

Gold sodium thiomalate selectivity inhibits interleukin-5-mediated eosinophil survival

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Gold sodium thiomalate (GST) has been used for treatment of patients with bronchial asthma. In this study we investigated the effects of GST on interleukin (IL)-5-mediated eosinophil survival in vitro. Blood cells were obtained from patients with bronchial asthma (n = 18). Eosinophils were purified from the blood samples by a Percoll discontinuous method and negative selection of neutrophils. Eosinophils (10^6 cells/ml) were incubated for 96 hours in 10% fetal bovine serum-RPMI 1640 (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) with various concentrations of recombinant human IL-5 (2×10^{-9} to 2×10^{-5} mmol/L) and with GST (0.01 to 100 μ mol/L) in 96-well, flat-bottomed microtiter plates at 37° C in a humidified 5% carbon dioxide atmosphere. Eosinophil viability was determined by trypan blue exclusion. IL-5 enhanced eosinophil survival in a dose-dependent manner. Higher concentrations of GST inhibited IL-5-mediated eosinophil survival. Moreover, GST induced apoptosis of eosinophils by antagonizing the effects of IL-5. However, GST did not inhibit IL-3- or granulocyte-macrophage colony stimulating factor-mediated eosinophil survival, suggesting that GST selectivity inhibits IL-5-mediated eosinophil survival. These results suggest that IL-5-mediated eosinophil survival is inhibited by GST and that GST blocks eosinophil apoptosis by IL-5. It is possible that GST may be capable of controlling eosinophil functions regulated by IL-5 in patients with bronchial asthma. (J ALLERGY CLIN IMMUNOL 1995;96:251-6.)

Key words: Bronchial asthma, eosinophils, interleukin-5, gold sodium thiomalate, apoptosis

Eosinophils play a prominent role in many inflammatory and allergic diseases. Eosinophils are considered to be main effector cells in the pathogenesis of bronchial asthma, destroying bronchial epithelium by eosinophilic cationic protein or major basic protein.¹⁻⁶ Various functions of eosinophils are known to be regulated by various cytokines such as granulocyte-macrophage colony stimulating factor (GM-CSF), interleukin (IL)-3, IL-5, and interferon- γ .⁷⁻¹¹ IL-5 has highly selective effects on eosinophils, such as differentiation, adhesion, effector functions, and survival.⁸⁻¹² IL-5 has therefore been considered the most important cytokine in the regulation of eosinophils.

Abbreviations used

FBS:	Fetal bovine serum
GM-CSF:	Granulocyte-macrophage colony stimulating factor
GST:	Gold sodium thiomalate
IL:	Interleukin

Serum levels of IL-5 are elevated in patients with bronchial asthma.¹³ One might speculate that inhibition or reduction of the effect of IL-5 on eosinophils may be beneficial in treating bronchial asthma. It has been reported that gold salts are relatively effective in the treatment of patients with bronchial asthma.¹⁴ Originally, gold salts were used to treat rheumatoid arthritis.¹⁵⁻¹⁷ To date, few reports concern the direct effect of gold salts on human cellular immunity, and fewer still on the effect on eosinophils. In this study the effects of gold sodium thiomalate (GST), one type of gold salt preparation, on IL-5-mediated eosinophil survival were investigated in vitro.

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METHODS

Purification of eosinophils

Informed consent was obtained from all subjects participating in the following studies. Heparinized venous blood was obtained from patients with bronchial asthma ($n = 18$) with moderate eosinophilia (range, 6% to 15% eosinophils; median, 10%). Patients did not have symptoms and had not taken any medication at the time of the study. The granulocyte layer was isolated from the blood by centrifugation for 15 minutes at 1800 g on a Ficoll-Hypaque gradient (Nycomed Pharma AS, Oslo, Norway). Contaminating erythrocytes were lysed, and the samples were washed in RPMI 1640 (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) with media containing 10% fetal bovine serum (FBS) (Cell Culture Laboratories, Cleveland, Ohio) (10% FBS-RPMI-1640). Eosinophils were purified from the samples by a Percoll (Pharmacia, Uppsala, Sweden) discontinuous method with gradient media (specific gravity: 1.100, 1.095, 1.090, 1.085, 1.080, and 1.070 gm/ml), as described by Gärtner.¹⁸ Eosinophils were collected from the layer between 1.100 and 1.095 gm/ml bands. Eosinophils (~80% pure after the initial purification) were resuspended in Hanks' balanced salt solution (Nissui Pharmaceutical). To remove contaminating neutrophils, negative selection was performed with immunomagnetic beads (Dynabeads M-450, sheep anti-mouse IgG, Dynal, Oslo, Norway) with an anti-CD16 monoclonal antibody (MG38, Nichirei, Tokyo, Japan).¹⁹ Then eosinophils (~95% purity on a Wright-Giemsa-stained cytocentrifuge preparation) were collected, and viability was examined by trypan blue exclusion (98% living eosinophils). The eosinophils were then washed twice in 10% FBS-RPMI-1640, and the number of cells was adjusted to 10^6 cells/ml in 10% FBS-RPMI 1640 for use in the following experiments.

Eosinophil survival assay

Purified eosinophils (10^6 cells/ml) were incubated in 10% FBS-RPMI 1640 with various concentrations of recombinant human IL-5 (2×10^{-9} to 2×10^{-5} mmol/L) (Genzyme, Cambridge, Mass.) and with GST (0.01 to 100 μ mol/L) (Shionogi Pharmaceutical Co. Ltd., Osaka, Japan) in 96-well, flat-bottomed microtiter plates (Nunc, Roskilde, Denmark) at 37° C in a humidified 5% carbon dioxide atmosphere. Control cultures of purified eosinophils were incubated in 10% FBS-RPMI 1640 alone. At the end of the culture periods (after 24, 48, 72, and 96 hours) the viability of eosinophils was determined by trypan blue exclusion. The percentage of eosinophil viability was calculated as follows: $[\text{No. of Viable Cells} \div (\text{No. of Viable Cells} + \text{No. of Dead Cells})] \times 100\%$. Each experiment was performed in duplicate.

Analysis of DNA fragmentation taken from eosinophils

Aliquots of purified eosinophils (10^6 cells) were incubated for 72 hours in 10% FBS-RPMI 1640 with either 2×10^{-6} mmol/L IL-5 plus 10 μ mol/L GST, 2×10^{-6} mmol/L IL-5 only, or medium alone at 37° C in a 5% carbon dioxide atmosphere. DNA fragmentation in the eosinophils was

detected by a method previously described for detecting apoptosis in thymocytes.²⁰ Briefly, the eosinophils were washed with cold phosphate-buffered saline solution and lysed with a buffer of 100 mmol/L NaCl, 50 mmol/L Tris, and 500 mmol/L ethylenediaminetetraacetic acid (pH 8.0) containing 0.5% sodium dodecylsulfate and 0.5 mg/ml proteinase K (Sigma Chemical Co., St. Louis, Mo.). After incubation at 50° C for 5 hours, a 10 μ l portion of 0.5 mg/ml ribonuclease (deoxyribonuclease-free) solution (Nippon Gene, Tokyo, Japan) was added to each sample, and the samples were incubated for 3 hours at 37° C. DNA was extracted with phenol-chloroform and precipitated with ethanol. DNA samples were then loaded on a 1.2% agarose gel containing 10 mg/ml of ethidium bromide, electrophoresed in a buffer of (90 mmol/L Tris, 90 mmol/L borate, and 2 mmol/L ethylenediaminetetraacetic acid) at 50 V for 90 minutes, and viewed under UV light. DNA extracted from murine thymocytes, in which spontaneous apoptosis was observed, was loaded as a positive control.

Statistical analysis

Data are shown as mean $\pm$ SD and were analyzed by paired or unpaired Student t test. A p value less than 0.05 was considered to be statistically significant.

RESULTS

Influence of GST on IL-5-mediated eosinophil survival in vitro

To assess the effect of IL-5 on eosinophil survival, eosinophils were incubated in 10% FBS-RPMI 1640 with various concentrations of IL-5. We observed increased survival of eosinophils that was dependent on the dose of IL-5 and reached a plateau at a concentration of 2×10^{-6} mmol/L IL-5. When eosinophils were incubated for 72 hours in medium with IL-5 at concentrations ranging from 2×10^{-9} to 2×10^{-5} mmol/L or without IL-5, the viability was $48.3\% \pm 3.4\%$, $58.3\% \pm 5.4\%$, $72.3\% \pm 6.2\%$, $71.3\% \pm 5.6\%$, $70.9\% \pm 3.5\%$, or $33.8\% \pm 4.5\%$, respectively (mean $\pm$ SD, $n = 6$) (Fig. 1).

Next we observed the effects of GST on eosinophil survival in the absence of IL-5. When eosinophils were incubated for 72 hours with GST at concentrations of 0.01, 0.1, 1, 10, and 100 μ mol/L and without GST, the viabilities were $33.6\% \pm 3.5\%$, $34.5\% \pm 4.2\%$, $32.8\% \pm 4.3\%$, $34.8\% \pm 5.1\%$, $34.3\% \pm 3.7\%$ or $33.8\% \pm 4.5\%$, respectively (mean $\pm$ SD, $n = 6$) (data not shown). Thus GST had no direct effect on eosinophil survival.

Finally, we studied the effect of GST on 2×10^{-6} mmol/L IL-5-mediated eosinophil survival. When eosinophils were incubated for 72 hours with IL-5 and GST at concentrations of 0.01, 0.1, 1, 10, and 100 μ mol/L, and without GST, the viability of eosinophils was $56.5\% \pm 8.5\%$, $50.4\% \pm 7.9\%$, $43.8\% \pm 9.8\%$, $38.5\% \pm 9.6\%$, $36.4\% \pm 9.6\%$, and

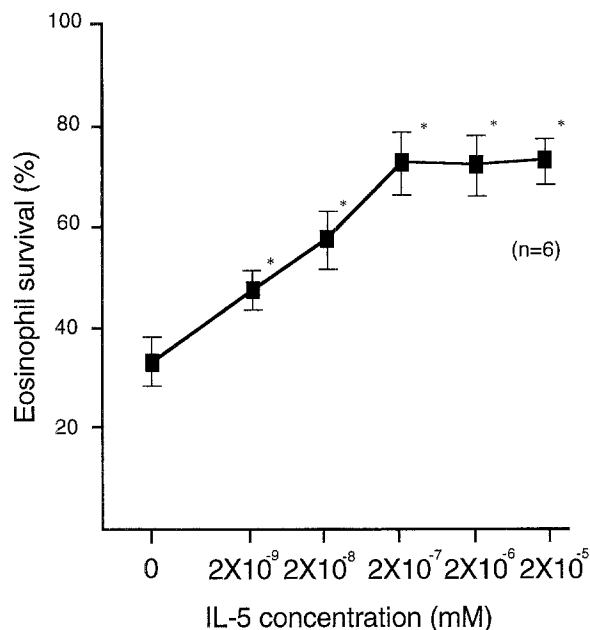


FIG. 1. Effect of IL-5 on eosinophil survival. Eosinophils were incubated for 72 hours with IL-5 at the concentrations indicated. Results are expressed as mean $\pm$ SD of six samples. Asterisk indicates significant increase in the survival rate of eosinophils incubated with IL-5 as compared with replicate cells incubated under the same conditions without IL-5 ($p < 0.01$).

72.6% $\pm$ 4.7%, respectively (mean $\pm$ SD, $n = 8$) (Fig. 2). When eosinophils were incubated for 24, 48, 72, and 96 hours with IL-5 and GST (10 μ mol/L), a concentration similar to the plasma concentration in human beings treated with GST,²¹ viability was significantly decreased compared with incubation with IL-5 alone (Fig. 3). These results demonstrate that higher concentrations of GST inhibit IL-5-mediated eosinophil survival in vitro. GST did not inhibit IL-3- or GM-CSF-mediated eosinophil survival from the same donors on the same day (Table I).

Influence of GST on eosinophil apoptosis

As visualized by agarose gel electrophoresis, DNA extracted from eosinophils that were incubated for 72 hours in medium alone showed a typical apoptotic "ladder" pattern of fragmentation, in which prominent fragments less than 1000 base pairs were observed with decrements of approximately 200 base pairs. In contrast, this pattern of fragmentation was not observed in IL-5-supplemented cell cultures. However, DNA extracted from replicate eosinophils incubated with IL-5 and GST showed a clear apoptotic ladder pattern (Fig. 4). These results demonstrate that GST inhibits the ability of IL-5 to prevent apoptosis in eosinophils.

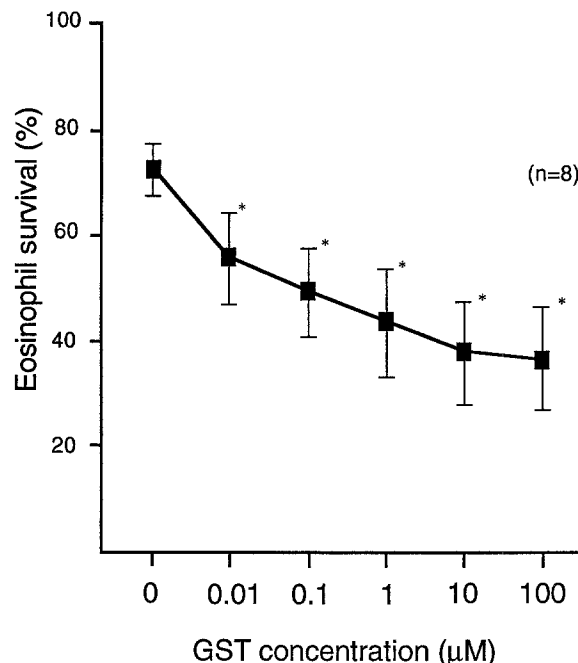


FIG. 2. Eosinophil survival mediated by 2×10^{-6} mmol/L IL-5 is inhibited by GST. Eosinophils were incubated for 72 hours with 2×10^{-6} mmol/L IL-5 and the indicated concentrations of GST. Results are expressed as mean $\pm$ SD of eight samples. Asterisk indicates statistically significant decrease in the survival rate of eosinophils incubated with 2×10^{-6} mmol/L IL-5 and GST, as compared with replicate eosinophils incubated under the same conditions with 2×10^{-6} mmol/L IL-5 alone ($p < 0.01$).

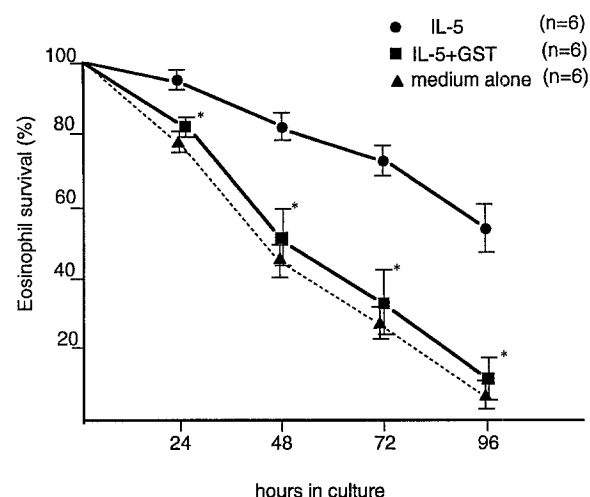


FIG. 3. Effect of GST on IL-5-mediated eosinophil survival. Eosinophils were incubated with 2×10^{-6} mmol/L IL-5 and 10 μ mol/L GST. Survival rate of eosinophils was assessed after 24, 48, 72, and 96 hours in culture. Results are expressed as mean $\pm$ SD of six samples. Asterisk indicates significant decrease in survival rate of eosinophils incubated with 2×10^{-6} mmol/L IL-5 and 10 μ mol/L GST, as compared with replicate eosinophils incubated under the same conditions with 2×10^{-6} mmol/L IL-5 alone ($p < 0.01$).

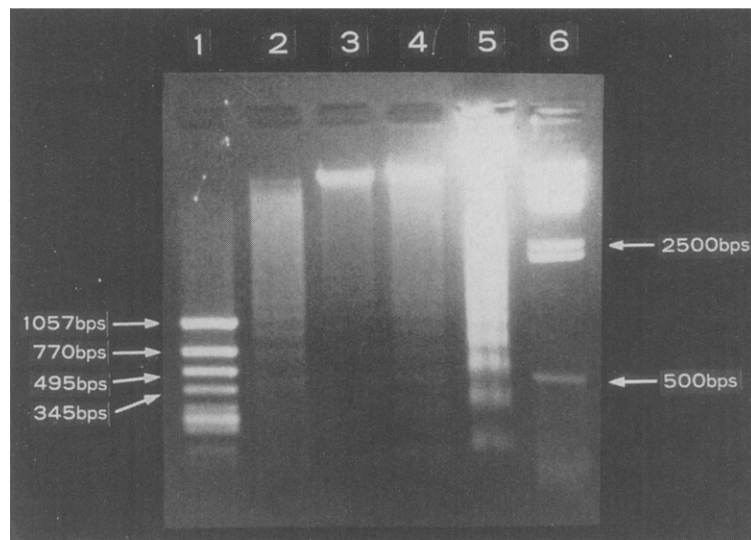


FIG. 4. Representative result of 1.2% agarose gel electrophoresis of DNA extracted from eosinophils. Lanes 1 and 6 are λ DNA markers. Lane 2, DNA from eosinophils incubated for 72 hours in medium alone. Lane 3, DNA from eosinophils incubated with 2×10^{-6} mmol/L IL-5. Lane 4, Eosinophils incubated with 2×10^{-6} mmol/L IL-5 and 10 μ mol/L GST. Lane 5 was DNA extracted from murine thymocytes as a positive control.

TABLE I. Effect of GST (10 μ mol/L) on eosinophil survival mediated by IL-5 (2×10^{-6} mmol/L), IL-3 (50 ng/ml), or GM-CSF (1 ng/ml)

Culture time (hr)	Eosinophil viability (%)						
	Control	IL-5	IL-5 with GST	IL-3	IL-3 with GST	GM-CSF	GM-CSF with GST
24	78.8 $\pm$ 2.6	92.6 $\pm$ 1.8	81.5 $\pm$ 3.4	90.6 $\pm$ 1.4	88.3 $\pm$ 2.4	91.7 $\pm$ 2.1	89.2 $\pm$ 2.8
48	49.2 $\pm$ 5.6	83.7 $\pm$ 2.4	53.8 $\pm$ 6.2	84.4 $\pm$ 4.8	85.2 $\pm$ 4.1	85.8 $\pm$ 3.6	82.1 $\pm$ 3.2
72	32.1 $\pm$ 4.8	75.4 $\pm$ 4.6	39.4 $\pm$ 4.4	76.2 $\pm$ 4.2	74.7 $\pm$ 6.2	78.6 $\pm$ 3.4	75.9 $\pm$ 4.8
96	8.6 $\pm$ 2.4	57.4 $\pm$ 3.8	13.5 $\pm$ 5.6	54.5 $\pm$ 6.4	53.1 $\pm$ 5.8	56.8 $\pm$ 2.8	57.3 $\pm$ 4.3

Results are expressed as mean $\pm$ SD of three samples, from same donor, taken on the same day. GST inhibited IL-5-mediated eosinophil survival. However, GST did not affect IL-3- or GM-CSF-mediated eosinophil survival.

DISCUSSION

IL-5 is considered to be the most important cytokine in the regulation of eosinophil function.^{8,13} Among its various functions, IL-5 is known to prolong eosinophil survival by preventing programmed cell death, or apoptosis.²²⁻²⁴ In this study we showed that GST (concentrations of 0.01 to 100 μ mol/L) inhibits IL-5-stimulated eosinophil survival without having a direct toxic effect on eosinophils. Moreover, when eosinophils were incubated for 72 hours with IL-5 (2×10^{-6} mmol/L) and GST (10 μ mol/L) apoptosis was observed. These results indicate that GST induces eosinophil apoptosis by antagonizing the effects of IL-5.

Apoptosis is characterized by the degradation of

DNA into oligonucleosome-sized fragments and has been observed in glucocorticoid-induced death of immature thymocytes.²⁵ Recently Savill et al.^{22,26} reported that neutrophils and eosinophils are destined to be excluded naturally by apoptosis. Although it remains unclear how eosinophil apoptosis is regulated in vivo, several investigators have reported that IL-5 prevents eosinophil apoptosis in vitro.²²⁻²⁴ The present studies confirm that IL-5 is capable of preventing apoptosis of eosinophils in vitro. Concerning the mechanisms by which IL-5 inhibits eosinophil apoptosis, Yamaguchi et al.²⁴ reported that IL-5-mediated eosinophil survival is associated with newly synthesized proteins induced by IL-5. In their report they revealed that actino-

mycin D, a transcription inhibitor, or cycloheximide, a protein synthesis inhibitor, abrogates the inhibition of eosinophil apoptosis by IL-5. The IL-5-induced proteins associated with eosinophil survival have not yet been characterized.

Gold salts have been accepted as antiinflammatory agents and have been used for treatment of patients with bronchial asthma since the 1930s.¹⁴ Gold salts inhibit inflammatory processes, such as phagocytosis by inflammatory cells, activation of macrophage lysosomal enzymes, and some types of triggers for lymphocyte DNA synthesis.^{21, 27-29} Recently, gold salts have been reported to exert immunomodulatory effects on T cells and macrophages.³⁰⁻³² Gold salts inhibit the proliferative responses of T cells stimulated by IL-2.³¹ Sfikakis et al.³² showed that the inhibitory effect of gold salts was associated with the suppression of both IL-2 and IL-2-receptor messenger RNA accumulation in T cells. In this study we showed that GST blocks the inhibition of eosinophil apoptosis by IL-5. It is possible that GST exhibits an inhibitory effect on the expression of the IL-5 receptor in eosinophils by a mechanism similar to that in T cells. With regard to the effect of gold salts on patients with bronchial asthma, it has been proposed that it inhibits the release of histamine in an anaphylactic reaction and reduces serum IgE levels in patients with asthma.³³ Nevertheless, the influence of GST on the pathophysiology of bronchial asthma remains obscure.

Several attempts have been carried out with various antiinflammatory agents to inhibit the effect of IL-5 on eosinophils. For example, it was shown that glucocorticoids inhibit IL-5-mediated eosinophil survival by blocking inhibition of eosinophil apoptosis.²² Although there are many differences in the chemical structures of GST and glucocorticoids, it is interesting to note that GST, like glucocorticoids, inhibits eosinophil survival prolonged by IL-5. With regard to the inhibitory effect of glucocorticoids on IL-5-mediated eosinophil survival,^{34,35} Lamas et al.³⁵ proposed that a steroid-dependent activation of an endonuclease that degrades nuclear DNA might exist in eosinophils. They also suggested that glucocorticoids might antagonize the actions of GM-CSF or IL-5 by stimulating degradation of the cytokines, reducing the number or function of cytokine receptors, or interfering with intracytoplasmic signaling events that occur after binding to the receptors. On the basis of our results, we speculated that GST may have an influence on intracytoplasmic signal transduction or protein synthesis in eosinophils.

Moreover, it is interesting that GST exerts selective inhibitory action on IL-5-mediated eosinophil survival. These results may reveal that differences exist in the structures of IL-3/GM-CSF and IL-5 receptors on eosinophils or that some discrepancies exist in the intracytoplasmic signaling events that occur after binding to each receptor.

Clinically it might be expected that an agent capable of controlling eosinophil activation by a cytokine such as IL-5 would be effective in the treatment of bronchial asthma. Future studies should investigate the effects of GST on lymphocytes and macrophages of patients with asthma.

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