INFECTION AND IMMUNITY, Nov. 1992, p. 4475–4482
0019-9567/92/114475-08$02.00/0

Vol. 60, No. 11

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

MEN-FANG SHAIO,[1]* PEY-RU LIN,[1] CHUNG-SHINN LEE,[1] SUN-CHIH HOU,[1] PETRUS TANG,[2] AND KUENDER D. YANG[3]

Department of Parasitology and Tropical Medicine, National Defense Medical Center, Taipei,[1] Department of Parasitology, Chang Gung Medical College, Taoyuan,[2] and Department of Pediatrics, Tri-Service General Hospital, Taipei,[3] Taiwan, Republic of China

Received 26 February 1992/Accepted 12 August 1992

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 10^7 *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. Chikunguwo 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% Panmede (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 (10^7) were incubated in 1 ml of HBSS at 37°C for 30 min. Motile flagellates were then removed by gentle centrifugation (500 × 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

* Corresponding author.

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-opsonized 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; T500; 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 10^7 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 (Neuropore 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 (2×10^6 cells per ml). A nitrocellulose micropore filter (5 μm pore size; Millipore Co., Bedford, Mass.) was set between the top 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 CO_2 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 chemoattractant 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 Amprep C18 minicolumn (Amersham). For the LTB4 assay, 100 μl of STV (with and without various treatments) was mixed with [^{3}H]LTB4 and then with 100 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, [^{125}I]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 10^7 organisms per ml and incubated with 0.1 μCi of [5,6,8,9,11,12,14,15-^{3}H]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 system and 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 ^{3}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 (1:0.5:0.03, by volume). After 5 min, the mixture was extracted with 1.4 volumes of diethyl ether (Merck, Darmstadt, Germany). The organic phase was concentrated under a nitrogen evaporator and resuspended in 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 R_f values of 0.45, 0.85, 0.95, and 1.0, respectively. To determine [^{3}H]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 nitrobenzooxadiazole (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 (2×10^6 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 lysolecithin 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 (10^6/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 anti-mouse 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, added 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 (5×10^6 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 methylester (Sigma) for 30 min. Loaded cells were washed twice, reconstituted in HBSS (with Ca^{2+} 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 (Spex 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 $CaCl_2$. Free Ca^{2+} concentrations were calculated from the 340 nm/380 nm ratio, assuming a K_d of 186 nM, as described previously (23).

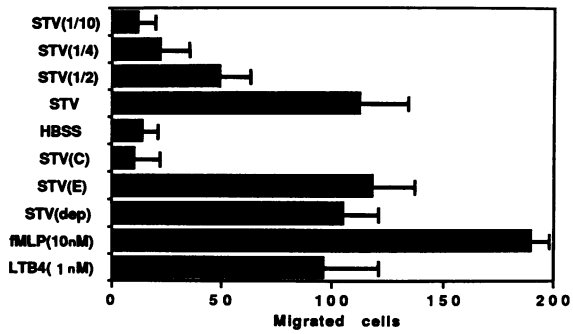

FIG. 1. Effect of STV on neutrophil chemotaxis. STV(C), concentrated STV (>3,000 Da); STV(E), effluent STV (<3,000 Da); STV(dep), STV deproteinized with perchloric acid. Both fMLP and LTB4 were used as positive controls. Error bars show 1 SD.

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 chemoattractant in STV was heat stable (up to 100°C) and not proteinaceous in nature (proteinase K resistant). There-

TABLE 1. Effect of proteinase K on chemotactic activity of STV

Chemoattractant (concn)	Proteinase K treatment	Mean no. of migrated cells ± SD[a]
STV	Yes	109 ± 12
	No	118 ± 16
fMLP(10nM)	Yes	9 ± 5
	No	186 ± 10
LTB4 (1nM)	Yes	105 ± 11
	No	98 ± 14

[a] Five separate experiments.

TABLE 2. Checkerboard analysis of STV-stimulated neutrophil chemotaxis[a]

STV concn (%) in bottom chamber	No. of neutrophils migrated through filters at STV concn (%) in top chamber of:				
	0	10	25	50	100
0	0				
10	12	8			
25	22		27		
50	51			38	
100	119				58

[a] Checkerboard analysis showed the chemokinetic component of total cell migration to be 49% ($P < 0.001$) at the highest STV concentration tested. Migrated cells were counted in five random HPFs per well, and results were expressed as the mean number of cells in five HPFs per triplicate well within a representative experiment.

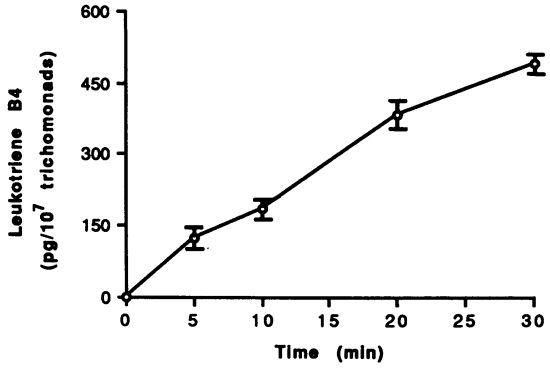

FIG. 2. Time-dependent release of LTB4 by *T. vaginalis*. Data points are means ± SD (error bars).

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.

Uptake and incorporation of arachidonic acid into *T. vaginalis* lipids. The time course of uptake of [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid is shown in Fig. 4. *T. vaginalis* incubated with 12 nM radio-labeled arachidonic acid exhibited time-dependent uptake. The uptake of arachidonic acid by trichomonads was most rapid within the first 5 min and reached a plateau after 15 min. The cessation of further uptake of exogenous fatty acids was not due to the death of cells, since 90% of the flagellates were viable at 60 min, as verified by trypan blue dye exclusion.

To determine whether the arachidonic acid taken up by the flagellates was metabolized, esterification of ^{3}H-fatty acid into neutral lipids and phospholipids was measured. The proportion of recovered tritium counts revealed that 74% of the fatty acid was metabolized to phospholipids (data not

TABLE 3. Effect of eicosanoid inhibitors on the release of eicosanoids by *T. vaginalis*[a]

Inhibitor (μM)	Stimulus[b]	Eicosanoid production (pg/10^7 trichomonads/ml)		
		LTB4	TXB2	PGE2
None	None	515 ± 34	<20	<8
	A23187	709 ± 34	<20	<8
	A.A.	1,188 ± 133	106 ± 33	483 ± 43
	A23187 + A.A.	1,114 ± 201	97 ± 28	432 ± 25
Indomethacin (1)	A23187	720 ± 84	ND[c]	<8
	A.A.	1,152 ± 184	ND	415 ± 25
	A23187 + A.A.	1,133 ± 179	86 ± 15	483 ± 23
Ibuprofen (100)	A23187	733 ± 110	ND	<8
	A.A.	1,313 ± 184	ND	495 ± 34
	A23187 + A.A.	1,306 ± 196	79 ± 16	416 ± 33
Esculetin (100)	A23187	783 ± 28	ND	<8
	A.A.	1,311 ± 179	ND	475 ± 38
	A23187 + A.A.	1,325 ± 246	81 ± 16	428 ± 42
Curcumin (100)	A23187	755 ± 168	ND	<8
	A.A.	1,314 ± 192	ND	431 ± 35
	A23187 + A.A.	1,443 ± 284	78 ± 12	464 ± 23

[a] Eicosanoid production was determined by radioimmunoassay.
[b] A.A., arachidonic acid. Stimulus concentrations were 10 and 100 μM for A23187 and A.A., respectively.
[c] ND, not determined.

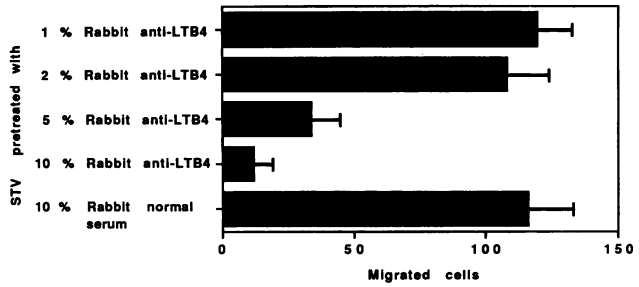

FIG. 3. Chemotactic activity of STV is abolished by anti-LTB4 antibody. Error bars show 1 SD.

shown). A small amount (18%) of the fatty acid was incorporated into neutral lipids (mainly diglyceride; data not shown). The incorporation of [^{3}H]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 cytometric 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, $[Ca^{2+}]_i$, in neutrophils stimulated with STV, LTB4, and fMLP was examined, and the results are shown in Fig. 6. The resting Ca^{2+} concentration was 90 ± 10 nM (n = 5), and the presence of undiluted STV promoted a transient rise in the intracellular Ca^{2+} level to 327 ± 36 nM. fMLP (10 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., 10^6 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 × 10^5 CFU/ml, and some patients had colony counts of more than 10^6 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

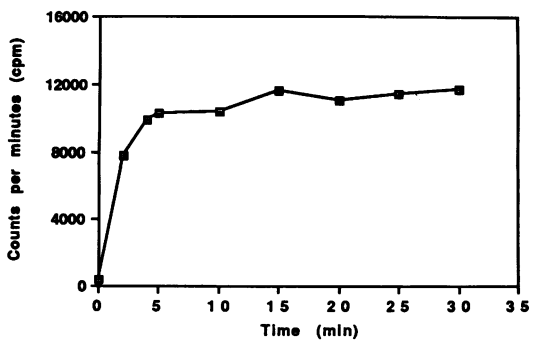

FIG. 4. Uptake of [^{3}H]arachidonic acid by *T. vaginalis*. Uptake occurred most rapidly within the first 5 min. The initial rate of uptake was about 2,000 cpm/min.

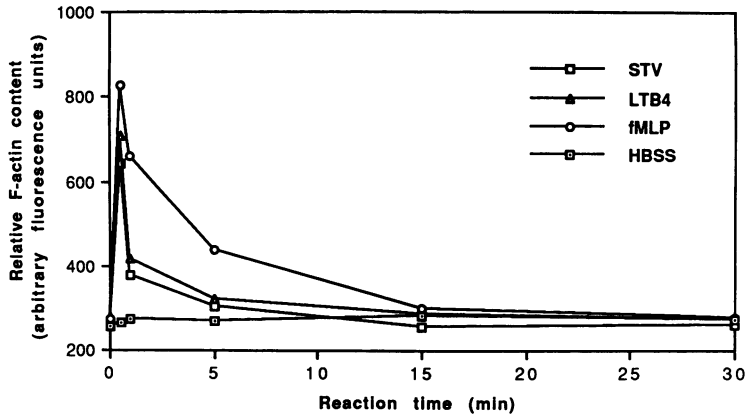

FIG. 5. Effect of STV on neutrophil actin polymerization. Actin polymerization, measured by fluorescence intensity, occurred within 30 s after the addition of STV. Baseline fluorescence intensity for resting neutrophils was 250 arbitrary units. Peak fluorescence intensity reached 650 arbitrary units.

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 evaluated. 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.

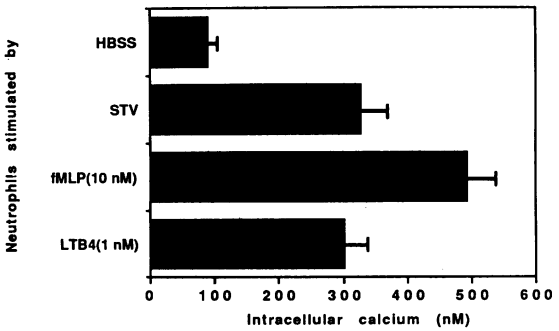

FIG. 6. Effect of STV on calcium mobilization in neutrophils. The intracellular calcium concentration of resting neutrophils was 90 ± 10 nM. STV induced a transient rise of $[Ca^{2+}]_i$ to 327 ± 36 nM. Error bars show 1 SD.

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

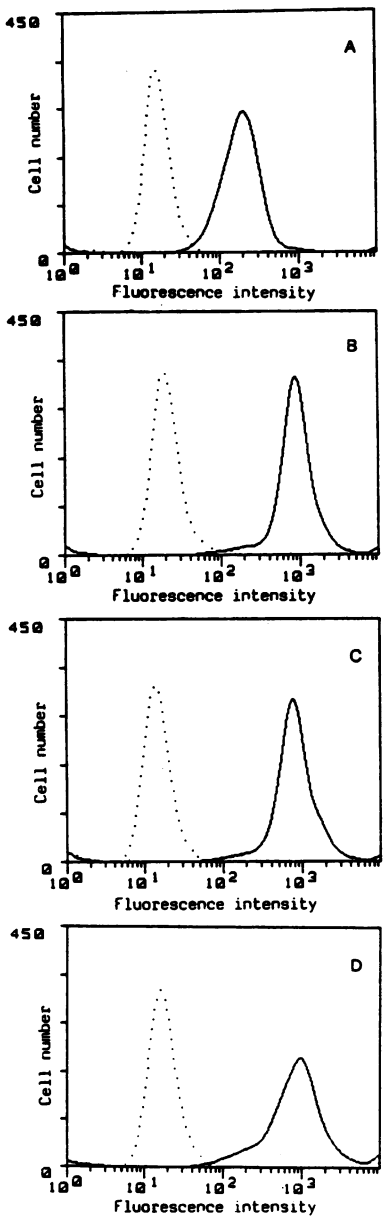

FIG. 7. Effect of STV on CR3 expression. Indirect immunofluorescence test with monoclonal antibody CD11b (solid lines) against CR3 on neutrophils revealed a mean fluorescence intensity of 180 for resting neutrophils (A) and 1,060 for STV-treated neutrophils (D). Both fMLP (B) and LTB4 (C) were used for comparison. Dotted lines indicate fluorescence background binding to control monoclonal antibody IgG2a.

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.

ACKNOWLEDGMENT

This work was supported by research grant NSC 81-0412-B-16-22 from the National Science Council, Taiwan, Republic of China.

REFERENCES

1. **Barak, L. S., R. R. Yocum, E. Nothnagel, and W. W. Webb.** 1980. Fluorescence staining of the actin cytoskeleton in living cells with 7-nitrobenz-2-oxa-1,3-diazole phallacidin. Proc. Natl. Acad. Sci. USA **77**:980–984.
2. **Blair, R. J., and P. F. Weller.** 1987. Uptake and esterization of arachidonic acid by trophozoites of *Giardia lamblia*. Mol. Biochem. Parasitol. **25**:11–18.
3. **Chang, J., J. H. Musser, and H. McGregor.** 1987. Phospholipase A2: function and pharmacologic regulation. Biochem. Pharmacol. **36**:2429–2436.
4. **Chikunguwo, S. M., P. R. Manson, and J. S. Read.** 1985. Investigations of *Trichomonas vaginalis* and chemotactic factor, p. 57. Abstr. Int. Symp. Trichomonads Trichomoniasis. Charles University, Prague, Czechoslovakia.
5. **Clancy, R. M., C. A. Dahinden, and T. E. Hugli.** 1983. Arachidonate metabolism by human polymorphonuclear leukocytes stimulated by *N*-formyl-Met-Leu-Phe or complement component C5a is independent of phospholipase activation. Proc. Natl. Acad. Sci. USA **80**:7200–7204.
6. **Clancy, R. M., and T. E. Hugli.** 1983. The extraction of leukotrienes (LTC4, LTD4, and LTE4) from tissue fluids: the metabolism of these mediators during IgE-dependent hypersensitivity reactions in lung. Anal. Biochem. **133**:30–39.
7. **Diamond, L. S., D. R. Harlow, and C. C. Cunnick.** 1978. A new medium for the axenic cultivation of *Entamoeba histolytica* and other entamoeba. Trans. R. Soc. Trop. Med. Hyg. **72**:431–432.
8. **Fitzharris, P., O. Cromwell, R. Moqbel, A. Hartnell, G. M. Walsh, C. Harvey, and A. B. Kay.** 1987. Leukotriene B4 generation by human neutrophils following IgG-dependent stimulation. Immunology **61**:449–455.
9. **Fouts, A. C., and S. J. Kraus.** 1980. *Trichomonas vaginalis*: reevaluation of its clinical presentation and laboratory diagnosis. J. Infect. Dis. **141**:137–143.
10. **Furlog, S. T.** 1991. Unique roles for lipids in *Schistosoma mansoni*. Parasitol. Today **7**:59–62.
11. **Fusco, A. C., B. Salafsky, and M. B. Kevin.** 1985. *Schistosoma mansoni*: eicosanoid production by cercariae. Exp. Parasitol. **59**:44–50.
12. **Goldman, D. W., and E. J. Goetzl.** 1982. Specific binding of leukotriene B4 to receptors on human polymorphonuclear leukocytes. J. Immunol. **129**:1600–1604.
13. **Goldman, D. W., and E. J. Goetzl.** 1984. Heterogeneity of human polymorphonuclear leukocyte receptors for leukotriene B4: identification of a subset of high affinity receptors that transduce the chemotactic response. J. Exp. Med. **159**:1027–1041.
14. **Hill, H. R., N. H. Augustine, J. A. Newton, A. O. Shigeoka, E. Morris, and F. Sacchi.** 1987. Correction of a developmental defect in neutrophil activation and movement. Am. J. Pathol. **128**:307–314.
15. **Hong, S. L.** 1988. The release of arachidonic acid from cellular lipids, p. 99–139. *In* L. Levine (ed.), Arachidonate metabolism in immunologic systems (Progress in Allergy, vol. 44). Karger, Basel.
16. **Honigberg, B. M.** 1990. Host cell-trichomonad interactions and virulence assays using *in vitro* systems, p. 155–212. *In* B. M. Honigberg (ed.), Trichomonads parasitic in humans. Springer-Verlag, New York.
17. **Howard, T. H., and W. H. Meyer.** 1984. Chemotactic peptide modulation of actin assembly and locomotion in neutrophils. J. Cell Biol. **98**:1265–1271.
18. **Klein, J.** 1990. Mediators and messengers, p. 246–268. *In* J. Klein (ed.), Immunology. Blackwell Scientific Publications, Boston.
19. **Korn, E. D.** 1982. Actin polymerization and its regulation by proteins from nonmuscle cells. Physiol. Rev. **62**:672–737.
20. **Krieger, J. N., P. Wolner-Hanssen, C. Stevens, and K. K. Holmes.** 1990. Characteristics of *Trichomonas vaginalis* isolates from women with and without colpitis macularis. J. Infect. Dis. **161**:307–311.
21. **Liu, L. X., and P. F. Weller.** 1990. Arachidonic acid metabolism in filarial parasites. Exp. Parasitol. **71**:496–501.

22. **Manson, P. R., and L. Forman.** 1980. *In vitro* attraction of polymorphonuclear leucocytes by *Trichomonas vaginalis*. J. Parasitol. **66:**888–892.
23. **Marks, P. W., and F. R. Maxfield.** 1990. Transient increases in cytosolic free calcium appear to be required for the migration of adherent human neutrophils. J. Cell Biol. **110:**43–52.
24. **McColl, S. R., N. P. Hurst, and L. G. Cleland.** 1986. Modulation by phorbol myristate acetate of arachidonic acid release and leukotriene synthesis by human polymorphonuclear leukocytes stimulated with A23187. Biochem. Biophys. Res. Commun. **141:**399–404.
25. **Nagy, L., T. H. Lee, E. J. Goetzl, W. C. Pickett, and A. B. Kay.** 1982. Complement receptor enhancement and chemotaxis of human neutrophils and eosinophils by leukotrienes and other lipoxygenase products. Clin. Exp. Immunol. **47:**541–547.
26. **Packman, C. H., and M. A. Lichtman.** 1990. Activation of neutrophils: measurement of actin conformational changes by flow cytometry. Blood Cells **16:**193–205.
27. **Palmblad, J., H. Gyllenhammar, B. Ringertz, E. Nilsson, and B. Cottell.** 1988. Leukotriene B4 triggers highly characteristic and specific functional responses in neutrophils: studies of stimulus specific mechanisms. Biochim. Biophys. Acta **970:**92–102.
28. **Palmblad, J., C. L. Malmsten, A.-M. Udén, O. Rådmark, L. Engstedt, and B. Samuelsson.** 1981. Leukotriene B4 is a potent and stereospecific stimulator of neutrophil chemotaxis and adherence. Blood **58:**658–661.
29. **Palmer, R. M. J., and J. A. Salmon.** 1983. Release of leukotriene B4 from human neutrophils and its relationship to degranulation induced by *N*-formyl-methionyl-leucyl-phenylalanine, serum-treated zymosan and the ionophore A23187. Immunology **50:** 65–73.
30. **Philip, A., P. Carter-Scott, and C. Rogers.** 1987. An agar culture technique to quantitate *Trichomonas vaginalis* from women. J. Infect. Dis. **155:**304–308.
31. **Psychoyos, S., S. Uziel-Fusi, M. M. Morrissey, V. Ganu, and C. W. Smith.** 1991. Thick filter neutrophil chemotaxis performed in the absence of albumin. J. Immunol. Methods **137:** 37–46.
32. **Rao, K. M. K., and J. Varani.** 1982. Actin polymerization induced by chemotactic peptides and concanavalin A in rat neutrophils. J. Immunol. **129:**1605–1607.
33. **Rein, M. F., J. A. Sullivan, and G. L. Mandell.** 1980. Trichomonacidal activity of human polymorphonuclear neutrophils: killing by disruption and fragmentation. J. Infect. Dis. **142:**575–585.
34. **Salafsky, B., and A. C. Fusco.** 1985. *Schistosoma mansoni*: cercarial eicosanoid production and penetration response inhibited by esculetin and ibuprofen. Exp. Parasitol. **60:**73–81.
35. **Samuelsson, B.** 1983. Leukotrienes: mediators of immediate hypersensitivity reactions and inflammation. Science **220:**568–575.
36. **Shaio, M. F., F. Y. Chang, S. C. Hou, C. S. Lee, and P. R. Lin.** 1991. The role of immunoglobulin and complement in enhancing the respiratory burst of neutrophils against *Trichomonas vaginalis*. Parasite Immunol. **13:**241–250.
37. **Shepherd, J. C., A. Aitken, and D. P. McManus.** 1991. A protein secreted in vivo by *Echinococcus granulosus* inhibits elastase activity and neutrophil chemotaxis. Mol. Biochem. Parasitol. **44:**81–90.
38. **Wallace, P. J., R. P. Wersto, C. H. Packman, and M. A. Lichtman.** 1984. Chemotactic peptide-induced changes in neutrophil actin conformation. J. Cell Biol. **99:**1060–1065.
39. **Walsh, C. E., L. R. Dechatelet, F. H. Chilton, R. L. Wykle, and M. Waite.** 1983. Mechanism of arachidonic acid release in human polymorphonuclear leukocytes. Biochim. Biophys. Acta **750:**32–40.