Immunomodulation by Gold Sodium Thiomalate in Mice

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ABSTRACT

Immunomodulatory effects of gold sodium thiomalate (gold), which is used as a therapeutic agent for rheumatoid arthritis, were examined in mice. Ten milligrams of gold was intraperitoneally administered once a week and the administration was continued up to 3 months. The number of lymphocytes yielded by the liver, spleen, and thymus decreased during the administration, especially in the early stage (up to one month). When the administration was continued up to 3 months, only accelerated thymic atrophy remained. The phenotypic characterization of lymphocytes after long-term administration (3 months) revealed that the most prominent change was a decrease in the proportion of conventional T cells (CD3⁺IL-2Rβ⁺) in various immune organs, including the liver and spleen. On the other hand, the proportion of extrathymic T cells (CD3⁺IL-2Rβ⁺) and NKT cells (CD3⁺NK1.1⁺) tended to increase due to the administration of gold. Other changes included a decrease in the proportion of granulocytes in all tested organs. The RT-PCR method confirmed that the relative increase in the sign of Vα14 mRNA which is used by NKT cells became prominent as a result of the administration. Moreover, functional activation of NKT cytotoxicity was induced at that time. These results suggest that gold mediates the immunomodulation in a different manner depending on lymphocyte subsets.

Gold sodium thiomalate (gold) is a widely used therapeutic agent for patients with rheumatoid arthritis. Various effects of gold treatment on the host defence system or the immune system of patients have been reported, for example, decreased release of lysosomal enzymes, altered activity of phagocytic cells, decreased synthesis of prostaglandins, and altered response of lymphocytes (3-6). Similar to the suspicion regarding the clinical efficacy of gold treatments (14), no definite information on the effects of gold on the host defence system or the immune system is available.

To more definitely determine the immunomodulatory effect of gold, we intraperitoneally administered gold into mice and examined the number, phenotype, and function of lymphocytes in various immune organs. The resulting immunomodulatory effects of gold were various, depending on lymphocyte subsets and on the immune organs. These results may be intimately associated with the indefinite effects of gold on the immune system.

MATERIALS AND METHODS

Mice and Gold Administration

C57BL/6 (B6) at the age of 8 to 20 weeks were used. These mice were originally purchased from Charles River Japan, Tokyo, Japan and maintained in the animal facility of Niigata University. Gold sodium thiomalate (10 mg/mouse) (Shionogi Pharmaceutical Co., Osaka,
Japan) was intraperitoneally administered once a week into the mice. In the case of the long-term administration, gold administration was continued for 3 months. At 3 days after the final administration, mice were sacrificed to examine the immunoparameters.

**Cell Preparation**

Mice anesthetized with ether were sacrificed by total exsanguination from incised axillary arteries and veins (13). The liver was removed, cut into small pieces with scissors, pressed through 200-gauge stainless steel mesh, and 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 once with medium, the cells were resuspended in 35% Percoll solution (Pharmacia Fine Chemicals, Piscataway, NJ) containing 100 U/mL heparin and centrifuged at 2000 rpm for 15 min at room temperature. The resulting pellet, which contained liver mononuclear cells (MNC), was resuspended in red blood cell (RBC) lysis solution (155 mM NH₄Cl, 10 mM KHCO₃, 1 mM EDTA-Na, 170 mM Tris, pH7.3). After being washed twice with the medium, the liver MNC were suspended in 1 mL of medium and the number of cells was counted. Thymocytes were obtained by forcing the thymus through 200-gauge steel mesh. Splenocytes were obtained by pressing the spleen through the steel mesh, followed by pellet treatment with a erythrocyte lysing solution.

**Immunofluorescence Tests**

The surface phenotype of cells was analyzed using monoclonal antibodies (mAbs) in conjunction with a two-color immunofluorescence test (15). The mAbs used here included fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, or biotin conjugated reagents of anti-CD3 (145-2C11), anti-IL-2Rβ (TM-β1), anti-NK1.1 (PK136), anti-CD4 (RM4-5), anti-CD8 (53-6.7), anti-Gr-1 (RA3-8C5), and anti-Mac-1 (M1/70) mAbs (PharMingen Co., San Diego, CA, USA). To prevent nonspecific binding of mAbs, pretreatment of cells with mAb against CD16/32 (2.4G2) (PharMingen) was always performed (i.e., the blocking of Fc receptors). Biotin-conjugated reagents were developed with PE-conjugated streptavidin (Becton-Dickinson, Mountain View, CA). The fluorescence positive cells were analyzed with a FACScan using LYSIS II software (Becton-Dickinson). Dead cells were excluded by forward scatter, side scatter, and PI gating.

**Cytotoxicity Assay**

Target cells for NKT cytotoxicity were thymocytes of syngeneic B6 origin (8). Thymocytes are NK-resistant but sensitive to TCRαβ cells. Cytotoxic activity was measured by a specific ⁵¹Cr-release assay. Labeled targets (2 × 10⁴/well) were incubated in a total volume of 200 µL with effectors in RPMI1640 medium supplemented with 10% FCS in a 96-well round-bottomed microculture plate. Incubation for 4 h was performed.

**RT-PCR Assay for Detection of mRNAs of Va14 Gene**

To detect mRNA of the Va14 gene, RNA was reversely transcribed (RT) using the oligo(dT)₁₈ primer, and such cDNA was further amplified by the PCR method as previously described (10). Briefly, total RNA was prepared from MNC of the liver and spleen by an acid guanidinium thiocyanate-phenol-chloroform method. cDNA was synthesized using 1 μg RNA with a first-strand cDNA synthesis kit (Clontech Laboratories, Palo Alto, CA).

For PCR amplification, 2 μL of cDNA was transferred to individual tubes that contained 10 pM of the primers for Va14 gene, Taq DNA polymerase (2.5 U; Toyobo Co., Osaka, Japan), dNTP (200 µM) in 10×PCR buffer (Toyobo Co.), and MgCl₂ (2 mM). Oligonucleotide primers used for PCR amplifications were Va14-5', 5'-TAAGCACAGCAGCTGCACA-3'; and CA, 5'-TGTTCCTGAGACCGAGGATC-3'. The samples were heated at 94°C for 5 min to denature DNA/RNA duplexes and then subjected to 30 amplification cycles of denaturation for 45 sec at 94°C, annealing for 45 sec at 60°C, and extension for 90 sec at 72°C, followed by a final elongation step for 7 min at 72°C, in a thermal cycler (Perkin-Elmer, Norwalk, CT).

**RESULTS**

**Immunosuppression Induced by the Administration of Gold**

We applied two protocols for the administration
of gold (Fig. 1). In the case of the short-term administration of gold (10 mg/week/mouse), the administration was conducted for a month. On the third day after the final administration, the number of lymphocytes were enumerated in various organs; the number of lymphocytes yielded by the liver, spleen, and thymus was observed to be decreased one month after the initial administration. The decreased level was especially prominent in the liver and thymus.

Administration (10 mg/week/mouse) was then conducted for a three month period (Fig. 1 right). Interestingly, the decrease in the number of lymphocytes disappeared in the liver and spleen. In the case of thymocytes, an age-associated decrease of the number was seen in control mice. An accelerated decrease in the number of thymocytes was induced in mice administered with gold.

**Phenotypic Characterization of Lymphocytes in Mice Administered with (Long Term) or without Gold**

Two-color staining for CD3 and IL-2Rβ was first conducted to identify NK cells (CD3IL-2Rβ⁺), extrathmic T cells (CD3highIL-2Rβ⁺) and conventional T cells (CD3high IL-2Rβ⁻) (Fig. 2). Two-color staining for CD3 and NK1.1 was conducted to identify NKT cells (11). Two-color staining for Mac-1 and Gr-1 was finally conducted. With the short-term administration of gold, no prominent changes at all were induced in comparison with control mice (data not shown).

The results from the long-term administration of gold were then represented. The most prominent change was seen in the proportion of conventional T cells both in the liver and spleen, namely, the decrease in the proportion of CD3highIL-2Rβ⁻ cells. The proportion of NK cells and extrathmic T cells was almost unchanged or slightly increased. NKT cells were observed to increase in the liver. The proportion of granulocytes decreased in the liver of mice administered with gold.

To confirm the increased level of NKT cells in the liver, the RT-PCR method was applied to detect Vα14 mRNA, which is preferentially used by NKT cells (Fig. 3). A prominent sign of Vα14 mRNA was induced in liver lymphocytes isolated from mice administered with gold.

**Functional Activation of NKT Cells in the Liver by the Administration of Gold**

Since the proportion of NKT cells in the liver increased in mice administered with gold, we examined whether functional activation accompanied this phenomenon. NKT cytotoxicity against syngeneic thymocytes was examined in mice administered with or without gold (Fig. 4). A prominent increase in the NKT cytotoxicity was demonstrated in mice administered with gold.

![Graph](image1.png)

**Fig. 1** Number of lymphocytes yielded by the liver, spleen, and thymus in mice administered or not administered with gold. Mice were administered with gold (10 mg/mouse) once a week for one month (short-term administration) and for up to 3 months (long-term administration). The mean and one SD of five mice are represented.
Fig. 2 Phenotypic characterization of lymphocytes in various immune organs of mice administered or not administered with gold. The administration was continued for 3 months. Two-color staining for CD3 and IL-2Rβ, that for CD3 and NK1.1, and that for Mac-1 and Gr-1 were conducted. Numbers in the figure are the percentages of fluorescence-positive cells in corresponding areas. The data shown here are representative of three experiments.

DISCUSSION

In the present study, we examined how the administration of gold modulated the immune system. Short-term administration (up to one month) induced immunosuppression in a nonspecific manner. Thus, the yield of lymphocytes by the liver, spleen, and thymus decreased, but phenotypic change of lymphocyte subsets was not seen in any organs. In sharp contrast, the long-term administration of gold induced unique immunomodulation, depending on leukocyte populations and lymphocyte subsets. The major change was the activation of extrathymic T cells (CD3IL-2Rβ+) in the liver. It is known that extrathymic T cells comprise both NK1.1+ and NK1.1− subsets (14), the NK1.1+ subset being called NKT cells (2,7). This NKT cell population was also found to increase in proportion in the liver of mice administered with gold. On the other hand, the proportion and the number of conventional T cells (CD3IL-2Rβ−) tended to decrease in almost all tested organs. These results suggest that the administration of gold induces unique immunomodulation depending on lymphocyte subsets.

Additional evidence indicating the involvement of the leukocyte populations was a decrease
in the proportion and number of granulocytes in the liver, spleen, and bone marrow. In the case of patients with rheumatoid arthritis, granulocyte-associated inflammation is known to occur in the joints (1). It is therefore speculated that the immunomodulation effect of gold on granulocytes may be important in the subsidence of joint inflammations in the patients. We previously showed that over-activated granulocytes are intimately associated with inflammation due to their release of superoxides (9).

The activation of extrathymic T cells and NKT cells was confirmed by the augmented sign of Vα14 mRNA of liver lymphocytes and by the augmented NKT cytotoxicity. In the case of mice, the majority of NKT cells use an invariant chain of Vα14Jα281 for TCRα(2, 7). At present, we do not know whether or not this effect of gold on NKT cells is beneficial for the treatment of rheumatoid arthritis. Namely, NKT cells are known to be immunoregulatory cells for the onset of certain autoimmune diseases (12, 16) but inversely they have an ability to induce tissue damage by their autoreactivity against self-cells (11). We cannot deny the possibility that some effects of gold are beneficial for the treatment of rheumatoid arthritis, although it is evident that other effects of gold worsen the disease.

Finally, definite effects of gold were seen as a profound immunosuppression against conventional T cells in the periphery. The proportion and the number of conventional T cells seen in the periphery might be induced due to the arrest in the mainstream of T cell differentiation in the thymus. Thus, both short- and long-term administration of gold was found to induce prominent thymic atrophy. Primarily, it is still controversial whether the immunomodulatory effect of gold is beneficial for the treatment of patients with rheumatoid arthritis. Namely, we were not able to obtain consistent good results for the patients by using gold in the clinic. In this regard, further study is necessary to determine whether the activation of NKT cells by gold is beneficial for the reduction of inflammation of the joints. However, the immunosuppressive effect of gold on granulocytes and conventional T cells may participate in the reduction of the inflammation of the joint in patients with rheumatoid arthritis.

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REFERENCES


