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AURANOFIN INHIBITS THE INDUCTION OF INTERLEUKIN 1β AND TUMOR NECROSIS FACTOR α mRNA IN MACROPHAGES

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Abstract—Gold compounds are widely used in the treatment of rheumatoid arthritis, but their mechanisms of action remain unclear. We demonstrate here that auranofin (AF) (0.1–3 μM), but neither the hydrophilic gold compounds aurothiomalate (ATM) and aurothioglucose nor methotrexate or D-penicillamine, inhibits the induction of interleukin 1β and tumor necrosis factor (TNF) α mRNA and protein by either zymosan, lipopolysaccharide (LPS), or various bacteria in mouse macrophages. The auranofin-mediated inhibition of the induction of TNF-α mRNA was stronger than that of interleukin (IL) 1β mRNA. AF, but not the other drugs, also inhibited zymosan-induced mobilization of arachidonate. The fact that AF inhibited the induction of mRNA for both these proinflammatory cytokines, irrespective of which stimulus was used, may indicate that it affects some common signal transduction step vital to their induction.

Key words: auranofin; aurothiomalate; methotrexate; interleukin 1; tumor necrosis factor; arachidonate

The gold compounds AF†, ATM, and aurothioglucose are widely used in the treatment of rheumatoid arthritis. There is still uncertainty concerning the cellular and molecular targets for the anti-inflammatory and disease-remittive effects of these compounds, in spite of many theories having been proposed. In a recent paper [1], we have demonstrated that all three of these gold compounds, as well as D-penicillamine, enhance protein kinase C-mediated activation of the arachidonate-mobilizing phospholipase A_2 in mouse macrophages. The present work deals with the effects of antirheumatic gold compounds, as well as D-penicillamine, methotrexate, and sulfasalazine, on the expression of IL-1β and TNF-α at the mRNA and protein level. It is not clear what stimuli are relevant in the afflicted synovium. A panel of stimuli known to induce expression of these cytokines has thus been used: LPS and zymosan, as well as Gram-positive and Gram-negative bacteria.

It is well known that interaction of zymosan particles with macrophages leads to activation of phospholipase C, activation of protein kinase C, and presumably a rise in cytosolic calcium concentration. Other kinases and/or phosphatases may also be involved, because the zymosan-induced phosphorylation response in macrophages is only partially dependent on protein kinase C [2]. The LPS-induced phosphorylation response occurs independently of down-regulation of the C-kinase [2], implying that the signal transduction pathway triggered by LPS does not involve the major isoforms of this kinase. To explore further the mechanisms involved, we have attempted to correlate the effects of the antirheumatic compounds on cytokine mRNA expression with those on zymosan-induced arachidonate release and changes in protein phosphorylation. A similar study on the antirheumatic compound tenidap has shown that it inhibits zymosan-induced protein phosphorylation, arachidonate release, and the induction of IL-1 and TNF-α in exactly the same concentration range [3, 4]. Phorbol ester sensitive isoforms of kinase C are not directly inhibited by tenidap [3]; indeed, these kinases do not appear to play a central role in the induction of proinflammatory cytokines, because an agonist of protein kinase C does not cause induction of either IL-1α/β or TNF-α in mouse peritoneal macrophages. These results suggest that tenidap inhibits an early step in zymosan-induced signalling to macrophage activation. Furthermore, both tenidap [3] and AF [1] themselves cause changes in the phosphoprotein pattern in macrophages. Finally, other compounds with anti-inflammatory properties have recently been shown to be useful in disclosing the role of individual protein kinases in the regulation of proinflammatory cytokine synthesis [5].

EXPERIMENTAL PROCEDURES

Materials and preparation of macrophages

Resident peritoneal cells were harvested in 4 mL of Medium 199 (Earle's salts, supplemented with 10 mM HEPES, 1% heat-inactivated fetal bovine serum and 20 units/mL of heparin) from female outbred NMRI mice (Bommice, Denmark), plated in either 12-well or 6-well tissue culture dishes, or in 25 cm^2 culture flasks, and incubated in an atmosphere of 5% CO_2 in air. Nonadherent cells were removed after 2 hr, and Medium 199 with 10% serum was added. Where indicated, the macrophages were treated with antirheumatic compound during this labelling time. After 22 hr, the cells were washed with PBS and allowed to equilibrate for 30 min in fresh serum-free medium before the various experiments.

AF (Ridaura) was dissolved in dimethyl sulphoxide, and ATM (Myocrisin) was dissolved in water. These compounds were obtained as gifts from SmithKline Beecham Pharmaceuticals and Rhône-Poulenc Pharma, respectively. Methotrexate, a gift from Lederle Laboratories, was dissolved in either 0.1 M NaOH or 1 M

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† Abbreviations: AF, auranofin; ATM, aurothiomalate; IL-1, interleukin 1; LPS, lipopolysaccharide; PBS, Dulbecco's phosphate buffered saline; TNF, tumor necrosis factor.

NaOH. Sulfasalazine, 5-amino-salicylic acid, and sulfapyridine were gifts from Pharmacia (Uppsala, Sweden); they were all dissolved in 0.1 M NaOH. D(-)-penicillamine and less commonly used hydrophilic gold compound aurothioglucose were purchased from Sigma; both were dissolved in water. Zymosan and LPS (from *Salmonella minnesota*) were also purchased from Sigma. The calcium ionophore A23187 was purchased from Boehringer, and dissolved in dimethyl sulphoxide. Bacteria were obtained as described [6], and were added as a suspension in Ca^{2+}/Mg^{2+}-free PBS.

Assessment of cytokine mRNA expression

In the cytokine mRNA assay, resident macrophages were isolated by adherence to plastic 25 cm^2 Nunclon culture flasks, using approximately 18×10^6 cells per flask. All drugs were added in 2–20 μL of a water or dimethyl sulphoxide solution, except sulfasalazine and its metabolites, which were added in a 0.1 M NaOH solution, and methotrexate, which was added in 1–5 μL of a 0.1 M or 1 M NaOH solution. In most experiments, antirheumatic drug was added 15 min before addition of the cell stimulant but, in some instances, overnight pretreatment with the drug was performed. After 90-min incubation with or without stimulant, the culture medium was removed and the cells washed with PBS.

Preparation of total RNA was performed according to Chomzynski and Sacchi [7]. The cells were lysed in guanidine isothiocyanate (4 M), sodium citrate (25 mM), 2-mercaptoethanol (0.1 M), and sarcosyl (0.5%, w/v). The lysate was transferred to a polypropylene tube and, sequentially, sodium acetate (2.0 M), water-saturated phenol, and a chloroform-isoamyl alcohol mixture (49:1) were added. The sample was mixed and centrifuged, and the upper liquid phase transferred to a fresh tube containing isopropanol. The mixture was incubated for at least 24 hr at −20°C to precipitate the RNA, which was then pelleted by centrifugation. The pellet was resuspended in the guanidine isothiocyanate-containing solution detailed above, reprecipitated, washed with ethanol, dried, and dissolved in 0.5% SDS.

Total RNA was separated on a formaldehyde-1.2% agarose gel and blotted onto a nylon membrane by 20 × SSC (1 × SSC = 0.15 M NaCl and 0.05 M sodium citrate, pH 7.0) by capillary transfer. The blots were stained with ethidium bromide, baked in a vacuum oven for 2 hr and prehybridized for 12–16 hr at 42°C in 50% formamide, 1% SDS, 5 × SSC, 1 × Denhart's solution (0.02% Ficoll, 0.02% BSA, 0.02% polyvinyl pyrrolidone) and 0.5 mg/mL denaturated salmon sperm DNA and 50 mM sodium phosphate buffer, pH 7.4. Synthetic oligonucleotide probes (Clontech Laboratories Inc., U.S.A.) with the following sequences were used; TNF-α: 5′-GCC GTT GGC CAG GAG GGC GTT GGC GCG CTG-3′, IL-1α: 5′-CTC TTC TTC AGA ATC TTC CCG TTG CTT GAC-3′, IL-1β: 5′-AGC TTT CAG CTC ATA TGG GTC CGA CAG CAC-3′, β-Actin: 5′-GGG TGT TGA AGG TCT CAA ACA TGA TCT GGG-3′. The probes were radiolabelled by 5′-labelling, using $\gamma(^{32}P)$-ATP, to a specific activity of approximately 10^9 cpm/μg DNA. Hybridization was carried out at 42°C for 12–16 hr with denaturated probe. After hybridization, blots were washed 4 times in 0.2 × SSC for 60 min followed by 4 additional washes at 55°C with 2 × SSC. The blots were then dried and exposed to Hyperfilm TM-MP (Amersham) at −30°C using intensifying screen. To approximate the amount of total mRNA in each experiment, an oligonucleotide probe for β-actin mRNA was used [8], and hybridization and autoradiography was performed as above.

Autoradiographs of blots were quantified by scanning with video densitometry and OD values were determined for each sample and normalized to values obtained for β-actin expression on the same blot. Because, in general, less TNF-α mRNA was expressed by the cells, it was necessary to analyze the Northern blots by a Fujix Bas 2000 digital imaging system, instead.

Western analysis of IL-1β and TNF-α

Equal aliquots of whole-cell extracts prepared in Laemmli sample buffer were boiled for 5 min and subjected to SDS-PAGE (12% acrylamide). Harvested culture medium was supplemented with bovine serum albumin and adjusted to 6% trichloroacetic acid followed by incubation at 4°C and centrifugation at 12 000 × g for 30 min. The precipitate was suspended in sample buffer and treated as described for the cell extract. Gels were then equilibrated in transfer buffer and the samples transferred to a nitrocellulose membrane. The membrane was blocked with 1% gelatin followed by incubation with rabbit antibodies against either IL-1β or TNF-α (Genzyme Diagnostics). Bound antibodies were detected with ^{125}I-labeled goat antirabbit antibodies (0.5×10^6 cpm/mL). Blots were then dried and analyzed by autoradiography or by digital imaging (Fujix Bas 2000). Extensive washing of membranes preceded each incubation step. Only the proform of IL-1β in the cell extracts and the 17 kDa processed TNF-α in the culture medium were detected.

Analysis of arachidonate release

Macrophages were cultured on 12-well tissue culture dishes (Costar) and labelled with 2 μCi (^3H)arachidonate (Amersham, U.K.; specific radioactivity 100–135 Ci/mmol). Where indicated, the antirheumatic drug was added 15 min before addition of zymosan or calcium ionophore. After incubation for 30 min, the culture medium was removed and an aliquot thereof, and of the cell lysate solubilized in 0.1% (w/v) of Triton X-100, were taken to radioactivity determination. High performance liquid chromatography analysis of eicosanoids in the medium was performed as described [1].

RESULTS

Effects of cytokine mRNA and protein expression

Control cells did not express mRNA for either IL-1α, IL-1β, or TNF-α, nor did cells treated with any of the antirheumatic compounds tested in themselves or cells stimulated with phorbol diester (not shown). LPS and zymosan both caused rapid expression of mRNA for all three proinflammatory cytokines, with a higher level of expression in response to LPS than to zymosan. We were able to detect both IL-1β and TNF-α mRNA after 30 min of stimulation with LPS (not shown). The level of mRNA for these cytokines increased in parallel and reached a plateau after approx. 90 min. There was no further increase when cells were stimulated for 2, 3, or 4 hr. It was possible to detect IL-1β mRNA, as well as trace amounts of IL-1α and TNF-α mRNA, after 30 min of zymosan stimulation, with increasing levels of mRNA after 60 and 90 min. Again, there was no further accu-

mulation of mRNA for these cytokines after zymosan stimulation for 2, 3, or 4 hr. Similar kinetics have been reported for the induction of mRNA for these cytokines in response to bacteria [9].

AF, in contrast to ATM, inhibited the LPS-induced expression of both IL-1β mRNA and TNF-α mRNA (Fig. 1, left 5 lanes). Kinetic experiments demonstrated that the inhibition of LPS-induced expression of these cytokines could be detected after 30 min of incubation and that there was no significant difference in the potency of the inhibition when the incubation time with LPS was prolonged to 4 h (not shown). The concentration dependence for these effects indicated that the expression of TNF-α mRNA was more sensitive to AF than that of IL-1β mRNA (Table 1). In agreement with these latter results, the LPS-induced production and release of mature TNF-α (Fig. 2) assessed by Western blotting was found to be inhibited by AF but not by ATM. Neither penicillamine nor methotrexate caused any significant inhibition of the LPS-induced expression of IL-1β mRNA or TNF-α mRNA (Table 1).

It has been demonstrated earlier that not only intact Gram-negative bacteria, but also Gram-positive bacteria, lacking LPS, are able to induce expression of mRNA for both IL-1α, IL-1β, and TNF-α (*e.g.* [9–11]). Because the signal transduction pathways involved here may differ from those utilized by LPS, it was considered of interest to examine whether AF could also inhibit the cytokine mRNA response induced by intact bacteria. As shown in Fig. 1 (right 3 lanes), AF inhibited the expression of both IL-1β and TNF-α mRNA induced by the Gram-positive *Peptostreptococcus anaerobius*. Table 2 demonstrates that the cytokine mRNA response induced by the Gram-negative species *Fusobacterium nucleatum* was somewhat more sensitive to inhibition by AF than that induced by the Gram-positive *P. anaerobius*. As noted above for LPS, the inhibitory effect on TNF-α mRNA was more pronounced than that on IL-1β mRNA when intact bacteria were employed as stimulus.

Table 1. Effect of gold compounds, penicillamine, and methotrexate on LPS-induced expression of mRNA for IL-1β and TNF-α

Drug conc. (μM)	IL-1β mRNA M ± S.E.M.	*n*	TNF-α mRNA M ± S.E.M.	*n*
AF				
0.01	93	2	58	2
0.03	100 ± 2.5	3	47 ± 14	3
0.1	74 ± 4.0	3	36 ± 1.8	3
0.3	22 ± 0.8	7	18 ± 4.5	8
1	21 ± 13	5	12 ± 5.0	6
3	4 ± 0.2	3	11 ± 0.5	3
ATM				
5	113 ± 11	4	122 ± 21	4
20	119 ± 13	4	139 ± 22	4
Penicillamine				
1	99 ± 7.6	4	102 ± 11	4
5	96 ± 13	4	92 ± 7.9	4
Methotrexate				
0.02	120 ± 18	4	123 ± 23	4
0.22	132 ± 19	4	132 ± 9.9	4
2.2	121 ± 20	4	100 ± 8.4	4
22	98 ± 5.9	4	97 ± 7.8	4

AF and methotrexate were added to cell cultures 15 min before LPS (3 μg/mL), and overnight preincubation was performed with ATM and penicillamine. After Northern blotting, scanning of autoradiograms or digital imaging and normalization of mRNA load by β-actin probing, results are expressed as a percentage of the IL-1β or TNF-α response induced by LPS alone. Incubation time with LPS was 90 min.

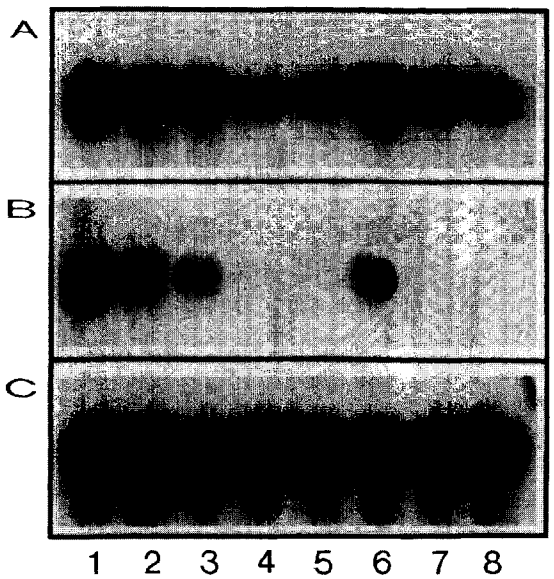

Fig. 1. Effect of AF and ATM on LPS-induced (Lanes 1–5) and *Peptostreptococcus anaerobius*-induced (Lanes 6–8) expression of IL-1β mRNA (A) and TNF-α mRNA (B). The probing of β-actin mRNA is shown in (C). Lane 1: LPS (3 μg/mL); Lane 2: 5 μM ATM overnight followed by LPS; Lane 3: 20 μM ATM overnight followed by LPS; Lane 4: 0.3 μM AF added 15 min before LPS; Lane 5: 1 μM AF added 15 min before LPS; Lane 6: *P. anaerobius* alone; Lanes 7–8: 0.3 and 1 μM AF, respectively, added 15 min before *P. anaerobius* (2×10^8/mL). Incubation time was 90 min.

Macrophages respond to zymosan in part by signalling via a phosphoinositide response and activation of protein kinase C. However, in contrast to the response to phorbol esters, a part of the changes in macrophage protein phosphorylation induced by zymosan is retained after downregulation of phorbol-ester-sensitive isoforms of protein kinase C [2]. This indicates that zymosan-induced activation of macrophages engages one or more additional signal transduction pathway(s). In accordance with this notion, zymosan, in contrast to phorbol esters (see above and [9]), induced expression of IL-1α mRNA (not shown), IL-1β mRNA, and also a low but clearly detectable level of TNF-α mRNA (Fig. 3). Furthermore,

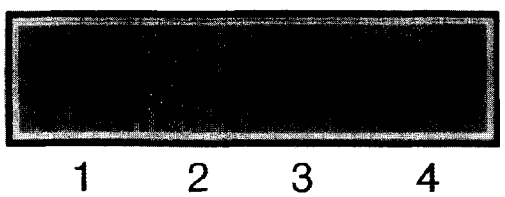

Fig. 2. Effect of AF and ATM on the formation of TNF-α in response to LPS (1 μg/mL), assessed by Western blotting. Incubation time with LPS was 4 hr. Lane 1: LPS; Lane 2: 1 μM AF added 15 min before LPS; Lanes 3 and 4: 5 and 20 μM ATM added 20 hr before LPS.

Table 2. Effect of AF on the expression of IL-1β and TNF-α mRNA in response by bacteria

Bacterium	AF (μM)	IL-1β mRNA (M ± SEM)	n	TNF-α mRNA (M ± SEM)	n
F. nucleatum	0.1	63 ± 11	5	33 ± 4.2	5
	1	12 ± 4.0	5	4.5 ± 1.9	5
	3	6.5 ± 2.8	5	5.4 ± 2.3	5
P. anaerobius	0.03	111	2	102	2
	0.3	41 ± 8.7	5	24 ± 7.6	5
	1	21 ± 4.9	4	14 ± 4.4	4

AF was added to cell cultures 15 min before the bacteria (2×10^8/mL). After Northern blotting, scanning of autoradiograms or digital imaging and normalization of mRNA load by β-actin probing, results are expressed as a percentage of the IL-1β or TNF-α response induced by bacteria alone. *F. nucleatum, Fusobacterium nucleatum; P. anaerobius, Peptostreptococcus anaerobius.*

AF was also found to inhibit the zymosan-induced expression of these cytokine mRNAs. Again, the zymosan-induced expression of TNF-α mRNA was more sensitive to inhibition by AF than that of IL-1β mRNA (Table 3), with 76% and 13% inhibition at 0.1 μM AF, respectively. Neither ATM, penicillamine, nor methotrexate affected the zymosan-induced expression of TNF-α mRNA or IL-1β mRNA (Table 3), nor did aurothioglucose (not shown).

To assess whether or not any additional effect of the antirheumatic compounds was exerted on the process of translation, particularly in the case of IL-1β where the inhibition of mRNA expression was generally incomplete, experiments were performed using Western analysis. As reported earlier (*e.g.* Ref [9]), the only immunoreactive form of IL-1β detected in stimulated macrophages was the unprocessed pro-form (Fig. 4). AF inhibited the formation of pro-IL-1β induced by either LPS or zymosan, and ATM was ineffective, even after a 22-hr preincubation. Nor did penicillamine or methotrexate affect the formation of pro-IL-1β in response to either LPS or zymosan (not shown). Any additional effect of these compounds on the translation of IL-1β mRNA could, therefore, not be substantiated.

Antirheumatic drugs and zymosan-induced arachidonate mobilization

In an earlier study [1], we reported that all three gold compounds tested, as well as D-penicillamine, enhanced phorbol-ester-induced arachidonate mobilization. Pre-

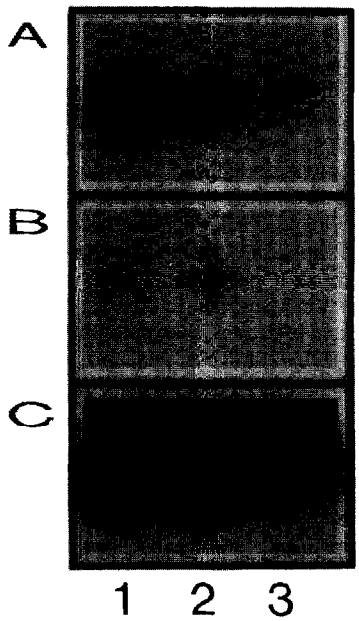

Fig. 3. Effect of AF on zymosan-induced expression of IL-1β mRNA (A) and tumor necrosis factor α mRNA (B). The probing of β-actin mRNA is shown in (C). Lane 1: zymosan (300 μg/ml); Lane 2: 0.3 μM AF added 15 min before zymosan; Lane 3: 1 μM AF added 15 min before zymosan. Incubation time with zymosan was 90 min.

Table 3. Effect of gold compounds, penicillamine, and methotrexate on zymosan-induced expression of IL-1β and TNF-α mRNA

Drug conc. (μM)	IL-1β mRNA (M ± SEM)	n	TNF-α mRNA (M ± SEM)	n
AF				
0.01	87	2	98	2
0.03	89	2	65	2
0.1	87 ± 5.2	4	24 ± 3.0	5
0.3	44 ± 5.2	12	15 ± 3.5	12
1	30 ± 5.9	9	7 ± 1.4	10
3	14 ± 4.5	4	5 ± 1.4	6
ATM				
5	108 ± 12	5	100 ± 8.3	5
20	99 ± 16	5	94 ± 8.7	5
Penicillamine				
1	95	2	96	2
3	106 ± 21	4	101 ± 22	4
10	89 ± 15	4	87 ± 25	4
Methotrexate				
0.02	111 ± 21	4	110 ± 12	4
0.22	124 ± 23	4	145 ± 26	4
2.2	100 ± 14	4	99 ± 9.6	4
22	94 ± 9.7	4	124 ± 12	4

AF and methotrexate were added to cell cultures 15 min before zymosan (300 μg/mL), and overnight preincubations with ATM and penicillamine were performed. After Northern blotting, scanning of autoradiograms or digital imaging and normalization of mRNA load by β-actin probing, results are expressed as a percentage of the IL-1β- or TNF-α response induced by zymosan alone. Incubation time with zymosan was 90 min.

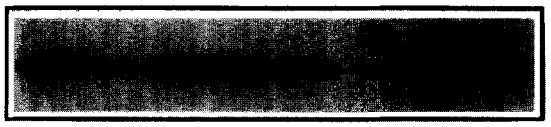

Fig. 4. Effect of AF or ATM on the formation of pro-IL-1β in response to LPS (1 μg/mL) or zymosan (100 μg/mL), assessed by Western blotting. Incubation time with either LPS or zymosan was 4 hr. Lane 1: LPS; Lane 2: 1 μM AF added 15 min before LPS; Lanes 3 and 4: 5 and 20 μM ATM added 20 hr before LPS; Lane 5: zymosan; Lane 6: 1 μM AF added 15 min before zymosan; Lanes 7 and 8: 5 and 20 μM added 20 hr before zymosan.

treatment for 4–22 hr was needed in the case of the water-soluble gold compounds and D-penicillamine. AF, but not the other drugs, also inhibited the calcium-ionophore-induced activation of the 5-lipoxygenase pathway. In view of these results and the effects of AF and ATM on cytokine expression, it was considered of interest to investigate whether or not the antirheumatic compounds affected the zymosan-induced signalling to phospholipase A_2 activation and arachidonate mobilization. AF was found to inhibit the mobilization of arachidonate induced by zymosan at quite low concentrations of the drug (0.1–1 μM; Fig. 5). Overnight pretreatment with AF gave similar results. As noted earlier [1, 12], higher concentrations of AF (5–20 μM) in the absence of other stimuli induced some mobilization of arachidonate. AF was a much more potent inhibitor of zymosan-induced mobilization of arachidonate than that induced by calcium ionophore [1]. Higher concentrations of AF (1–5 μM) exerted a strong inhibitory influence on the formation of all eicosanoids after stimulation of the cells with zymosan (not shown), which argues for an inhibitory effect at the level of the arachidonate-mobilizing phospholipase A_2. Even after overnight pretreatment, sulfasalazine (1–100 μM), or combinations of its two main metabolites sulfapyridine and 5-amino-salicylic acid (1–100 μM each), or methotrexate (0.02–22 μM), did not affect arachidonate mobilization induced by zymosan. Nor did they alter the pattern of eicosanoids formed in response to this stimulus (not shown).

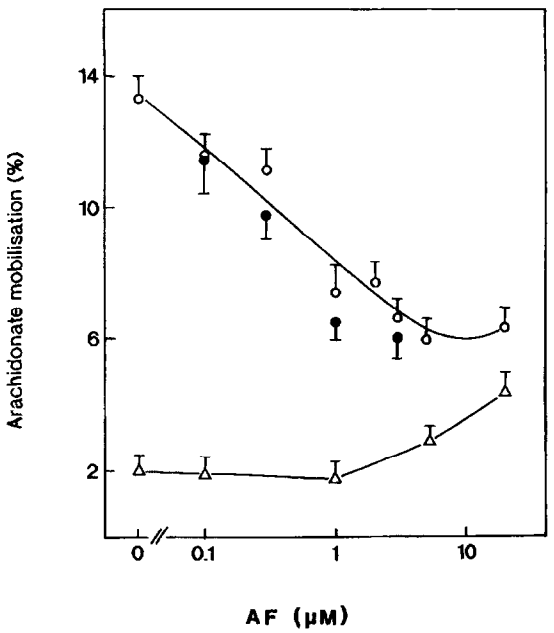

Fig. 5. Arachidonate release induced by AF alone (△) and effect of AF with (●) or without (○) overnight preincubation on the arachidonic-mobilization of arachidonate induced by zymosan (600 μg/mL). Means and SEM are indicated (n = 5–8).

Effects on protein phosphorylation

Earlier experiments have revealed that both AF and ATM are capable of inducing increased phosphorylation of several proteins both in neutrophils [13] and macrophages [1]. In macrophages, the hydrophilic compounds require an incubation for 4 hr or longer to exert this effect. Neither compound was, however, capable of altering the phosphorylation response to an activator of protein kinase C [1]. In further experiments, performed as described [1], AF did not significantly alter the zymosan-induced increases in whole cell protein phosphorylation, which are known to be to a large extent dependent on kinase C [2], nor did the hydrophilic gold compounds, even after a 22-hr preincubation (not shown). On the other hand AF, but not the hydrophilic compounds (not shown), was found to strongly inhibit the LPS-induced changes in protein phosphorylation, known to be independent of protein kinase C [2].

DISCUSSION

To our knowledge, studies on the effect of gold compounds on the expression of the proinflammatory cytokines IL-1α/β and TNF-α at the mRNA or protein level in macrophages have not been previously reported. There have been reports on the formation and/or release of these cytokines assessed by bioassay primarily in human monocytes, but the results are somewhat contradictory. In several studies AF, but not ATM, was found to inhibit LPS-induced production and/or release of IL-1 or IL-1-like activity from peripheral blood mononuclear cells [14–16]. Chang *et al.* [17] also reported that although both ATM and AF inhibited the action of IL-1, only AF inhibited IL-1 production in zymosan-stimulated human monocytes. In contrast, others suggested that ATM also inhibited IL-1 production in monocytes [18], and that both AF and ATM affected IL-1 release in a biphasic manner; enhancing at lower and inhibiting at higher concentrations [19]. In a later report where cell-associated IL-1 was also determined, evidence was presented that AF but not ATM inhibited the formation of IL-1 [20]. Similar results with regard to production of TNF-α in LPS-stimulated macrophages and monocytes have also been reported [21].

Our results support the notion that AF, but not ATM or aurothioglucose, inhibits IL-1β production and demonstrate that this inhibition is exerted as early as the mRNA level. Furthermore, we present evidence that AF inhibits the expression of TNF-α mRNA even more potently. This occurs irrespective of which of 4 different stimuli, probably utilizing different cell surface receptors, is used, suggesting that auranofin affects some central, post-receptor signal transduction step common to the induction of both IL-1 and TNF-α. A similar effect, somewhat more potent on the expression of TNF-α than IL-1β mRNA, has recently been reported for the flavonoid quercetin [9]. Cyclosporin A, acting as an inhib-

itor of protein phosphatase 2b, also exerts a similar inhibitory effect [9]. Our previous finding of a potentiating effect of AF on protein-kinase-C-mediated signalling to the arachidonate-mobilizing phospholipase A_2 [1] would certainly be consistent with an inhibitory effect on one or more protein phosphatases. However, the target for this effect and the inhibitory effect on cytokine mRNA expression is not likely to be the same, because the potentiating, in contrast to the inhibitory effect, was also shared by hydrophilic gold compounds and penicillamine. On the other hand, the ability of AF in itself to cause enhanced phosphorylation of several macrophage phosphoprotein bands [1], as well as its ability to inhibit LPS-induced increases in protein phosphorylation, are likely to be related to the inhibitory effect on cytokine mRNA expression. In addition to transcriptional regulation of IL-1β and TNF-α expression, it is becoming increasingly clear that regulation can also occur at the level of translation [5, 22, 23]. Although we cannot exclude that AF exerts an additional inhibitory effect at this level, in particular in the case of TNF-α, the hydrophilic gold compounds and penicillamine show no demonstrable inhibition.

With regard to mobilization of arachidonate, it is remarkable that AF, although potentiating the macrophage response to phorbol diester, inhibits the response to zymosan. This provides a further piece of evidence, in addition to those alluded to above (see Results), for additional signalling to arachidonate mobilization initiated by zymosan. Because AF does not cause any detectable effects on the protein phosphorylation cascade initiated in response to either zymosan or a direct activator of protein kinase C, it would appear that one or more event in the additional signal chain, unrelated to protein kinase C, is targeted by AF.

The strong inhibition of both IL-1β and TNF-α expression demonstrated here could provide a partial explanation for the antirheumatic effects of AF because these cytokines are generally considered of prime importance for disease maintenance in rheumatoid arthritis [24]. It is peculiar, however, that the hydrophilic gold compounds, which are at least as potent as AF in clinical studies, do not affect the expression of these proinflammatory cytokines. It could be that the hydrophilic compounds enter the cells slowly and/or that they need to undergo intracellular metabolism to become active metabolites. However, it has been demonstrated that ATM is able to act intracellularly under the same experimental conditions, at least after an exposure time of ⩾4 hr [1]. Although the results of Chang *et al.* [17] argue that methotrexate inhibits the action of IL-1, but not its production, these authors found that penicillamine partially inhibited IL-1 production in monocytes. Our data imply that the expression of IL-1β and TNF-α at the mRNA level in macrophages is not the primary target for these drugs.

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