*Immunology* 1992 **76** 242–250

# *Entamoeba histolytica* alters arachidonic acid metabolism in macrophages *in vitro* and *in vivo*

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*Accepted for publication 22 January 1992*


## SUMMARY

*Entamoeba histolytica* infections are associated with a state of transient suppression of cell-mediated immunity. Macrophages, the most important cells in host defence and control of invasive amoebiasis, in infected animals have been found to be deficient in effector functions and accessory cell potential. However, little is known of the cellular mechanisms responsible for the down-regulation. This study investigated whether macrophage dysfunction in amoebiasis is associated with altered macrophage arachidonic acid (AA) metabolism. Resident peritoneal macrophages (PMO) from naive gerbils produced enhanced levels of prostaglandin $E_2$ ($PGE_2$) and leukotriene $C_4$ ($LTC_4$) in response to live *E. histolytica* trophozoites, to diffusible excretory/secretory products released from live amoebae in millicells and to freeze-thawed soluble amoebic proteins that were inhibitable by indomethacin (INDO) and nordihydroguaiaretic acid (NDGA), respectively. In contrast to PMO from naive gerbils, PMO from animals with amoebic liver abscesses at 10, 20 and 30 days post-infection (p.i.) released high basal levels of $PGE_2$ and $LTC_4$. In response to zymosan stimulation, PMO from infected animals produced two- and fourfold less $PGE_2$ and $LTC_4$, respectively, as compared to uninfected controls. AMO showed high constitutive basal release of $PGE_2$ and $LTC_4$. In response to amoebic and zymosan stimulation, AMO at 10 days p.i. produced significantly higher levels of $PGE_2$ than AMO at 20 days p.i., while AMO at 30 days p.i. were refractory to stimulation to produce higher than basal levels of $PGE_2$. Early (10 days) and late (20–30 days) AMO were refractory to amoebic and zymosan stimulation for enhanced $LTC_4$ release. Pretreatment of AMO with AA substrate restored optimal $PGE_2$ and $LTC_4$ biosynthesis, but the cells were generally unresponsive to zymosan stimulation to produce augmented levels of $LTC_4$. These results strongly suggest that intrinsic or secreted products or both from *E. histolytica* can induce profound alteration of eicosanoid formation in cyclooxygenase and lipoxygenase pathways in macrophages from naive and infected animals and that AA metabolism by AMO is sequentially modified during the course of the disease.


## INTRODUCTION

Amoebiasis is the disease caused by the protozoan parasite *Entamoeba histolytica*, which results in approximately 50 million cases of invasive colitis or liver abscess and 50,000 to 100,000 deaths per year.[1] Cell-mediated immunity has been described in both human and animal models of the disease in control of invasive amoebiasis and host resistance to reinfection.

Abbreviations: AA, arachidonic acid; AMO, amoebic liver abscess-derived macrophages; INDO, indomethacin; LT, leukotriene; NDGA, nordihydroguaiaretic acid; PG, prostaglandin; p.i., post-infection; PMO, peritoneal macrophages; RIA, radioimmunoassay; TX, thromboxane.

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Macrophages are important effector cells in resistance against *E. histolytica* infection.[2-4]

Human monocyte-derived macrophages from uninfected individuals, and from patients treated for amoebic liver abscesses activated with lymphokines *in vitro*, kill virulent amoebic trophozoites through a contact-dependent, antibody-independent mechanism involving oxidative-dependent and independent processes. After a cure of an amoebic liver abscess, specific cell-mediated immune mechanisms develop that are effective *in vitro* against the parasite.[3] With murine bone marrow-derived macrophages, recombinant interferon-gamma (rIFN-$\gamma$), recombinant tumour necrosis factor-alpha (rTNF-$\alpha$) and recombinant colony-stimulating factor-1 (rCSF-1) alone or in combination were shown to activate macrophages for amoebicidal activity. The mechanism of amoebicidal activity was 45% $H_2O_2$ dependent and 61% inhibitable with protease inhibitors.[4] More recently,[5] the amoebicidal activity of human neutrophils acti-

vated with rIFN-$\gamma$ and rTNF-$\alpha$ was shown to occur by both contact and non-contact modes involving the secretion of $H_2O_2$.

During an infection with *E. histolytica*, transient immunosuppression occurs. A decreased number of helper (CD4) and an increased number of suppressor (CD8) lymphocyte phenotypes in peripheral blood, and decreased T-lymphocyte proliferative responses to amoebic antigens, were found in patients with active disease.[3] Because T-lymphocyte functions are macrophage dependent, infections with *E. histolytica* may induce macrophage dysfunctions that can interfere with their ability to modulate T-cell functions.

In a gerbil model of amoebic liver abscess, the early stages of the liver lesions are characterized by an acute inflammatory response which progresses rapidly to become granulomatous within 5 days after inoculation. Neutrophils are the predominant cell type early in the infection and at 5 days the connective tissue is infiltrated around the lesion site by macrophages.[6] Late in the infection, organized amoebic granulomas (20–30 days) predominate and the cellular infiltrates are now comprised of macrophages and lymphocytes. Amoebic liver abscess-derived macrophages (AMO)[3] (5–20 days) were found to be deficient in a variety of effector and accessory cell functions. Defects were shown in macrophage respiratory burst potential, in secreting and expressing membrane-bound interleukin-1 (IL-1)-like activity, and in the killing of *E. histolytica* trophozoites *in vitro*.[7] However, macrophage populations distant from the abscess site (e.g. peritoneal and spleen) were not affected in the above functions. Similar results were obtained when elicited murine macrophages were treated with amoebic proteins *in vitro*. Amoebic proteins (10–100 $\mu$g/ml) down-regulated the release and expression of IL-1-like activity, the release of $H_2O_2$ and Ia antigen expression. Moreover, amoebic-conditioned medium (secretory/excretory products released by *E. histolytica*) also caused a similar effect. These results strongly suggest that intrinsic or secreted products or both from *E. histolytica* are actively regulating macrophage functions at the abscess site. However, little is known of the cellular mechanisms responsible for the down-regulation of macrophage functions in amoebiasis.

Eicosanoids produced by macrophages are known to modulate both inflammatory and immune responses.[8,9] Prostaglandin $E_2$ ($PGE_2$) has potent immunosuppressive effects on immune cells, including inhibition of monocyte/macrophage and granulocyte functions, inhibition of T-lymphocyte activation and lymphokine production, and induction of B-lymphocyte unresponsiveness.[10–12] Alteration of macrophage arachidonic acid (AA) metabolism has been reported to occur in some parasite diseases, including schistosomiasis,[13] leishmaniasis[14] and African trypanosomiasis,[15] which could play a role in pathogenesis and modulation of the immune response.

The purpose of this study was to determine whether macrophage dysfunction is amoebiasis is associated with altered macrophage AA metabolism. We quantified the eicosanoid produced by naive macrophages in response to *E. histolytica* stimulation, and in a gerbil model of amoebic liver abscess, the eicosanoid produced by inflammatory macrophages during the course of the infection. Our results demonstrate that live *E. histolytica*, secretory/excretory products released from live amoebae and soluble *E. histolytica* proteins (cell lysates) can stimulate AA metabolism in resident macrophages for enhanced $PGE_2$ and leukotriene $C_4$ ($LTC_4$) release, whereas inflammatory peritoneal macrophages (PMO) and AMO from infected animals showed altered $PGE_2$ synthesis and release as the infection progressed with profound suppression of $LTC_4$.

## MATERIALS AND METHODS

*Cultivation and harvesting of* E. histolytica

The pathogenic *E. histolytica* strain HM1-IMSS amoebae grown axenically in TYI-*S*-33 medium[16] were used for the experiments. Amoebic trophozoites in mid-log phase (48–60 hr) were harvested, washed three times in ice cold Hanks' balanced salt solution (HBSS) (Gibco Laboratories, Grand Island, NY), and suspended in HBSS. The amoebae were lysed by three freeze–thaw cycles. The amoebic suspensions were centrifuged at 15,000 *g* at 4° for 15 min to remove cellular debris and the supernatant used as soluble amoebic protein. The protein concentration was determined by the method of Bradford[17] using bovine serum albumin as a standard. Fresh amoebic proteins were made daily for the experiments to minimize degradation during storage.

*Animals and infection procedures*

Male inbred gerbils (*Meriones unguiculatus*), 50–60 days old and weighing between 55 and 60 g (Tumblebrook Farms, West Brookfield, MA), were used in all experiments. Gerbils were infected intrahepatically with $5 \times 10^5$ amoebic trophozoites in mid-log phase as described previously.[6]

*Preparation of peritoneal and spleen macrophages*

The peritoneal cavities of infected and normal gerbils were washed with 10 ml of cold medium, and the harvested cells were washed twice and adjusted at $3 \times 10^6$ per well in 1 ml of completed medium [RPMI-1640 medium (Gibco), with 5% heat-inactivated foetal calf serum (Hyclone Laboratories, Logan, UT), 24 mM HEPES (Sigma Chemical Co., St Louis, MO) and 100 U/ml of penicillin and 100 $\mu$g/ml of streptomycin sulphate (Sigma)] in 24-well plates. Cells were incubated for 2 hr at 37° in 5% $CO_2$, then washed six times with warm medium and incubated overnight before use in experiments. Spleen macrophages were prepared as described previously.[7] In brief, the spleens of gerbils were removed aseptically and a cell suspension was made. Cells were washed thrice in medium, free of erythrocytes with 0·17 M $NH_4Cl$ (Sigma), and plated at $3 \times 10^7$ cells in 1 ml of complete medium in 24-well plates. After 2 hr of incubation at 37° in 5% $CO_2$, cells were washed 10 times with warm medium and incubated overnight before the assays.

*Isolation of amoebic liver abscess-derived macrophages (AMO)*

The method was mainly according to the procedures described by Denis and Chadee.[7] Briefly, liver abscesses of gerbils killed at various times after infection were aseptically harvested and transferred to culture dishes containing RPMI complete medium. A cell suspension was prepared by teasing the tissues apart with forceps. This suspension, after being washed, was incubated in HBSS containing 0·5% collagenase (type IA, from *Clostridium histolyticum*; Sigma) and 100 $\mu$g/ml of deoxyribonuclease (DNase) (type 1, Sigma) and agitated in a water-bath at 37° for 40 min. Red blood cells were removed by osmotic lysis and the cells were washed four times. $8 \times 10^6$ cells/ml/well in 24-

well plates were incubated at 37° for 4 hr, washed twice gently with warm medium and then incubated overnight. The cells were then washed vigorously 10–12 times with warm medium to remove non-adherent cells. This procedure allowed the recovery of cells which were >97% macrophages as determined by Giemsa stain (Sigma) and differential cell counts of cytospin preparations.

*Generation of macrophage-conditioned media*

Adherent macrophages were incubated with different stimuli at 37° in 5% $CO_2$. Macrophages were incubated for 2 hr with live trophozoites ($2 \times 10^4$ or $2 \times 10^5$/ml) and the culture supernatant harvested. At this concentration of trophozoites, no significant loss of macrophages was observed (>95% live cells as determined by trypan blue exclusion assay). Zymosan was boiled for 15 min and opsonized with fresh normal human serum for 30 min at 37°, then washed three times with RPMI medium, resuspended in medium and used at a concentration of 100 μg/ml for cell cultures. The complete medium used for cell cultures contained polymyxin B (1 μg/ml polymyxin B sulphate, Sigma) except for the cultures with LPS as a stimulus. After incubation, cell culture media were harvested and centrifuged. The supernatants (conditioned media) were either used fresh or stored at −70° before use. Because the percentage of adherent cells varied for the different macrophage populations, the number of adherent cells was determined as follows. Macrophage monolayers were kept on ice for 1 hr and the cells were flushed vigorously with a pipet and any remaining adherent cells were scraped off the plate with a cell scraper (Falcon, NJ). Cells were then enumerated with a haemocytometer.

*Eicosanoid extraction and radioimmunoassay (RIA)*

Eicosanoids from macrophage-conditioned media were extracted by passing through C2 ethyl and PH phenyl Amprep columns (Amersham, Oakville, Ontario, Canada) using different solvents according to the type of eicosanoid species following the manufacturer's protocol. The extracts were evaporated to dryness under a $N_2$ stream and stored at −70°. Extracts of cell culture supernatants were reconstituted in RIA sample buffer prior to the assay. Quantitative RIA kits for $PGE_2$ [$^3$H] were purchased from Advanced Magnetics Inc. (Cambridge, MA), and $LTC_4$ [$^3$H] RIA kits from New England Nuclear (Du Pont, Mississauga, Canada Inc). The level of cross-reactivity for the anti-$PGE_2$ was 50% with $PGE_1$, 3% with $PGA_2$, 1·3% with $PGF_{2\alpha}$, and 1% with 6-Keto-$PGE_1$, 15-Keto-$PGF_{2\alpha}$ and 15-KEto-$PGE_2$. Cross-reactivity with all other eicosanoids is less than 1%. Anti-$LTC_4$ cross-reacts by 60·5% with 11-trans-$LTD_4$, 55·3% with $LTD_4$, 10·1% with $LTD_4$-sulphone, 9·5% with $LTC_4$-sulphone and 8·6% with $LTE_4$.

*Statistical analysis*

Student's *t*-test was used to analyse the difference between control and experimental groups. ANOVA was used to determine the difference of means from more than two groups and Duncan's multiple range test was used to determine significant difference between means. Values of $P > 0.05$ were not considered significant. Data are presented as means ±standard deviation ($x \pm SD$) of the means.

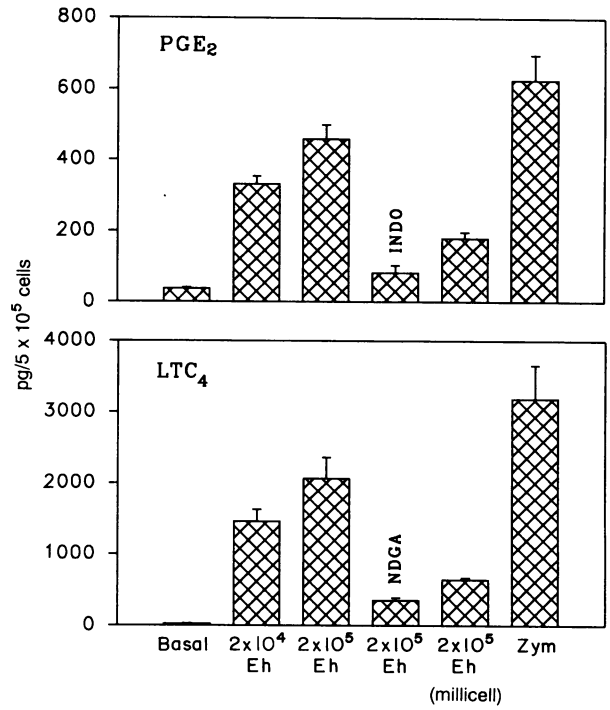


**Figure 1.** The effect of *E. histolytica* (Eh) trophozoites on $PGE_2$ and $LTC_4$ production by gerbil PMO. PMO were prepared as described in Materials and Methods and incubated with amoebic trophozoites ($2 \times 10^4$ and $2 \times 10^5$/ml) in direct contact and trophozoites ($2 \times 10^5$/ml) separated from the PMO monolayers by minicells, and opsonized zymosan (100 μg/ml) for 2 hr at 37 . In some conditions, PMO were pretreated with INDO (1 μM) or NDGA (3 μM) prior to stimulation. Basal release of $PGE_2$ and $LTC_4$ by unstimulated PMO was used as a control. Experiments were preformed in triplicate ($n = 12$). In all cases, basal release versus experimental groups $P < 0.01$.

## RESULTS

### The effects of *E. histolytica* trophozoites and soluble amoebic proteins on $PGE_2$ and $LTC_4$ production by macrophages from naive gerbils *in vitro*

Resting resident PMO released very low basal levels of $PGE_2$ and $LTC_4$ *in vitro*, similar to those observed in other studies.[14,18] In contrast, *E. histolytica* trophozoites in direct contact with PMO monolayers for 2 hr significantly ($P < 0.01$) enhanced the production of $PGE_2$ and $LTC_4$ in a dose-dependent manner with values similar to the positive control, opsonized zymosan (Fig. 1). By comparison, PMO produced about four times higher amounts of $LTC_4$ than $PGE_2$ when stimulated with trophozoites or opsonized zymosan (Fig. 1). As expected, PMO pretreated with the cyclooxygenase inhibitor indomethacin (INDO) (1 μM, Sigma) or the lipoxygenase inhibitor nordihydroguaiaretic acid (NDGA) (3 μM, Sigma) specifically inhibited $PGE_2$ and $LTC_4$ production by 82 and 83%, respectively, in response to amoebic stimulation. When trophozoites were prevented from making direct contact with the macrophage monolayers but their excretory/secretory products were allowed to diffuse out using millicells (Culture plate insert with 0·45 μM membranes, Millipore Ltd, Harrow, Middlesex, U.K.), PMO again secreted high levels of $PGE_2$ and $LTC_4$ ($P < 0.01$). However, the levels of $PGE_2$ and $LTC_4$ induced by the

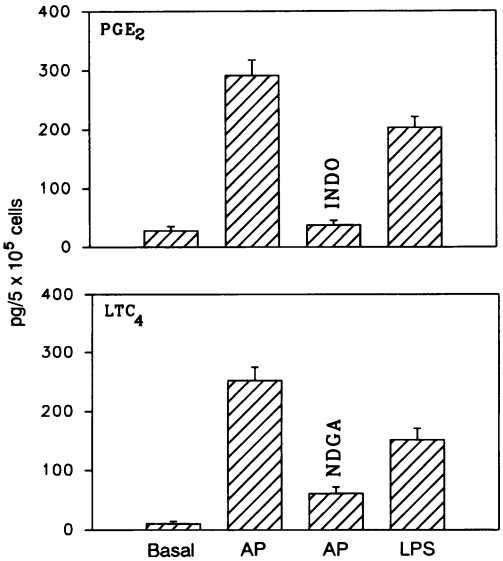


**Figure 2.** The effect of soluble amoebic proteins (AP) on $PGE_2$ and $LTC_4$ production by naive PMO. Resident PMO from naive gerbils were incubated with AP (50 μg/ml) or LPS (100 ng/ml) as a positive control. In some conditions, PMO were pretreated with INDO (1 μM) or NDGA (3 μM) prior to stimulation. After 2 hr of incubation, the supernatants were collected and $PGE_2$ and $LTC_4$ levels determined. Experiments were preformed in triplicate ($n=12$). Basal release versus experimental groups $P<0{\cdot}01$.

substances released from $2\times10^5$ trophozoites were about 61 and 78% lower, respectively, than those induced by equivalent numbers of trophozoites in direct contact with the macrophages (Fig. 1).

Our results also indicate that amoebic proteins can stimulate PMO to produce high levels of $PGE_2$ and $LTC_4$ as compared to basal levels released by control cells and to the positive control, lipopolysaccharides (LPS) ($P<0{\cdot}01$; Fig. 2). Concentrations of amoebic proteins as low as 10 μg/ml stimulated PMO to produce significant amounts of $PGE_2$ and $LTC_4$ (data not shown) with maximum release occurring with 50 μg/ml. Both $PGE_2$ and $LTC_4$ production induced by amoebic proteins was specifically inhibited by INDO (85%) and NDGA (82%), respectively. We then determined whether amoebic proteins injected i.p. into gerbils would elicit a similar effect. Peritoneal cells were harvested from animals injected i.p. (hr previously) with amoebic proteins (50 μg in 0·3 ml HBSS) or HBSS alone as a control. As shown in Table 1, PMO from gerbils injected with amoebic proteins produced significantly ($P<0{\cdot}01$) higher basal and opsonized zymosan-stimulated levels of $PGE_2$ than HBSS-injected controls. PMO from animals injected with amoebic proteins produced remarkably different amounts of $PGE_2$ in response to zymosan (16-fold increase) as compared to HBSS-injected controls (22-fold increase). These results strongly suggest that resident macrophage populations were being studied. Inflammatory macrophages at the site of an infection are refractory to zymosan stimulation to high $PGE_2$ release.[19]

Spleen macrophages from naive animals were less responsive for the production of $PGE_2$ and $LTC_4$ than PMO. The production of $PGE_2$ and $LTC_4$ by spleen macrophages was significantly ($P<0{\cdot}01$) lower than that produced by PMO, when spleen macrophages were stimulated with amoebic proteins or zymosan (Table 2, Figs 1 and 2). These results suggest that

**Table 1.** $PGE_2$ production by peritoneal macrophages from gerbils injected intraperitoneally with soluble amoebic proteins

| | $PGE_2$ (pg/5 × $10^5$ cells) | |
|---|---|---|
| Conditions | HBSS control | Amoebic proteins |
| PMO (basal) | 29±5 | 50±9* |
| PMO+zymosan | 636±24 | 815±24* |
| Fold increase | 22 | 16 |

Naive gerbils were injected i.p. with 50 μg of amoebic proteins (AP) suspended in HBSS or HBSS alone as a control. PMO were harvested after 2 hr and incubated for 2 hr *in vitro*. $PGE_2$ in the culture supernatants was extracted and detected by RIA. Results are presented as mean ±SD of triplicate experiments ($n=12$).

* $P<0{\cdot}01$ compared with basal or zymosan-stimulated controls by Student's *t*-test.

**Table 2.** $PGE_2$ and $LTC_4$ production by naive spleen macrophages in response to *E. histolytica* proteins

| Conditions | $PGE_2$ (pg/5 × $10^5$ cells) | $LTC_4$ (pg/5 × $10^5$ cells) |
|---|---|---|
| SMO | 0 | 0 |
| SMO+AP | 34±4* | 59±4* |
| SMO+zymosan | 115±8 | 167±14 |

Spleen macrophages (SMO) from naive gerbils were incubated with soluble amoebic proteins (AP) (50 μg/ml) or opsonized zymosan (100 μg/ml) for 2 hr and $PGE_2$ and $LTC_4$ levels in the culture supernatants determined by RIA. Experiments were performed in duplicate ($n=6$).

* $P<0{\cdot}01$ compared with basal controls by Student's *t*-test.

different resident macrophage populations in gerbils have the capacity to produce different amounts of cyclooxygenase and lipoxygenase metabolites in response to a phagocytic (zymosan) or soluble (amoebic protein) stimulus.

### $PGE_2$ and $LTC_4$ production by macrophages isolated from gerbils with amoebic liver abscesses

*Peritoneal macrophages (PMO)*

Since $PGE_2$ and $LTC_4$ production by macrophages was enhanced in response to amoebic stimulation *in vitro* we then investigated whether $PGE_2$ and $LTC_4$ production by PMO would be altered from gerbils with amoebic liver abscesses at 10, 20 and 30 days p.i. We reasoned that inflammatory macrophages would dominate the peritoneal cavities of gerbils during the course of an infection. As shown in Table 3, PMO isolated from infected gerbils regardless of the day p.i. produced significantly ($P<0{\cdot}01$) enhanced basal levels of $PGE_2$ *in vitro* as compared to PMO from naive animals. Despite the high basal release, PMO from infected animals were still able to release significantly ($P<0{\cdot}01$) higher levels of $PGE_2$ when incubated with amoebic proteins or zymosan. The amounts of $PGE_2$

**Table 3.** Basal and stimulated release of $PGE_2$ and $LTC_4$ by peritoneal macrophages isolated from gerbils with amoebic liver abscesses

| | $PGE_2$ (pg/5 × $10^5$ cells) | | | |
|---|---|---|---|---|
| Conditions | Control | 10 days | 20 days | 30 days |
| $PGE_2$ | | | | |
| Basal | 31±4 | 186±8* | 116±8 | 107±27 |
| AP | 291±26* | 434±33 | 350±27 | 206±39 |
| Fold increase | 9 | 2 | 3 | 2 |
| Zymosan | 625±44* | 779±20 | 514±23 | 357±26 |
| Fold increase | 20 | 4 | 4 | 3 |
| $LTC_4$ | | | | |
| Basal | 56±14 | 362±47* | 341±48 | 339±37 |
| AP | 322±28* | 809±64 | 647±35 | 501±42 |
| Fold increase | 6 | 2 | 2 | 1·5 |
| Zymosan | 3016±47* | 1313±144 | 957±73 | 716±47 |
| Fold increase | 54 | 4 | 3 | 2 |

PMO isolated from gerbils with amoebic liver abscesses at 10, 20 and 30 days post-infection were prepared as described in Materials and Methods. PMO were incubated *in vitro* with amoebic proteins (AP) (50 μg/ml) or opsonized zymosan (100 μg/ml) for 2 hr. PMO from age-matched sham-operated gerbils were used as control. Experiments were performed in triplicate.

*$P<0·01$ compared to basal release in control cells and for $PGE_2$ and $LTC_4$ release stimulated with AP or zymosan compared to homologous controls by Student's *t*-test.

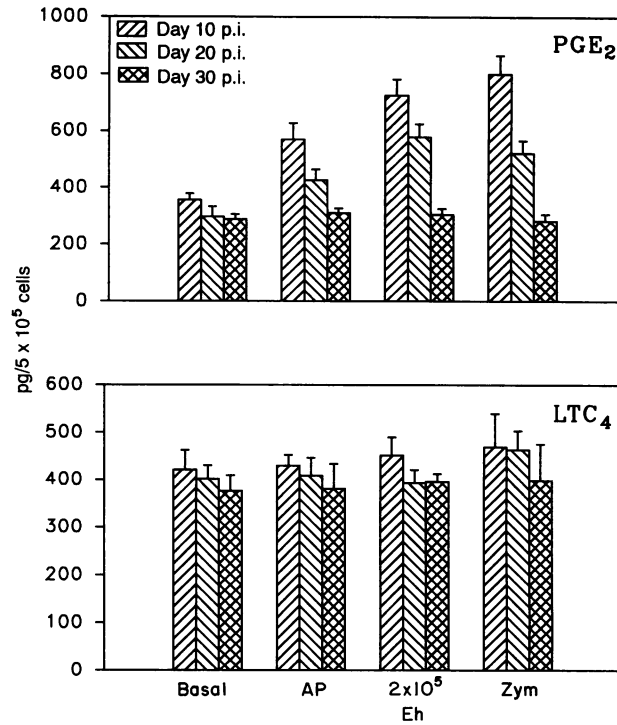


**Figure 3.** Basal and stimulated release of $PGE_2$ and $LTC_4$ by AMO. AMO at 10, 20 and 30 days post-infection were stimulated as indicated for 2 hr and $PGE_2$ and $LTC_4$ levels in the supernatants determined by RIA. Experiments were performed in triplicate ($n=12$). $P<0·05$ for Day 10 $PGE_2$ release compared to Days 20 and 30. $P<0·01$ for $PGE_2$ release stimulated with amoebae or zymosan at 10 and 20 days compared with homologous controls for basal $PGE_2$ release.

produced by PMO in response to zymosan were 85, 79 and 68% above basal levels at 10, 20 and 30 days p.i., respectively. These results indicate that $PGE_2$ production by PMO was decreased as the infection progressed.

PMO from infected gerbils also produced significant basal levels of $LTC_4$ *in vitro* (Table 3). PMO at 10 days p.i. released the highest amount of $LTC_4$ when exposed to amoebic proteins or zymosan as compared to PMO at 20 and 30 days p.i. Similar to the $PGE_2$ production by PMO from infected animals, the production of $LTC_4$ by PMO was decreased during the course of the infection. The levels of $PGE_2$ and $LTC_4$ produced by PMO from infected animals in response to zymosan stimulation were two to four times lower than those produced by PMO from naive animals (Fig. 1, Table 3). However, compared to homologous uninfected animals, PMO from infected gerbils produced 16 an 50-fold less $PGE_2$ and $LTC_4$, respectively, in response to zymosan stimultaion.

*Abscess macrophages*

AMO were isolated from gerbils at Days 10, 20 and 30 p.i. and $PGE_2$ and $LTC_4$ levels determined. As shown in Fig. 3, AMO spontaneously released $PGE_2$ *in vitro*. AMO from animals at 10 and 20 days, but not at 30 days, were hyper-responsive to amoebic proteins, trophozoites and zymosan stimulation to produce enhanced levels of $PGE_2$. AMO at 10 days p.i. released the highest levels of $PGE_2$. Regardless of the day p.i., AMO were unresponsive to stimulation to produce higher than basal levels of $LTC_4$ (Fig. 3).

These results indicate that the defect for $LTC_4$ biosynthesis could be at the substrate (AA) or enzyme (phospholipase and/or lipoxygenase) level. Since exogenous AA can be utilized by macrophages to synthesize eicosanoids,[20] AMO were preincubated with AA (50 μg/ml) for 2 hr, and $PGE_2$ and $LTC_4$ levels determined. Our results show that AMO pretreated with AA substrate, regardless of the day p.i., produced enhanced levels of $PGE_2$ and $LTC_4$ as compared to AMO without pretreatment with AA (Fig. 4). Interestingly, even though $LTC_4$ biosynthesis was restored, the cells remained unresponsive to zymosan stimulation to produce higher levels of $LTC_4$. In contrast, $PGE_2$ production was increased from AMO at 20 and 30 days p.i. in response to zymosan stimulation (Fig. 4). PMO from naive and infected animals preincubated with AA substrate also released higher basal levels of $PGE_2$ and $LTC_4$. However, PMO from infected animals consistently produced lower amounts of the eicosanoids.

## DISCUSSION

*E. histolytica* infections are associated with impairment of the immune response during the acute phase of the disease. Evidence for this is supported by the apperance of suppressor (CD8) lymphocyte phenotypes in peripheral blood of individuals with amoebic liver abscess and decreased lymphocyte proliferative response to amoebic antigen.[3] More recently, soluble factors in the sera of amoebiasis patients have been shown to suppress the *in vitro* lymphocyte response to *E. histolytica* antigens.[21] These studies, coupled with the facts that amoebic extracts are immunosuppressive when injected into animals[22] and that amoebic extracts induce the proliferation of a

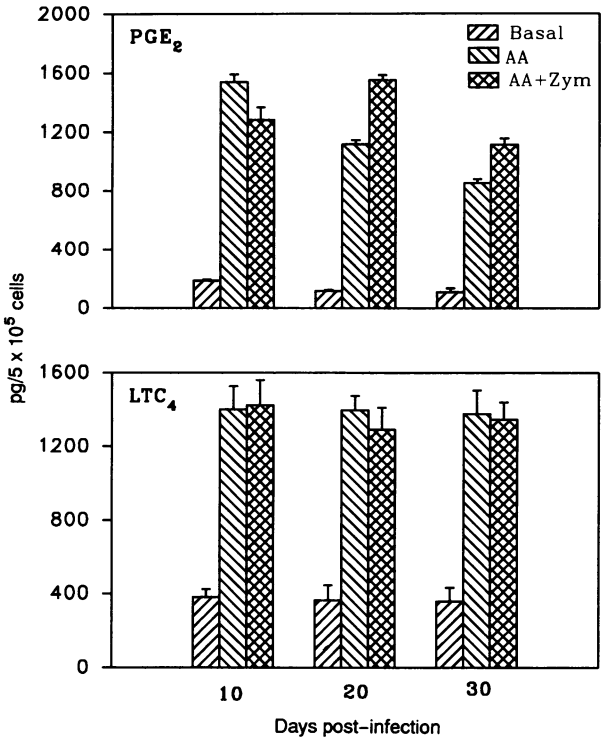


**Figure 4.** $PGE_2$ and $LTC_4$ production by AMO preincubated with AA substrate. AMO were pretreated with AA (50 μg/ml) for 2 hr, washed three times with warm medium and incubated with fresh medium without AA or stimulated with opsonized zymosan (100 μg/ml) for 2 hr. $PGE_2$ and $LTC_4$ levels in the supernatants were determined by RIA. Experiments were performed in triplicate ($n = 12$).

subset of non-T cells by polyclonal activation,[23,24] suggest that there are many ways by which defects in cell-mediated immune responses may occur in amoebic infections. In order to explore the mechanisms associated with macrophage dysfunction and immunosuppression in *E. histolytica* infection, the effect of *E. histolytica* on macrophage AA metabolism and eicosanoid formation was investigated in this study.

The results presented here demonstrate that *E. histolytica* induces profound alteration in macrophage eicosanoid formation in both cyclooxygenase and lipoxygenase pathways. PMO from naive animals produced enhanced levels of $PGE_2$ and $LTC_4$ in response to live *E. histolytica* trophozoites and amoebic proteins *in vitro* in a parasite dose-dependent manner. In contrast, inflammatory PMO and AMO from infected animals showed high constitutive basal levels of $PGE_2$ and $LTC_4$ *in vitro*. Enhanced spontaneous release of $PGE_2$ by macrophages was also shown in *Listeria monocytogenes*-infected mice.[25] Augmentation of $PGE_2$ synthesis in response to stimulation was detected in PMO and AMO from infected animals. Macrophages at 10 days p.i. produced the highest amount of $PGE_2$ compared with those at 20 and 30 days p.i. However, $PGE_2$ synthesis by macrophages in response to exogenous stimulation was decreased as the infection progressed. In contrast, PMO from infected animals produced dramatically low levels of $LTC_4$ when exposed to amoebic proteins or zymosan as compared to PMO from naive animals. Moreover, AMO were refractory to stimulation to produce higher than basal levels of $LTC_4$. This finding is in agreement with the results that $LTC_4$ synthesis is reduced in inflammatory macrophages,[26] and that macrophages obtained after a single i.p. infection of *C. parvam* failed to release $LTC_4$ after exposure to a maximal phagocytic load of zymosan.[19] Our results also demonstrate a marked difference in $PGE_2$ and $LTC_4$ production by macrophages from naive and infected gerbils in response to stimulation, suggesting that *E. histolytica* infection induces alteration of macrophage eicosanoid formation both *in vitro* and *in vivo*. The changing pattern of eicosanoid formation by macrophages in infection with *E. histolytica* may have effects on modulating immune and inflammatory reactions in amoebiasis.

Resident macrophage populations are very responsive for enhanced PG and LT release in response to zymosan, thrombin and AA stimulation.[27] An unexpected finding in our study was the high levels of $PGE_2$ and $LTC_4$ produced by naive PMO following stimulation with live *E. histolytica* trophozoites, and diffusible excretory/secretory products released by amoebae. This was not restricted to PMO only, as SMO also produced elevated levels of $PGE_2$ and $LTC_4$ in response to amoebic stimulation. Membrane perturbation is a known stimulus for $PGE_2$ release by macrophages during phagocytosis of zymosan and bacteria,[28] especially if they are opsonized. Live amoebae may induce eicosanoid formation by direct contact by damaging macrophage membranes[28] or by the mitogenic effects of the amoebic Gal/GalNAc adherence lectin.[29] Soluble amoebic protein and secretory/excretory amoebic product-induced PG and LT release is difficult to explain, but clearly shows that macrophage AA metabolism is altered in response to these products. Perhaps the secreted amoebic Gal/GalNAc lectin,[30] pore-forming protein[31] or neutral proteinase[32] alone or in combination may cause sufficient macrophage membrane perturbation to elicit phospholipase activity for AA metabolism.

PGE has been shown to play an important role in the regulation of cellular and humoral immune responses.[33-35] Of particular interest is the ability of $PGE_2$ to regulate the production of varius monocyte/macrophage peptide signals necessary to promote fully an immune response. These studies include the suppression of macrophage-derived TNF production and gene expression, IL-1 production,[36,37] and I-region-associated antigen expression.[38] The *in vivo* suppression of I-region-associated antigen and other signal peptides necessary for cell-to-cell communication would alter macrophage/lymphocyte driven reaction(s) and 'down-regulate' the local immune response.

At present, there are no studies on macrophage AA metabolism in *E. histolytica* infection. In comparison, AA metabolism by macrophages in *Leishmania donovani* and *Trypanosoma brucei brucei*[14,15] infections has shown enhanced $PGE_2$ production. Our finding is similar to the observation that macrophages from acute phase lesions of *Schistosoma mansoni* egg-induced granulomas produced high levels of PG. Unstimulated macrophages from actue granulomas also produced significant amounts of PG consisting primarily of $PGE_2$.[39] Thus, $PGE_2$ may act as an early non-specific regulator of macrophage functions involved in the acute phase of infection. Recent studies from our laboratory[7] have shown that AMO were deficient in reactive oxygen intermediate release, IL-1 release and lymphokine responsiveness as well as in killing *E. histolytica* trophozoites *in vitro*. Furthermore, since murine macrophage functions were also impaired *in vitro* by amoebic proteins (from pathogenic amoebae), but not from the non-pathogenic *E.*

*histolytica*-like Laredo strain, this indicates that the inhibition is probably specific to pathogenic *E. histolytica*. Immunosuppression in amoebiasis might occur during early stages of the infection.[22] Different factors may be involved in macrophage dysfunction in amoebiasis, including the factors from both parasite and host. Our results indicate that enhanced production of $PGE_2$ by macrophages in *E. histolytica* infections might be one of the factors which contribute to macrophage dysfunction and immunosuppression in amoebiasis. Elevated levels of $PGE_2$ at 10 and 20 days after infection may play a role in disease exacerbation by altering T-cell function or macrophage activation. Maximum growth of the amoebic liver abscess occurs between 10 and 15 and 20 and 30 days after infection in gerbils.[6] $PGE_2$ from AMO or PMO may alter macrophage recruitment or macrophage function which favours growth of the abscess and increased parasite multiplication. $PGE_2$ may also lead to the development of *E. histolytica*-specific T-suppressor cells,[40,41] which is common during active disease.[3] More recently,[42] $PGE_2$ has been shown to selectively inhibit production Thl lymphokines (IL-2 and IFN-$\gamma$) but to have no effect on Th2 lymphokine release (IL-4 and IL-5) and may, therefore, favour an immune response dominated by the production of Th2-associated lymphokines. This would have important implications in the control of amoebic infections, as IFN-$\gamma$ is the most important lymphokine for activating macrophages for the *in vitro* killing of *E. histolytica*.[4,5]

Macrophages are the major source of LT in acute inflammation.[43] $LTC_4$ and its metabolities $LTD_4$ and $LTE_4$ collectively make up the biological activity known as slow-reacting substance of anaphylaxis. They may mediate inflammatory reaction by producing changes in blood flow and increasing vascular permeability. LT are capable of providing the helper signal for IFN-$\gamma$ production.[44] The most prominent cell type in amoebic liver abscess of gerbils after 5–10 days are neutrophils, which are found diffusely distributed throughout the granuloma walls.[6] Late in the infection at 20–30 days, lymphocytes and macrophages predominate in the granuloma walls. $LTC_4$ directly increases microvascular permeability and adherence of neutrophils to surfaces,[45,46] and may play a role in the pathogenesis of amoebic liver abscess. In this study, AMO at 10, 20 and 30 days after infection produced the same basal levels of $LTC_4$ and were refractory to stimulation for enhanced $LTC_4$ release. In contrast, PMO from infected animals produced significantly higher basal levels of $LTC_4$ and even higher levels in response to zymosan stimulation. Interestingly, PMO from uninfected animals produced 54-fold more $LTC_4$ in response to zymosan than PMO from infected animals (two- to four-fold increases). A similar trend was noted for $PGE_2$ release, whereby uninfected animals produced 20-fold more $PGE_2$ in response to zymosan than cells from infected animals (three- to four-fold increase).

At present, all the factors that down-regulate cellular eicosanoid synthesis are not completely known. Humes *et al.*[47] suggested that elicited and resident macrophages may produce different amounts of PG because of the rate of AA release by the cells. Thioglycollate-elicited macrophages produced less $^3H$[AA] than resident macrophages in response to zymosan. Recent studies[27] now show that resident macrophages produce large quantities of AA metabolities, whereas monocytes in the blood and those that migrate to the site of an infection (*L monocytogenes*) have a decreased capacity to produce $PGE_2$, $PGI_2$ and $LTC_4$, while thromboxane $A_2$ ($TXA_2$) synthesis is conserved. Other studies have shown that altered macrophage AA metabolism occurs because there is inactivation of cyclooxygenase,[47] cAMP-mediated decrease in phospholipases A2 activity,[48] and IFN-$\alpha$, IFN-$\beta$ and IFN-$\gamma$ inhibition of phospholipase activity.[49,50] Lymphokine production and subsequent suppression of phospholipase activity may explain the observed decrease in production of $PGE_2$ and $LTC_4$ from macrophages as the infection progresses. Evidence for this was shown when AMO were pretreated with AA substrate to bypass the need for phospholipase activation by which both $PGE_2$ and $LTC_4$ levels were significantly increased. Studies with *S. mansoni* egg-induced liver granulomas during the acute (8 weeks) and chronic (20 weeks) phases have shown high levels of $TXA_2$ and low amounts of $PGE_2$, but not $LTC_4$, in response to zymosan stimulation or when incubated with exogenous AA substrate.[51] Other workers[44] have similarly reported the lack of LT synthesis by pulmonary granuloma macrophages in response to zymosan stimulation; however, the addition of AA substrate restored $LTB_4$, $LTC_4$ and $LTD_4$ synthesis to the macrophages.

This study has clearly shown that *E. histolytica* products can modulate AA metabolism in naive macrophages *in vitro* and *in vivo*. $PGE_2$ and $LTC_4$ production by abscess macrophages was typical of granuloma macrophages demonstrating an initial high production of $PGE_2$ and diminished production later as the infection progressed. These results are consistent with the fact that during the acute phase of the disease (10–20 days p.i.), amoebae-induced $PGE_2$ production favours a state of transient immunosuppression that allows proliferation of the abscess mass and survival of the parasite.

## ACKNOWLEDGMENTS

This work was supported by a grant from the Natural Sciences and Engineering Research Council of Canada (NSERC). W.W. is a recipient of a studentship from the Medical Research Council of Canada. Research at the Institute of Parasitology is partially funded by NSERC and the Fonds pour la Formation de Chercheurs et l'aide à la Recherche du Québec (FCAR). The authors thank Kathy Keller for excellent technical assistance and Dr John Gillard from Merck Frosst, Dorval/Pointe Claire, Canada for critical review of the manuscript.

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