A Novel Neutrophil-Activating Factor Released by *Trichomonas vaginalis*

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We have investigated the effects of a novel neutrophil-activating factor released by *Trichomonas vaginalis* (TV-NAF) on neutrophil chemotaxis. TV-NAF was present in the supernatant from 107 *T. vaginalis* (STV) cultured in 1 ml of serum-free Hanks' balanced salt solution (HBSS) at 37°C for 30 min. With a multichamber chemotactic assay, we found that there were 112 ± 15 migrated neutrophils (mean ± standard deviation, n = 7) for STV and 11 ± 4 for HBSS per high-power field (×400). STV was also able to induce neutrophil actin assembly (increased 1.5-fold), enhance expression of complement receptor type 3 (increased 5-fold), and promote intracellular calcium mobilization (increased 2.5-fold). There was no chemotactic activity in the preparation of STV from killed trichomonads. The fact that heating up to 100°C or deproteinization by treatment with proteinase K at 65°C for 1 h did not abolish its chemotactic activity suggests that the TV-NAF involved was not a protein. The chemotactic activity of TV-NAF was associated with the fraction containing small molecules of less than 3,000 Da. Therefore, the possibility that eicosanoid production by trichomonads is responsible for neutrophil activation was investigated. Leukotriene B4 (LTB4; 500 pg/ml) but not thromboxane B2 (<20 pg/ml) or prostaglandin E2 (<8 pg/ml) was found in the STV by radioimmunoassay. Production of LTB4 by trichomonads was time dependent and increased twofold when arachidonic acid (100 μM) was added but was not decreased when eicosanoid inhibitors were present. Evidence for the presence of LTB4 in STV was further provided by the fact that rabbit anti-LTB4 antiserum could abolish the chemotactic activity of STV. These studies suggest that the spontaneous release of TV-NAF, which is most likely LTB4, may activate neutrophils, presumably through a different arachidonate metabolic pathway than that in mammalian cells.

Trichomoniasis is one of the common sexually transmitted diseases in humans. Although it is known that infection elicits a profuse, acute, inflammatory discharge containing many neutrophils and trichomonads (9), the pathogenetic mechanisms of *Trichomonas vaginalis* have not been well characterized. The current concept that trichomonad-neutrophil interactions constitute an integral part of the interaction between *T. vaginalis* and the host system has been clearly elucidated by Rein et al. (33) and Shaio et al. (36). Chemotaxis is the first step for neutrophils to phagocytose invading microorganisms. Therefore, the presence of chemoattractants will enhance phagocytosis by neutrophils. Manson and Forman (22) reported a heat-labile chemoattractant produced by *T. vaginalis* in the presence of human serum. Chikungwuwo et al. (4) also demonstrated that a heat-labile chemotactic factor was probably a peptide, with a molecular mass of about 900 Da, and was chymotrypsin sensitive but trypsin resistant. Recently, in our laboratory, we found that in the absence of serum, the supernatant collected from *T. vaginalis* (STV) in Hanks' balanced salt solution (HBSS) possesses chemotactic activity which is heat stable. Obviously, our finding is a novel one.

In this study, an attempt was made to identify the nature of the chemoattractant in STV. The influence of STV on chemotaxis by neutrophils was examined. Assessments were made by a chemotactic assay, actin polymerization, expression of complement receptor type 3 (CR3), and intracellular calcium mobilization. The results from these studies provide more information about the inflammatory response induced by *T. vaginalis*.

**MATERIALS AND METHODS**

**Organism.** Seven local isolates, axenically cultivated, were maintained in a modified medium identical to the TYI-S-33 medium of Diamond et al. (7), except that 0.5% Panemode (Paines & Byrne Limited, Greenford, England) was added. The number of organisms per culture was determined with a Coulter counter (model D industrial; Coulter Electronics, Inc., Hialeah, Fla.) with a 70-μm aperture tube.

**Preparation of STV.** Each isolate of *T. vaginalis* grown at 37°C in 15-ml tubes containing TYI-S-33 medium was harvested during the logarithmic growth phase after 36 h of cultivation. They were centrifugally washed three times in HBSS (GIBCO, Grand Island, N.Y.), and then viable cells were counted in a hemacytometer with trypan blue-saline. HBSS without phenol red was used as the wash buffer and diluent throughout this study. The medium-free flagellates (107) were incubated in 1 ml of HBSS at 37°C for 30 min. Motile flagellates were then removed by gentle centrifugation (500 x g for 10 min at 4°C), and the supernatant was filter sterilized (Minisart; 0.22 μm; Sartorius, Goettingen, Germany). This preparation is referred to as STV, and various treatments were done depending on the experimental designs. STV was deproteinized either by treatment with 4 M perchloric acid at 4°C for 30 min and adjustment of the
pH with 5 N NaOH or by enzymatic digestion with 50 μg of proteinase K (Boehringer, Mannheim, Germany) per ml at 65°C for 1 h.

To determine eicosanoid production by trichomonads, either stimulants such as 100 μM arachidonic acid (Sigma Chemical Co., St. Louis, Mo.), 10 μM calcium ionophore A23187 (Sigma), serum-opsinzed zymosan (1 mg/ml; Sigma), phorbol myristate acetate (40 ng/ml; Sigma), and formyl-methionyl-leucyl-phenylalanine (FMLP, 10 nM; Sigma) or eicosanoid inhibitors such as 100 μM ibuprofen (Sigma), 100 μM esculetin (Sigma), and 100 μM curcumin (Sigma) were added (24, 34). In addition, to determine whether leukotriene B4 (LTB4) was the factor responsible for the chemotactic activity of STV, STV was reacted with heat-inactivated rabbit anti-LTB4 antiserum (TRK 940; batch no. 34B, lot 12; Amersham, Buckinghamshire, England) at room temperature for 2 h before the chemotactic assay. Anti-prostaglandin E2 (anti-PGE2) and anti-thromboxane B2 (anti-TXB2) antisera (Amersham) as well as normal rabbit serum were used as controls.

Preparation of neutrophils. Venous blood from healthy young students was drawn into plastic syringes containing 10 IU of heparin per ml. Dextran (4.5%, wt/vol; TS500; Pharmacia, Uppsala, Sweden) was added in a ratio of 1:5, and the syringe was incubated nozzle upward at 37°C for 30 min. The upper fraction of leukocyte-enriched plasma was then layered onto Ficoll-Paque (Pharmacia) at a ratio of 2:1 and centrifuged at 1,500 g for 10 min. The supernatant was discarded. The pellet was washed, and residual erythrocytes were lysed by distilled-water treatment for 15 s. This hypotonic shock was stopped by adding an equal volume of 1.8% sodium chloride solution. The neutrophils were finally suspended to 107 cells per ml in HBSS. With this method, the purity and viability of neutrophils consistently exceeded 95 and 98%, respectively.

Assay of chemotaxis. Chemotaxis was assessed in a 48-well microchemotaxis chamber (Neuroprobe Inc., Cabin John, Md.) by a method modified from one described previously (14). The lower chambers were filled with 29 μl of HBSS or various amounts of STV. FMLP (10 nM) and LTB4 (1 nM; Amersham) were used as positive controls. The upper chambers were filled with 50 μl of neutrophil suspensions (2 × 106 cells per ml). A nitrocellulose microprobe filter (5 μm pore size; Millipore, Bedford, Mass.) was set between the bottom and bottom chambers. Nitrocellulose filters were pretreated with 2% albumin (Sigma) so that neutrophil chemotaxis could take place in albumin-free medium (31). Triplicate samples were run in each experiment. Reaction mixes were incubated in a humidified CO2 incubator for 2 h. After incubation, the filters were fixed with methanol and stained with hematoxylin. In addition, the contents taken from the bottom chambers were examined microscopically by cytocentrifugation preparation. It has been shown that no neutrophils can cross the filter to reach the bottom chamber. The possibility that migrated cells fell off and adhered to the walls of the chambers could be excluded by simply cooling the bottom chambers on ice for 30 min or by adding trypsin-EDTA to the bottom chambers before cytocentrifugation. Therefore, the neutrophils that migrated to the bottom surface of the filter were counted in a microscope with a 10× ocular, 40× objective glass field (37). Migrated cells were counted in five random high-power fields (HPFs) per well, and the results were expressed as the mean number of cells in five HPFs per triplicate well.

An experimental protocol in the form of a checkerboard was used to test for both directional (chemotactic) migration and also nondirectional (chemokinetic) migration. For the latter, STV was put on both sides of the filter to eliminate the chemotactrant gradient across the filter. The ratio of chemokinetic to chemotactic cell migration was calculated to obtain the chemokinetic value.

Determination of eicosanoids. LTB4, TXB2, and PGE2 levels were determined by radioimmunoassay with commercial kits (Amersham). Prior to assay, solid-phase extraction procedures for sample purification were carried out with an Amrprep C18 minicolumn (Amersham). For the LTB4 assay, 100 μl of STV (with and without various treatments) was mixed with [3H]LTB4 and then with 10 μl of rabbit anti-LTB4 antibody and incubated at 25°C for 2 h. At the end of incubation, 200 μl of dextran-coated charcoal was added to the mixtures and incubated at 25°C for 5 min. After centrifugation, 250 μl of the supernatant was removed for counting of radioactivity. A similar procedure was also used for the TXB2 assay. For the PGE2 assay, [252P]PGE2 was used as a tracer.

Uptake of exogenous arachidonic acid. An axenic culture of T. vaginalis at the logarithmic phase of growth was harvested by centrifugation and then washed with HBSS three times. The washed parasites were then resuspended in HBSS to a final concentration of 105 organisms per ml and incubated with 0.1 μCi of [5,6,8,9,11,12,14,15-3H]arachidonic acid (87 Ci/mmol; Amersham) per ml. At different times, aliquots of the reaction mixture were taken, filtered through 3.0-μm nitrocellulose filter paper, and washed with three portions of 15-ml of ice-cold HBSS containing a 10-fold-higher concentration of unlabeled arachidonic acid. The filter papers were then dried under a vacuum and then soaked in 10 ml of scintillation liquid (Ultrafluor; National Diagnostics) to determine the radioactivity with a beta scintillation counter (LS 3801; Beckman Instruments, Inc., Fullerton, Calif.).

Incorporation of arachidonic acid into T. vaginalis lipids. Lipid extracts of T. vaginalis were analyzed for H-fatty acid incorporation into neutral lipids and phospholipids. Ultrasonically disrupted parasites and supernatant fluid were extracted by a modification of the method of Clancy and Hugli (6). Isopropanol (Sigma) and 5 M citric acid (Sigma) were added in the ratio sample-isopropanol-citrate (1:0.5: 0.05, by volume). After 5 min, the mixture was extracted with an equal volume of chloroform-methanol-water (2:1:0.01). Incorporation of arachidonic acid into parasite neutral lipids and amounts of residual free fatty acids were quantitated after thin-layer chromatography (TLC) resolution. Polysilicic acid gel TLC sheets were developed with petroleum ether-diethyl ether-glacial acetic acid (90:15:1). Each lane was cut transversely into 5-mm sections, which were eluted with methanol in scintillation vials, and radioactivity was counted in a beta scintillation counter after addition of scintillation fluid. Reference diglyceride, arachidonic acid, triglyceride, and cholesterol ester standards migrated with Rf values of 0.45, 0.85, 0.95, and 1.0, respectively. To determine [3H]arachidonic acid incorporation into different classes of phospholipids, parasite phospholipids were resolved and identified by normal-phase high-pressure liquid chromatography (HPLC) with a Waters HPLC system (model 590; Waters Associates, Milford, Mass.) at a flow rate of 1 ml/min.

Assay of actin polymerization. Actin in nonmuscle cells exists mainly in the globulin form (G-actin) at the resting state (19). Upon leukocyte activation, actins were polymer-
ized into filamentous form (F-actin) (32). Thus, changes in F-actin content can reflect the state of cytoskeleton organization resulting from cell activation or locomotion (26). A flow cytometric analysis of nitrobenzoxadiazole (NBD)-phallacidin staining, in which phallacidin specifically binds to F-actin (1), was used. F-actin content can be measured by NBD fluorescence at a 520-nm emission wavelength and a 488-nm excitation wavelength (17, 38). For experiments, neutrophils (2 × 10⁶ cells per ml) were put into a microcentrifuge tube (Treff Laboratory Inc., Schweiz, Switzerland) and incubated with STV at 10, 25, 50, and 100% (vol/vol). STV was heated at 56, 80, and 100°C for 30 min. Mixtures containing neutrophils and various treatments of STV were incubated at 37°C with rotation at 12 cycles per min. Results were checked at time zero, 30 s, 1 min, and 5 min and then at 5-min intervals for up to 30 min. Portions (200 µl) of reaction mixtures were permeabilized, fixed, and stained in an equal amount of the NBD-phallacidin (Molecular Probes, Eugene, Ore.) staining cocktail containing 100 µg of lysoctethin per ml, 7.4% formaldehyde, and NBD-phallacidin (110 nM) at 37°C for 10 min. After being washed twice in cold phosphate-buffered saline (PBS), cells were suspended in 0.5 ml of PBS and analyzed by a flow cytometer (FACScan; Becton Dickinson, Mountain View, Calif.). Forward scatter was used simultaneously to gate cell population sizes. The results were recorded as histograms of fluorescence channel versus cell number.

Measurement of CR3 expression. Neutrophils (10⁶/ml) were suspended in HBSS and various STV preparations containing 2% bovine serum albumin (BSA) at 37°C for 20 min. Portions (100 µl) were put on ice immediately. The cells were incubated with 10 µl of monoclonal antibody to CD11b (CR3; Immunotech S.A., Marseille, France) or control monoclonal antibody immunoglobulin G2a (IgG2a) on ice for 30 min. After three washes with 500 µl of PBS-BSA, 20 µl of fluorescein isothiocyanate (FITC)-conjugated goat antimouse IgG F(ab')₂ antibody (Organon Teknika, Malvern, Pa.) diluted 1:30 (vol/vol) in PBS-BSA was added to the cell suspension, which was incubated for 30 min and washed again. The cells were finally suspended in 100 µl of PBS-BSA, with 100 µl of 2% (wt/vol) paraformaldehyde (Sigma) in water, pH 7.2, as a fixative, and stored at 4°C. Fluorescence was analyzed with a FACScan, and the data are expressed as the percentage of cells demonstrating an increase in fluorescence above the background.

Measurement of free calcium in the cytosol. Neutrophils (5 × 10⁶ cells per ml) in HBSS supplemented with 20 mM HEPES (N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid, pH 7.4) were incubated at 37°C with 2 µM fura-2-acetoxy methyl ester (Sigma) for 30 min. Loaded cells were washed twice, reconstituted in HBSS (with Ca²⁺ at 1.27 mM) with 20 mM HEPES in a quartz cuvette, maintained at 37°C, and held in the light path of a dual-wavelength fluorescence apparatus (Spx Fluorolog 3) set at an emission wavelength of 505 nm. Fluorescence excitation at 340 nm and 380 nm was monitored during the addition of 10 nM fMLP, 1 nM LTB4 and various STV preparations. The assay was controlled by the addition of EGTA (ethylene glycol tetraacetic acid), Tris buffer, Triton X-100, and CaCl₂. Free Ca²⁺ concentrations were calculated from the 340 nm/380 nm ratio, assuming a K₅ of 186 nM, as described previously (23).

FIG. 1. Effect of STV on neutrophil chemotaxis. STV(C), concentrated STV (>3,000 Da); STV(E), effluent STV (<3,000 Da); STV(dep), STV deproteinized with perchloric acid. Both fMLP and LTB4 were used as positive controls. Error bars show 1 SD.

RESULTS

Effect of STV on neutrophil chemotaxis. STV was prepared at various times. STV from a 30-min incubation exhibited the most prominent chemotactic activity for neutrophils. We found that 112 ± 15 neutrophils (mean ± standard deviation [SD], n = 7) and 11 ± 4 cells were attracted per HPF for STV and HBSS, respectively. Although STV was chemotactic for neutrophils in a dose-dependent manner, its half-concentration showed a dramatic reduction in chemotaxis (Fig. 1). The chemotactic activity of STV disappeared when a 1:10 dilution of STV was used (Fig. 1). Concentrations of STV below a 1:10 dilution, ranging from 10⁻² to 10⁻⁴ dilution, were also examined, and no chemotactic activity was found (data not shown). This suggests that STV contained a chemoattractant with a critical level for attracting neutrophils. Heating of STV up to 100°C (data not shown) and deproteinization of STV by treatment with perchloric acid (4 M) or proteinase K did not abolish the chemotactic activity of STV (Fig. 1 and Table 1). fMLP pretreated with proteinase K did lose its chemotactic activity, while LTB4 did not (Table 1). These results suggested that the chemoattractant involved was not a protein. By centrifugation, concentrated substances [STV(C)] over 3 kDa in size showed no chemotactic activity, while the effluent fraction [STV(E)] containing small molecules of less than 3 kDa retained intact chemotactic activity (Fig. 1). Both fMLP (10 nM) and LTB4 (1 nM) were used as positive controls.

Checkerboard analysis of STV-stimulated chemotaxis across an albumin-treated nitrocellulose filter in albumin-free medium indicated a chemokinetic component of 49% at the peak STV concentration (Table 2).

Eicosanoid production by T. vaginalis. The neutrophil chemotaxant in STV was heat stable (up to 100°C) and not proteinaceous in nature (proteinase K resistant). Where-

<table>
<thead>
<tr>
<th>TABLE 1. Effect of proteinase K on chemotactic activity of STV</th>
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<tbody>
<tr>
<td>Chemotaxant (conc)</td>
</tr>
<tr>
<td>--------------------</td>
</tr>
<tr>
<td>STV</td>
</tr>
<tr>
<td>fMLP (10 nM)</td>
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<td>LTB4 (1 nM)</td>
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* Five separate experiments.
fore, the possibility that eicosanoid release by *T. vaginalis* was responsible for neutrophil chemotaxis was investigated. Among seven preparations of STV from different isolates of *T. vaginalis* tested, all were found to give a peak spontaneous release of LTb4 ranging from 360 to 550 pg/ml. However, none of these preparations produced PGE2 (<8 pg/ml) or TXB2 (<20 pg/ml) (Table 3). Production of LTb4 by trichomonads was time dependent (Fig. 2) and reached a maximum after 30 min of incubation (data not shown). The chemotactic activity of STV was completely abolished by the addition of 10% rabbit anti-LTB4 antiserum, but it was not altered by normal rabbit serum (Fig. 3) or by anti-PGE2 or anti-TXB2 antiserum (data not shown). In a control experiment, this anti-LTB4 antiserum did remove the chemotactic activity of LTb4 but had no effect on that of fMLP (data not shown). This indicates that the LTb4 in STV is the component responsible for the chemotactic activity.

When arachidonic acid (100 μM) was used as a stimulus, LTb4 production exhibited a twofold increase, and both TXB2 and PGE2 became measurable (Table 3). Although calcium ionophore A23187 (10 μM) increased LTb4 production by trichomonads to some degree, it did not alter TXb2 or PGE2 production. The combination of arachidonic acid and A23187 did not increase eicosanoid production by trichomonads over the increase with arachidonic acid alone. Other stimuli such as serum-opsonized zymosan, phorbol myristate acetate, and fMLP had no enhancing effect on LTb4 production by trichomonads. On the other hand, none of the eicosanoid inhibitors used, indomethacin (1 μM), ibuprofen (100 μM), esculetin (100 μM), or curcumin (100 μM), was found to block eicosanoid production by trichomonads.

**TABLE 3. Effect of eicosanoid inhibitors on the release of eicosanoids by *T. vaginalis*"}

<table>
<thead>
<tr>
<th>Inhibitor (μM)</th>
<th>Stimulus</th>
<th>LTb4 (Eicosanoid production (pg/10⁷ trichomonads/ml))</th>
<th>TXB2 (Eicosanoid production (pg/10⁷ trichomonads/ml))</th>
<th>PGE2 (Eicosanoid production (pg/10⁷ trichomonads/ml))</th>
</tr>
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<tr>
<td>None</td>
<td>None</td>
<td>515 ± 34</td>
<td>&lt;20</td>
<td>&lt;8</td>
</tr>
<tr>
<td></td>
<td>A23187</td>
<td>709 ± 34</td>
<td>&lt;20</td>
<td>&lt;8</td>
</tr>
<tr>
<td></td>
<td>A.A.</td>
<td>1,188 ± 133</td>
<td>106 ± 33</td>
<td>483 ± 43</td>
</tr>
<tr>
<td></td>
<td>A23187 + A.A.</td>
<td>1,114 ± 201</td>
<td>97 ± 28</td>
<td>432 ± 25</td>
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<tr>
<td></td>
<td>A.A.</td>
<td>720 ± 84</td>
<td>ND</td>
<td>&lt;8</td>
</tr>
<tr>
<td></td>
<td>A.A.</td>
<td>1,152 ± 184</td>
<td>ND</td>
<td>415 ± 25</td>
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<tr>
<td></td>
<td>A23187 + A.A.</td>
<td>1,133 ± 179</td>
<td>86 ± 15</td>
<td>483 ± 23</td>
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<tr>
<td></td>
<td>A23187</td>
<td>733 ± 110</td>
<td>ND</td>
<td>&lt;8</td>
</tr>
<tr>
<td></td>
<td>A.A.</td>
<td>1,313 ± 184</td>
<td>ND</td>
<td>495 ± 34</td>
</tr>
<tr>
<td></td>
<td>A23187 + A.A.</td>
<td>1,306 ± 196</td>
<td>79 ± 16</td>
<td>416 ± 33</td>
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<tr>
<td></td>
<td>A23187</td>
<td>783 ± 28</td>
<td>ND</td>
<td>&lt;8</td>
</tr>
<tr>
<td></td>
<td>A.A.</td>
<td>1,211 ± 179</td>
<td>ND</td>
<td>475 ± 38</td>
</tr>
<tr>
<td></td>
<td>A23187 + A.A.</td>
<td>1,325 ± 246</td>
<td>81 ± 16</td>
<td>428 ± 42</td>
</tr>
<tr>
<td></td>
<td>A23187</td>
<td>755 ± 168</td>
<td>ND</td>
<td>&lt;8</td>
</tr>
<tr>
<td></td>
<td>A.A.</td>
<td>1,314 ± 192</td>
<td>ND</td>
<td>431 ± 35</td>
</tr>
<tr>
<td></td>
<td>A23187 + A.A.</td>
<td>1,443 ± 284</td>
<td>78 ± 12</td>
<td>464 ± 23</td>
</tr>
</tbody>
</table>

* Eicosanoid production was determined by radioimmunoassay.
* A.A., arachidonic acid. Stimulus concentrations were 10 and 100 μM for A23187 and A.A., respectively.
* ND, not determined.

**FIG. 2. Time-dependent release of LTb4 by *T. vaginalis*. Data points are means ± SD (error bars).**
production was increased (data not shown). A small amount (18%) of the fatty acid was incorporated into neutral lipids (mainly diglyceride; data not shown). The incorporation of [3H]arachidonic acid into various classes of phospholipid was identified by normal-phase HPLC and compared with the standard chromatogram of different phospholipid classes. Radiolabel from fatty acid was found principally in phosphatidylcholine and phosphatidylethanolamine after 60 min of incubation at 37°C. Label was also detected in the phosphatidylinositol and phosphatidylserine fractions, but no radioactivity was found associated with phosphatidylglycerol (data not shown).

**Effect of STV on actin assembly and calcium mobilization in neutrophils.** Actin polymerization, expressed as fluorescence intensity as measured by cytomteric analysis, occurred within seconds after the addition of STV (Fig. 5). The mean fluorescence intensity reached a peak after 30 s. It subsequently declined sharply, approaching background values after approximately 5 min. Both fMLP (10 nM) and LTB4 (1 nM) were used as positive controls. In contrast to fMLP, LTB4 initiated a rapidly appearing but transient effect in the neutrophils. STV initiated actin polymerization with a pattern similar to that with LTB4.

The intracellular concentration of free calcium, 

\[ \text{Ca}^{2+} \]

in neutrophils stimulated with STV, LTB4, and fMLP was examined, and the results are shown in Fig. 6. The resting 

\[ \text{Ca}^{2+} \]

concentration was 90 ± 10 nM (n = 5), and the presence of undiluted STV promoted a transient rise in the intracellular 

\[ \text{Ca}^{2+} \] level to 327 ± 40 nM. fMLP (1 nM) and LTB4 (1 nM), used as positive controls, induced intracellular calcium mobilization up to 496 ± 38 and 302 ± 31 nM, respectively.

**Effect of STV on CR3 expression.** STV induced a time-dependent increase in CR3 expression by neutrophils incubated at 37°C. Maximal expression was seen by 20 min (data not shown), when there was a mean fivefold rise in fluorescence intensity over control values (Fig. 7A and D; 180 and 1,060 arbitrary units for HBSS and STV, respectively). Incubation of neutrophils with 10 nM fMLP and 1 nM LTB4 for 20 min resulted in a fourfold-higher increase in CR3 expression (Fig. 7B and C, respectively). On the other hand, the percentage of the cell population bearing CR3 was not increased in the presence of stimuli, whether STV, fMLP, or LTB4 (data not shown).

**DISCUSSION**

This study has shown that *T. vaginalis* can spontaneously release a novel neutrophil-activating factor (TV-NAF). Evidence that this novel TV-NAF is most likely LTB4 includes: (i) it is heat stable and not proteinaceous in nature; (ii) production was increased by addition of arachidonic acid; (iii) it was identified in a radioimmunoassay specific for LTB4; and (iv) its chemotactic activity was abolished by anti-LTB4 antiserum.

Whether this TV-NAF in STV is a specific product of *T. vaginalis* is a major concern. Several lines of evidence clearly disassociate it from the products or combined products of *Escherichia coli* and *Enterobacter aerogenes*. First, all these isolates have been maintained axenically in this laboratory. Second, scanning electron microscopic examination revealed that no cocci or bacilli were attached on the surface of the trichomonads (data not shown). Third, regrowth of *T. vaginalis* from the STV preparation showed no bacterial contamination. Fourth, the addition of lipopolysaccharides from *E. coli* K235 to the trichomonad suspension did not enhance LTB4 production (data not shown). Finally, the supernatant from a *Giardia lamblia* (an intestinal flagellate) suspension did not attract neutrophils, as assayed by chemotaxis (data not shown).

While all seven isolates tested in this study were from patients with symptomatic trichomoniasis, a recent axenic isolate of *T. vaginalis* from an asymptomatic patient produced up to 450 pg of LTB4 per ml (data not shown). This indicates that LTB4 production may not be unique to the pathogenic trichomonads. Variations in host response to infection with *T. vaginalis* have previously been ascribed to variations in the pathogenicity of the infecting organism (16). Although it is difficult to establish the relationship between the number of *T. vaginalis* in patient discharges and their clinical status (9, 20, 30), the infectious dose of invading organisms is always considered one of the major factors responsible for the pathogenesis of infection. No significant LTB4 production could be detected when low concentrations of trichomonads (e.g., 10⁶ flagellates per ml) were used (data not shown). Philip et al. (30) showed that most patients with trichomoniasis had colony counts ranging from 40 to 9 × 10³ CFU/ml, and some patients had colony counts of more than 10⁶ CFU/ml. Fouts and Kraus (9) reported that trichomonal growth occurred as high as the eighth logarithmic dilution. However, this study had no data available to show the number of trichomonads in the patient’s vagina.

Manson and Forman demonstrated that a heat-labile chemoattractant is produced by *T. vaginalis* in the presence of human serum (22). Obviously, our heat-stable chemoattractant, produced by *T. vaginalis* in the absence of serum, is not related to complement activation. The generation of LTB4 by *T. vaginalis* in complement-lacking microenvironments, such as the male urogenital tract, may be of considerable biological importance. LTB4 has broad and potent

![FIG. 3. Chemotactic activity of STV is abolished by anti-LTB4 antibody. Error bars show 1 SD.](http://iai.asm.org/Downloaded from http://iai.asm.org/)

![FIG. 4. Uptake of [3H]arachidonic acid by *T. vaginalis*. Uptake occurred most rapidly within the first 5 min. The initial rate of uptake was about 2,000 cpm/min.](http://iai.asm.org/Downloaded from http://iai.asm.org/)
biological activities, especially neutrophil activation (25, 27, 28, 35). These activities result in positive-feedback mechanisms that are probably important for recruitment and stimulation of neutrophils at sites of infection. Neutrophils not only respond when exposed to LTB4 but also are a major source of this factor when stimulated by the calcium ionophore A23187 (29) or following IgG-dependent stimulation (8). We believe that such mechanisms may be partially responsible for the previously described interaction between T. vaginalis and neutrophils in vitro (33, 36), though the activation of complement is the major contribution (33, 36). A study on LTB4 production by neutrophils that have interacted with trichomonads is under way.

It has been reported that LTB4 is a significant chemoattractant at 1 to 10 nM (5) and evoked chemotaxis with a linear dose response from 1 to 100 nM (25). Goldman and Goetzl (12) have demonstrated that human neutrophils possess a subset of receptors for LTB4 that are distinct from those specific for peptide chemotactic factors. The fact that anti-LTB4 antiserum abolished neutrophil chemotaxis by STV suggests that the epitope of LTB4 responsible for neutrophil chemotaxis could be blocked selectively but may not necessarily mean that the other biological activities of LTB4 were lost. It has been shown that high-affinity receptors for LTB4 on normal human neutrophils appear to transduce the chemotaxis evoked by LTB4 without substantially modifying lysosomal degranulation (13). Since trichomonads produce LTB4 and respond to some of the same stimuli as neutrophils, the possibility of specific binding sites for LTB4 on T. vaginalis remains to be established. Nevertheless, our observation that up to 500 pg/ml (1.5 nM) was spontaneously produced by 10^7 trichomonads could be an important finding which would facilitate the elucidation of the pathogenesis of trichomonad infections.

Under normal circumstances, arachidonic acid is bound as an integral part of phospholipids. Only when a cell is injured or stimulated is arachidonic acid cleaved off and released. There are two pathways for arachidonic acid release: a nonspecific one, liberating small amounts of the acid upon cell injury (18), and a specific one, liberating large amounts upon cell stimulation (39). The nonspecific disturbance of the plasma membrane may range from simple stretching to a frank rupture (18). Although trypan blue exclusion revealed that up to 95% of the trichomonads were viable after 1 h of incubation, the possibility that nonspecific mechanisms partly contribute to LTB4 release by T. vaginalis cannot be excluded.

Until recently, lipid metabolism in human parasites has not received much attention (2, 10, 11, 21, 34). It has been suggested that arachidonate metabolism and subsequent eicosanoid production are required for successful cercarial penetration (11, 34). Blair and Weller demonstrated the metabolic incorporation of arachidonic and palmitic acids by Giardia trophozoites but did not show that eicosanoids were subsequently generated (2). Our findings have clearly shown that T. vaginalis can take up and incorporate arachidonic acid into trichomonad lipids and that the release of LTB4 increases twofold when exogenous arachidonic acid is present. However, little is known about the pathways of biosynthesis or acquisition of trichomonad phospholipid compared with mammalian pathways. Phospholipase A2 has been isolated from particulate membrane fractions with specificity toward phosphatidylcholine and phosphatidylethanolamine (3). It has also been postulated that granulocyte stimulation activates membrane phospholipase A2 to release arachidonic acid, which is then enzymatically metabolized by the 5-lipoxygenase pathway to form LTB4 as well as a host of other bioactive lipid metabolites (15, 18). Whether phospholipase A2 is present and plays a similar role in T. vaginalis remains to be established. The fact that LTB4 but not PGE2 or TXB2 is generated by these trichomonads

![Graph](image-url)

**FIG. 5.** Effect of STV on neutrophil actin polymerization. Actin polymerization, measured by fluorescence intensity, occurred within 30 s after the addition of STV. Baseline fluorescence intensity for resting neutrophils was 250 arbitrary units. Peak fluorescence intensity reached 650 arbitrary units.

![Graph](image-url)

**FIG. 6.** Effect of STV on calcium mobilization in neutrophils. The intracellular calcium concentration of resting neutrophils was 90 ± 10 nM. STV induced a transient rise of [Ca^{2+}], to 327 ± 36 nM. Error bars show 1 SD.
indicates that *T. vaginalis* could have a different arachidonic acid-metabolic pathway than mammalian cells. Moreover, the lack of inhibition by eicosanoid inhibitors may also reflect differences in this parasite’s enzymes compared with the described mammalian systems. Clarification of these potential differences will await future isolation and characterization of the trichomonad enzymes.

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**REFERENCES**