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The Importance of Leukotrienes in Mast Cell–Mediated *Toxoplasma gondii* Cytotoxicity

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Mast cells participate in the host defense against parasites. Mast cells release leukotrienes (LTs), potent 5-lipoxygenase (LO) products of arachidonic acid well-known to be involved in the inflammatory process. After incubation with *Toxoplasma gondii,* mast cells were found to degranulate and release LTB_4; this interaction damages the tachyzoites. This mast cell activity against the tachyzoites was inhibited by the 5-LO inhibitor A-63162 and the 5-LO–activating protein inhibitor MK-886 but not by the cyclooxygenase inhibitor indomethacin. Reactive oxygen species were not implicated in the mast cell–mediated toxoplasmacidal activity. The generation of LTs is important for mast cell secretion, and LTB_4 released by mast cells and other inflammatory cells may be a key factor in the host defense against *T. gondii.*

Toxoplasma gondii, the protozoal pathogen that causes toxoplasmosis, inhibits respiratory burst activity and phagolysosomal fusion in macrophages replicating within these cells to the detriment of the host [1]. Effector cells against *T. gondii* include monocytes [2], interferon-γ (IFN-γ)–activated macrophages [1, 3], neutrophils [2], and platelets [4]. Mast cells exert cytotoxic activity against certain target cells, such as mammalian tumor cells [5] and schistosomula of *Schistosoma mansoni* [6], but their role in the host defense against *T. gondii* has not been examined previously. We investigated the role of mast cells against tachyzoites of *T. gondii.*

Materials and Methods

Special reagents. The selective 5-lipoxygenase (LO) inhibitor A-63162 (*N*-hydroxy-*N*-[1-(4-phenylmethoxyphenyl)ethyl]acetamide; provided by R. L. Bell and G. W. Carter, Abbott Laboratories, Abbott Park, IL) [7] and 5-lipoxygenase–activating protein (FLAP) inhibitor MK-886 (3-[1-(4-chlorbenzyl)3-t-butyl-thio-t-isopropyl-indol-2-yl]-2-2-dimethylpropanoic acid; provided by A. W. Ford-Hutchinson, Merck Frosst Centre for Therapeutic Research, Pointe Claire-Dorval, Québec, Canada) [8] were dissolved in DMSO at 10^{-2} *M* concentrations and diluted in Tyrode's buffer with a final concentration of 0.1% dimethyl sulfoxide (DMSO). The CO inhibitor indomethacin (Sigma, St. Louis) was dissolved in ethanol at a concentration of 10^{-2} *M* and diluted with PBS.

Mast cell isolation. Mast cells were obtained by peritoneal lavage of 200-g male Sprague-Dawley rats and purified (>90%) using 38% bovine serum albumin gradients [5]. The purified mast cells were >95% viable as determined by trypan blue dye exclusion [5].

Macrophage isolation. Resident peritoneal cells were collected by lavage of 6- to 8-week-old BALB/c mice [2] and resuspended in RPMI 1640 medium buffered with 10 m*M* HEPES (Whittaker MA Bioproducts, Walkersville, MD) and supplemented with 2 m*M* L-glutamine, 25 U/mL penicillin G, and 25 μg/mL streptomycin (GIBCO BRL, Gaithersburg, MD). At a concentration of 1×10^6 macrophages/mL, the cells were added to sterile 35×10 mm tissue culture plates (Falcon 3801 Primaria; Becton Dickinson, Oxnard, CA) and allowed to adhere for 1 h at 37°C in 5% CO_2–95% air with humidity. The plates were washed with sterile PBS to remove nonadherent cells, and adherent macrophages were used immediately in interactions with *T. gondii* with or without mast cell reaction mixtures as described below.

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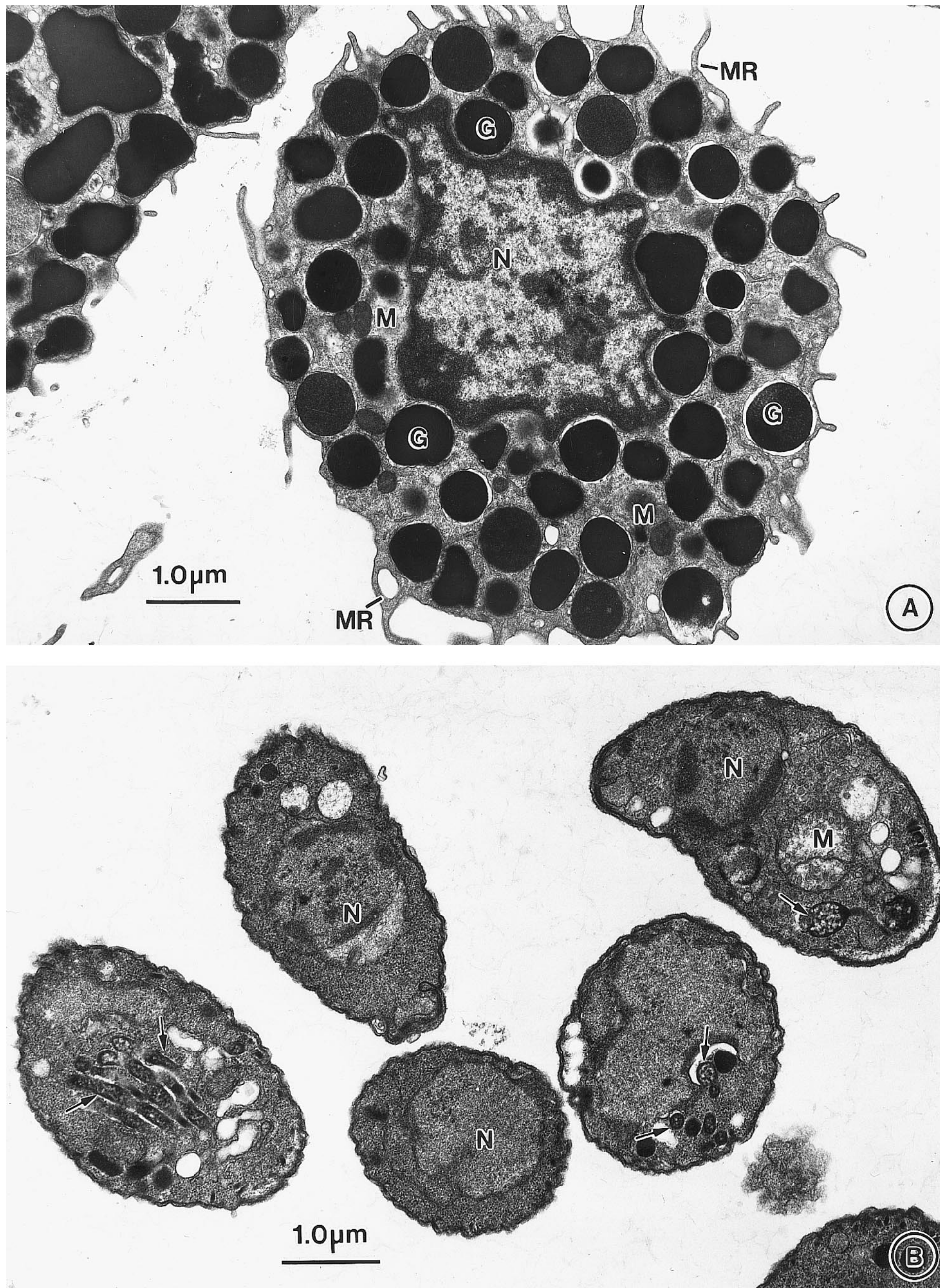

Figure 1. Morphology of mast cell–induced *T. gondii* cytotoxicity. **A**, **B**: Ultrastructure of mast cells and T. gondii tachyzoites as examined by transmission electron microscopy. **A**: Unstimulated rat peritoneal mast cells contain nucleus (N), electron-dense granules (G), mitochondria (M), and surface microridges (MR) of typical appearance. **B**: *T. gondii* tachyzoites contain nucleus (N), mitochondria (M), and characteristic rhoptry organelles (arrows).

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T. gondii isolation. *T. gondii* RH strain, maintained in BALB/c mice by intraperitoneal passage, was isolated by peritoneal lavage and filtration through a 3-μm polycarbonate filter (Nuclepore, Pleasanton, CA) [4]. The *T. gondii* were >95% viable when assayed by trypan blue dye exclusion [4].

Mast cell–T. gondii interaction. Duplicate samples of mast cells and *T. gondii* (1:6 ratio; mast cell to organism) were incubated in Tyrode's buffer for 30–60 min at 37°C. The reaction mixtures were then centrifuged at 700 *g* for 10 min at 4°C. The supernatants and cell pellets were collected and stored at −70°C until assay of histamine or eicosanoids. For electron microscopy, cell pellets were fixed in 2% glutaraldehyde in 0.1 *M* sodium cacodylate buffer, pH 7.4. To assess the role of eicosanoids and reactive oxygen species in mast cell–mediated cytotoxicity against *T. gondii,* in some studies the mast cell–*T. gondii* reaction mixtures were made in the presence of the 5-LO inhibitor A-63162, the FLAP inhibitor MK-886, the CO inhibitor indomethacin, or the hydrogen peroxide (H_2O_2) scavenger catalase.

To assess whether *T. gondii* after incubation with mast cells were able to infect and replicate in other cells, the macrophage monolayers were incubated for 30 min at 37°C with *T. gondii* that had been previously incubated in the absence or presence of mast cells (1:6 ratio; mast cell to organism) for 60 min at 37°C. Noningested organisms were then removed by washing, and the cell

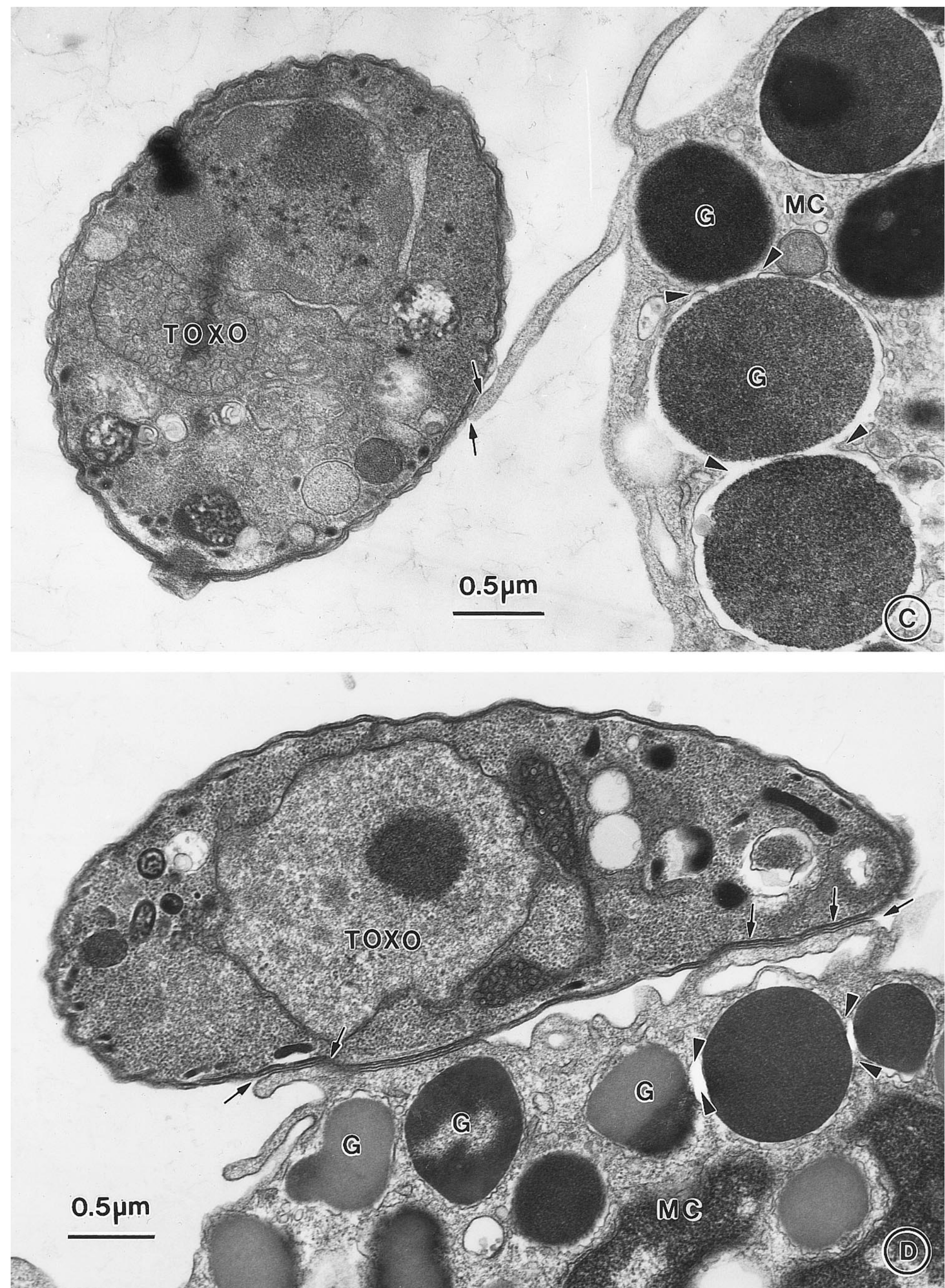

Figure 1 *(continued).* **C**, **D**: Adherence of *T. gondii* to mast cells. Mast cells (MC; 2×10^6) were incubated with *T. gondii* (1.2×10^7; TOXO) in 1 mL of Tyrode's buffer, pH 7.4, for 30 min at 37°C. **C**: Initial contact (arrows) between mast cells and *T. gondii* tachyzoites is by extension of mast cell microvillus projection to surface of *T. gondii.* **D**: Extensive area of close apposition (arrows) of *T. gondii* and mast cell surface membrane structures is seen. In **C** and **D**, initial mast cell secretory changes noted after interaction with *T. gondii* include membrane fusion (arrowheads) between adjacent cytoplasmic granules (G).

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monolayers were either fixed with 5% formalin (time 0) or incubated in RPMI medium for an additional 6 h before fixation in 5% formalin. After staining with Giemsa, the number of intracellular *T. gondii* per macrophage was determined by light microscopy [1].

Histamine assay. Histamine in the supernatant and cell pellet fractions was assayed fluorometrically between 360 and 480 nm with *o*-phthaldialdehyde [5].

Eicosanoid assays. The reaction mixture supernatants underwent reverse-phase high-pressure liquid chromatography on a 5-μm particle size analytical C_{18} column (Ultrasphere; C_{18}-IP, 4.6 $\times$ 250 mm; Beckman Instruments, Fullerton, CA) with methanol-water–acetic acid (75:25:0.1) at a flow rate of 1 mL/min [1]. Eluting peaks were monitored at 235 and 270 nm, with rapid spectral scanning for identification of compounds that coeluted with authentic hydroxyeicosatetraenoic acid (HETE), hydroxyoctadecadienoic acid (HODE), and leukotriene (LT) standards [1]. In addition, the extracted supernatants were assayed for 5-LO (LTB_4, LTC_4) and CO (prostaglandin [PG] D_2, PGE_2, thromboxane [TX] B_2) arachidonate products by RIA and ELISA [1, 9].

Morphologic studies. After glutaraldehyde fixation, the samples were processed for transmission electron microscopy and examined on an electron microscope (model 100B; JEOL, Tokyo)

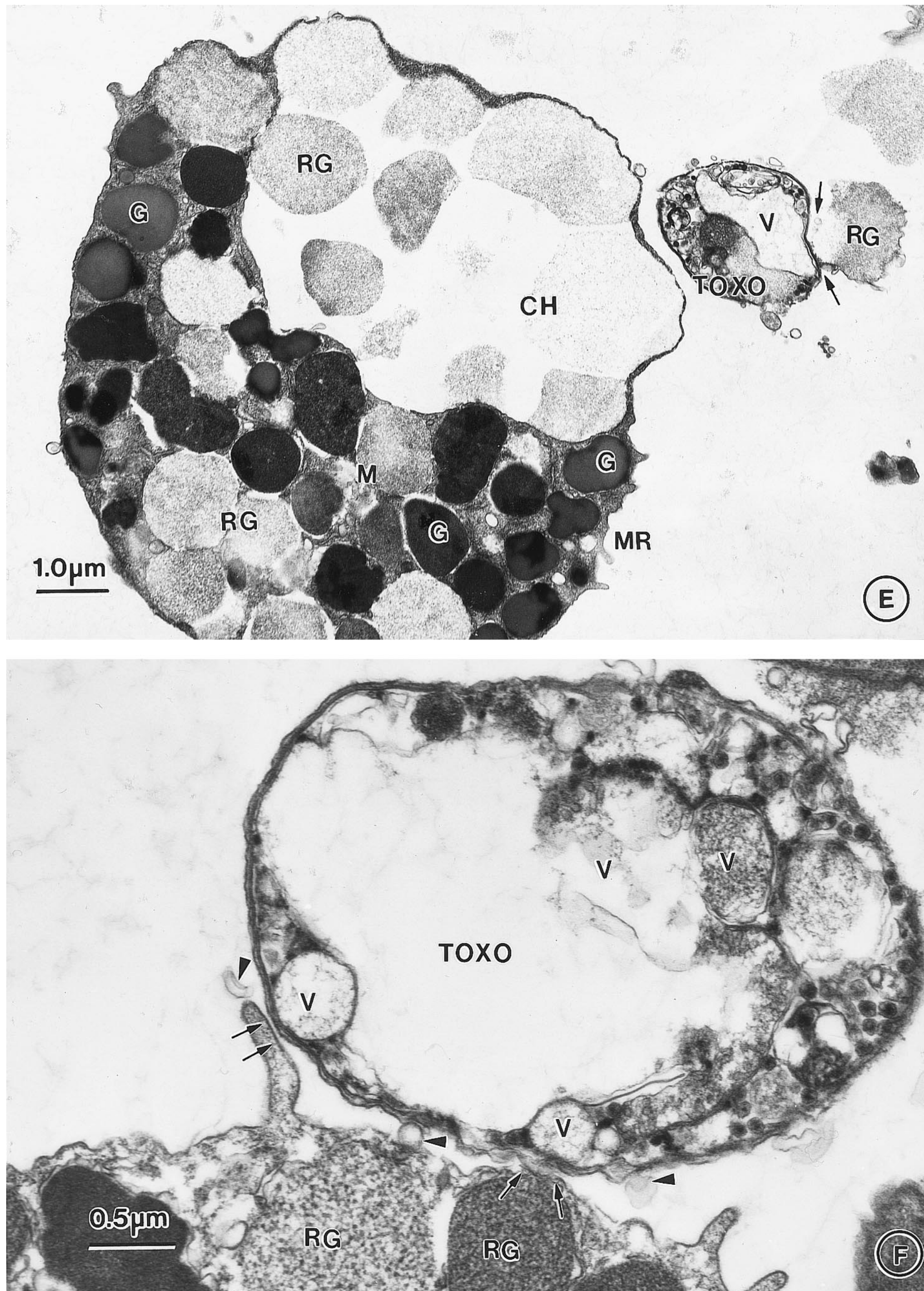

Figure 1 *(continued).* **E**, **F**: Mast cell–induced extracellular *T. gondii* cytotoxicity. **E**: Mast cells showed evidence of degranulation, with formation of secretory channels (CH) containing released granules (RG) of decreased electron density compared with intact cytoplasmic granules (G). Released mast cell granule material (arrows) is attached to parasite surface; vacuole (V) formation in *T. gondii* (TOXO) is observed. **F**: At higher magnification, close contact of mast cell surface membrane projections to *T. gondii* tachyzoite is seen (arrows). Cytotoxic changes noted in organisms after their interaction with mast cells include surface membrane vesiculation (arrowheads) and intracytoplasmic vacuolization (V) with loss of intracellular rhoptry organelles and mitochondria.

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at 60 kV [1, 5]. Ten randomly selected fields in each grid were photographed at a magnification of 7000 for morphometric analysis of the percentage of degranulated mast cells [10] and damaged *T. gondii* [1, 11].

Statistical analysis. Data are reported as means ± SEs of combined experiments. Differences were analyzed for significance ($P < .05$) by Student's two-tailed *t* test for independent means.

Results and Discussion

The interaction of mast cells and *T. gondii* tachyzoites was examined ultrastructurally and biochemically. Purified rat peritoneal mast cells incubated in the absence of *T. gondii* exhibited their typical large electron-dense cytoplasmic granules by transmission electron microscopy (figure 1A). The crescent-shaped *T. gondii* incubated in buffer alone have a characteristic surface membrane structure composed of closely apposed inner and outer membrane units (figure 1B). When the two were incubated together in the absence of antibody or other agents, the tachyzoites adhered to the surface microvillus projections of the mast cells (figure 1C, D). This interaction was followed by mast cell degranulation (figure 1E–G). Large intracytoplasmic secretory channels

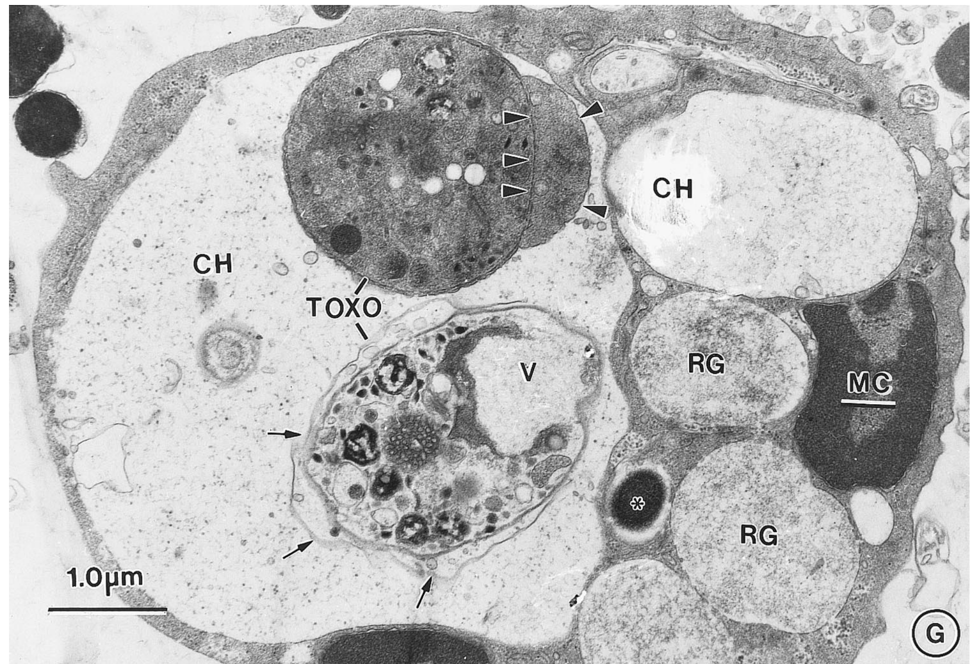

Figure 1 *(continued).* **G**: Mast cell–induced intracellular *T. gondii* cytotoxicity. Extensive mast cell degranulation is noted as consequence of interaction of mast cells (MC) with *T. gondii* (TOXO). Few electron-dense granules (*) are observed, and swollen, released granules (RG) are present. Disrupted *T. gondii* are noted within intracellular secretory channels (CH) in mast cells. Extrusion of cytoplasmic contents between inner and outer surface membrane units (arrowheads) and surface membrane vesiculation (arrows) in tachyzoites are observed.

containing swollen mast cell granules of decreased electron density were observed.

Extracellularly released mast cell granule material attached to the surface of the parasites (figure 1E). Mast cell–induced *T. gondii* cytotoxic changes included a marked disruption of the surface membrane structures of the organisms; intracellular vacuolation and leakage of cytoplasmic contents into the space between the separate inner and outer membrane units occurred (figure 1E–G). Damaged parasites were also seen intracellularly within secretory channels of the degranulated mast cells (figure 1G). A possible mechanism of how *T. gondii* trigger mast cell degranulation may be through release of lysophospholipids from the tachyzoites' apical organelles, termed rhoptries [12], since lysophospholipids potentiate mast cell histamine secretion [13].

Killing of *T. gondii* by mast cells was confirmed by the inability of the mast cell–treated tachyzoites to infect and replicate in murine peritoneal macrophages. *T. gondii* after incubation in the presence or absence of mast cells for 60 min at 37°C were incubated with macrophage monolayers for 30 min at 37°C. After washing, the incubations were either terminated by formalin fixation of the monolayers (0 h) or continued in media alone for an additional 6 h before fixation. In monolayers incubated with buffer-treated *T. gondii,* the number of *T. gondii* per macrophage was increased ~3-fold (4.9 ± 0.4 *T. gondii*/macrophage, $P = .001$) at 6 h compared with 0 h (1.7 ± 0.3 *T. gondii*/macrophage). In contrast, there was no significant increase in the number of *T. gondii* present in the macrophages incubated with the mast cell–treated *T. gondii* at 6 h (0.6 ± 0.2 *T. gondii*/macrophage) compared with 0 h (0.4 ± 0.1 *T. gondii*/macrophage).

Mast cell histamine release increased from a background level of 9% to 72% after incubation with the tachyzoites. Exogenous histamine, however, had no effect on *T. gondii* ultrastructure. Only 3% of *T. gondii* tachyzoites ($n = 106$) and 2% of *T. gondii* tachyzoites ($n = 119$) had evidence of cytotoxic changes after incubation with, respectively, buffer alone or buffer containing 10^{-6} *M* histamine for 30 min at 37°C.

5-LO arachidonate products were not detected in supernatants from mast cells (figure 2A) or *T. gondii* (figure 2B) tachyzoites incubated in buffer alone for 60 min. When mast cells were incubated with *T. gondii* (1:6 ratio, mast cell to organism), release of LTB_4 was demonstrated by reverse-phase high-pressure liquid chromatography (figure 2C) and quantitated by RIA (219.8 ± 82.2 pg of LTB_4/5 × 10^6 mast cells, $n = 5$). No significant release of LTC_4, LTD_4, LTE_4, 5-HETE, 12-HETE, 15-HETE, 9-HODE, 13-HODE, PGD_2, PGE_2, or TXB_2 above baseline (mast cells alone) was detected in the mast cell–*T. gondii* reaction mixture supernatants. The LTB_4 levels (~0.7 × 10^{-9} *M*) found in the mast cell–*T. gondii* supernatants are comparable to those previously shown to induce *T. gondii* cytotoxicity (10^{-10}–10^{-6} *M*).

LTB_4-induced *T. gondii* cytotoxicity was characterized by vesiculation of the surface membrane and loss of the cytoplasmic contents of the organisms [1]. These are the same cytotoxic changes as seen in *T. gondii* after incubation with mast cells. The CO arachidonate acid product TXA_2 [4] and the LO linoleic acid product 13-HODE released by human platelets [11] also induce similar cytotoxic changes after interaction with *T. gondii,* but neither of these products was found in the mast cell–*T. gondii* reaction mixture supernatants.

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Both 5-LO and FLAP are necessary for LT synthesis [8]. The effect of the selective 5-LO inhibitor A-63162 [7] and the selective FLAP inhibitor MK-886 [8] on *T. gondii*–mediated mast cell degranulation was studied. By morphometric analysis, ~70% of the mast cells exhibited secretory changes ultrastructurally after interaction with *T. gondii* (table 1). A-63162 and MK-886 at 10^{-6} *M* concentrations, which block LT release by >90% [8], each inhibited mast cell degranulation induced by *T. gondii* by 89% ($P = .001$) and 83% ($P = .001$), respectively. Histamine release by mast cells incubated with *T. gondii* in the presence of either A-63162 or MK-886 at a 10^{-6} *M* concentration was not significantly different from that by mast cells incubated in buffer alone. Further, mast cell–mediated *T. gondii* cytotoxicity was also significantly inhibited by these LT inhibitors but not by the CO inhibitor indomethacin (table 1), indicating the importance of 5-LO arachidonate products in this cytotoxic process. Similarly, we recently found that LTs play an important role in human macrophage IFN-γ–induced antitoxoplasma activity. LTB_4 promotes intracellular killing of *T. gondii* tachyzoites ingested by non–IFN-γ–treated monocyte-derived macrophages [1]. In addition, the selective 5-LO inhibitor zileuton, but not the CO inhibitor indomethacin, inhibits IFN-γ–induced antitoxoplasma activity in these phagocytes [1].

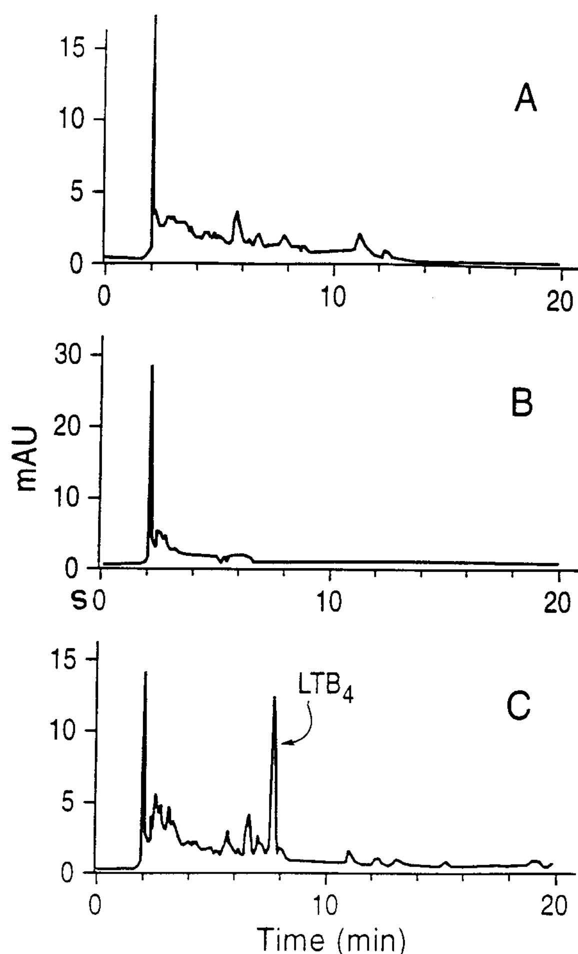

Figure 2. Role of leukotrienes in mast cell degranulation and *T. gondii* cytotoxicity: *T. gondii*–activated mast cells release LTB_4. Mast cells (5×10^6) were incubated for 60 min at 37°C in absence (**A**) or presence (**C**) of *T. gondii* tachyzoites (3×10^7) in 1 mL of Tyrode's buffer. In **B**, *T. gondii* (3×10^7) were incubated in 1 mL of buffer alone for 60 min at 37°C. Supernatants underwent reverse-phase high-pressure liquid chromatography with UV absorbance measured at 270 nm.

Table 1. Leukotriene inhibitors block mast cell degranulation and *T. gondii* cytotoxicity.

Reaction mixture	Mast cell degranulation, degranulated cells/ total cells (%)	*T. gondii* cytotoxic changes, damaged *T. gondii*/ total *T. gondii* (%)
Mast cells	27/305 (8.9)	—
T. gondii	—	42/580 (7.2)
Mast cells + *T. gondii*	278/398 (69.8)	134/306 (43.8)
Mast cells + *T. gondii* + A-63162	71/341 (20.8)*	32/234 (13.4)*
Mast cells + *T. gondii* + MK-886	44/206 (21.4)*	36/300 (12.0)*
Mast cells + *T. gondii* + indomethacin	252/399 (63.2)	144/389 (37.0)

NOTE. Mast cells (2×10^6) and *T. gondii* (1.2×10^7) were incubated in absence or presence of A-63162 (10^{-6} *M*), MK-886 (10^{-6} *M*), or indomethacin (10^{-5} *M*) in 1 mL of Tyrode's buffer for 30 min at 37°C. % of degranulated mast cells and damaged *T. gondii* was determined by morphometry. Data are means of 2–3 experiments for each condition.

* $P < .05$ vs. mast cells + *T. gondii*.

In prior work, we found that exogenous H_2O_2 was required to produce mast cell degranulation [5] and cytotoxic activity against tumor cells [5] and schistosomula [6]; cytotoxicity occurred through the interaction of H_2O_2 and a halide with endogenous mast cell granule peroxidase [5, 6]. We could not demonstrate a role of reactive oxygen species in mast cell toxoplasmacidal activity. The addition of catalase (60 μg/mL), which degrades H_2O_2, to the mast cell–*T. gondii* reaction mixtures, did not affect *T. gondii* cytotoxicity as assessed morphometrically (data not shown).

Our finding that mast cells produce toxic changes in *T. gondii* without exogenous H_2O_2 or antibody demonstrates a new role for mast cells in the host defense against pathogens and suggests as well that LTB_4 could be an important cytotoxic mediator in this inflammatory process. Mast cell–derived chemoattractant LTs previously have been demonstrated to be key mediators of the early leukocyte influx into inflammatory sites induced by immune complexes [14, 15]. Further, inhibition of mast cell secretion by drugs that block 5-LO or FLAP may be an important antiinflammatory action of these agents in treatment of diseases such as asthma.

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Direct Isolation of DNA from Patient Stools for Polymerase Chain Reaction Detection of *Cryptosporidium parvum*

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Although polymerase chain reaction (PCR) can sensitively detect parasitic or other infections, its use with fecal samples is extremely limited, primarily because of the presence of substances that inhibit DNA extension. Here an improved protocol is reported for directly isolating DNA from aged or fresh formalin-fixed stools, which can then be used to detect *Cryptosporidium parvum* by nested PCR. This method is highly reproducible, sensitive, and specific. It detects $<$1 pg of *C. parvum* DNA in human stool, and there are no cross-reactions with other parasites commonly found there.

The polymerase chain reaction (PCR) amplification of a unique DNA segment belonging to an organism can be used for its specific detection in clinical samples. Because of increased sensitivity and specificity, PCR (or PCR-related technologies) has been used to improve the detection of *Cryptosporidium parvum* in clinical and environmental samples. In order to provide a method for the molecular diagnosis of *C. parvum* from patient stools, we began a study to evaluate published PCR techniques for detection of this opportunistic pathogen in clinical samples [1–4]. Previously described methods for molecular detection of *C. parvum* usually relied upon DNA extraction from purified oocysts [1–4], and some even required 100 ng of DNA for a nested PCR ($\sim$1 million oocysts [5]). In addition, most suffered from the major problem encountered when isolating DNA from bacteria, viruses, and other parasites in stool [6–9], that is, inhibitors copurified with DNA preventing reliable detection by PCR. None of these proved sufficiently reliable, sensitive, or specific for PCR detection of *C. parvum* in stool.

In this study, a highly reliable DNA extraction method was combined with nested PCR that enables femtogram quantities of *C. parvum* DNA to be detected in aged or fresh formalin-fixed human stools. Using the small subunit rRNA (srDNA) as the target sequence, the nested reaction is *C. parvum*–specific, that is, no cross-reactions were observed when purified genomic DNA (gDNA) of other parasites, or DNA extracted from human stools containing enteric microorganisms, were tested. Unlike previously published techniques, this PCR method is highly reproducible, specific, and sensitive and does not require prior isolation, concentration, or purification of oocysts for detecting *C. parvum* in human stool.

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