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Paradoxical Effects of Glycyrrhizin on the Induction of Granulocytes and Extrathymic T Cells between Normal Mice and Mice with Preexisting Granulocytosis

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Summary. **Some traditional Chinese medicines are known to have paradoxical effects. For example, one such medicine (i.e., glycyrrhizin) is effective for the treatment of allergic diseases (i.e., lymphocyte-associated inflammation), and is also sometimes effective for the treatment of suppurative diseases (i.e., granulocyte-associated inflammation). To investigate the mechanisms underlying this paradox, we conducted a study in mice. When normal mice were administered with one i. p. injection of glycyrrhizin, granulocytes and extrathymic T cells increased in proportion and number in various immune organs. However, an opposite response of glycyrrhizin was induced with such an injection in mice with preexisting granulocytosis. These mice included those treated with G-CSF and AIDS mice infected with murine leukemia virus. In other words, glycyrrhizin reduced the levels of granulocytes and extrathymic T cells in various organs of mice with preexisting granulocytosis. These results suggest that some drugs have the ability to exert paradoxical effects, depending on the conditions of the host immune system. The underlying mechanisms are discussed.**

Key words—Chinese medicine, glycyrrhizin, lymphocytes, granulocytes, extrathymic T cells.

INTRODUCTION

The host defense system consists of both phagocytes and lymphocytes. The major cells of the former are granulocytes and macrophages while those of the latter are T cells and B cells. Since granulocytes and macrophages carry a high concentration of adrenergic receptors on the surface among leukocytes, they are activated under conditions of sympathetic nerve strain, for example, anger, stress, exercise, overwork, heavy consumption of alcohol, bacterial infection, etc.[1-7] Under these conditions, hosts tend to fall victim to inflammatory diseases due to the interaction of granulocytes with resident bacteria or invading bacteria.[8] On the other hand, lymphocytes express cholinergic receptors on the surface and are, therefore, activated under conditions of parasympathetic nerve dominance, for example, the absence of stress, viral infection (early phase), allergic states, etc.[9-14] Pulse rate is a good indicator for determining the state of the autonomic nervous system. Namely, tachycardia occurs under sympathetic nervous strain, while bradycardia is observed with parasympathetic nerve dominance.

Based on these findings and various indicators, attention has been focused on glycyrrhizin, an aqueous extract from licorice root *(Glycyrrhiza radix)*.[15,16] This Chinese medicine is often effective for the treatment of both allergic diseases (e.g., urticaria, hypersensitivity to pollen, and atopic dermatitis) and suppurative diseases (e.g., chronic phase of viral infection, periproctal abscess, and gastric ulcer).[17-25] The former diseases are caused by the overactivity of lymphocytes (accompanied by granulocytopenia), whereas the latter are caused by the overactivity of granulocytes (accompanied by lymphocytopenia). It is, therefore, possible that glycyrrhizin may have paradoxical effects even on the host defense system.

In the course of studies on glycyrrhizin, we have

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encountered an interesting result; namely, that glycyrrhizin induces granulocytosis in normal mice while it reduces the levels of granulocytes in mice with preexisting granulocytosis. Recent studies have revealed that some oxidized substances (e.g., NO) also have some paradoxical effects as reductants or oxidants; namely, they prevent tissue damage under certain conditions while inversely damaging tissue under other conditions.[26-28] The present results have revealed that glycyrrhizin seems to exert similar paradoxical effects on the host defense system.

MATERIALS AND METHODS

Mice

C3H/HeN and C57BL/6 mice at the age of 5 to 8 wks, fed under specific pathogen-free conditions in our laboratory, were used for this study. All mice were originally obtained from Charles River Japan, Inc. (Atsugi, Japan).

Drugs and experimental design

Glycyrrhizin (GL), an aqueous extract of licorice root known as an anti-inflamatory substance in Chinese medicine,[15,16] was used. This compound consists of one molecule of glycyrrhetinic acid and two molecules of glucuronic acid. In the present experiments, we used GL supplemented with glycine and cysteine (i.e., Stronger Neo-Minophagen C, SNMC). As a single high dose, SNMC was i.p. injected at a concentration of 10 mg/mouse. SNMC (2 mg/0.2 ml) was also administered intraperitonealy twice a week, as chronic low doses.

Induction of granulocytosis by G-CSF

Recombinant human G-CSF was kindly provided by Chugai Pharmacentical Co., Tokyo, Japan. 10^4 U/mouse was i.p. injected twice a week.

Infection with LP-BM5 murine leukemia virus (MuLV)

Cell-free supernatants of LP-BM5 MuLV were prepared according to established methods by culturing G6 SC-1 clones with normal SC-1 cells for 5 days.[16] Mice were i.p. inoculated with 150 μl of stock solution containing $10^{5.4} \times$C plaque-forming units and $10^{2.7}$ mink-cell focus-forming units per milliliter. The titer of LP-BM5 MuLV components in the mixture was determined as reported previously.[16]

Cell preparations

Mononuclear cells (MNC) were harvested from various organs of mice. Hepatic MNC were isolated by an improved method described elsewhere.[29] Briefly, mice anesthetized with ether were sacrificed by total exsanguination via a cardiac puncture. To obtain MNC, the liver was removed, pressed through 200-gauge stainless steel mesh, and then suspended in Eagle's MEM supplemented with 5 mM HEPES (Nissui Pharmaceutical Co., Tokyo, Japan) and 2% heat-inactivated newborn calf serum. After being washed with the medium, the cell pellet was sometimes resuspended in the medium. MNC were isolated from parenchymal hepatocytes, the nuclei of hepatocytes, and Kupffer cells by Percoll (35% Percoll containing 100 U/ml heparin) gradient centrifugation.[30]

Splenocytes and thymocytes were obtained by pressing the spleen MNC and thymus through 200-gauge steel mesh; splenic erythrocytes were disrupted by 0.83% NH_4Cl-Tris buffer (pH 7.6).[29] Bone marrow cells were obtained by flushing bilateral femurs with the medium. Blood MNC were collected by 2% dextran sedimentation for 40-50 min at room temperature, and then the supernatant was harvested. Contaminated erythrocytes were also eliminated by the NH_4Cl-Tris buffer.

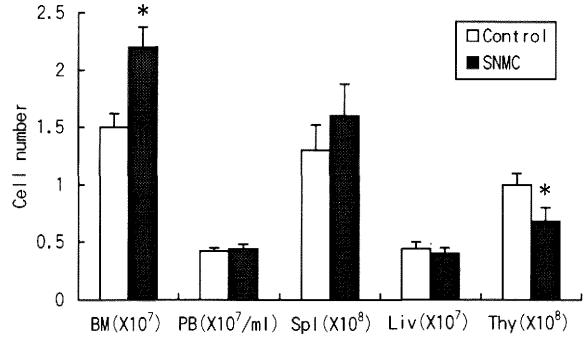


Fig. 1. Changes in the numbers of MNC in various organs of mice treated with a single injection of SNMC. SNMC (10 mg/0.2 ml) was i.p. injected and MNC were prepared on Day 3. Mean and SD of three experiments are represented. The number of MNC in the bone marrow has increased but that in the thymus decreased. $*p<0.05$.

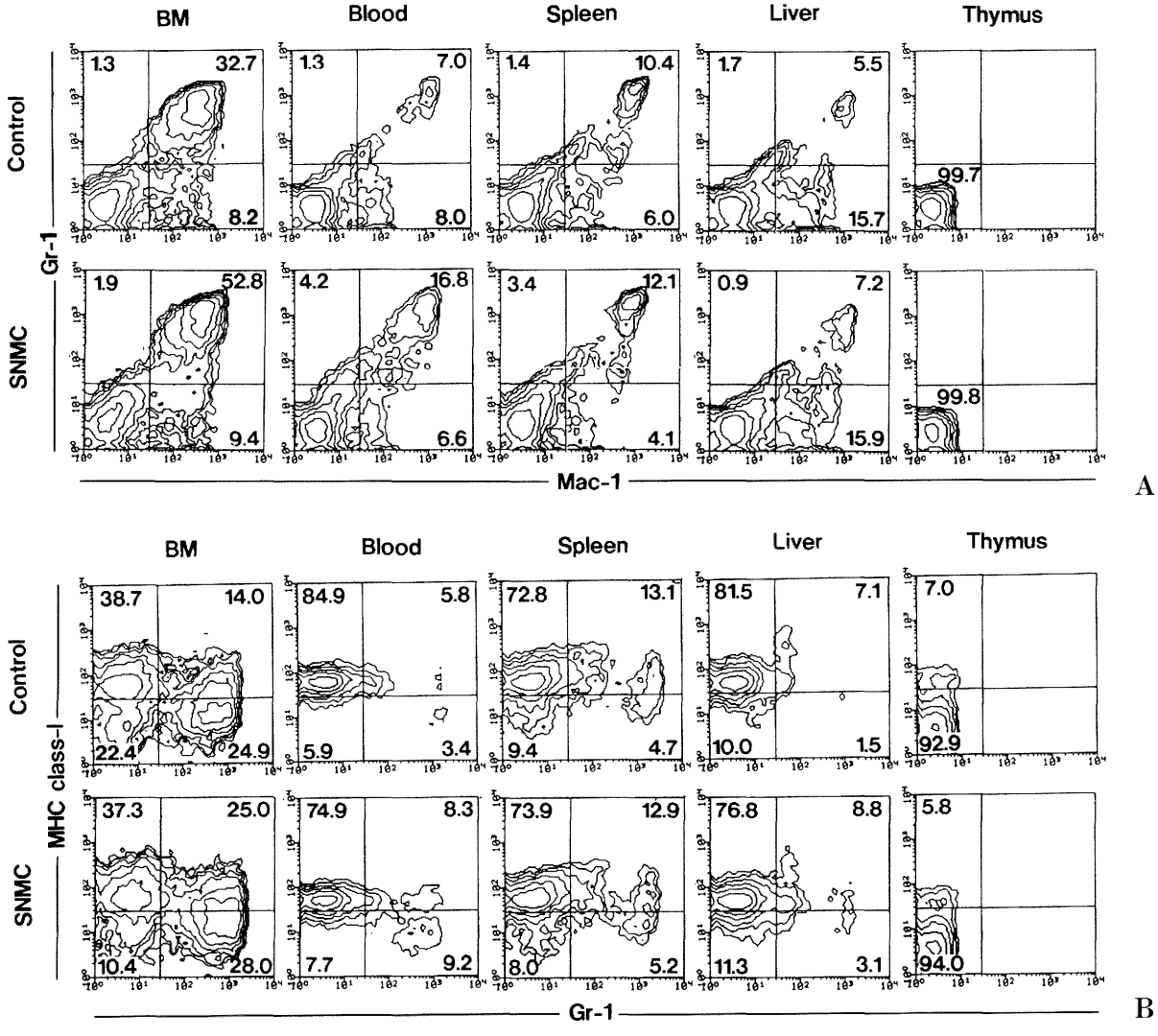


Fig. 2. Identification of granulocytes among MNC in various organs before and after the administration of glycyrrhizin, **A.** Two-color staining for Gr-1 and Mac-1, **B.** Two-color staining for Gr-1 and MHC class I antigens. The characterization was done on Day 3. Numbers in the figure represent the percentages of fluorescence-positive cells. Representative results of three experiments are depicted. The proportion of granulocytes has increased in the bone marrow and blood by the administration of SNMC.

Immunofluorescence test

The surface phenotypes of cells were analyzed using mAbs in conjunction with a two-color immunofluorescence test.[31] FITC, R-Phycoerythrin (R-PE), or biotin-conjugated anti-CD3 (145-2C11), anti-IL-2Rβ, anti-granulocyte (Gr-1, RB6-8C5), anti-erythrocyte (TER119), and anti-MHC class I (anti-mouse H-2K^k) mAbs were obtained from PharMingen Co. (San Diego, CA, U.S.A.). FITC-conjugated anti-monocyte (Mac-1) mAb (Caltag Laboratories, San Francisco, CA, U.S.A.) was also used. Biotin-conjugated reagents were developed with PE-conjugated streptavidin (Caltag Laboratories, San Francisco, CA, U.S.A.). The fluorescence-positive cells were analyzed with a FACScan (Becton-Dickinson Co., Mountain View, CA, U.S.A).

Statistical analysis

Statistical significance was analyzed by using Student's *t*-test.

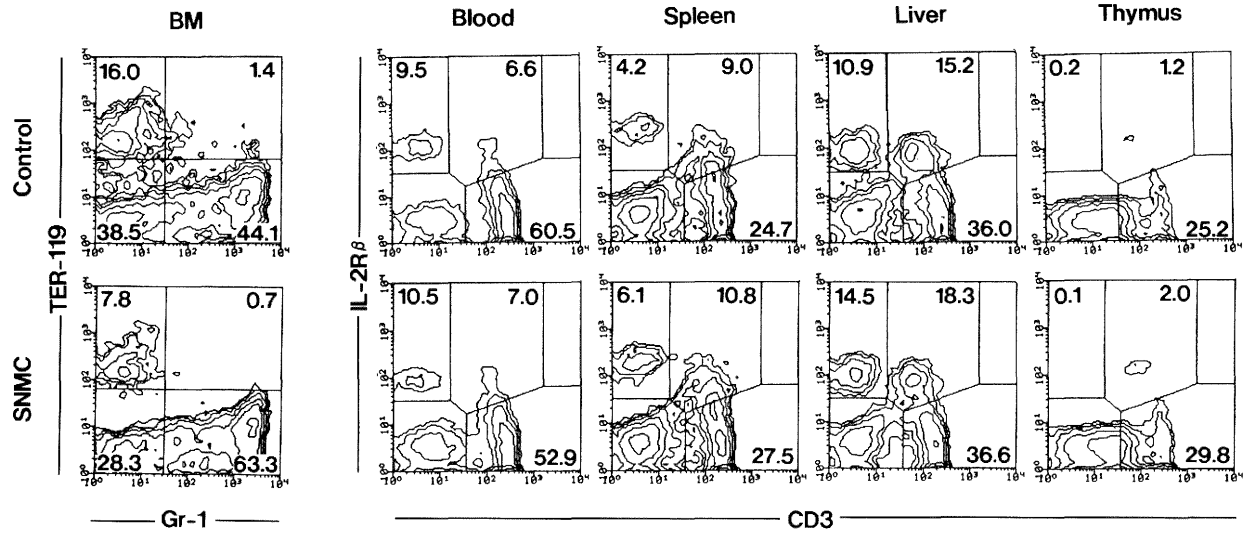


Fig. 3. Further phenotypic characterization of MNC in various organs. Two-color staining for Gr-1 and TER-119 (erythroid antigen) was performed in the bone marrow, while two-color staining for CD3 and IL-2Rβ was done in the other immune organs. Representative results of three experiments are depicted. The proportion of erythroid cells has decreased in the bone marrow by the administration of SNMC. IL-2Rβ^+ CD3^{int} cells tend to increase in all tested organs.

RESULTS

Effects of an acute high dose of glycyrrhizin on granulocytes

Mice were administered with a single i.p. injection of SNMC and the numbers of MNC yielded by the various immune organs were enumerated on Day 3 (Fig. 1). The number of MNC in the bone marrow increased and that in the thymus decreased ($P<0.05$), while the numbers of MNC in other organs remained unchanged ($P>0.05$).

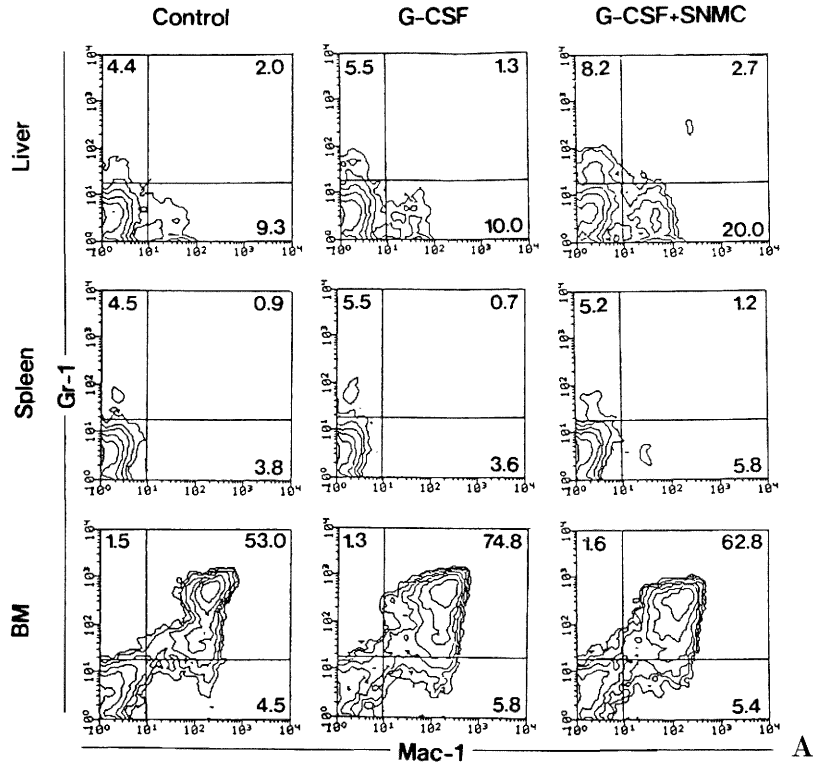
To determine the variation of granulocytes, two-color staining for Gr-1 and Mac-1 and that for Gr-1 and MHC class I were performed (Fig. 2). In these stainings, granulocytes were estimated to be Mac-1$^+$ Gr-1$^+$ (Fig. 2A) and these granulocytes, especially in the bone marrow, were a mixture of MHC class I$^+$ and class I$^-$ (Fig. 2B). It was found that such granulocytes in the bone marrow, as well as in the blood, increased markedly after the injection of SNMC.

Erythroid antigen$^+$ (TER-119) cells inversely decreased in the bone marrow (Fig. 3). In this figure, two-color staining for CD3 and IL-2Rβ was also carried out to determine the variation of IL-2Rβ^+ CD3^{int} cells (i.e, extrathymic T cells). It was confirmed that IL-2Rβ^+ CD3^{int} cells increased, especially in the liver, by the injection of SNMC. Namely, an acute high dose of glycyrrhizin simultaneously induced an increase in the number of granulocytes in the bone marrow and an increase in the proportion of CD3^{int} cells in the liver.

Effects of an administration of glycyrrhizin in mice with preexisting granulocytosis

In this experiment, mice were initially injected with G-CSF to induce granulocytosis. The purpose of this study was to determine whether glycyrrhizin enhances or suppresses the levels of granulocytes in these mice. In this protocol, a low-dose administration of SNMC(2 mg/one time/mouse) was repeated twice a week in conjunction with the treatment with G-CSF (also twice a week) (Fig. 4).

The number and phenotype of granulocytes were examined on Day 10. Reflecting the increase in the number of granulocytes due to the treatment with G-CSF, the number of MNC in the bone marrow increased up to 200% (data not shown). It was demonstrated that the simultaneous injection of SNMC tended to decrease the proportion of granulocytes in mice with G-CSF (75%→63%) in the bone marrow (Fig. 4A). Since the number of bone marrow (BM) cells in mice treated with G-CSF and SNMC also decreased (30% decrease in comparison with mice treated with G-CSF alone), the decrease in the abso-



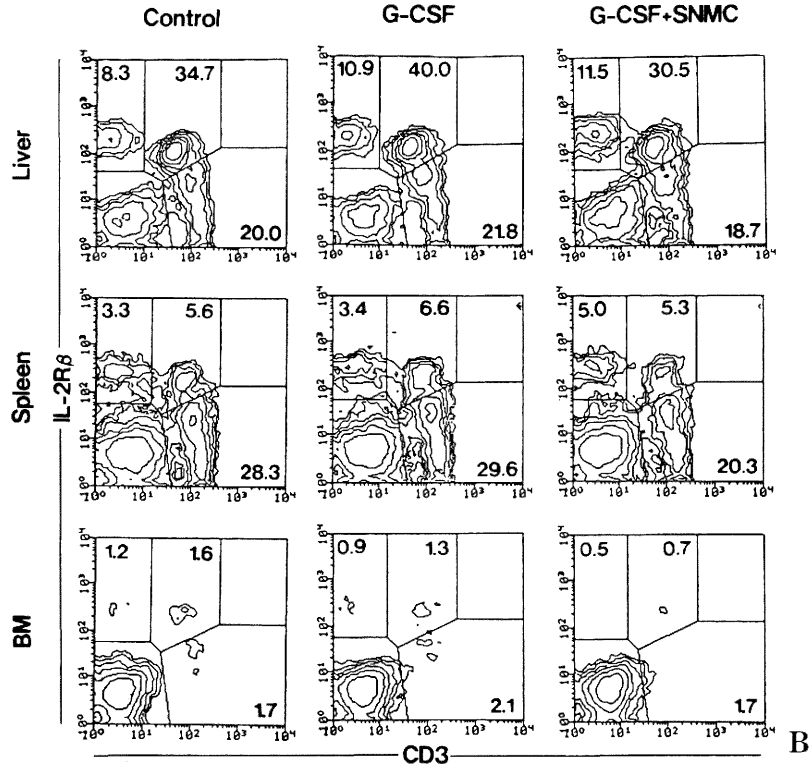


Fig. 4. SNMC suppressing the increase in the proportion of granulocytes and extrathymic T cells in mice treated with G-CSF, **A.** Two-color staining for Gr-1 and Mac-1, **B.** Two-color staining for CD3 and IL-2Rβ. Representative results of three experiments are depicted. G-CSF simultaneously increases the proportion of granulocytes and extrathymic T cells. However, such an increase is considerably suppressed by the chronic injections of a low dose of SNMC (2 mg/one time/mouse).

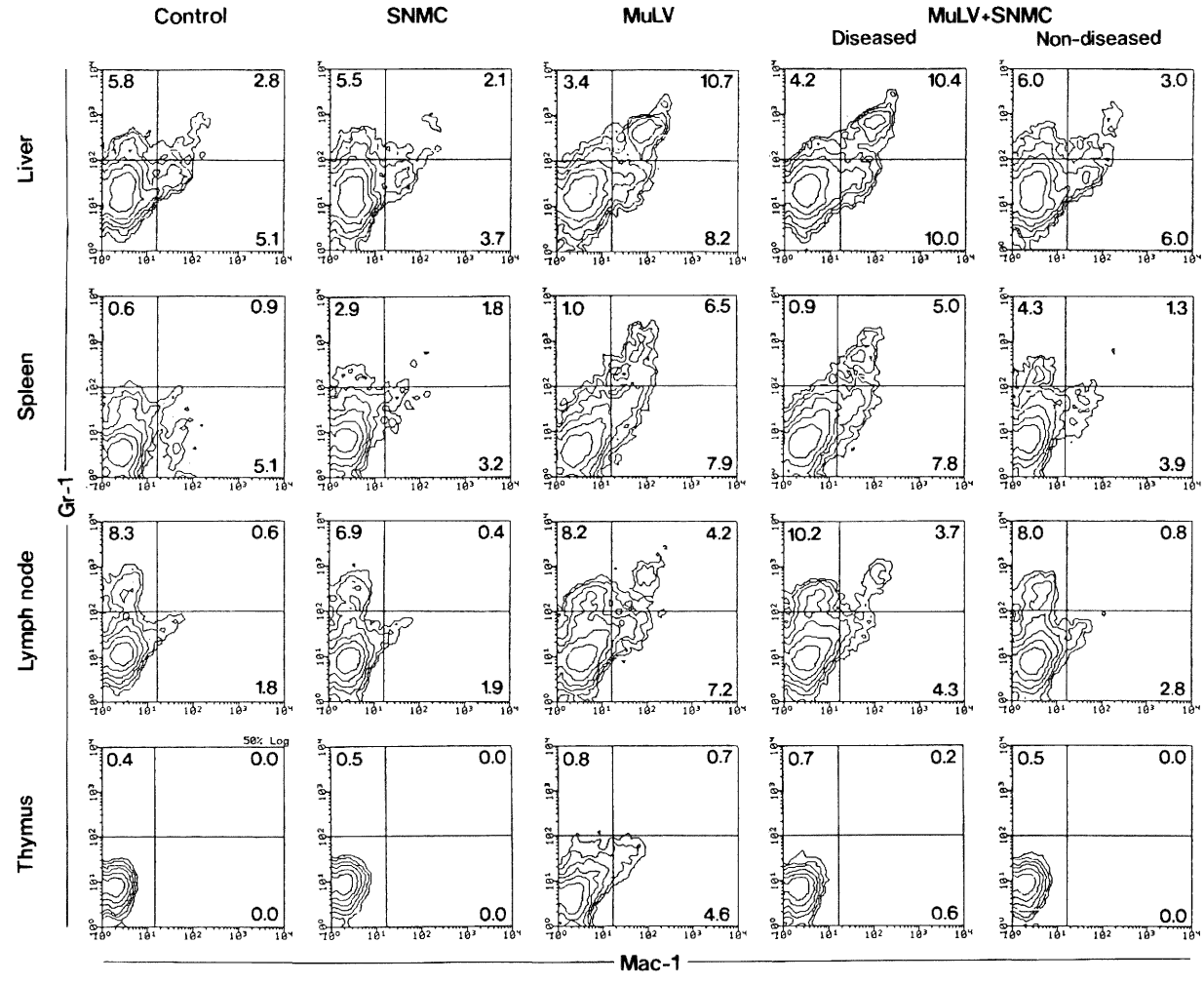


Fig. 5. SNMC also suppressing the increase in the proportions of granulocytes and extrathymic T cells in mice infected with MuLV. Representative results of three experiments are depicted. Mice infected with MuLV show increased levels of granulocytes and extrathymic T cells in various organs. However, the chronic injections of SNMC (2 mg/one time/mouse) have suppressed such elevation.

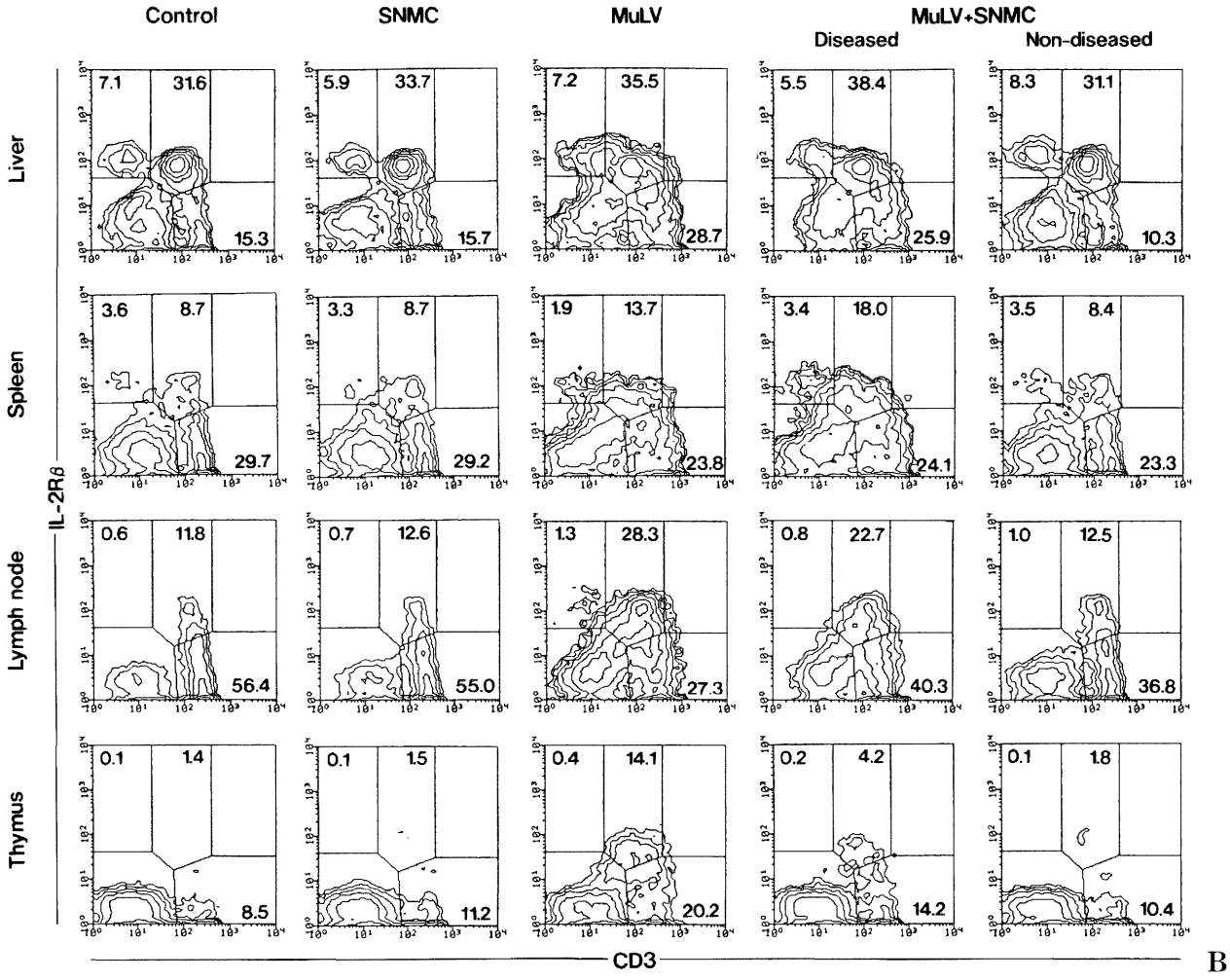
lute number of granulocytes was substantial in the bone marrow. The numbers of MNC in the organs other than the bone marrow remained stable.

G-CSF increased the proportion of IL-2Rβ^+ CD3^{int} cells in the liver (Fig. 4B). The injection of SNMC suppressed such an elevation as well.

Suppression of granulocytosis by the administration of glycyrrhizin in AIDS mice

We previously reported that, in mice infected with MuLV (i.e., mouse AIDS), CD3^{int} cells proliferated extensively and finally fell victim to acquired immunodeficiency disease.[16] In the course of these experiments, we noticed that granulocytosis was induced in parallel with the progress of the disease in these mice (Fig. 5). Since granulocytosis is also seen in AIDS patients (our unpublished observation), the overactivity of granulocytes in humans and mice with AIDS seems to be associated with tissue destruction as well as with oxidation of generalized molecules in the body.

In these respects, it was investigated whether the administration of SNMC modulated the levels of granulocytes in mice infected with MuLV (Fig. 5A, *right column*). From the beginning of the infection with MuLV, SNMC (1/5 of acute high dose) was injected twice a week. Five weeks later, mice were classified into a diseased group and a disease-free group. In other words, approximately 60% of the



mice infected with MuLV did not show the onset of the disease (e.g., wasting syndrome) when SNMC were administered. It was demonstrated that the levels of SNMC (e.g., Mac-1$^+$Gr-1$^+$ cells) in the liver, spleen, and lymph nodes decreased prominently in the disease-free group of mice treated with SNMC. Control mice and mice treated with SNMC alone did not show granulocytosis.

Two-color staining for CD3 and IL-2Rβ is represented in Fig. 5B. As already reported, a massive expansion of CD3^{int} cells was seen in various immune organs in mice infected with MuLV. On the other hand, the disease-free group of mice treated with SNMC showed an almost normal pattern similar to that of control mice. In other words, the granulocytosis and massive expansion of CD3^{int} cells were always seen as parallel events after MuLV infection.

DISCUSSION

In the present study, we demonstrated that glycyrrhizin has paradoxical effects: the increase of granulocytes and extrathymic T cells in number when injected into normal mice, and their decrease in number when injected into mice with preexisting granulocytosis. It is presumed that the present phenomenon is intimately associated with the characteristics of this Chinese medicine seen in clinical use. Namely, glycyrrhizin is used for the treatment of

allergic diseases such as urticaria, atopic dermatitis, and acute hepatitis. In these diseases, an elevated level of lymphocytes and a decreased level of granulocytes are primarily seen. On the other hand, glycyrrhizin is sometimes effective for the treatment of suppurative diseases (or granulocyte-associated diseases), including the chronic phase of viral infections (e.g., chronic active hepatitis and AIDS), periproctal abscess, and gastric ulcer.[17-22] In these diseases, an elevated level of granulocytes and a decreased level of lymphocytes are inversely seen. It is concluded that glycyrrhizin has a normalization effect on the host immune system.

In answer to the question of why one substance has such contrary effects on the immune system in hosts, the following speculation is possible. Many Chinese medicines, hormones (especially steroid hormones), and NO are intermediate oxidized substances themselves and, therefore, function as both oxidants and reductants. If they function as oxidants, they simulate the sympathetic nervous system and result in granulocytosis. This is due to adrenergic receptors on granulocytes.[1-7] However, if they function as reductants, they stimulate the parasympathetic nervous system and result in lymphocytosis. This is due to cholinergic receptors on lymphocytes.[9-14] This speculation seems to be reasonable if we consider NO. NO itself is an oxidant, stimulates the tissue function, and induces granulocytosis or tissue damage when injected or produced at a high concentration.[26-28] On the other hand, NO becomes a reductant (i.e., $NO \rightarrow NO_2 \rightarrow NO_3^-$), protects against tissue damage, and induces lymphocytosis when injected at chronic low doses. In the case of glycyrrhizin, if it is injected into mice with sympathetic nerve strain (i.e., granulocytosis), it tends to act as a reductant in the body. Under such conditions, many free radicals and superoxides are present (i.e., symathetic nerve dominance). The intermediate oxidized drugs could further absorb such free radicals and superoxides.

As a rule, the levels of granulocytes and extrathymic T cells increase or decrease simultaneously. This is because both of them carry a high level of adrenergic receptors on the surface.[8] Inversely, when granulocytes and extrathymic T cells decrease, conventional T cells and B cells increase. There is a tendency for primitive leukocytes to carry a higher level of adrenergic receptors, while phylogenetically developed leukocytes carry a higher level of cholinergic receptors on their surface. It seems that their inverted regulation by autonomic nervous system may be a very important factor in the host defense system of animals and humans.

In any case, it should be understood that many drugs, hormones, cytokines, etc., have varying characteristics as intermediate oxidative states. Therefore, depending on the conditions of the host and on the doses administered, they have the ability to function as either oxidants or reductants. This is the first report of a Chinese medicine having inverted functions in the host immune system as shown by the indicators of granulocytes and lymphocytes.

Finally, we confess that *in vitro* experiments investigating whether glycyrrhizin directly moduletes the differentiation of granulocytes from their precursors remain to be done. Since there are some reports (written in Japanese) about the *in vitro* effects of glycyrrhizin on immunologic functions (e.g., PHA response), such *in vitro* experiments in the presence or absence of G-CSF should be carried out in a future study.

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