Alteration of leukotriene release by macrophages ingesting Toxoplasma gondii

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ABSTRACT Mouse resident peritoneal macrophages incubated with ionophore A23187 or opsonized zymosan released leukotrienes (LT) B4 and C4 (LTB4 and LTC4) and LTD4 and LTE4, respectively. In contrast, incubation with Toxoplasma gondii, an obligate intracellular protozoan, led to the formation of 11-, 12-, and 15-hydroxyicosatetraenoic acids (HETEs) together with an unidentified compound, designated compound X. Each of these compounds incorporated [3H]arachidonic acid from the macrophage during phagocytosis of T. gondii. Compound X migrated immediately prior to 15-HETE by reverse-phase HPLC and was distinct from authentic monoHETE, monohydroperoxyicosatetraenoic acid (mono-HPETE), and dihydroxyicosatetraenoic acid (dHETE) standards. The generation of compound X by macrophages correlated with the extent of phagocytosis of T. gondii and with intracellular survival of the organisms. Prior antibody-coating of T. gondii or activation of macrophages, either of which inhibited survival and replication of ingested organisms, was associated with production of LTD4 but not compound X. Killed organisms also stimulated LTD4 release only. Although T. gondii concentrated arachidonic acid, they did not metabolize the compound to identifiable lipoxigenase products. Preincubation of macrophages with the relative lipoxigenase inhibitors nordihydroguaiaretic acid or 5,8,11,14-eicosatetraenoic acid inhibited the formation of compound X. The absence of leukotriene production by macrophages ingesting T. gondii may explain the relative lack of a neutrophil inflammatory response in diseases due to obligate intracellular organisms. Alternatively, compound X may have functional activities that might mediate some of the host responses to cellular parasitism.

The leukotrienes (LT) have been identified as potentially important inflammatory mediators of host defense (1, 2). LTB4 is a potent neutrophil chemoattractant and stimulus for aggregation, degranulation, and adherence to endothelium. The sulfidopeptide leukotrienes LTC4, LTD4, and LTE4, which collectively constitute the slow-reacting substance of anaphylaxis (SRS), promote vasoconstriction and enhance permeability across the postcapillary venule. Resident tissue macrophages are major sources of the LT (3–5). The plasma membrane of these cells is rich in esterified arachidonate that is released during membrane perturbation and is then available to Ca2+-dependent 5-lipoxygenase that regulates the formation of the LT (6, 7). Traditional stimuli for LT production by macrophages in vitro include both soluble (calcium ionophore A23187, endotoxin, phorbol myristate acetate) and particulate (zymosan, immunocomplexes) stimuli; LT production in response to the latter has been related directly to the degree of phagocytosis (3–5).

Resident tissue macrophages are the initial target of many obligate intracellular pathogens that infect man, but the capacity of viable organisms to trigger the release of inflammatory mediators from macrophages has not been systematically investigated. The pathogenic protozoa are particularly adept at survival within macrophages (8). Because a significant neutrophilic response is infrequent in such infections, we examined the ability of macrophages to generate LT while ingesting the obligate intracellular protozoan Toxoplasma gondii.

MATERIALS AND METHODS

Special Reagents. Hydroxyl[3H]icosatetraenoic acid ([3H]HETE) and hydroperoxy[3H]icosatetraenoic acid ([3H]HPETE) (5-8, 9-11, 12- and 15-HETEs and HPETEs), and 5,15-dihydroxy- and 8,15-dihydroxy[3H]icosatetraenoic acid ([3H]5,15- and [3H]8,15-dHETE) standards were gifts of W. C. Hubbard (National Institutes of Health, Bethesda, MD). LTB4, 20-OH-LTB4, LTC4, LTD4, LTE4, and (5S,12S)-5,12-diHETE were provided by J. Rokach, Merck Frosst Labs, Pointe Claire, Quebec.

Murine Peritoneal Macrophages. Resident peritoneal cells were collected in Ca2+/Mg2+-free Hanks’ balanced salt solution (GIBCO) by lavage of 5- to 8-week-old BALB/c or C57BL/6 mice as described (9). Corynebacterium parvum suspension (Wellcome) intraperitoneally 12 days previously. Macrophage monolayers were established by adherence followed by washing in 35-mm tissue culture dishes as described (9). The addition of 6 × 10^6 macrophages per dish yielded 300 μg of adherent protein when using resident cells and 400 μg of adherent protein when using C. parvum-activated cells. The monolayers, composed of 98% nonspecific esterase-positive cells, were incubated overnight for 18 hr. In selected experiments, 1 μg/ml of [3H]arachidonic acid (AA; specific activity, 62.2 Ci/mmol; New England Nuclear; 1 Ci = 37 GBq) was added to the overnight cultures.

T. gondii. T. gondii RH strain was maintained by intraperitoneal passage in BALB/c mice. The organisms were harvested by peritoneal lavage, separated from leukocytes by filtration through a 3-μm polycarbonate filter (Nuclepore), washed three times, and suspended at 10^7 T. gondii per ml in Krebs–Ringer phosphate buffer (pH 7.4) containing 5.5 mM glucose (KRPG buffer) as described (9). Organisms prepared in this fashion were >90% viable as assessed by acidine orange–ethidium bromide fluorescence (9). In selected studies, T. gondii were heat-killed by incubation at 100°C for 5 min, glutaraldehyde-fixed (2% glutaraldehyde for 30 min at room temperature), or antibody-coated by incubation at 37°C

Abbreviations: LT, leukotrienes(s); LTB4, LTC4, LTD4, leukotrienes B4, C4, and D4; AA, arachidonic acid; HETE, hydroxyicosatetraenoic acid; dHETE, dihydroxyicosatetraenoic acid; HPETE, hydroperoxyicosatetraenoic acid; RP- and SP-HPLC, reverse-phase and straight-phase high-performance liquid chromatography.
for 30 min with 50% heat-inactivated human Sabin–Feldman dye-test-positive serum (anti-Toxoplasma titer, >1:128). Dye-test-negative serum was used as a control. Antibody-coated T. gondii were 84 ± 3% viable.

Preparation of Macrophage Supernatants. Macrophage monolayers were washed with KRPG buffer and overlayed with 3 ml of that buffer at 37°C containing 3 x 10⁷ T. gondii, 3 mg of opsonized zymosan (ICN), or 30 μg of A23187 (Sigma). After the contents were allowed to settle for 10 min, dishes were incubated at 37°C on a plate shaker for 20 min (A23187) or 90 min (zymosan, T. gondii). Supernatants were collected, cleared by centrifugation (250 x g), and stored at -70°C until assayed by HPLC (see below). In designated experiments, monolayers were preincubated with nordihydroguaiaretic acid (Sigma), indomethacin (Sigma), or 5,8, 11,14-icosatetraynoic acid (provided by W. E. Scott, Hoffmann-La Roche) at indicated concentrations in KRPG buffer for 2 hr prior to and during the subsequent incubation with stimuli.

Generation of Supernatone Anion and H₂O₂ by Mononuclear Cells. Similarly defined monolayers were challenged with the same stimuli in 3 ml of KRPG buffer with the addition of either 80 μM ferricytochrome c (Sigma) in the presence or absence of 75 μg of superoxide dismutase (2500 units/mg, Sigma) or 20 μM scopeotin (Sigma) with 3 units of horse-radish peroxidase (Sigma). After allowing the mixture to settle and then shaking them for 60 min at 37°C, supernatants were harvested into ice tubes and cleared by centrifugation. Supernatone dismutase-inhibitable ferricytochrome c reduction was used to calculate supernatone anion (O₂⁻) generation (9), and the oxidation of scopeotin was used to calculate H₂O₂ production as described (10).

Phagocytosis. Macrophage monolayers were established on 15-mm glass coverslips as described (9) and challenged with zymosan or T. gondii at the same concentrations used for the generation of LT. At the indicated time points, coverslips were washed to remove nonadherent particles, fixed in 2.5% glutaraldehyde, and stained with Diff-Quik. The number of ingested particles per cell was assessed microscopically under oil immersion. Intracellular survival and replication of T. gondii were determined by light microscopy from coverslips examined 20 hr after infection of monolayers using a ratio of 2 or 3 T. gondii per macrophage as described (9).

Phagolysosomal Fusion. Macrophage monolayers on glass coverslips were incubated at 37°C in Hanks’ balanced salt solution containing 20 μg of acridine orange per ml, washed, and reincubated in RPMI medium for 1 hr before challenge with zymosan or T. gondii. After 30 min, coverslips were washed and inverted onto a drop of glycerol on a microscope slide. The percentage of ingested particles promoting phagolysosomal fusion was quantitated by using fluorescence microscopy as described (11).

Assay for Lipoygenase Products of Arachidonate. Supernatants underwent solid-phase extraction using 3 ml octadecyl Baker-10 columns (J. T. Baker Chemical, Phillipsburg, NJ) as described (12). Macrophages were evaporated to dryness under nitrogen, suspended in methanol/water/acetic acid, 75:25:5.01 (vol/vol), pH 7.4, clarified by centrifugation (3000 x g for 5 min at 4°C), and applied to a 5-μm UltraspHERE ODS C₁₈ column (4.6 x 250 mm; Beckman Instruments, Berkeley, CA). Reverse-phase HPLC (RP-HPLC) was subsequently performed with the same solvent at a flow rate of 1 ml/min. Substances eluted in peaks at 280 nm and 235 nm were rechromatographed by RP-HPLC on a Beckman UltraspHERE ODS C₁₈ column (4.6 x 75 mm) with the same solvent at a flow rate of 1.6 ml/min and by straight-phase HPLC (SP-HPLC) on a µPorasil column (3.9 x 300 mm; Waters Associates) with hexane/isopropanol, 100:0.45 (vol/vol), at a flow rate of 1 ml/min. Peaks were identified by coelution with authentic standards, UV spec- troscopy, and biologic activity as described (13). The amount of each compound was determined by using the extinction coefficient of the identified arachidonate product. The radioactivity of eluted peak material was measured by a FLO-ONE model HP radioactivity flow detector (Radiomatic, Tampa, FL) using Radiomatic FLO-SCINT II at a scintillant/HPLC solvent ratio of 2:1. The slow-reacting substance of anaphylaxis activity of the sulfidopeptide leukotrienes was determined by the guinea pig ileal bioassay, and chemotactic activity of peak material that rechromatographed with LTB₄ was measured by the leading-front assay with human neutrophils as described (14).

Uptake of AA and AA-Derived Products by T. gondii. Aliquots of T. gondii in 150 μl of KRPG buffer were placed in flat-bottom 96-well microtiter trays (Falcon), and various concentrations of tritiated AA, LTB₄, LTC₄, LTD₄ or 15-HETE were added. Trays were incubated on an orbital shaker (Belloco Glass), and at specified times the contents of the cells were deposited on glass-fiber filter paper by using a multiple automated sample harvester. Dried filters were counted in a liquid scintillation counter.

RESULTS

Resident BALB/c peritoneal macrophages were challenged with either the calcium ionophore A23187 or opsonized zymosan and were incubated for 20 min or 90 min, respectively; supernatants from this treatment were analyzed by HPLC (Fig. 1). LT were identified by their comigration in RP- and SP-HPLC with authentic standards, their characteristic UV spectra, and their biologic activity (chemotactic activity for LTB₄ and slow-reacting substance of anaphylaxis activity for LTC₄ and LTD₄). After A23187 challenge (Fig. 1A), macrophages released LTC₄ (1.3 μg/mg of monolayer protein) and LTB₄ (0.3 μg/mg). After ingestion of zymosan (Fig. 1B), LTD₄ (0.6 μg/mg) was the major product released. However, challenge with viable T. gondii (Fig. 1C) resulted in a marked alteration in the products released. No LTB₄, LTC₄ or LTD₄ was present by HPLC analysis. The predominant peak at 235 nm was a novel compound, designated compound X, that migrated immediately prior to 15-HETE. The compound was rechromatographed by RP- and SP-HPLC; it migrated separately from 5-, 8-, 9-, 11-, 12-, and 15-HETEs or -HPETEs and 5,15- or 8,15-diHETEs in both RP- and SP-HPLC. The scanning UV absorption spectrum of compound X showed a peak at 206 nm with shoulders at 224 and 276 nm. Three monohETEs, 11-HETE (0.1 μg/ml), 12-HETE (0.8 μg/mg), and 15-HETE (0.2 μg/mg) were also recovered, as assessed by coelution with rechromatographed HETE standards and characteristic UV spectra. When T. gondii were killed by heating prior to incubation with resident macrophages, the major product recovered was LTD₄ (0.7 μg/mg) (Fig. 1D). Glutaraldehyde-fixed T. gondii gave an elution chromatogram identical to that of heat-killed T. gondii. Compound X and the monoHETEs were not present. Macrophages from C57BL/6 mice produced comparable findings for each of the stimuli. Incubation of macrophages alone under these conditions resulted in no detectable release of lipoygenase products.

Although ingestion of heat-killed T. gondii was not accompanied by the alteration in lipoygenase products that occurred during phagocytosis of viable T. gondii (Fig. 1C and D), this was not due to impaired phagocytosis of heat-killed organisms. Phagocytosis of viable and heat-killed organisms was comparable under these conditions (Fig. 2). Further, the generation of compound X correlated directly with the extent of phagocytosis of viable organisms as assessed microscopically.

Resident macrophages are unable to kill ingested T. gondii, organisms that effectively elude triggering the respiratory
burst of the phagocyte (15) and impede the fusion of potentially toxic lysosomes with the phagosome (16). As previously reported (9, 15, 17), resident macrophages produced little O_2^- or H_2O_2 after ingestion of T. gondii, and only 12% of ingested organisms showed evidence of phagolysosomal fusion (Table 1). T. gondii readily replicated in these cells, with 4.9 organisms per vacuole 20 hr after infection. In contrast, opsonized zymosan was a potent inducer both of the respiratory burst and of phagolysosomal fusion. The respiratory burst provoked by heat-killed T. gondii was poor, although ingested organisms did demonstrate phagolysosomal fusion.

Prior incubation of T. gondii with specific antibody is nontoxic in the absence of complement but renders the organisms susceptible to the microbicidal systems of resident macrophages (18). Antibody-coated T. gondii induced a readily detectable respiratory burst and did not impede phagolysosomal fusion during ingestion by resident macrophages (Table 1). More than 75% of ingested organisms were killed, and T. gondii surviving at 20 hr replicated poorly (1.3 ± 0.2 Toxoplasma per vacuole). Supernatants from macrophages ingesting antibody-coated T. gondii contained LTD_4 but no monoHEtEs or compound X (data not shown).

Activation of macrophages also enables these cells to kill T. gondii (19). C. parvum-activated macrophages phagocytized viable T. gondii with a readily measured respiratory burst and demonstrated significantly greater phagolysosomal fusion than did resident macrophages (Table 1). More than 60% of ingested organisms were killed, and the replication of surviving organisms was limited (1.2 ± 0.2 Toxoplasma per vacuole 20 hr). Although resident macrophages released compound X and monoHEtEs during phagocytosis of viable T. gondii (Fig. 1C), activated macrophages released predominantly LTD_4 (0.3 μg/mg of protein).

T. gondii incorporated increasing amounts of [3H]AA up to 60 min; uptake was proportional to both cell number (Fig. 3) and AA concentration (data not shown). Heat-killed organisms did not incorporate the labeled compound. The oxidation products of AA found in the supernatant after 15–60 min of incubation with T. gondii were similar to those caused by spontaneous oxidation of AA in the absence of organisms. No LT, HETE, or compound X was detected. Incubation of [3H]AA-labeled organisms with A23187 resulted in no release of AA products. Tritiated LTD_4, LTC_4, LTD_4, or 15-HETE, in contrast to [3H]AA, was not incorporated by T. gondii under these conditions.

To determine whether viable T. gondii could modify typical macrophage-derived lipoxygenase products, supernatants from resident macrophages incubated with A23187 (20 min) or zymosan (90 min) were incubated further for 60 min with viable T. gondii in the absence of macrophages. After incubation, the supernatants were extracted and examined by RP-HPLC. No modification of the macrophage lipoxygenase profile was detected; LTD_4, LTC_4, and LTD_4 were each recovered in expected amounts (data not shown).

Resident macrophages were incubated with inhibitors of AA metabolism before and during phagocytosis of viable T. gondii, then the supernatants were extracted and analyzed by HPLC to determine effects on the formation of compound X. As assessed by the height of peak X absorbance, the generation of compound X was significantly less in the presence of the relative lipoxygenase inhibitors 5,8,11,14-eicosatetraynoic acid and nordihydroguaiaretic acid at concentrations of both 100 μM (2.5 ± 5.4% and 4.9 ± 4.3% of control, respectively) and 10 μM (7.4 ± 2.4% and 12.2 ± 6.0%, respectively). The relative cyclooxygenase inhibitor indomethacin had little effect on the formation of compound X (97.6 ± 0.1% of control at 100 μM).

Resident macrophages that had been preincubated with [3H]AA were washed and challenged with viable T. gondii. The fractions corresponding to compound X and the monoHEtEs were collected; each demonstrated incorpo-
Table 1. Interactions of macrophages with T. gondii and zymosan

<table>
<thead>
<tr>
<th>Macrophages</th>
<th>Stimulus</th>
<th>( \text{O}_2^\cdot ) ( \text{nmol/mg of protein} )</th>
<th>( \text{H}_2\text{O}_2^\cdot ) ( \text{nmol/mg of protein} )</th>
<th>Phagolysosomal fusion, %</th>
<th>T. gondii per vacuole at 20 hr</th>
</tr>
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<tbody>
<tr>
<td>Resident</td>
<td>T. gondii</td>
<td>4.1 ± 1.9</td>
<td>6.0 ± 4.2</td>
<td>12 ± 5</td>
<td>4.9 ± 0.4</td>
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<tr>
<td></td>
<td>Opsonized zymosan</td>
<td>46.3 ± 7.2</td>
<td>49.1 ± 6.3</td>
<td>88 ± 5</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Heat-killed T. gondii</td>
<td>6.2 ± 0.9</td>
<td>7.1 ± 1.1</td>
<td>71 ± 11</td>
<td>—</td>
</tr>
<tr>
<td>C. parvum-activated</td>
<td>T. gondii</td>
<td>84.1 ± 8.1</td>
<td>110 ± 9.6</td>
<td>57 ± 11</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Opsonized zymosan</td>
<td>287 ± 29.2</td>
<td>338 ± 36.3</td>
<td>92 ± 3</td>
<td>—</td>
</tr>
</tbody>
</table>

*Assessed by superoxide dismutase-inhibitable ferricytochrome c reduction at 60 min. Responses to phorbol 12-myristate 13-acetate at 100 ng/ml were 110 and 376 nmol of \( \text{O}_2^\cdot \) per mg of protein per 60 min for resident and C. parvum-activated macrophages, respectively. Results shown are means ± SEM (n = 3–7).

†Assessed by fluorescence quenching of scopoletin at 60 min. Responses to phorbol 12-myristate 13-acetate at 100 ng/ml were 80 and 465 nmol of \( \text{H}_2\text{O}_2^\cdot \) per mg of protein per 60 min for resident and C. parvum-activated macrophages, respectively. Results shown are means ± SEM (n = 3).

‡Percentage of ingested particles showing diffuse orange fluorescence. Results are means ± SEM of duplicate determinations (n = 2–3).

Discussion

These studies demonstrate that resident murine macrophages release a novel profile of lipoxygenase products when phagocytizing the obligate intracellular protozoan T. gondii. Whereas macrophages stimulated by A23187 or zymosan particles generated LTB₄, LTC₄, and LTD₄, macrophages phagocytizing T. gondii released no detectable LT as assessed by HPLC. The major products released after the ingestion of T. gondii were 11-, 12-, and 15-HETE, together with an unidentified compound designated “compound X.” In RP- and SP-HPLC, compound X migrated separately from all available HETE, HPETE, and diHETE standards. The compound’s incorporation of \(^{3}H\) AA, together with its inhibition by the relative lipoxygenase inhibitors nordihydroguaiaretic acid and 5,8,11,14-icosatetraynoic acid, suggests that compound X may be a macrophage lipoxygenase product. Although nordihydroguaiaretic acid can also inhibit esterification of AA, attempted base hydrolysis of compound X (NaOH in methanol at 45°C for 12 hr) did not alter its elution time in RP-HPLC, suggesting that the unknown compound is not an acyl ester of AA (data not shown).

Three conditions were necessary for the T. gondii-induced shift in lipoxygenase products released from macrophages. First, macrophages and T. gondii had to be incubated together long enough for phagocytosis to occur. Neither macrophages nor T. gondii alone produced detectable lipoxygenase products under these conditions. Products...
could be recovered in the supernatant within 30 min of incubation of macrophages with *T. gondii*, and peak levels were obtained between 90 and 120 min, a process that correlated with the ingestion of organisms as assessed microscopically (Fig. 2). Second, organisms had to be viable. Boiling or fixing *T. gondii* in glutaraldehyde prior to incubation with resident macrophages provoked subsequent LT release that was comparable to that released by ingestion of zymosan. Third, organisms had to survive and replicate intracellularly after phagocytosis. Prior antibody-coating of *T. gondii*, which enables resident macrophages to kill these protozoa (18), did not provoke a lipoxygenase shift in resident macrophages. Further, *C. parvum*-activated macrophages, which significantly impaired both *T. gondii* survival and replication, also did not generate compound X during phagocytosis of viable *T. gondii*.

Although viable *T. gondii* were required to generate the unique profile of lipoxygenase products recovered, there was no evidence that the organisms themselves produced compound X, either directly or from substances released from zymosan-triggered macrophages. *T. gondii* readily incorporated AA but formed no detectable oxidation products either spontaneously or after ionophore stimulation. *T. gondii* presumably share with other intracellular protozoa the capacity to utilize free fatty acids as an energy source via two-carbon β-oxidation (20, 21). However, under the same experimental conditions, *T. gondii* neither incorporated nor metabolized LTB₄, LTC₄, LTD₄, or 15-HETE. The incorporation of the radiolabel from *T. gondii*-derived [³H]AA into macrophage HETEs (Fig. 4B) was an unexpected finding. Although macrophage utilization of exogenous AA supplied by lymphocytes has been reported (22), the capacity of these cells to utilize AA supplied by an ingested microorganism has not been described to our knowledge.

The mechanism by which viable *T. gondii* induce a shift in macrophage AA metabolism is unknown. Lipoxygenase pathways in macrophages are not constitutive, but are presumably activated by the Ca²⁺ fluxes accompanying membrane perturbation (23, 24). Generation of compound X and the monoHETEs correlated directly with the extent of phagocytosis of viable *T. gondii*. These organisms parasitize macrophages using an incompletely characterized process that evaporates activation of the respiratory burst (13) and impedes subsequent phagolysosomal fusion (16). The use of antibody-coated *T. gondii* or activated macrophages, each of which promotes phagolysosomal fusion, was not associated with the shift in AA metabolism induced by viable organisms. Distinct macrophage pools of AA have been described, and their availability after membrane events may be stimulus dependent (5). The ability to label compound X with macrophage-derived but not *T. gondii*-derived [³H]AA (Fig. 4) would be consistent with the concept of different AA pools. A lysosomal location of at least one phospholipase A₂ has been suggested (25); it is possible that this enzyme would not be available to viable *T. gondii* during phagocytosis.

The role of the parasite-mediated shift in macrophage lipoxygenase products in establishing *in vivo* infection is unknown. LT mediates multiple important inflammatory mechanisms, including the attraction and localization of circulating neutrophils and monocytes. Of note, both neutrophils and blood monocytes readily kill *T. gondii* (9, 15, 26) but are not a prominent feature of the host inflammatory response to this infection (27). The inability of resident macrophages to generate LT during the phagocytosis of viable *T. gondii* would be consistent with these *in vivo* observations. It is also possible that compound X might directly mediate some of the cellular or systemic host responses that occur in toxoplasmosis. Our current efforts are directed at production of sufficient amounts of this compound to determine its structure and to investigate its functional activity. Reiner and Malemud (28) recently have reported altered cyclooxygenase and lipoxygenase activities in macrophages infected with *Leishmania donovani*, suggesting that the capacity for obligate intracellular organisms to affect host cell AA metabolism may be a more generalized phenomenon. These studies emphasize the necessity of examining the interaction of phagocytes with viable biologic agents to understand the nature of the host–parasite interaction.

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