Effects of Gold on Cytokine Production *in Vitro*; Increase of Monocyte Dependent Interleukin 10 Production and Decrease of Interferon-γ Levels

JON LAMPA, LARS KLARESKOG, and JOHAN RÖNNELID

ABSTRACT. ***Objective***. To investigate the effects of gold salt on the differential production of proinflammatory and antiinflammatory cytokines *in vitro*.

Methods. Heparinized blood from 10 blood donors and 10 patients with polyarthritis was density separated and incubated with various concentrations of gold salt [Myocrisin®, gold sodium thiomalate (GSTM) plus phenyl mercury nitrate]. Cytokine production was measured after incubation for 16–20 h using an Elispot method detecting interleukin 10 (IL-10), IL-6, tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ) producing cells. In addition, parallel cell culture supernatants were collected and analyzed by ELISA for IL-10, IL-6, TNF-α, IFN-γ, and IL-2. In some cultures phytohemagglutinin A (PHA) was added in predefined concentrations.

Results. GSTM increased the number of cells producing IL-6 and IL-10 in a dose dependent manner, both with and without simultaneous addition of PHA. These effects were seen in samples from both healthy blood donors and patients with polyarthritis. The increase in IL-10 production was inhibited when monocytes were depleted. No effects of GSTM were seen on IFN-γ or TNF-α producing cells. Parallel supernatant cultures displayed a GSTM dose dependent decrease in IFN-γ levels after mitogen stimulation, whereas no changes were seen in IL-6 or TNF-α levels.

Conclusion. The differential effects of gold salt on cytokine production, with a marked stimulatory effect on IL-10 and IL-6, indicate that gold salt may act as a relatively selective immunostimulator rather than as a general immunosuppressant. (J Rheumatol 2002;29:21–8)

Key Indexing Terms:
RHEUMATOID ARTHRITIS GOLD INTERLEUKIN 10
INTERFERON MONOCYTES

Production of certain proinflammatory cytokines such as interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), and interleukin 1 (IL-1) constitutes an important part of the pathogenesis of polyarthritis in rheumatoid arthritis (RA).

Treatment with parenteral gold [presently Myocrisin®, gold sodium thiomalate (GSTM) plus phenyl mercury nitrate] is a very old but still used therapy for RA, which in selected cases can be remarkably efficient, even sometimes causing complete remission[1,2].

Several studies have implicated the suppressant effects of gold on T cell[3] and monocyte[4] function, as well as downregulation of production of several proinflammatory cytokines[5,6]. However, the existence of common side effects such as eosinophilia, and increase in IgE[7], often preceding the beneficial effects of the drug, suggests that the action of gold in RA may be more complex than immunosuppression alone. These observations make it reasonable to believe that, instead of suppressing immune functions by general inhibition of cytokine production, gold might have divergent effects on the production of both pro and antiinflammatory cytokines in RA. To explore this possibility we investigated the effects of GSTM on the differential production of cytokines in RA as well as in healthy individuals.

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MATERIALS AND METHODS

Patients and healthy blood donors. Ten patients (7 men and 3 women, median age 51 yrs, range 22–82) were included, 8 of whom were recruited from the early arthritis program at Karolinska Hospital. No patient had a history of treatment with disease modifying antirheumatic drugs (DMARD). The remaining 2 RA patients, recruited from the outpatient clinic, had not been treated with DMARD for at least 4 weeks. Of the early arthritis patients, all but one (diagnosed with seronegative polyarthritis) also subsequently fulfilled the American Rheumatology Association criteria for RA[8]. Healthy blood donors (7 men and 3 women, median age 51 yrs, range 29–66) were recruited through the transfusion unit at Karolinska Hospital. The ethics committee of northern Stockholm, Sweden, approved the study.

Cell preparation. Peripheral blood was collected into heparinized tubes and

diluted 1:2 with phosphate buffered saline. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation (Ficoll, Hypaque, Pharmacia Amersham, Uppsala, Sweden) and diluted to 1×10^6/ml in RPMI-1640 (Flow Laboratories, Irvine, Scotland, UK) supplemented with glutamine, HEPES buffer, penicillin, streptomycin, and 10% of a defined batch of fetal calf serum (Flow) (complete medium). Cell viability was assessed by trypan blue exclusion and always exceeded 95%. The gold compound used in the study was Myocrisin® (Aventis, Strasbourg, France), 1 ml consisting of 20 mg gold sodium thiomalate and 20 μg phenyl mercury nitrate (GSTM). The concentrations of GSTM in cell cultures were 0, 3, 12.5, and 40 μg/ml. Concentrations in this range have been used in previous *in vitro* studies and proven non-cytotoxic[9]. The lowest concentration in our study is in the same range as measured immediately before GSTM injection during weekly maintenance therapy[10].

Elispot analysis of cytokine-producing PBMC. Numbers of cytokine-producing cells were analyzed by Elispot according to our method using plastic ELISA plates[11] coated with primary antibody, 50 μl/well, 15 μg/ml overnight (16–20 h) at 4°C. PBMC were added (10^5 cells/well for IFN-γ, 2.5×10^4/well for IL-10, 4000/well for IL-6, and 500/well for TNF-α; duplicate wells) in complete medium and incubated overnight (16–20 h). For the detection of IFN-γ, phytohemagglutinin A (PHA) was added in titrated suboptimal concentrations. Wells were washed and biotinylated secondary antibodies were added at 1 μg/ml overnight. Avidine-alkaline phosphatase (Dako, Glostrup, Denmark) was added at a dilution of 1:250 and allowed to bind for 2 h and BCIP 710-3 (Sigma, St. Louis, MO, USA) was added for 5 h after washing to visualize the spots. The number of cytokine-producing cells was counted using an inverted microscope. Antibodies used were 1-D1K (MabTech, Stockholm, Sweden) and biotinylated 7-B6-1 (MabTech) for IFN-γ; 19F1 (Pharmingen, San Diego, CA, USA) and biotinylated 12G8[12] for IL-10, IL-6-I (MabTech) and biotinylated 39C3 (Pharmingen) for IL-6, and 20-A4[12] and biotinylated secondary antibodies derived from Duokit (Genzyme, Cambridge, MA, USA) for TNF-α.

Depletion studies. PBMC were prepared as described above and diluted in complete medium to 10^6 cells/ml. Dynabeads (Dynal®, Oslo, Norway) were used for the depletion of CD3, CD14, and CD19 positive cells, respectively. The resulting cell suspensions were incubated in duplicate wells with/without 40 μg/ml GSTM as described above and subsequently analyzed with Elispot for IL-10 production.

Analysis of cell culture supernatants. PBMC (see above) at a concentration of 10^6/ml were incubated in duplicate wells with different concentrations of GSTM, with or without PHA 0.5 μg/ml for 20–24 h. Cell culture supernatants were isolated and analyzed with ELISA for contents of IL-2, IL-6, IL-10, IFN-γ, and TNF-α. Dilutions of the supernatants 1/5 to 1/10 were used to optimize the results of the method. The same paired antibodies as for Elispot were used for ELISA measurements of IL-6, IL-10, TNF-α, and IFN-γ levels, whereas primary and secondary biotinylated antibodies (Duokit, Genzyme) were used for measurements of IL-2 in supernatants.

IL-10 levels were analyzed using an ultrasensitive ELISA (Bio-Source, Camarillo, CA, USA). In 3 subjects, cell viability was checked after incubation with 40 μg/ml GSTM, always exceeding 95%.

Statistics. Nonparametric methods (Wilcoxon signed rank test) were used throughout the study to record differences in cytokine production with various GSTM concentrations. The Mann-Whitney nonparametric test was used for comparisons between patients and healthy blood donors.

RESULTS

GSTM induces IL-6 and IL-10 production in PBMC from patients with polyarthritis and healthy blood donors. PBMC were isolated as described above and incubated 16–20 h with or without PHA and different concentrations of GSTM. Cytokine production was analyzed with Elispot. There was a dose-dependent increase in the IL-10 and IL-6 production (Figures 1a, 1c, 2a, 2c). The production of IFN-γ was not changed by the addition of GSTM (Figures 3a, 4a). Neither was the TNF-α production changed (data not shown). The results were similar with or without PHA and for both healthy blood donors and patients.

GSTM induced IL-10 production is monocyte dependent. Depletion studies were performed to determine which cell population is responsible for GSTM induced IL-10 production. Depletion of cells expressing CD19 had no influence on IL-10 production. In contrast, depletion of CD3 expressing cells reduced IL-10 production markedly; however, there was still increased IL-10 production when GSTM was added. Depletion of CD14 positive monocytes resulted in complete inhibition of IL-10 production, which could not be increased with added GSTM (Figure 5). The effect of GSTM on T cell dependent IL-10 production varied between different experiments, but the monocyte effect was always present.

GSTM reduces IFN-γ levels in supernatants in a dose-dependent manner. PBMC isolated as described above were incubated 16–20 h with or without PHA and different concentrations of GSTM. The supernatants were analyzed for cytokine content with ELISA. PHA stimulated IFN-γ levels were dose-dependently reduced with GSTM added (Figures 3b, 4b). IFN-γ was not detectable without PHA (data not shown). Also, PHA stimulated IL-2 levels were significantly decreased with GSTM ($p < 0.05$; data not shown). IL-6 levels (Figures 1d, 2d) and TNF-α levels (data not shown) were not changed. Similar results were also noted after PHA stimulation (data not shown). IL-10 concentrations with 3 respective 40 μg/ml GSTM were significantly reduced in the PBMC cultures of patients with polyarthritis (Figure 1b) but not for the healthy blood donors (Figure 2b).

DISCUSSION

To our knowledge this is the first study to show that gold has diverse effects on the production of pro- and antiinflammatory cytokines. We show that the numbers of IL-10 and IL-6 producing cells were dose dependently increased with GSTM added in cultures. However, GSTM did not affect the number of IFN-γ producing cells. The GSTM induced IL-10 production was monocyte dependent. The effect of the GSTM increased IL-10 production was studied in parallel supernatants. There was a dose-dependent decrease of IFN-γ levels in cell supernatants with GSTM, but the levels of IL-6 and TNF-α remained unchanged.

We used 2 parallel methods to investigate GSTM induced cytokine production *in vitro*. In the Elispot technique, which enumerates the number of cytokine producing cells, the cytokines are immediately bound to the detection surface directly after secretion from the producing cells. Thus, this technique mirrors actual cytokine production. The supernatant measurements by ELISA, on the other hand, detect the net cytokine concentrations. Theoretically, increased cellular activity causing increased cytokine production might result in

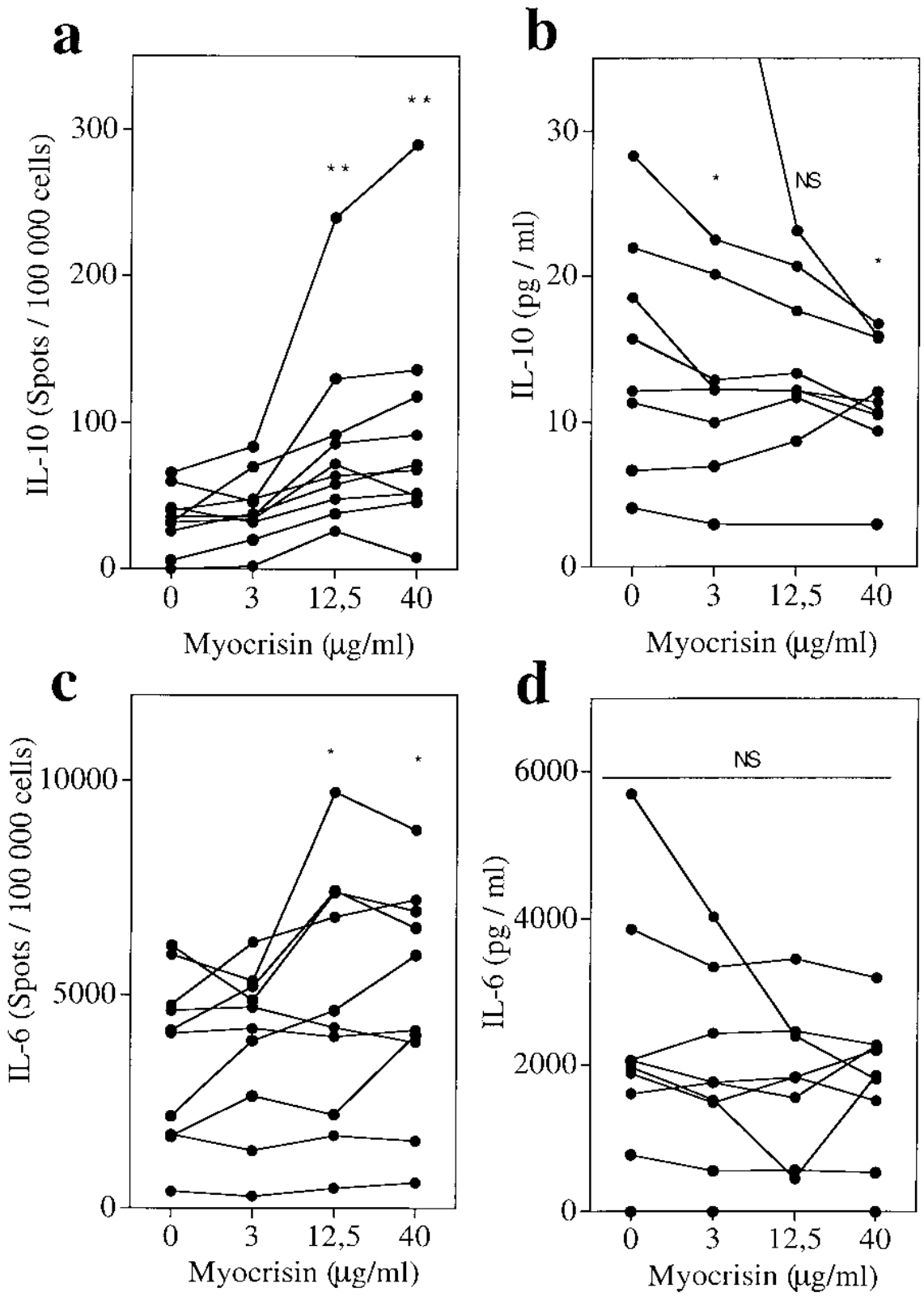

Figure 1. PBMC from polyarthritis patients (n = 10) were incubated 16–20 hours with different concentrations of GSTM (Myocrisin). In a and b effects on IL-10 production are displayed, whereas c and d depict influences on IL-6. Methods used for cytokine detection were Elispot (a, c) and ELISA of cell culture supernatants (b, d). In b, the highest lines represent values of 50 and 48 μg/ml for the GSTM (Myocrisin) concentrations 0 and 3 μg/ml. Data from one patient were excluded from the graph due to very high values for all samples in all the wells. All p values represent comparisons with GSTM (Myocrisin) 0 μg/ml. NS: not significant; *p < 0.05; **p = 0.005.

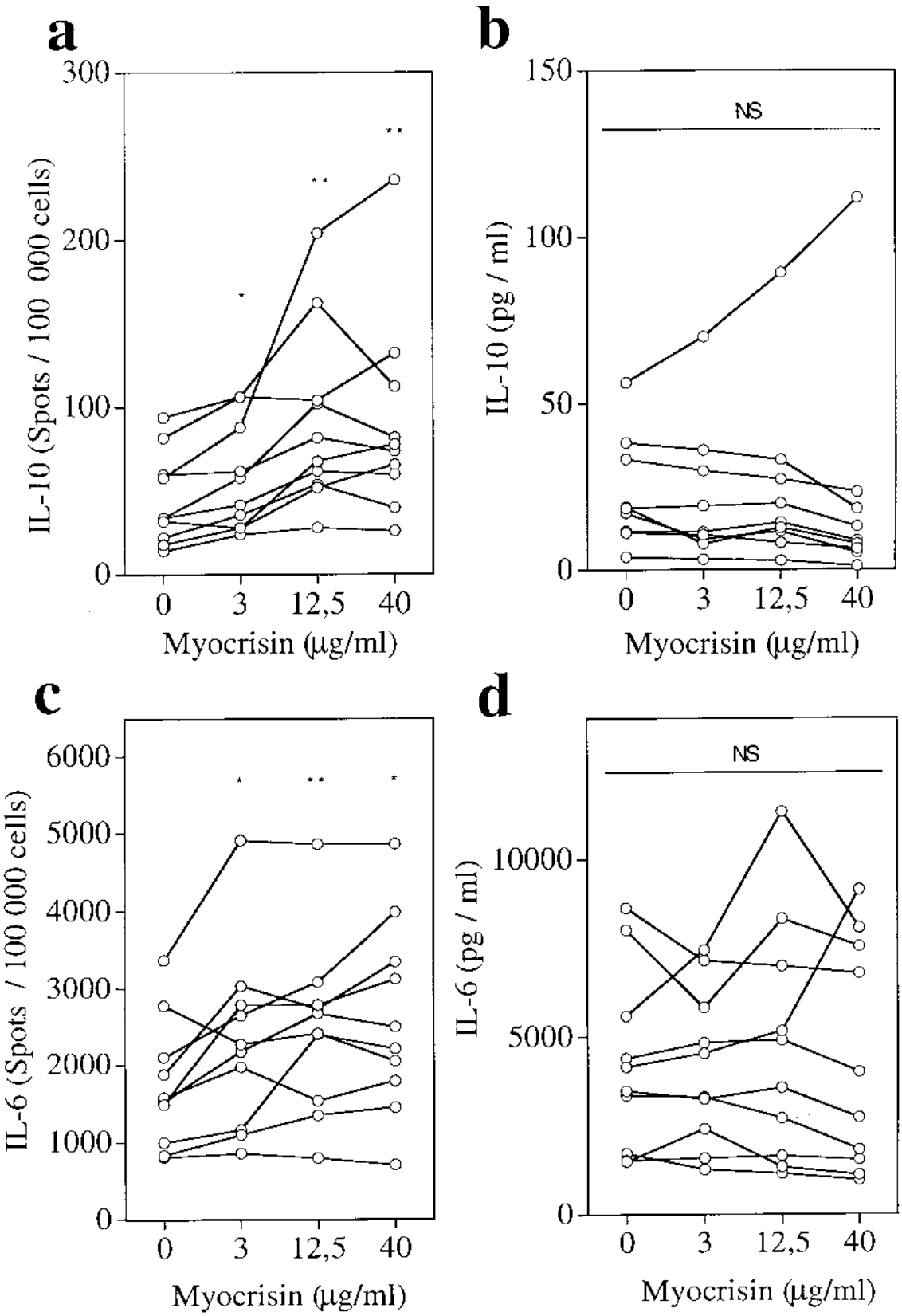

Figure 2. PBMC from healthy blood donors (n = 10) were incubated 16–20 h with different concentrations of GSTM. In a and b effects on IL-10 production are shown, whereas c and d depict influences on IL-6. Methods used for cytokine detection were Elispot (a, c) and ELISA of cell culture supernatants (b, d). All p values represent comparisons with GSTM (Myocrisin) 0 μg/ml. NS: not significant; *$p < 0.05$; **$p = 0.005$.

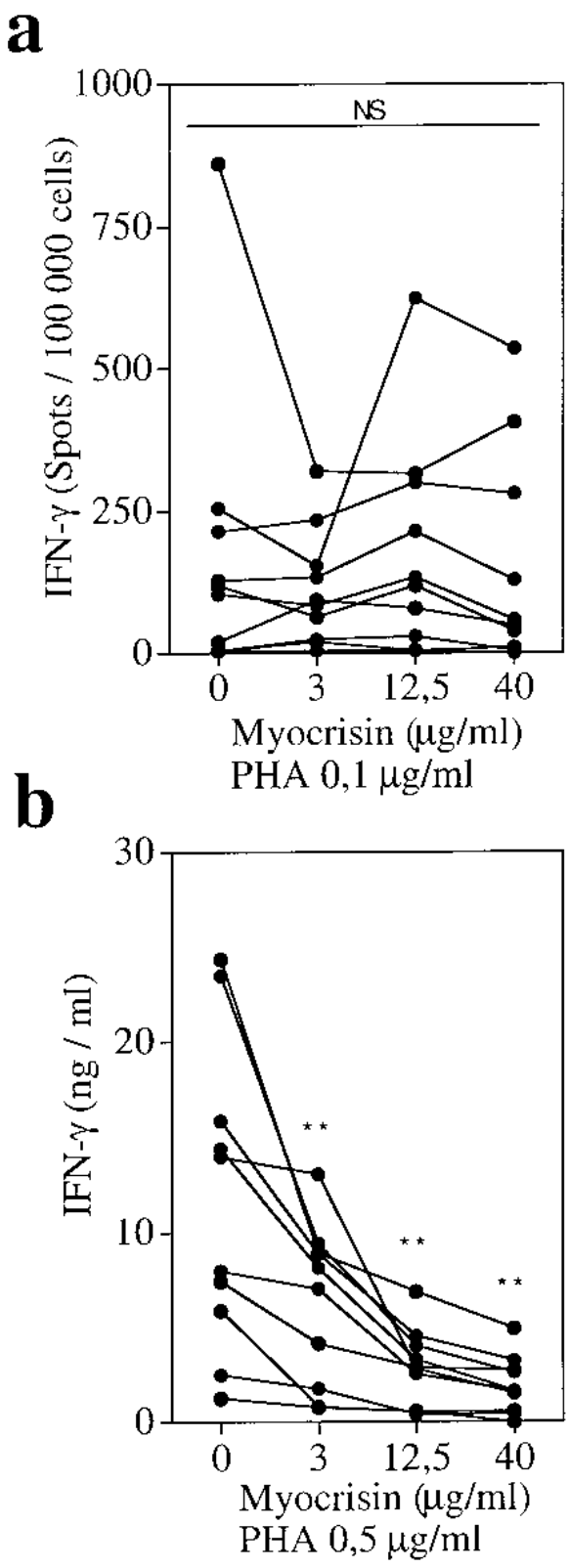

Figure 3. PBMC from patients with polyarthritis (n = 10) were incubated 16–20 h with different concentrations of GSTM (Myocrisin). IFN-γ production was determined with Elispot (a). IFN-γ content in cell culture supernatants was analyzed with ELISA (b). All p values represent comparisons with GSTM (Myocrisin) 0 µg/ml. NS: not significant; *p < 0.05; **p = 0.005.

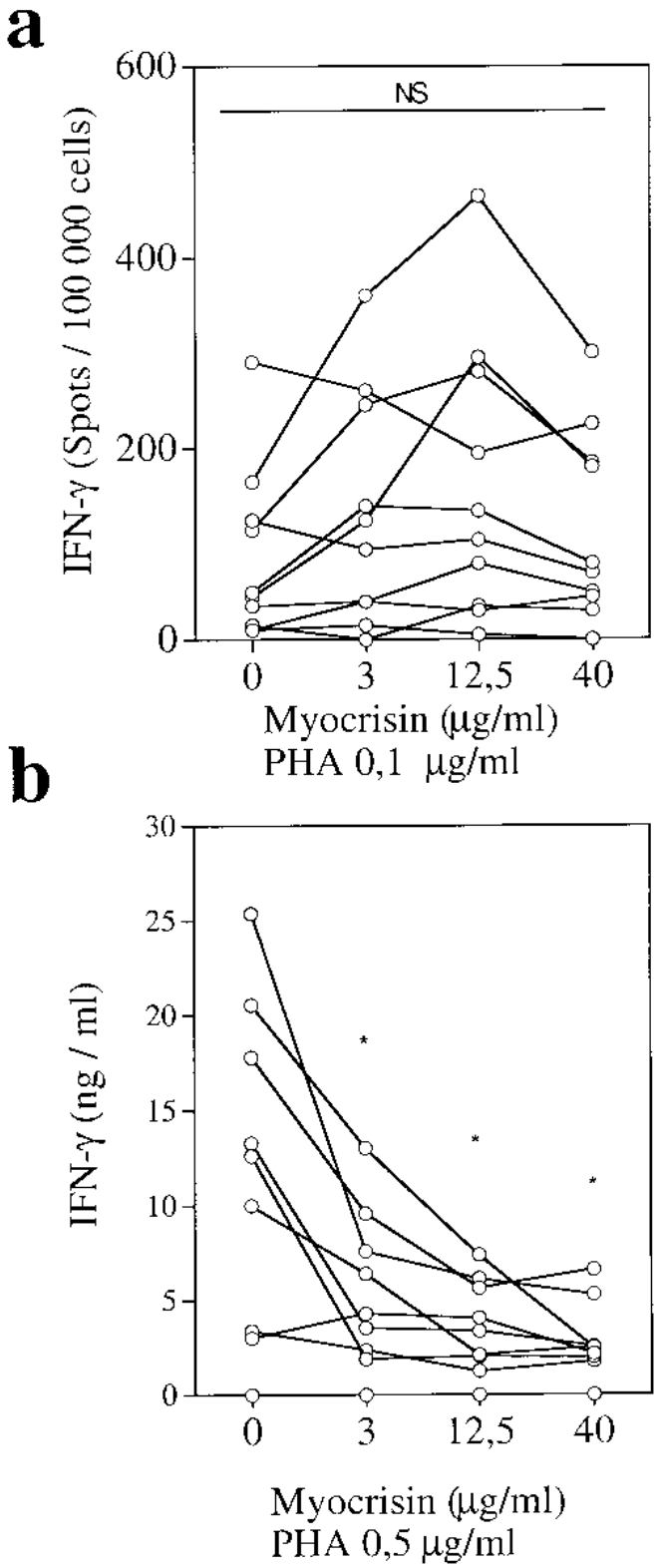

Figure 4. PBMC from healthy blood donors (n = 10) were incubated 16–20 h with different concentrations of GSTM. IFN-γ production was determined with Elispot (a). IFN-γ content in cell culture supernatants was analyzed with ELISA (b). Two of the controls included in 4a had nondetectable levels of IFN-γ for all GSTM (Myocrisin) concentrations. All p values represent comparisons with GSTM (Myocrisin) 0 µg/ml. NS: not significant; *p < 0.05; **p = 0.005.

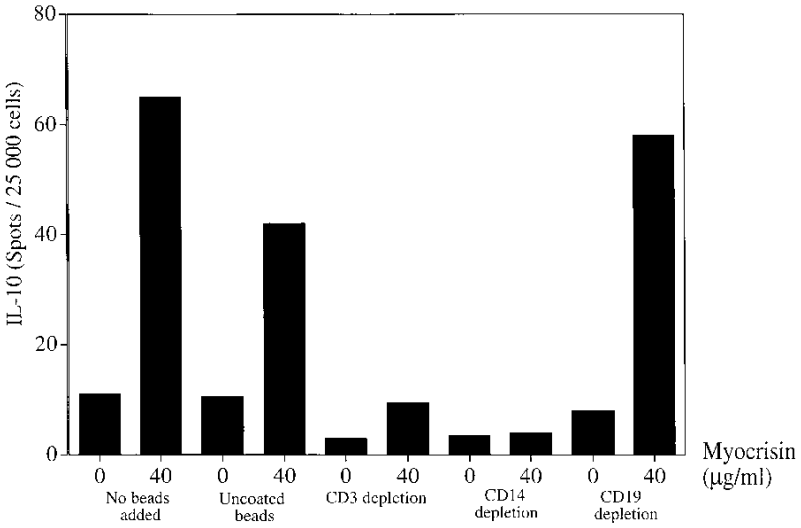

Figure 5. Following depletion of specific cell subsets PBMC were incubated 16–20 h with 0 or 40 μg/ml of GSTM (Myocrisin). IL-10 production was detected with Elispot. This experiment is representative of 3 showing comparable results.

augmented consumption of the same cytokine, implying an advantage of using techniques such as Elispot for direct measurement of cytokine production.

Comparisons between the 2 techniques have also shown the Elispot to be 10–200 times more sensitive than ELISA measurements[13]. For our studies of gold effects we used Myocrisin®, which is the trademark of GSTM for parenteral administration.

Parenteral gold has mainly been reported to have inhibiting properties on cytokine production and on cytokine dependent events. Thus GSTM may decrease the proliferation-inducing activity of human IL-1 on murine thymocytes[14] and also inhibits the production of IL-8 from synoviocytes[6]. Koda and collaborators reported a decreased IL-1ß dependent production of IL-6 after incubation *in vitro* with GSTM[12], whereas Crilly, *et al* did not detect any effect of GSTM on IL-6 production from macrophages in another system[15]. GSTM has also been shown to reduce the chemotactic activity of monocytes[16]. The effects of GSTM on T cells include inhibition of proliferation induced by mitogens or IL-2[17,18]. Blitstein-Willinger and collaborators detected a stimulation of low dose GSTM on proliferation and IL-1 production from adherent PBMC, but doses over 3 μg/ml had an inhibitory effect[19]. In addition, GSTM has been reported to inhibit several IFN-γ dependent effects such as induction of MHC class II expression in monocytes[20]. However, no studies have reported stimulatory effects of GSTM on the production of antiinflammatory cytokines.

IL-10, formerly known as cytokine synthesis inhibitory factor, is an immunoregulatory cytokine produced by T cells[21,22] and monocytes/macrophages[23], and it has inhibitory effects on several proinflammatory cytokines such as IL-1, IL-2, IL-4, IL-5, IL-6, IL-8, TNF-α, IFN-γ, and granulocyte monocyte-colony stimulating factor[21,24-26]. Increased IL-10 production in a cell culture may thus influence the production of other cytokines and cytokine receptors, which can be measured by analyzing the cytokine content in the supernatants of the cell cultures. In our study, GSTM induced IL-10 production was paralleled by a dose dependent downregulation of the IFN-γ concentrations in the supernatants. These results support studies showing the T cell suppressive effects of GSTM, although the mechanisms underlying these effects remain undetermined. Conversely, in the Elispot analysis there was no change of the number of IFN-γ spots with GSTM. One explanation for the discrepancy between the 2 methods in this context may be that a decreased production of IFN-γ per cell might solely affect the intensity of a spot, but without significant alteration of the number of IFN-γ producing cells. This phenomenon has earlier been noted in our laboratory with measurement of other cytokines such as TNF and IL-6 (our unpublished observations).

Differentiation between GSTM effects on Th1 or Th2 was not feasible in our study as IL-4 could not be detected in any experiment (data not included).

The IL-6 and TNF-α levels in the supernatants were not changed with GSTM, possibly explained by inhibitory effects on these cytokines by increased IL-10 production. However, using Elispot we observed a GSTM induced increase in IL-6 production, but not of TNF-α. We could find no correlation between the increase in IL-10 production and IFN-γ or IL-6 changes (data not shown). There was a downregulation of IL-10 levels in supernatants of patients with polyarthritis but this was not evident in the healthy blood donors. We cannot explain this finding, but speculate that this may be due in part to the self-inhibitory effects by IL-10[27].

We performed depletion studies to investigate which cells

were responsible for the GSTM induced IL-10 production. We determined that although both T cells and monocytes contributed to the unstimulated IL-10 production, GSTM induced IL-10 production was only abolished when monocytes were depleted. Thus CD14+ cells, i.e., monocytes and macrophages, appear to constitute the main source of GSTM induced IL-10 production. Microscopically we have observed that these cells digest gold granula during incubation (data not shown). According to our results, this phagocytosis of gold thus may be paralleled by increases in IL-10 and IL-6 production.

Our results indicate that GSTM stimulates antiinflammatory mechanisms, and speculatively this may be important for its effect in the treatment of RA. However, the panorama of advantageous and adverse effects of parenteral gold is different in different individuals. We believe that this may be due to differences in the cytokine profile, perhaps particularly of IL-10 production. In this context it may be noted that GSTM may increase the severity of systemic lupus erythematosus, a systemic inflammatory disease, which among other features is characterized by increased IL-10 production[28]. A better understanding of the differential effects of gold on cytokine production might thus contribute to better knowledge of both the positive and adverse effects of parenteral gold in RA. This knowledge may provide new clues concerning the use of drugs with more selective actions than gold to stimulate immunological events with beneficial effects on RA.

We observed that parenteral gold has monocyte stimulating properties *in vitro* and also inhibits T cell activity. The differential effects of GSTM on cytokine production, with a marked stimulatory effect on IL-10 and IL-6, indicate that parenteral gold may act on inflammatory diseases as a relatively selective immunostimulator rather than as a general immunosuppressant.

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