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STUDIES ON THE INHIBITORY EFFECTS OF CAFFEOYLQUINIC ACIDS ON MONOCYTE MIGRATION AND SUPEROXIDE ION PRODUCTION

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ABSTRACT.—Three caffeoylquinic acids, isolated from the Peruvian plants *Tessaria integrifolia* and *Mikania cordifolia* that are used medicinally as anti-inflammatory agents, were tested for their activities on monocyte migration and superoxide anion production. 3,5-Di-*O*-caffeoylquinic and 4,5-di-*O*-caffeoylquinic acids exhibited an appreciable anti-inflammatory activity in vitro, while the tricaffeoyl derivative was inactive.

The leaf decoctions of the Peruvian species *Tessaria integrifolia* R. et P. and *Mikania cordifolia* Willd. (Asteraceae) are used in traditional medicine as anti-inflammatory agents (1). The isolation of caffeoylquinic acid derivatives from *T. integrifolia* (2) and *M. cordifolia* (3) has been reported, and 3,5-di-*O*-caffeoylquinic, 4,5-di-*O*-caffeoylquinic, and 3,4,5-tri-*O*-caffeoylquinic acids were shown to exhibit potential anti-inflammatory effects in preliminary studies (4). Indeed, caffeoylquinic acid derivatives are known for their inhibitory activities on lipid peroxidation in mitochondria and microsomes of the liver (5) and on histamine release from mast cells (6). 3,4,5-Tri-*O*-caffeoylquinic and 4,5-di-*O*-caffeoylquinic acids have recently been noted to inhibit HIV replication (7), and 3,4,5-tri-*O*-caffeoylquinic acid inhibits DNA polymerase (8). Furthermore, the synthesis of leukotriene B4 induced by calcium ionophore A 23187 in human polymorphonuclear leukocytes is inhibited by 3,5-, 4,5-, and 3,4-di-*O*-caffeoylquinic acids (9), and 3,4-di-*O*-caffeoylquinic acid had a strong inhibitory effect on platelet aggregation, platelet thromboxane biosynthesis, and hydrogen peroxide-induced endothelial cell injury (10). On the other hand, the role played by the caffeoylquinic acids in the modulation of chemotaxis and activation of immunocompetent cells, such as monocytes, normally involved in the inflammatory reactions, is well known. For this reason, we have studied the in vitro effects of these compounds on some human leukocyte functions related to inflammatory mechanisms such as monocyte migration and superoxide ion production.

Because of their diversity of functions, cells of monocyte/macrophage lineage are key elements in a large number of acute and chronic inflammatory diseases. In fact, besides being the major component responsible for delayed sensitivity reaction, they also play an important role in acute inflammation. These cells influence the activity of other inflammatory cells in large measure via the production of an array of secreted proteins such as the interleukins. For example, mononuclear phagocytes release large amounts of interleukin-8 (IL-8) in response to a wide variety of inflammatory conditions. IL-8 acts on neutrophils to induce chemotaxis, H_2O_2 production, and exocytosis of intracellular granules. The latter is associated with increases in a number of neutrophil receptors, including the CR3 (C3bi) adhesion receptor, and increased adhesion receptor expression may be the basis for neutrophil margination (11).

The locomotion of mononuclear phagocytes is a critical determinant of their ability to fulfil their role in inducing an inflammatory response. There may be many reasons for the accumulation of these immunocompetent cells at the inflammatory site, including chemotaxis and the inhibition of migration of monocytes away from the site by lymphokines. Once phagocytes have reached the site of inflammation, the presence of several proinflammatory factors may stimulate their "respiratory burst" activity, a physiological response in which oxygen is rapidly metabolized to yield a series of highly toxic intermediates. The initial product of the respiratory burst, formed when oxygen accepts a single electron from NAPDH via a plasma membrane-associated oxidase system, is the superoxide anion (O_2^-), a molecule able to induce a variety of phlogistic effects. Indeed, the oxygen metabolites released from the mononuclear phagocytes into the environment may induce tissue damage. Toxic oxygen species released from activated leukocytes have been reported to injure the membranes of cultured human endothelial cells (12), and hydroxyl radicals which attack the leukocyte itself could be responsible for the death of these cells during phagocytosis and contribute to the inflammatory response by releasing hydrolytic enzymes (13).

Thus, there appears to be a link between the ability of various agents to release oxygen radicals from monocyte cultures in vitro and to elicit acute and chronic inflammation in vivo. In fact, most drugs that inhibit both leukocyte migration and release of free radicals (e.g., glucocorticoids), also show a strong anti-inflammatory activity.

RESULTS AND DISCUSSION

Specific bioassays were performed designed to demonstrate whether the caffeoylquinic acids isolated from *T. integrifolia* and *M. cordifolia,* having potential anti-inflammatory activity, are able to inhibit monocyte migration and oxygen metabolism. The results of these experiments showed that all the cell cultures were very healthy after exposure to the different test compounds at concentrations normally used for the assay. Indeed, they did not differ from the negative control cultures in terms of LDH activity in the conditioned media.

Figure 1 shows the 3,5-di-*O*-caffeoylquinic acid and 4,5-di-*O*-caffeoylquinic acid inhibition of monocyte migration, in the following order of potency: 3,5-di-*O*-caffeoylquinic acid inhibited the peak of chemotactic index at a concentration of 10^{-11} M, but revealed a significant inhibitory activity at a concentration as low as 10^{-13} M; 4,5-di-*O*-caffeoylquinic acid blocked the chemotaxis only at a concentration of 10^{-7} M; 3,4,5-tricaffeoylquinic acid was completely inactive at any of the tested concentrations. To distinguish between chemotaxis and chemokinesis, a checkboard analysis was performed in which different concentrations of the putative chemoattractant were balanced on both sides of the filter. Our results show that monocytes migrate in response to a positive gradient of the chemoattractants, thus indicating a true chemotactic effect. Moreover, monocyte incubation with the active substances at 37° for 45 min amplifies their inhibitory effects. The inhibitory chemotactic effects of a preincubation of 3,5- and 4,5-di-*O*-caffeoylquinic acids are shown in Figure 2. From these results, it is more evident that the chemotactic block, especially for 3,5-di-*O*-caffeoylquinic acid occurs at concentrations (10^{-15}–10^{-16}) much lower than those necessary for the same substances utilized without preincubation. Furthermore, a time of incubation greater than 45 min did not induce any increase in the inhibitory effects of these compounds.

In order to determine whether the caffeoylquinic acid derivatives alter the zymosan-induced activation of O_2^- production by macrophages, the remaining cells were incubated with the three caffeoylquinic acids. The respiratory burst was triggered by the addition of opzonized zymosan (1 mg/ml). In our previous experiments, it was demon-

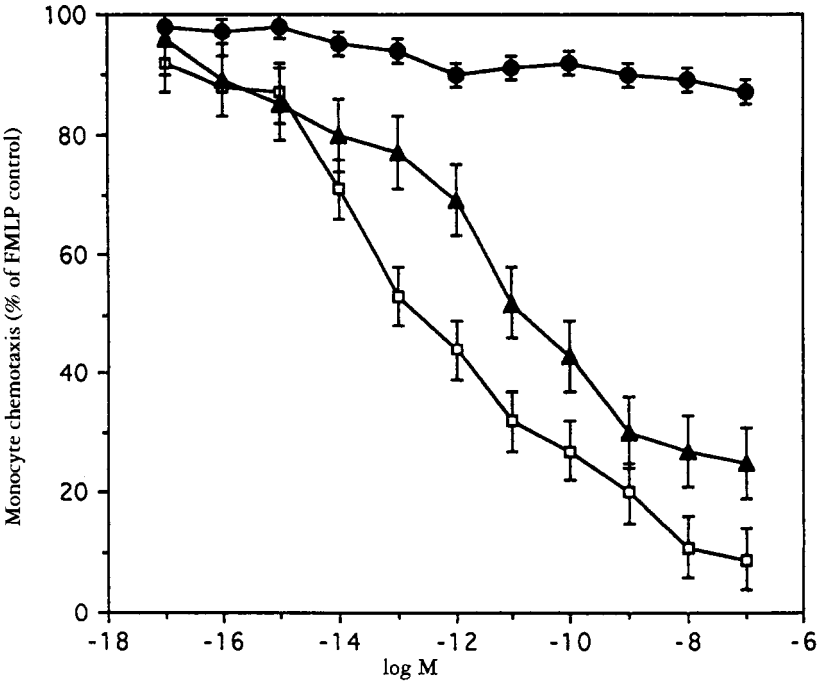

FIGURE 1. Chemotactic response to fMLP of monocytes obtained from healthy volunteers in the presence of different amounts of 3,5-di-*O*-caffeoylquinic acid (—□—), 4,5-di-*O*-caffeoylquinic acid (—▲—), and 3,4,5-tri-*O*-caffeoylquinic acid (—●—). Data are means±SD from six experiments.

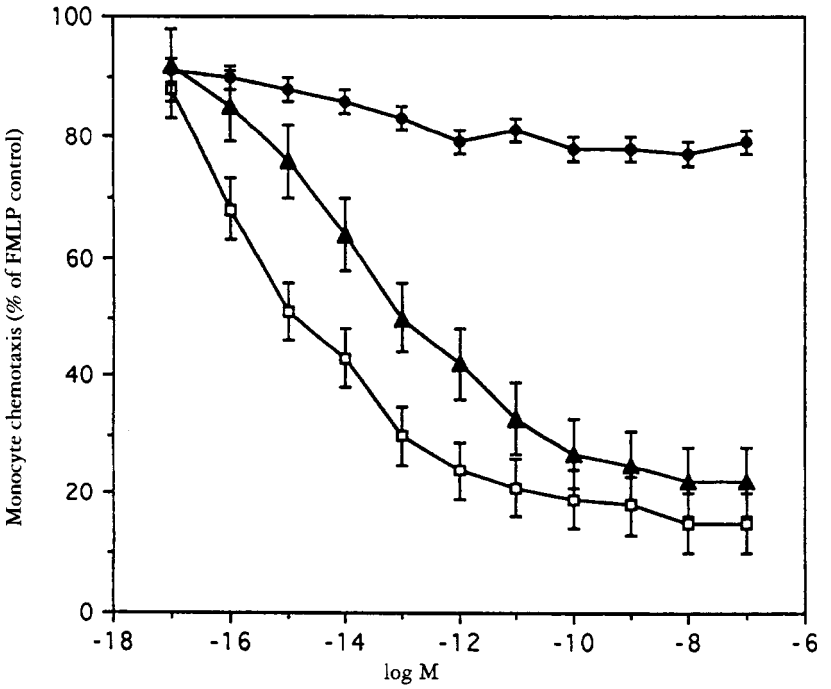

FIGURE 2. Chemotactic response to fMLP of monocytes obtained from healthy volunteers preincubated with different amounts of 3,5-di-*O*-caffeoylquinic acid (—□—), 4,5-di-*O*-caffeoylquinic acid (—▲—), and 3,4,5-tri-*O*-caffeoylquinic acid (—●—). Data are means±SD from six experiments.

strated that this concentration of zymosan elicits maximal production of O_2^-. Figure 3 illustrates that in the absence of caffeoylquinic acids, opsonized zymosan produces an increase in O_2^- production, while the simultaneous presence of 3,5-di-*O*-caffeoylquinic acid and 4,5-dicaffeoylquinic acid in the incubation medium resulted in a decrease in the liberation of O_2^-, which was dependent on the concentration of the inhibitory molecules. Under the conditions used, a maximal inhibitory effect was found at 500 μl of 3,5-di-*O*-caffeoylquinic acid, with the inhibitory effect increased by preincubation of the macrophages with the molecule for 45 min at 37°, and abolished by washing the cells

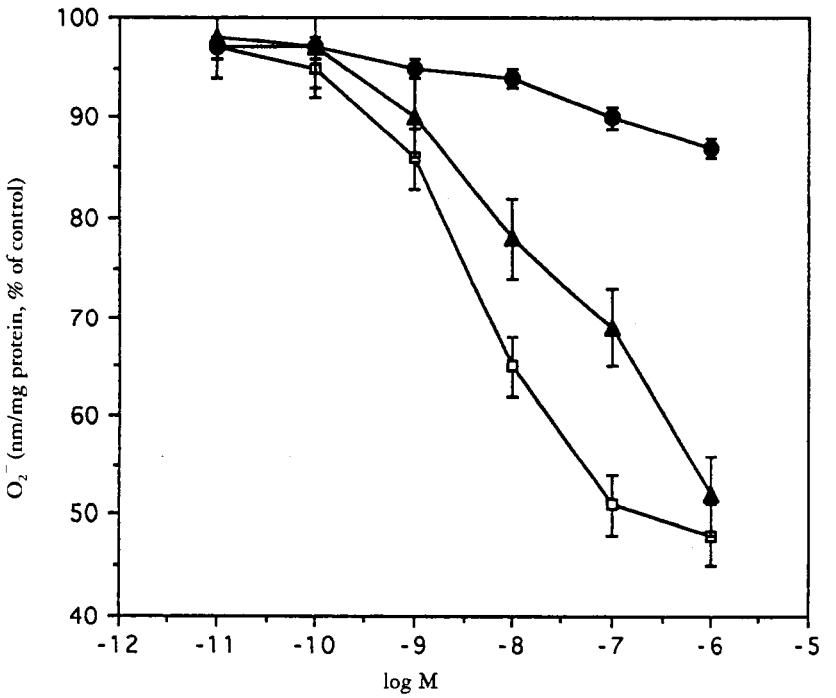

FIGURE 3. Effects of different amounts of 3,5-di-*O*-caffeoylquinic acid (—□—), 4,5-di-*O*-caffeoylquinic acid (—▲—), and 3,4,5-tri-*O*-caffeoylquinic acid (—●—) on O_2^- production by zymosan-stimulated monocytes. Experiments were performed with monocytes from five donors; values are means±SD.

after the incubation time (Figure 4). Although the absolute values of O_2^- production differed among the various experiments conducted, the degrees of inhibition at different concentrations of 3,5-di-*O*-caffeoylquinic acid were consistent. Figure 4 also reveals that the presence of this compound shifts the hyperbolic profile observed in the control cells to a sigmoid curve, indicating a delay in the initiation of response to opsonized zymosan. Finally, the effect of 3,5-di-*O*-caffeoylquinic acid plus different doses of zymosan, showed a degree of inhibition constant, suggesting that the competition between opsonized zymosan and this compound to occupy some putative receptor(s) is unlikely (data not shown). Several possibilities may be considered in order to explain the inhibition of zymosan-stimulated O_2^- synthesis. A possible explanation for the decrease in the production of O_2^- could be an enhanced rate of dismutation of O_2^- to H_2O_2. This hypothesis was tested by measuring both the rate of H_2O_2 production during the first 1 min of incubation, and the total production of H_2O_2 after 15 min of incubation of cells

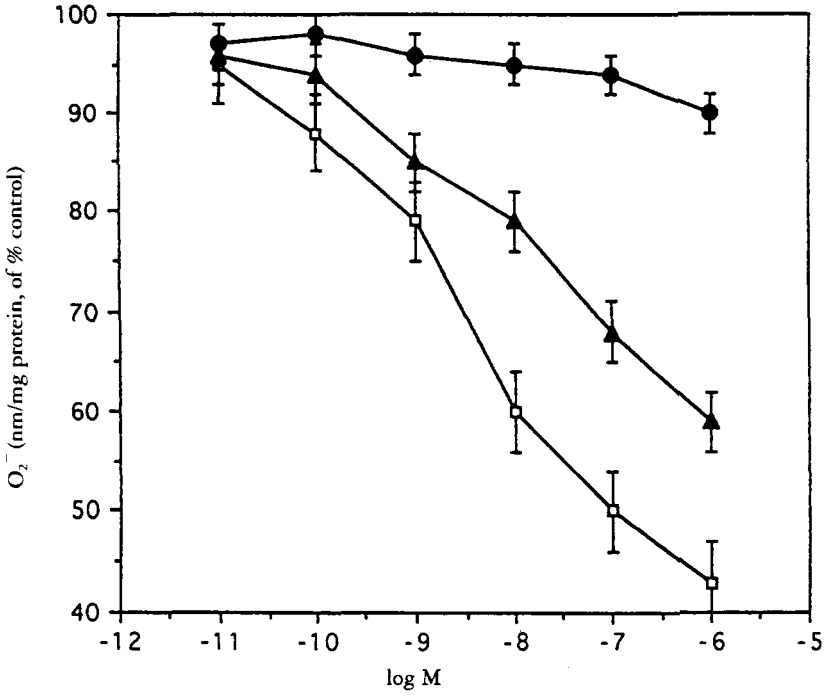

FIGURE 4. Effects of monocyte preincubation with different amounts of 3,5-di-*O*-caffeoylquinic acid (—□—), 4,5-di-*O*-caffeoylquinic acid (—▲—), and 3,4,5-tri-*O*-caffeoylquinic acid (—●—) on O_2^- production by zymosan-stimulated monocytes. Experiments were performed with monocytes from five donors; values are means ± SD.

with increasing doses of 3,5-di-*O*-caffeoylquinic acid. Our data showed that this compound produces a concomitant inhibition of H_2O_2 production.

Furthermore, because the effects of 3,5-di-*O*-caffeoylquinic acid disappeared in the absence of the substance in the medium, this could indicate that this compound did not produce stable changes in the essential component(s) of NADPH oxidase, and that its effect might be related to the disturbance in some step(s) in the transduction of the signal due to opsonized zymosan interaction with the membrane. One possible mechanism could be related to the recent finding concerning the electrogenic nature of NADPH oxidase and to the respiratory control exhibited by the enzyme (14,15). 3,5-Di-*O*-caffeoylquinic acid could produce rapid changes in membrane potential by influencing either the membrane binding or the translocation of some constituents of the macrophage membrane. In connection with this, a correlation between membrane potential changes and O_2^- production in human granulocytes has been described (16).

The observed biological effects of the dicaffeoylquinic acids were similarly impaired by the tricaffeoylquinic derivative, where three caffeoyl residues are present.

On the basis of the data obtained, it is possible to conclude that the caffeoylquinic acids isolated from the leaves of *T. integrifolia* and *M. cordifolia* could affect migration and O_2^- secretion in activated human macrophages. The order of the potency of the inhibitory effects on macrophage biological functions among the dicaffeoylquinic acids (3,5-di-*O*-caffeoylquinic>4,5-di-*O*-caffeoylquinic) is similar to that observed by Kimura *et al.* in the inhibition of lipid peroxidation induced by ADP plus ascorbic acid in liver mitochondria (5), and in the inhibition of Con A plus phosphatidylserine-induced histamine release from mast cells (6).

These results may suggest the importance both of the number of caffeoyl groups in the molecule and of their substitution pattern. Although the caffeoyl residues are important for biological activity, the complete substitution of the (-OH) groups of quinic acid interfered with the activity of the molecule. Indeed, the observation that the inhibitory effects of dicaffeoylquinic acids were stronger than those of the tricaffeoylquinic derivative could be related either to steric hindrance, determined by the presence of three caffeoyl residues in the same molecule, or to the chemical "inactivation" (complete substitution) of quinic acid.

Finally, studies concerning the mechanism(s) of action of these compounds are in progress. In particular, we are determining if the caffeoylquinic acids derivatives, inducing a perturbation of the monocyte plasma membrane, are able to inhibit specific pathway(s) of membrane signal transduction involved in cell activation.

EXPERIMENTAL

PLANT MATERIAL.—*Tessaria integrifolia* leaves were collected in August 1988, in Ayabaca Province, Piura Department, Peru, and identified by Dr. V. De Feo. *Mikania cordifolia* aerial parts were collected in September 1988, in Cuzco Department, Peru, and identified by Dr. E. Szeliga, of the Instituto Peruano de Investigación Fitoterápica Andina. Voucher specimens of the two plants are deposited at the Herbarium of the Dipartimento di Chimica delle Sostanze Naturali, Naples University (Nos. DFP/88-47 and DFP/88-121, respectively).

EXTRACTION AND ISOLATION.—The ground, air-dried leaves of *T. integrifolia* (300 g) and the air-dried aerial parts of *M. cordifolia* (420 g) were each sequentially extracted, at room temperature, with petroleum ether (bp 40°–70°), $CHCl_3$, $CHCl_3$-MeOH (9:1) mixture, and MeOH. Aliquots of the MeOH extracts, in 2-g lots, were chromatographically fractionated on a Sephadex LH-20 column (80×4 cm), using MeOH as eluent. Fractions of 10 ml each were collected and combined on the basis of tlc similarity [*n*-BuOH-HOAc-H_2O (12:5:3), detection cerium sulfate in H_2SO_4]. Fraction 16 (of 27 pooled fractions) from the *T. integrifolia* MeOH extract was separated by reversed-phase hplc [C_{18} μ-Bondapak column, 30×0.78 cm, flow rate 3 ml/min, MeOH-H_2O (4:6)] to give 3,4,5-tri-*O*-caffeoylquinic acid (22 mg). Fraction 21 yielded pure 4,5-di-*O*-caffeoylquinic acid (26 mg).

From the MeOH extract of *M. cordifolia*, fractions 37–52, purified by hplc [column C_{18} μ-Bondapak, flow rate 2.5 ml/min, MeOH-H_2O (55:45) as eluent] afforded 3,5-di-*O*-caffeoylquinic acid (18 mg).

The identification of the isolated compounds was performed by comparison of spectroscopic data (^{1}H-nmr, ^{13}C-nmr, ^{13}C DEPT nmr) with those reported in the literature (17). The isolates used in the assays were endotoxin free as determined by the Limulus amebocyte lysate test. This test permits the detection and quantitation of endotoxins in fluids (E-Toxate kit, Sigma, Milano, Italy).

BIOLOGICAL ASSAYS.—Basal chemotaxis experiments were performed with peripheral blood obtained from healthy volunteers. Mononuclear cells were separated by sedimentation over a 46% Percoll solution (285 mOs, Pharmacia, Milan, Italy). The time elapsing between collection of a blood sample and separation never exceeded 4 h. In preliminary studies we observed that at an elapsed time of 6 h, the chemotactic activity in response to the well-known chemotactic peptide f-Met-Leu-Phe (fMLP) is preserved (100%). The cells, after washing, were resuspended in Dulbecco's Modified Eagle's Medium (DMEM) to which 1% BSA and 20 nM HEPES were added and finally diluted to a concentration of 10^7 monocytes/ml. The chemotaxis assays were performed using a 48-well microtaxis chamber (Neuroprobe, Bethesda, MD). A 5-μm pore polycarbonate filter separated the upper and lower chambers in order to allow only the actively migrating cells to pass through the pores, thus minimizing random movements. Cells (50,000/well) were placed in the upper compartment, and different concentrations of chemoattractant substances were placed in the lower one. After incubation (90 min at 37°), the migrated cells adhering to the distal part of the filter were fixed and stained. Migrated cells were quantitated by counting three fields in triplicate using an optical image analyzer (IBAS, Zeiss). Results expressed as the chemotactic index, i.e., the ratio between migration toward test attractants and that toward buffer alone. The number of migrating cells in the buffer controls generally ranged from 20–30 cells/field. As positive control, the migration to the fMLP was assessed. A chemotactic index of 5–6 was observed at 10^{-8} M of fMLP.

In the in vitro experiments, monocytes from healthy volunteers were incubated at 37° for 45 min with the test compounds, washed with phosphate-buffered saline at room temperature, and counted. The test compounds were dissolved in Krebs-Ringer phosphate buffer (pH 7.4) as previously described (5). The same amount of buffer, but without the test substances, was added to the control cultures. The chemotactic responses were evaluated on the entire dose-response curve by two-way analysis of variance.

CELL VIABILITY.—The viability of the cells was routinely determined by the trypan blue exclusion test. Another test (lactate dehydrogenase activity evaluation) was also employed to measure the cellular response to the test compounds. Lactate dehydrogenase (LDH) is an enzyme that is released from the cell when it is injured. Therefore, the amount of LDH present in the media at the completion of the test correlates to the health of the cell (18). The control cultures included in the experiments were used either as positive or negative controls for LDH analysis. Positive controls were those in which the cell suspension was given 100 μl of Triton×100 and held at 37° for 2 h. These cultures were labeled C+. The untreated controls were labeled C− and served as negative controls for LDH analysis.

The amount of LDH in each culture was measured in Units (U) by using a specific Sigma chemical kit (OG 1340-K, Sigma, Milano, Italy), and was corrected for background activity by substracting the average activity of the negative controls, U_{C-}. The LDH activity of the cultures was then expressed as a percentage of the total possible activity in the cultures given in the formula:

$$\%\text{LDH Activity} = 100 \times (U_{spec.} - U_{C-})/(U_{C+} - U_{C-})$$

Therefore, by definition, the positive control cultures, C+, have 100% LDH activity and the negative control cultures, C−, have 0% LDH activity. Expressing the data in this manner allows for comparison between the different experiments.

SUPEROXIDE ION PRODUCTION.—Superoxide ion (O_2^-) production was measured using the method described by Pick and Mizel (19). Briefly, monocytes were suspended in DMEM at 5×10^6/ml and 0.1 ml/well was plated in 96-well tissue culture plates. After adherence of the macrophages for 2 h at 37° and washing to remove nonadherent cells, 0.1 ml of the following reaction mixtures in phenol red-free DMEM was added to each of the wells containing: 1) cytochrome c (160 μM, Sigma); 2) cytochrome c and superoxide dismutase (300 U/ml, Sigma); 3) cytochrome c, superoxide dismutase and activated zymosan (Sigma); 4) cytochrome c and activated zymosan; and 5) cytochrome c, activated zymosan and the test compound.

The plates were incubated for 1 h at 37° in 5% CO_2. The A_{550} (absorbance at 550 nm) was determined using a multiscan microplate reader (Flow Laboratories). The concentration of O_2^-/well in nmol was calculated using the formula $A_{550}\times100/6.3$. This formula is derived from the extinction coefficient for the adsorption of reduced-oxidized cytochrome c measured at 550 nm using the formula E550 nm $=21\times10^3$ M^{-1} cm^{-1} and a light path of 3 mm. Monocytes were solubilized with 1 N NaOH and the protein concentrations were measured by the bicinchonic acid method (BCA protein assay, Pierce Chemical Co., Rockford, IL). Results are expressed as nmol O_2/mg of macrophage protein.

Synthesis of H_2O_2 was measured using the scopoletin assay (20). The oxidation of scopoletin by H_2O_2 was followed fluorimetrically at $\lambda_{excitation}$ 366 nm and $\lambda_{emission}$ 460 nm at 37°. The reaction mixture contained 2 μM scopoletin, 1 mM NaN_3, 45 units of horseradish peroxidase/ml, 10 mM glucose, 1 mg/ml opsonized zymosan, and $1–1.5\times10^6$ cells/ml.

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