fig. 6, dark circles) compared to before treatment (open circles). Interestingly, the T lymphocytes harvested from the perfused G-1 column still retained reactivity to PPD antigen-pulsed APCs (black squares), although it was somewhat less than that of preadsorption cells.

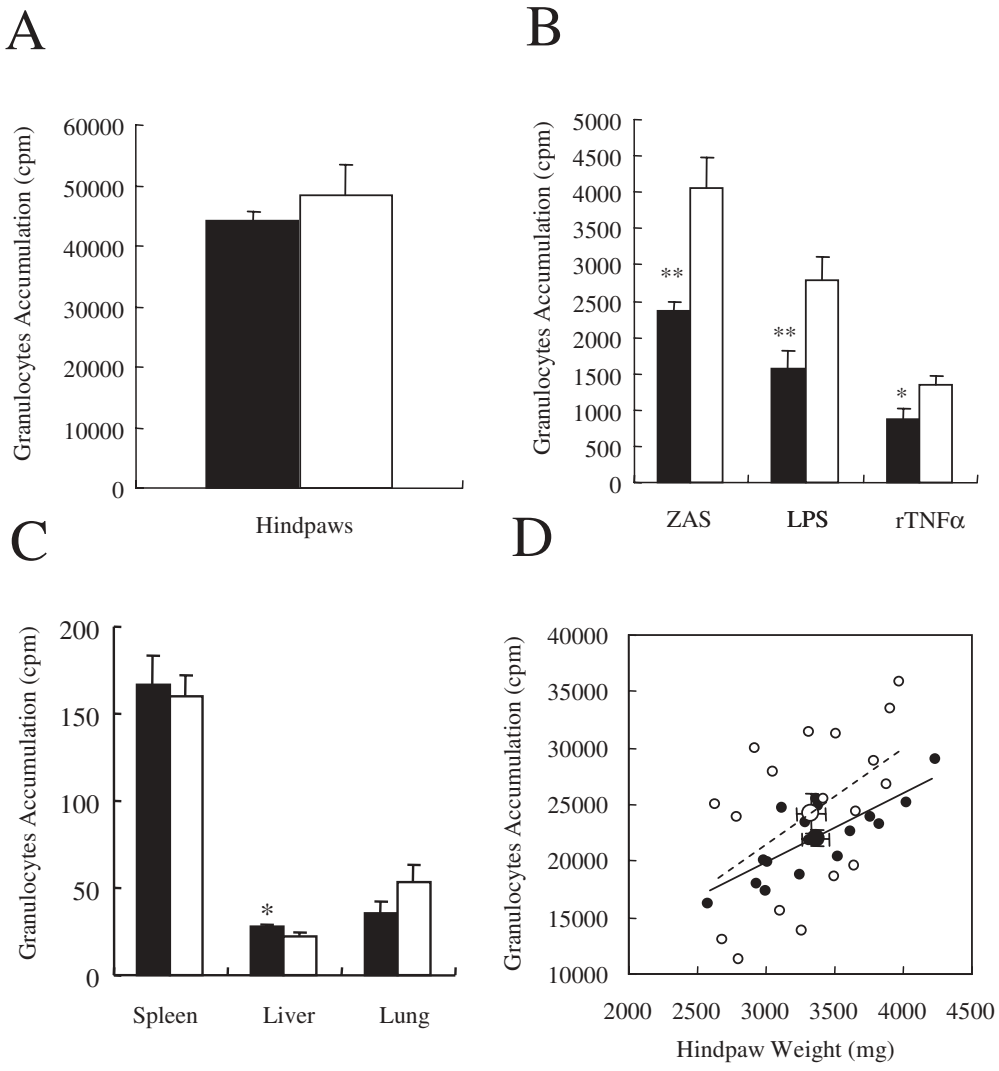

Fig. 1A–D. The effect of G-1 column treatment on granulocyte migration to hindpaw (**A**), dermal inflammation (**B**) and spleen, liver, and lung (**C**). ^{111}In-labeled granulocytes (10^6 cpm) treated with closed-circuit G-1 (*black bar*) or sham (*white bar*) column for 60 min were injected intravenously into rats suffering from adjuvant arthritis and allowed to circulate for 2 h. The accumulation of ^{111}In-labeled granulocytes was expressed as cpm per total dissected hindpaw joints (**A**) or per dissected skin tissue (**B**), and per milligram tissue (**C**). Dermal inflammation was induced by intradermal (id) injection of 25% zymosan-activated serum (*ZAS*), *Escherichia coli* 0111 endotoxin (*LPS*) (1 ng/site), or recombinant rat TNF-α (*rTNFα*) (10 ng/site) immediately after intravenous injection of ^{111}In-labeled granulocytes. Granulocyte ^{111}In accumulation in normal uninflamed joints and skin averaged <1500 cpm in the hindpaw and <120 cpm at skin sites). In the correlation diagram between granulocyte accumulation and hindpaw weight (**D**), *closed circles* represent G-1 column-treated ($r = 0.79$, $P < 0.0001$) and *open circles* sham-treated ($r = 0.51$, $P = 0.03$), respectively. *Large circles* indicate average values. The values are the mean ± SEM of nine animals (18 hindpaws) except rTNFα (six animals). Student's *t* test was used to determine the significance of differences between the values of the G-1 and sham groups (*$P < 0.05$; **$P < 0.01$)

Discussion

In the first set of studies, the most significant finding was that after G-1 column treatment of the circulating blood of adjuvant-arthritic rats for 60 min, the migration of granulocytes and monocytes to sites of acute inflammatory reactions in the skin (Fig. 1B) induced by C5a, LPS, or TNFα was reduced. This result may be related to findings that granulocytes exposed to the G-1 column shed their surface L-selectin but upregulated expression of CD11b/CD18 adhesion molecules.[8,11–14] L-selectin is a key molecule for mediating the capture and rolling of granulocytes on the endothelium prior to their transendothelial migration.[20] The upregulated CD11b/CD18 does not participate in this initial phase of the leukocyte adhesion/migration cascade, and, therefore, it does not compensate for the loss of L-selectin.[21] Thus, these G-1 column-exposed granulocytes may fail to engage in the primary interaction with the endothelium, at least at acutely inflamed sites.

The lack of a significant effect of G-1 treatment on granulocyte migration to arthritic joints (Fig. 1A) may be due in part to the multi-mediator driven, chronic inflammation of arthritis, during which redundant mechanisms for

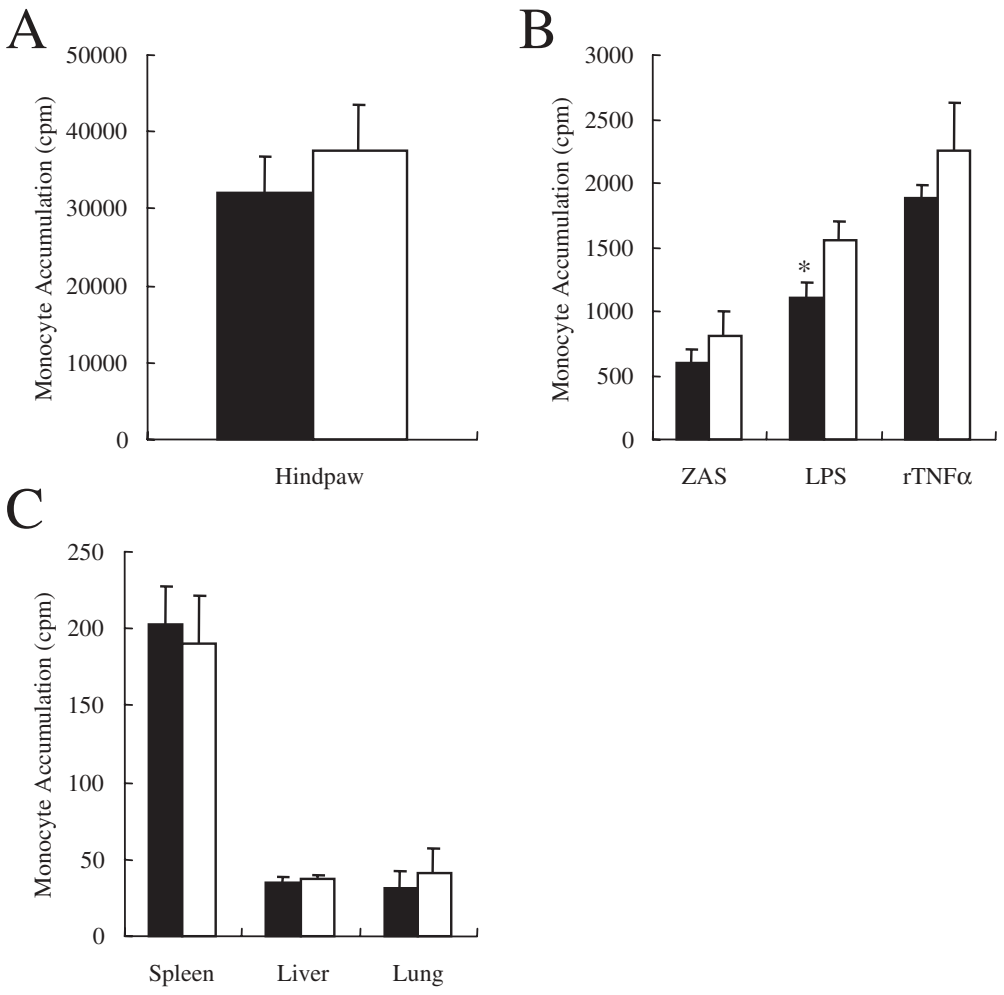

Fig. 2A–C. The effect of G-1 column treatment on monocyte migration into hindpaw (**A**), dermal inflammation (**B**), and spleen, liver, and lung (**C**). ^{51}Cr-labeled monocytes (10^6 cpm) treated with the closed-circuit G-1 (*black bar*) or sham (*white bar*) column were injected intravenously into the adjuvant-arthritic rats. The same six animals that were used for the data shown in Fig. 1A were used. The accumulation of ^{51}Cr-labeled monocytes was determined as described in Fig. 1. The values are mean ± SEM of six animals in each group except for rTNFα (three animals). The differences between the G-1-treated group and the sham group are significant ($*P < 0.05$). Values of monocyte accumulation in uninflamed normal joints averaged 1400 cpm in the hindpaw and 130 cpm in control diluent-injected skin sites

leukocyte recruitment are activated by, for example, other adhesive molecules, all of which can mediate capture and rolling.[17,21–23] In the arthritic synovium there is also neovascularization, in which granulocytes may not engage in a rolling phase owing to low shear forces, thus proceeding readily to firm adhesion and emigration.[24] All these factors likely play a role in the variability of onset and severity from joint to joint.

When we compensated for some of this variability by analyzing the data for each hindpaw on the basis of joint weight and granulocyte accumulation (Fig. 1D), the correlation between these parameters was much stronger in the animals treated even just once with G-1 perfusion because of a marked decrease in the grade and variability of granulocyte accumulation. This suggests an acute modulating effect of the G-1 treatment on the migration activity of the granulocytes, aside from the complex changes in the inflamed synovium.

There are many reports describing adverse effects on the lung associated with extracorporeal perfusion.[25,26] Therefore, lung dysfunction can be a serious side effect of extracorporeal devices, especially via activation of the complement system. However, there was no increase in localization of granulocytes into the lung after the G-1 column treatment (Fig. 1C). Perhaps most of the complement activation products were inactivated in the column or bound to the leukocytes.[11–14] Our results suggest that extracorporeal perfusion with a G-1 column should be as safe as or safer than the usual hemodialysis methods.

In contrast to the lung, granulocyte localization into the reticuloendotheliar system, especially the liver, was slightly increased (Fig. 1C), suggesting that some of the G-1-treated granulocytes may be cleared more rapidly from circulation, perhaps contributing to the antiarthritic effect of G-1 column treatment. However, granulocytopenia was not observed, so the level of accelerated clearance was easily

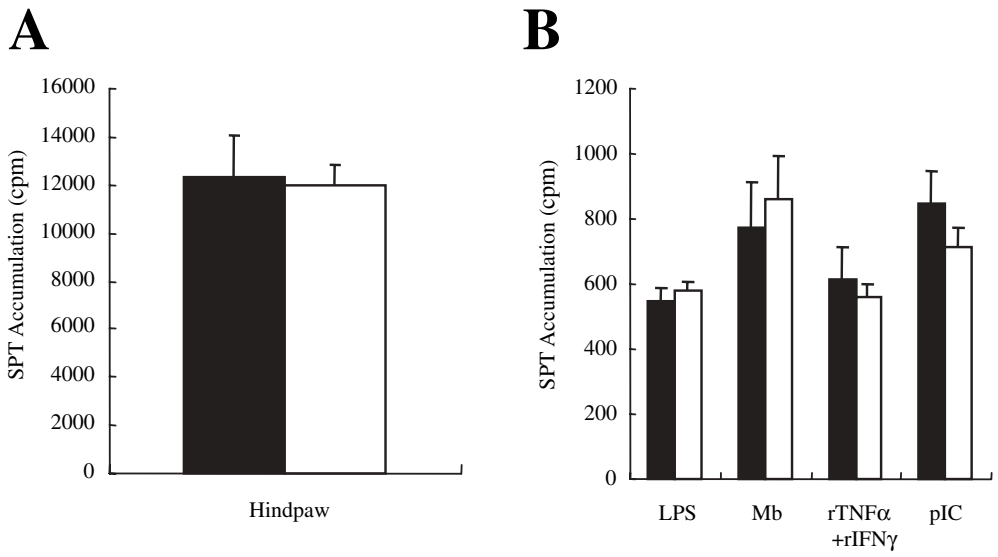

Fig. 3A,B. The effect of G-1 column treatment on splenic T-lymphocyte (*SPT*) migration to hindpaw (**A**) or sites of dermal inflammation (**B**). ^{51}Cr-labeled SPTs (10^6 cpm) treated with G-1 column (*black bar*) or sham column (*open bar*) in a closed circuit were injected intravenously into the rats with adjuvant arthritis and allowed to circulate for 20 h. The accumulation of ^{51}Cr-labeled SPTs was determined as cpm per total hindpaw joints (**A**) and per dissected skin tissue (**B**). Dermal inflammation was induced by intradermal injection of 100 ng LPS, 20 μg heat-killed *Mycobacterium*, the combination of 300 U rat rIFNγ and 10 ng rTNFα and 200 ng poly I:C (*pIC*) immediately after intravenous injection of labeled SPT. The values are mean ± SEM of six animals in each group

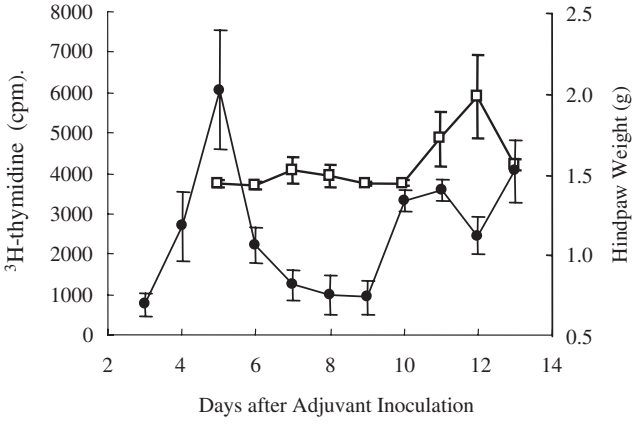

Fig. 4. The time course of weight of the nonimmunized hindpaw and purified protein derivative (PPD) reactivity of peripheral mononuclear cells (MNCs) after induction of adjuvant arthritis. Rats were immunized with adjuvant into the foot pad of the left hindpaw, and, at various times as indicated, peripheral MNCs were isolated and stimulated with 3 μg/ml PPD (maximum reactivity concentration) for 72 h and pulsed with ^{3}H-thymidine for another 18 h. ^{3}H-thymidine incorporation is expressed as the cpm per starting T-lymphocyte number (*closed circles*). The weights of the nonimmunized paw were also monitored (*open squares*). The values are the mean ± SEM of five animals in each group

compensated in vivo by the eightfold reserve population of granulocytes in the bone marrow sinuses.[27]

The migration of monocytes after G-1 column treatment showed almost the same trend as that of the granulocytes, with no significant effect on migration to the hindpaw, but some decrease in migration to sites of acute dermal inflammation, especially by LPS (Figs. 2A and B).

In the T-lymphocyte migration experiments, G-1 column treatment had no significant effect on accumulation in joints or skin inflammation (Figs. 3A and B). It has been shown that the G-1 column does not adsorb T lymphocytes in arthritic rats or in human patients.[11–14] In this series of experiments, similar results were obtained. Taken together, these findings indicate that acute G-1 treatment has an effect at least on granulocyte migration to sites of inflammation but that the cellular interactions and mediators generated by exposure to the G-1 beads do not affect the migration of lymphocytes. It is noteworthy, however, that with repeated treatment in clinical trials in rheumatoid arthritis or ulcerative colitis, the number of lymphocytes, especially resting $CD4^+$ T lymphocytes in the circulation of G-1-treated patients with a better prognosis, gradually increased in number after treatment.[10] The disease activity in such patients subsided within 6 months afterward. Thus, there is likely an effect of G-1 column treatment on the activity of lymphocytes with prolonged treatment. In fact, we reported previously that with G-1 column treatment of adjuvant arthritic rats, surface expression of CD44[14] and CD134 (OX40)[28] cell-activation markers on T cells adherent to the G-1 column are always higher than that on circulating T lymphocytes at the inlet as well as at the outlet of the column. Furthermore, the cells, including those $CD44^{high}$ and $CD134^{high}$ T cells recovered from the G-1 beads, induced granuloma and lymphoid follicle formation when injected into the skin of normal rats,[29] suggesting that these were T lymphocytes with chronic inflammatory activities.

In the current studies on adjuvant-arthritic rats, blood T lymphocytes from the arthritic rats showed high reactivity to PPD, an antigenic component of *Mycobacterium*. This reactivity was highest on the fifth day after adjuvant

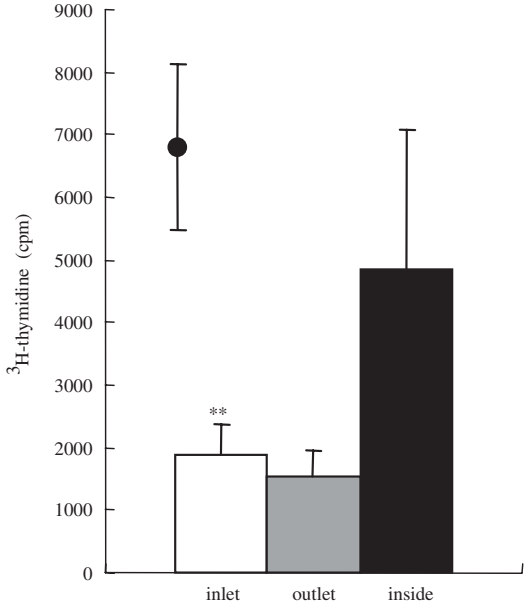

Fig. 5. The effect of G-1 column treatment on proliferative response of MNCs upon PPD stimulation. Arthritic rats on the fifth day after adjuvant inoculation underwent G-1 column treatment. Blood MNCs in the inlet (*white bar*) and outlet (*gray bar*) of the G-1 column and the adsorbed MNCs in the column (*black bar*) were collected at 60 min after G-1 treatment. The ^{3}H-thymidine incorporation into MNCs was determined as described in Fig 4. The proliferation value of MNCs in peripheral blood just before G-1 column treatment is shown by the *closed circle* as an indicator of initial reactivity. Values are expressed as mean ± SEM of five animals except for before G-1 column treatment (n = 10). The differences between the value before and that on the inlet side of the column after the 60-min G-1 treatment is significant (**$P < 0.01$)

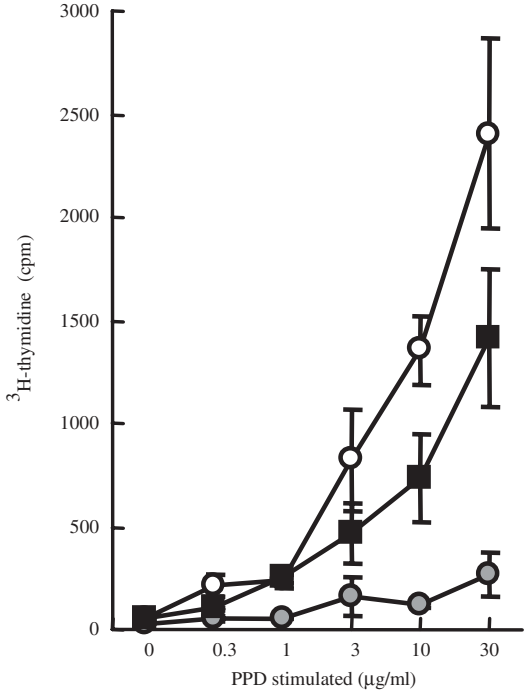

Fig. 6. The effect of G-1 column treatment on T-lymphocyte proliferation to PPD-stimulated normal splenocytes (antigen-presenting cells, APCs). T lymphocytes (>95%) were isolated from the peripheral blood before (*open circles*) and after (*gray circles*) extracorporeal G-1 column treatment, or recovered from the G-1 column after G-1 treatment (*closed squares*). T cells from five animals were pooled and tested. Adherent cells from normal splenocytes, which were stimulated with stepwise increasing amounts of PPD and served as PPD-stimulated APCs, were cocultured with T lymphocytes for 72 h, followed by pulse-labeling with ^{3}H-thymidine for another 18 h. The vertical axis expresses the rate of ^{3}H-thymidine incorporation in cpm and the horizontal axis expresses the stepwise increasing concentration of PPD stimulation of APCs. Experiment was carried out in triplicate, and each point represents the mean ± SD of values of five animals

immunization, and was a transient but remarkable response. Appearance of these reactive T cells preceded clinical arthritis and appeared to be important for the development of arthritis afterward (Fig. 4). This is supported by the findings that immunosuppressive therapy with methotrexate at around this time abolishes arthritis development.[30] Therefore, we assessed the antigen reactivity of T lymphocytes after G-1 column treatment at this point in disease development.

During G-1 column treatment for 60 min on the fifth day after adjuvant, the G-1 beads appeared to preferentially retain the antigen-reactive MNCs present in circulation, since the antigen reactivity of the MNCs in the efferent blood was considerably decreased throughout the G-1 apheresis (Fig. 5). Furthermore, the MNCs recovered from the G-1 beads had greater PPD reactivity than cells in the effluent from the column. However, since mixed lymphocytes and monocytes were recovered from the column, it is possible that the increase in reactivity was caused by adsorbed antigen-presenting cells, such as monocytes, B lymphocytes, or even dendritic cells. To address this possibility, we purified T lymphocytes from the blood, using an RTE column (CD3 affinity-column) yielding 95% T cells. Normal splenic macrophages, obtained from syngeneic normal Lewis rats were pulsed with PPD, and a constant number were cocultured with a standardized number of the purified T lymphocytes prepared from the afferent, G-1 bead-adsorbed, and efferent blood, respectively. The CD3$^+$ T lymphocytes in the efferent (outflow) blood virtually lacked PPD reactivity. The reactivity of the bead-adsorbed lymphocytes was higher than that of the efferent cells, and approximated that of the blood T lymphocytes prior to extracorporeal circulation (Fig. 6).

These findings indicate that when the presence of antigen-specific T lymphocytes in the peripheral blood peaks, the G-1 beads preferentially adsorb such T lymphocytes and can remove them from circulation. That this is a selective process is supported by the fact that there was no significant decrease in the number of T lymphocytes in the blood of G-1-perfused rats or indeed even between the afferent and efferent blood in the circuit. It is possible that the PPD-specific clones of circulating T lymphocytes activated by Mb antigens (probably less than a few percent) were bound by *Mycobacterium* antigen-presenting cells (especially monocytes but possibly also other APCs) adherent to the G-1 beads, similar to an immunoadsorbent process.

The removal of PPD antigen-specific T lymphocytes from the circulation of adjuvant-arthritic rats by G-1 col-

umn perfusion was an unexpected but important new finding in these studies. Whether the T lymphocytes, which preferentially adhered on the G-1 beads during perfusion,[14] are identical to the CD44-positive and/or CD134-positive $CD4^+$ T cells reported by some authors as arthrogenic[31] is not yet clear. However, in our previous studies,[12–14] we demonstrated that three successive G-1 treatments between the fifth and tenth day after adjuvant immunization attenuated arthritis severity and significantly accelerated the remission phase with even complete recovery in some animals. This suggests that a direct effect on the immunocompetent cells likely occurred. These dramatic effects of G-1 therapy on the antigen responsiveness of T lymphocytes in adjuvant arthritis warrant further investigations, including passive transfer studies and comparative studies between antigen-specific and nonspecific T lymphocytes. However, we believe that the novel findings reported here on the effect of G-1 treatment on leukocyte migration and on selective removal of antigen-reactive T lymphocytes achieve new insights into the mechanisms of action of G-1 therapy and provide a foundation for further such investigations.

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