

Production of Interleukin-8 by Human Neutrophils Stimulated with *Trichomonas vaginalis*

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Neutrophils are the predominant inflammatory cells found in the vaginal discharges of patients infected with *Trichomonas vaginalis*. Although chemoattractants, such as leukotriene B₄ and interleukin-8 (IL-8), are found in the vaginal discharges of symptomatic trichomoniasis patients, little is known about the mechanism of how neutrophils accumulate or mediate initial inflammatory response after acute *T. vaginalis* infection. We examined IL-8 production in neutrophils activated by *T. vaginalis* and evaluated the factors involved in *T. vaginalis* adherence that might affect IL-8 production. When human neutrophils were stimulated with live trophozoites, *T. vaginalis* lysate, or *T. vaginalis* excretory-secretory products, the live trichomonads induced higher levels of IL-8 production than the lysate or products did. When live trichomonads were pretreated with various inhibitors of proteinase, microtubule, microfilament, or adhesin (which are all known to participate in the adherence of *T. vaginalis* to vaginal epithelial cells), IL-8 production significantly decreased compared with the untreated controls. Furthermore, an NF- κ B inhibitor (pyrrolidine dithiocarbamate), a mitogen-activated protein (MAP) kinase (MEK) inhibitor (PD98059), and a p38 MAP kinase inhibitor (SB203580) significantly suppressed IL-8 synthesis in neutrophils. These results suggest that live *T. vaginalis*, particularly adherent trophozoites, can induce IL-8 production in neutrophils and that this action may be mediated through the NF- κ B and MAP kinase signaling pathways. In other words, *T. vaginalis*-induced neutrophil recruitment may be mediated via the IL-8 expressed by neutrophils in response to activation by live *T. vaginalis*.

Trichomonas vaginalis commonly causes vaginitis and perhaps cervicitis in women as well as urethritis in both sexes (9). In pregnant women, trichomonads are implicated in the premature rupture of membranes, premature delivery, and the delivery of low-birth-weight infants (24, 34). In addition, trichomoniasis has been implicated as a risk factor of human immunodeficiency virus transmission (18). More than 180 million people worldwide are annually infected by this parasite (13), and the prevalence rate was recently found to be 10.4% in the area of Kuri, Korea (28).

Although neutrophil infiltration has been considered to be primarily responsible for cytological change (9, 10), the pathogenesis of *Trichomonas vaginalis* infection has not yet been clearly characterized. Moreover, little is known about the exact mechanism of how neutrophils accumulate or mediate the initial inflammatory response after acute *T. vaginalis* infection. Nevertheless, it is generally believed that chemoattractants generated at reaction sites may have an important role. Chemoattractants reported to be possibly involved in the inflammatory response of *T. vaginalis* include leukotriene B₄ and interleukin-8 (IL-8), both of which are found in the vaginal discharges of symptomatic trichomoniasis patients (31, 32).

IL-8 is the best characterized member of the α -chemokine or CXC subfamily. IL-8 acts primarily on polymorphonuclear cells (PMNs) but also has potent chemotactic and stimulatory

effects on T cells, basophils, and eosinophils. Upon exposure to inflammatory stimuli, such as lipopolysaccharide, IL-1, or tumor necrosis factor, IL-8 is released by a wide variety of cell types, including monocytes/macrophages, neutrophils, T lymphocytes, fibroblasts, endothelial cells, and epithelial cells (19). Human monocytes are known to produce IL-8 after stimulation with *T. vaginalis* (33); however, to date, no reports have described IL-8 production by neutrophils after stimulation with *T. vaginalis*. In this study, to elucidate the mechanisms of neutrophil infiltration in *T. vaginalis* infection, we examined IL-8 production in neutrophils activated by *T. vaginalis* and evaluated factors involved in adherence of *T. vaginalis* which subsequently might affect IL-8 production. Furthermore, the involvement of nuclear factor NF- κ B and mitogen-activated protein (MAP) kinase signaling pathways in the IL-8 production by neutrophils was also investigated.

MATERIALS AND METHODS

Reagents. Leupeptin (acetyl-Leu-Leu-Arg-al), TLCK (*N* α -*p*-tosyl-L-lysine chloromethyl ketone), TPCK (*N*-tosyl-L-phenylethyl chloromethyl ketone), EDTA, colchicine, cytochalasin D, cycloheximide, PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid)], and Histopaque 1077 were purchased from Sigma (St. Louis, Mo.). E-64 {*N*-[*N*-(1-3-transcarboxyoxirane-2-carbonyl)-L-leucyl]-agmatine} was from Boehringer (Mannheim, Germany). Pyrrolidone dithiocarbamate (PDTC), PD98059, and SB203580 were from Calbiochem (Nottingham, United Kingdom), dextran T500 was from Pharmacia (Uppsala, Sweden), and fetal bovine serum and Trizol reagent were from Gibco BRL (Gaithersburg, Md.).

***T. vaginalis* culture and antigen preparation.** The *T. vaginalis* isolate used in this study, KT4, was isolated from a Korean female with acute vaginitis (29). Trichomonads were grown in a complex Trypticase-yeast extract-maltose (TYM) medium supplemented with 10% heat-inactivated horse serum (7). *T. vaginalis*

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lysates were prepared by harvesting at the log phase of *T. vaginalis* growth, sonicating in 0.1 M phosphate-buffered saline (PBS), and centrifuging at $3,500 \times g$ for 1 h at 4°C. Excretory-secretory products (ESP) were obtained by suspending trophozoites (10^7 /ml) in RPMI 1640 medium, culturing them at 37°C for 1 h and then centrifuging at $10,000 \times g$ for 30 min. The resulting supernatants were passed through a 0.22- μ m-pore-size filter. In some experiments, to determine the involvement of virulence of *T. vaginalis* on IL-8 production, we used low-virulence strains, such as KT9, KT-Kim, KT-12, and CDC85, whose virulence levels had previously been determined (29).

Isolation of human neutrophils. Fresh human blood samples were drawn from healthy donors and treated with heparin, and neutrophils were isolated from the blood samples by a method previously described (11) with minor modifications. Briefly, 10 volumes of blood were mixed with 2 volumes of dextran {4.5% dextran T500 suspended in PIPES buffer (25 mM PIPES, 50 mM NaCl, 5 mM KCl, 25 mM NaOH, 5.4 mM glucose [pH 7.4])}, and neutrophils were obtained by layering on Ficoll-Hypaque (Histopaque 1077) after 30-min sedimentation at 37°C. After centrifugation at $385 \times g$ for 30 min at 4°C, the supernatant and mononuclear cells at the interface were carefully removed. The wall inside the centrifuge tube was wiped twice with sterile gauze to remove adhering mononuclear cells. Erythrocytes in the sediment were lysed twice with sterile distilled water. Cell viability was determined by using the trypan blue exclusion test (>99%). The purity of neutrophils was confirmed morphologically (>98%), and monocytic contamination was examined by phenotypic analysis using flow cytometry (Becton Dickinson, San Jose, Calif.) after staining with fluorescein isothiocyanate (FITC)-conjugated anti-CD14 antibody (<0.02%).

Culture conditions of neutrophils. Freshly isolated neutrophils were suspended and cultured in RPMI 1640 medium supplemented with 10 mM HEPES and 10% fetal bovine serum. After the neutrophils (2×10^5) were cocultured with live trophozoites at various neutrophil/trichomonad ratios (20:1 to 5:1) for 1 to 24 h, IL-8 protein expression was determined, and the optimal neutrophil/*T. vaginalis* ratio for enhanced expression of IL-8 was found to be 5:1 to 10:1. In the subsequent experiments, therefore, 2×10^5 neutrophils were stimulated with 2×10^4 trichomonads. Also, neutrophils were stimulated with trichomonad lysate (200 and 400 μ g/ml) or ESP (25 and 50 μ l) for 3 to 24 h at 37°C. After incubation, cell-free culture supernatants were collected and stored at -20°C until the IL-8 enzyme-linked immunosorbent assay (ELISA) was performed.

To determine whether the adherence of *T. vaginalis* to neutrophils is critical for induction of IL-8 production by neutrophils, we used the 24-well Transwell insert system (Costar, Cambridge, Mass.). Transwell inserts with a porous bottom (pore size, 3 μ m) serve as the upper chamber, and ordinary tissue culture plate wells serve as the lower chamber. Medium containing trophozoites (2×10^6) was placed in the upper chamber, neutrophil suspension (2×10^5) was added to the lower chamber, and the plates were then incubated for 24 h. After 24 h of incubation, culture supernatants from the lower chamber were collected for IL-8 measurement.

Competitive RT-PCR for IL-8 mRNA expression in neutrophils. After neutrophils (10^7) were stimulated with *T. vaginalis* (10^6) at 37°C for 1 to 4 h in a propylene tube to prevent adherence, total neutrophil RNA was extracted by the guanidium thiocyanate-phenol-chloroform method described previously (4). The total RNA was pelleted, washed in 75% ethanol, dried, resuspended in diethyl pyrocarbonate-treated water, and quantitated spectrophotometrically. After reverse transcription (RT), cDNA was amplified using IL-8-specific primers (12) (5'-ATG ACT TCC AAG CTG GCC GTG GCT-3' and 5'-TCT CAG CCC TCT TCA AAA ACT TCT C-3') in a thermal cycler (PTC-100; MJ Research Inc., Watertown, Mass.) under the following amplification conditions: 35 cycles, with 1 cycle consisting of denaturation (45 s at 95°C) and annealing and extension (2.5 min at 56°C). The PCR products were electrophoresed, visualized by ethidium bromide staining, and quantitated by the method previously described (14). Briefly, 1 μ g of RNA extracted from neutrophils was reverse transcribed with various synthetic standard RNA transcripts (1×10^5 to 5×10^8 molecules/ μ g) in the reaction tube. (The synthetic standard RNA was kindly provided by M. F. Kagnoff at the University of California, San Diego.) After cDNA PCR, the PCR products obtained were electrophoresed, and the intensities of their bands were quantified by imaging densitometry (GS-670; Bio-Rad, Hercules, Calif.). The ratios of the band intensities were then obtained from standard and target RNA and plotted against the starting number of standard RNA molecules; i.e., when the ratio of band intensities was 1, the number of target RNA molecules was equivalent to the number of standard RNA molecules.

Effects of proteinase, microtubule, microfilament, and adhesin synthesis inhibitors on the production of IL-8. Previous studies on the specificity of the adherence of *T. vaginalis* to vaginal epithelial cells (VECs) demonstrated that adherence is a multifactorial process, in which microtubules, microfilaments, four adhesins, and cysteine proteinases participate (1, 17, 21). In order to inves-

tigate whether cytoadherence of live trichomonads to neutrophils is critical for IL-8 production, live *T. vaginalis* trophozoites were pretreated with proteinase inhibitors for 30 min at 37°C as described previously (23), washed three times, and then allowed to react with isolated neutrophils. The proteinase inhibitors included cysteine proteinase inhibitors, such as E-64 (500 μ g/ml); serine and cysteine inhibitors, such as leupeptin (1.5 mM), TLCK (1 mM), and TPCK (1 mM); and a metalloproteinase inhibitor, EDTA (1 mM). These reagents were prepared in appropriate stock solutions, and TLCK and TPCK were dissolved in methanol and ethanol, respectively. In addition, live *T. vaginalis* trophozoites were also preincubated with colchicine (5 μ M) and cytochalasin D (5 μ g/ml in dimethyl sulfoxide [DMSO]) for 30 min at 37°C, which are microtubule and microfilament inhibitors, respectively (17). To inhibit adhesin synthesis, *T. vaginalis* were incubated with cycloheximide (20 μ g/ml) for 2 h at 37°C (2). After preincubation, the trichomonads were gently washed three times with culture medium before incubation with neutrophils. The pretreated *T. vaginalis* (2×10^4) were then cocultured for 24 h with neutrophils (2×10^5).

Pretreatment of neutrophils with NF- κ B inhibitor or MAP kinase inhibitors. To determine the involvement of the NF- κ B and MAP kinase signaling pathways in *T. vaginalis*-induced IL-8 production by neutrophils, the neutrophils were pretreated with a NF- κ B inhibitor, PDTC (25, 50, and 100 μ M), a MAP kinase (MEK) inhibitor, PD98059 (50 and 100 μ M), and a p38 MAP kinase inhibitor, SB203580 (25, 50, and 100 μ M) for 1 h (15, 19), and the neutrophils were then cocultured for 24 h with live *T. vaginalis*. After incubation, IL-8 levels in culture supernatants were measured by ELISAs. PDTC was dissolved in PBS, and PD98059 and SB203580 were dissolved in 0.1% DMSO.

IL-8 ELISA. Commercial kits were used to quantify IL-8 (Endogen, Woburn, Mass.) and CXCL chemokine GRO- α (R&D Systems, Minneapolis, Minn.), according to the manufacturer's recommendations. The minimum detectable concentrations of IL-8 and GRO- α were 2.0 ng/ml. The specificity of the assay was confirmed by the manufacturer.

Scanning electron microscopy. Scanning electron microscopy was used to observe the morphological changes of neutrophils after stimulation with *T. vaginalis*. Thus, neutrophils (2×10^6) and *T. vaginalis* (2×10^5) were cocultured for 24 h at 37°C and then fixed in 3% glutaraldehyde in 0.13 M Millonig's phosphate buffer (0.13 M NaH₂PO₄, 0.1 M NaOH [pH 7.3]) at 4°C. They were then washed three times with PBS, allowed to adhere to poly-L-lysine-coated glass coverslips, postfixed for 1.5 h at room temperature in 1% OsO₄ in Millonig's phosphate buffer, dehydrated in ethanol, dried in a critical point dryer (HCP-2; Hitachi, Hitachinaka, Japan), and mounted on stubs. The neutrophils were then coated with gold using an ion sputtering coater (E-1010; Hitachi) and observed with a scanning electron microscope (S-2380N; Hitachi) at an accelerating voltage of 25 kV (16).

Statistical analysis. The results are expressed as means \pm standard errors of the means (SEMs) of three to five independent experiments. The Mann-Whitney U test was used for statistical analysis, and a *P* value of <0.05 was considered statistically significant.

RESULTS

Expression of IL-8 mRNA and protein by neutrophils. To investigate whether *T. vaginalis* can induce IL-8 production of neutrophils, we first examined IL-8 mRNA expression by neutrophils stimulated with live trophozoites of *T. vaginalis*. RT-PCR for IL-8 in freshly isolated neutrophils revealed constitutive mRNA expression. As shown in Table 1, quantitative analysis of mRNA using synthetic standard RNA demonstrated that the number of IL-8 mRNA transcripts expressed in neutrophils stimulated with live *T. vaginalis* was 50 times higher than the number expressed by the control neutrophils (incubated with medium alone) when it peaked 2 h after stimulation. In contrast, the β -actin mRNA levels remained relatively constant in each experiment.

To confirm whether IL-8 mRNA expression closely reflected its protein release, we measured the IL-8 protein level in culture supernatants of neutrophils. As shown in Fig. 1, the amount of IL-8 protein released from neutrophils that had been stimulated with live *T. vaginalis* was dependent on the number of live trichomonads used. When 2×10^5 neutrophils

TABLE 1. Quantification of IL-8 mRNA in neutrophils stimulated with live *T. vaginalis*^a

Incubation time (h)	No. of IL-8 transcripts/ μ g of cellular RNA in neutrophils		Ratio of IL-8 transcripts (stimulated/not stimulated)
	Not stimulated	Stimulated	
1	7×10^5	2×10^7	28.6
2	5×10^5	2.5×10^7	50.0
3	5×10^5	5×10^6	10.0
4	7.5×10^5	2×10^6	2.7

^a Neutrophils (10^7 /well) were stimulated with *T. vaginalis* (10^6 /well) in six-well plates. Total RNA was extracted at the indicated times by the guanidium thiocyanate-phenol-chloroform method, and mRNA levels for IL-8 were determined by competitive RT-PCR with synthetic standard RNA (1×10^5 to 5×10^8 molecules/ μ g). The results from a representative experiment are shown; three replicate experiments were performed.

were stimulated with 1×10^4 trichomonads for up to 24 h, the amount of IL-8 protein produced was similar to that of cells incubated with medium alone. However, when 2×10^4 or 4×10^4 trichomonads were incubated with the same number of neutrophils, IL-8 production by neutrophils was potently induced. The stimulatory effects of *T. vaginalis* on IL-8 production became obvious 2 h after stimulation and were particularly strong between 6 and 24 h after stimulation (Fig. 1). In contrast, the amount of IL-8 protein in the presence of trichomonal lysate (200 and 400 μ g/ml) and ESP (25 and 50 μ l) was less than 1,000 pg/ml, which was markedly lower than that with live *T. vaginalis* (Fig. 2).

We also measured GRO- α production under the same conditions as those used for IL-8 induction. When 2×10^5 neutrophils were stimulated with 2×10^4 trichomonads for up to 24 h, the amount of GRO- α produced by neutrophils was 723.3 ± 65.06 pg/ml compared with 32.5 ± 0.43 pg/ml without stimulation (data not shown).

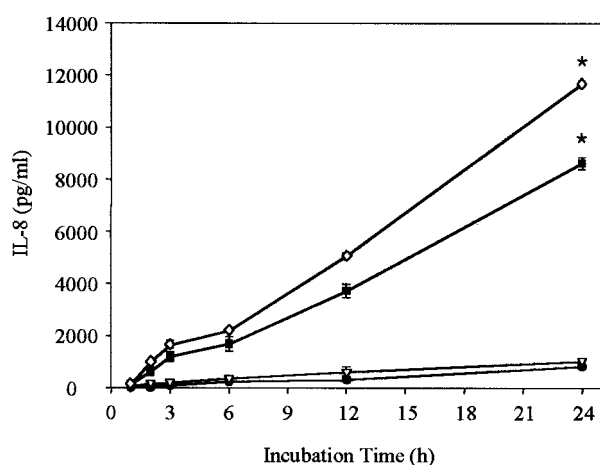


FIG. 1. IL-8 production by human neutrophils treated with live *T. vaginalis*. Neutrophils (2×10^5 /well) were stimulated with 1×10^4 (▽), 2×10^4 (■), and 4×10^4 (◇) *T. vaginalis* or medium alone (●) for 1 to 24 h at 37°C. Culture supernatants were collected at the indicated times, and secreted IL-8 was measured by an ELISA. Data are the means \pm SEMs (error bars) of three replicate experiments. Values that are significantly different ($P < 0.05$) from the control value (medium alone) are indicated by an asterisk.

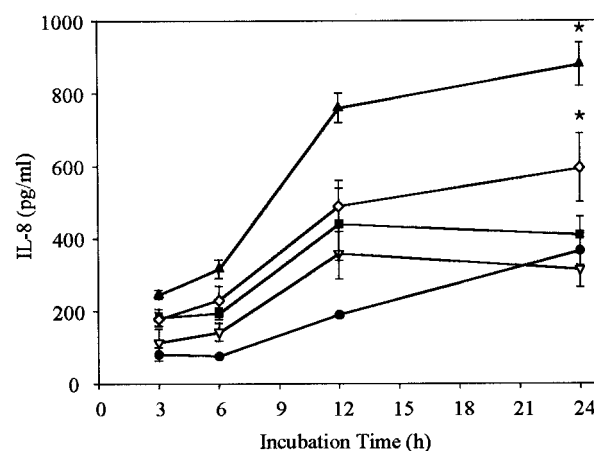


FIG. 2. IL-8 production by neutrophils treated with *T. vaginalis* lysate and ESP. Neutrophils (2×10^5 /well) were incubated with *T. vaginalis* lysate (400 [▲] or 200 [◇] μ g/ml), ESP (50 [■] or 25 [▽] μ l/well), or medium alone (●) for 3 to 24 h at 37°C. Culture supernatants were collected at the indicated times, and secreted IL-8 was measured by an ELISA. Data are the means \pm SEMs (error bars) of three replicate experiments. Values that are significantly different ($P < 0.05$) from the control value (medium alone) are indicated by an asterisk.

As shown in Fig. 3, the amounts of IL-8 protein produced by neutrophils treated with five isolates of *T. vaginalis*, including a highly virulent isolate, KT4, and trichomonads of low virulence, such as KT9, KT-Kim, KT-12, and CDC85, were similar, indicating that IL-8 production was not related to the virulence of the trichomonad.

Effects of proteinase inhibitors on IL-8 production. Previous studies on the specificity of the adherence of *T. vaginalis* to VECs demonstrated that adherence is a multifactorial process, in which microtubules, microfilaments, four adhesins, and cysteine proteinases participate (1, 17, 21). In support of these observations, *T. vaginalis* pretreated with proteinase inhibitors produced much lower levels of IL-8 protein than trichomonads that were not pretreated. The cysteine proteinase inhibitor E-64 (500 μ g/ml) and cysteine and serine proteinase inhibitors,

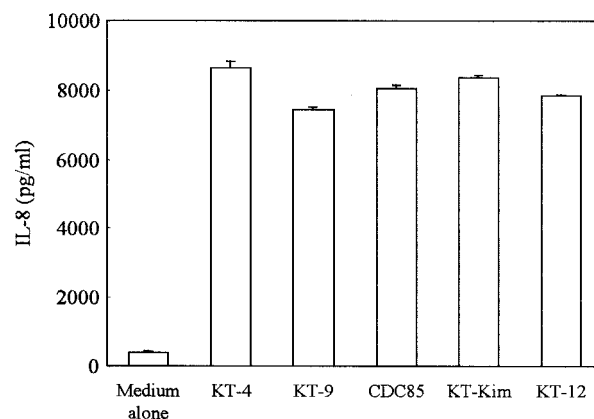


FIG. 3. IL-8 production by human neutrophils treated with five isolates of *T. vaginalis*. Neutrophils (2×10^5 /well) were stimulated with live trophozoites (2×10^4) for 24 h at 37°C.

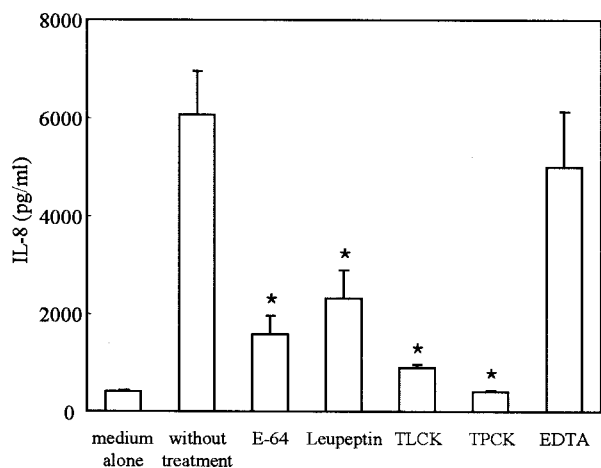


FIG. 4. IL-8 production by human neutrophils cocultured with *T. vaginalis* that had been pretreated with proteinase inhibitors. *T. vaginalis* organisms were preincubated for 30 min at 37°C with 500 µg of E-64 per ml, 1.5 mM leupeptin, 1 mM TLCK, 1 mM TPCK, and 1 mM EDTA. For a control, neutrophils were similarly preincubated but without inhibitor. Preincubated *T. vaginalis* organisms were washed three times and allowed to react with human neutrophils for 24 h at 37°C. Culture supernatants were collected after 24 h of incubation, and IL-8 secreted was measured by an ELISA. Data are the means \pm SEMs (error bars) of three replicate experiments. Values that are significantly different ($P < 0.05$) from the control value (not pretreated with a proteinase inhibitor) are indicated by an asterisk.

such as leupeptin (1.5 mM), TLCK (1 mM), and TPCK (1 mM), significantly reduced IL-8 production by neutrophils. In contrast, the metalloproteinase inhibitor EDTA (1 mM) did not affect IL-8 production (Fig. 4).

Effects of microtubule and microfilament inhibitors on IL-8 production. When colchicine (5 µM), a microtubule inhibitor, and cytochalasin D (5 µg/ml), a microfilament inhibitor, were added to a live *T. vaginalis* suspension, the neutrophils secreted significantly reduced amounts of IL-8 (Fig. 5). Lower levels of IL-8 were produced by neutrophils treated with cycloheximide (20 µg/ml), an adhesin synthesis inhibitor, than in control neutrophils (Fig. 5).

To determine whether the adherence of *T. vaginalis* to neutrophils is critical for induction of IL-8 production by neutrophils, we used the 24-well Transwell insert system. When suspensions containing trophozoites and neutrophils were placed in the upper and lower wells, respectively, and incubated for 24 h, the IL-8 produced by neutrophils in the lower well was 709.4 pg/ml (Fig. 5).

IL-8 protein levels after pretreatment with specific inhibitors of NF-κB and MAP kinase. To investigate the involvement of the NF-κB and MAP kinase signaling pathways in *T. vaginalis*-induced IL-8 production of neutrophils, we investigated the effect of pretreatment of neutrophils with PDTC, PD98059, or SB203580 on IL-8 production. As shown in Fig. 6, pretreatment of neutrophils with PDTC, PD98059, or SB203580 significantly suppressed IL-8 production induced by live *T. vaginalis*.

Scanning electron microscopy. The surfaces of normal neutrophils have a few microvilli (small spherical extensions of cell membrane, some on a short process or stalk) (Fig. 7B). Normal

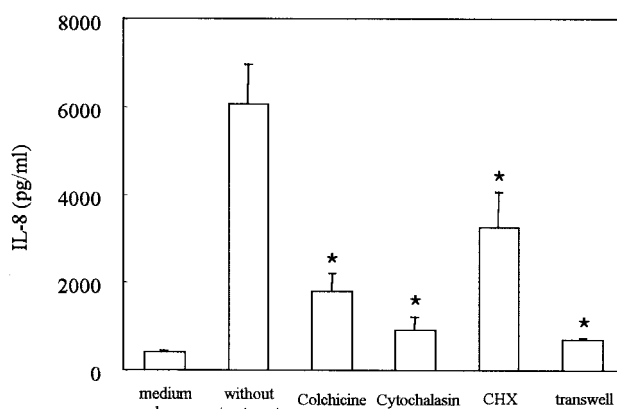


FIG. 5. IL-8 production by human neutrophils cocultured with *T. vaginalis* that had been pretreated with microtubule, microfilament, and adhesin synthesis inhibitors. *T. vaginalis* was preincubated with colchicine (5 µM) or cytochalasin D (5 µg/ml) for 30 min at 37°C. *T. vaginalis* was incubated with cycloheximide (CHX) (20 µg/ml) for 2 h at 37°C. Preincubated *T. vaginalis* was washed three times and allowed to react with human neutrophils for 24 h at 37°C. The Transwell chamber was also used to prevent adhesion. The suspensions containing trophozoites and neutrophils were placed in the upper and lower wells, respectively, and incubated for 24 h, and the IL-8 production by neutrophils in the lower well was measured. Data are the means \pm SEMs (error bars) of three replicate experiments. Values that are significantly different ($P < 0.05$) from the control value (not pretreated with a proteinase inhibitor) are indicated by an asterisk.

T. vaginalis have flagella. Each trophozoite contacts other trichomonads through flagella (Fig. 7A). When neutrophils and *T. vaginalis* were cocultured at a neutrophil/trophozoite ratio of 10:1, several activated neutrophils surrounded one trophozoite, and many filopodia extended toward the trichomonad. The surfaces of the stimulated neutrophils were completely covered by the plasma membrane, elaborating into irregular ridges or small ruffles (Fig. 7C).

DISCUSSION

Neutrophils are known to play an important role in inflammatory responses by virtue of their ability to perform a series of effector functions that collectively represent a major mechanism of innate immunity against injury or infection. In recent years, however, it has become obvious that the contribution made by neutrophils to host defense and natural immunity extends well beyond their traditional role as professional phagocytes (30).

A few studies have been conducted on the cellular response of neutrophils to *T. vaginalis* infection. Groups of polymorphonuclear leukocytes surrounding individual large trichomonads are able to fragment the trophozoite and phagocytose the pieces (27). *T. vaginalis* trophozoites that interacted with neutrophils secrete proteins that are chemotactic to PMNs (10, 20, 26).

IL-8, a cytokine with potential proinflammatory effects, has chemoattractant activity and is able to activate and degranulate neutrophils. In vivo, IL-8 is an important regulator of neutrophil activation and migration (11). IL-8 maintains its biological activity under significant pH changes and resists mild proteo-

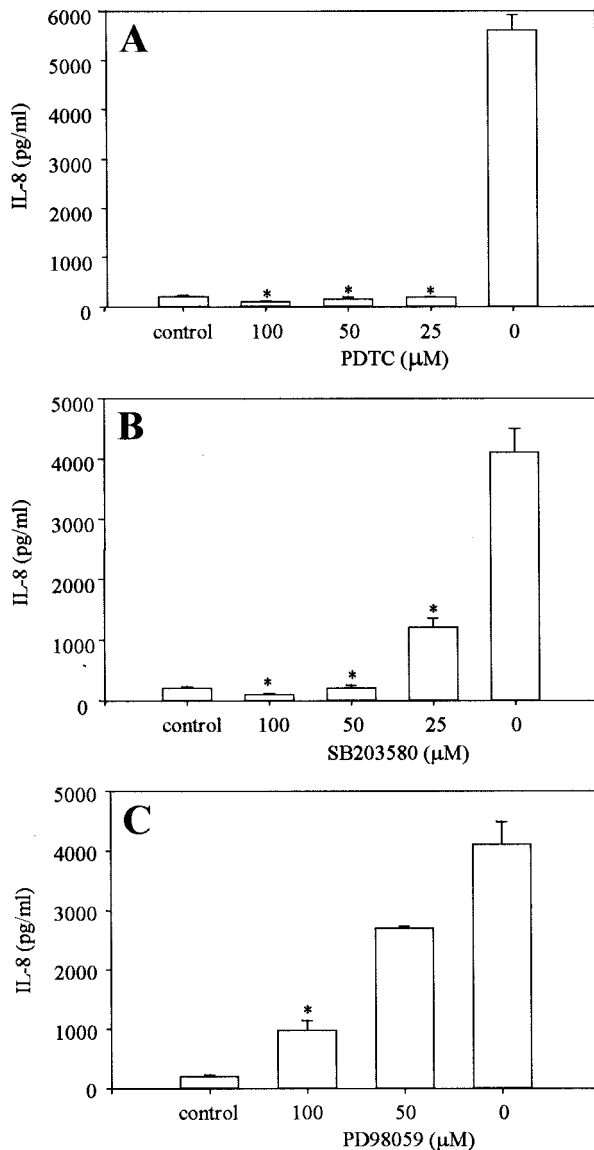


FIG. 6. Effects of NF- κ B and MAP kinase pathway-specific inhibitors on IL-8 production by human neutrophils. Neutrophils were pretreated for 1 h at 37°C with NF- κ B inhibitor PDTC (A), p38 MAP kinase inhibitor SB203580 (B), and MAP kinase inhibitor PD98059 (C). DMSO (1%) was used as the solvent control. Pretreated neutrophils (2×10^5) were incubated with *T. vaginalis* (2×10^4) for 24 h at 37°C, and IL-8 secreted was measured by an ELISA. Data are the means \pm SEMs (error bars) of three replicate experiments. Values that are significantly different ($P < 0.05$) from the control value (no inhibitor) are indicated by an asterisk.

lytic degradation compared with other known chemotactic factors (37). This suggests that the production of IL-8 at in vivo sites of acute inflammation may have a prolonged biological effect upon the recruitment of neutrophils (36).

Little is known about IL-8 production by neutrophils stimulated with *T. vaginalis*, although Shaio et al. (33) demonstrated that membrane components of *T. vaginalis* induce IL-8 production by monocytes. This was the first study to report the ability of *T. vaginalis* to induce IL-8 secretion by neutrophils. In

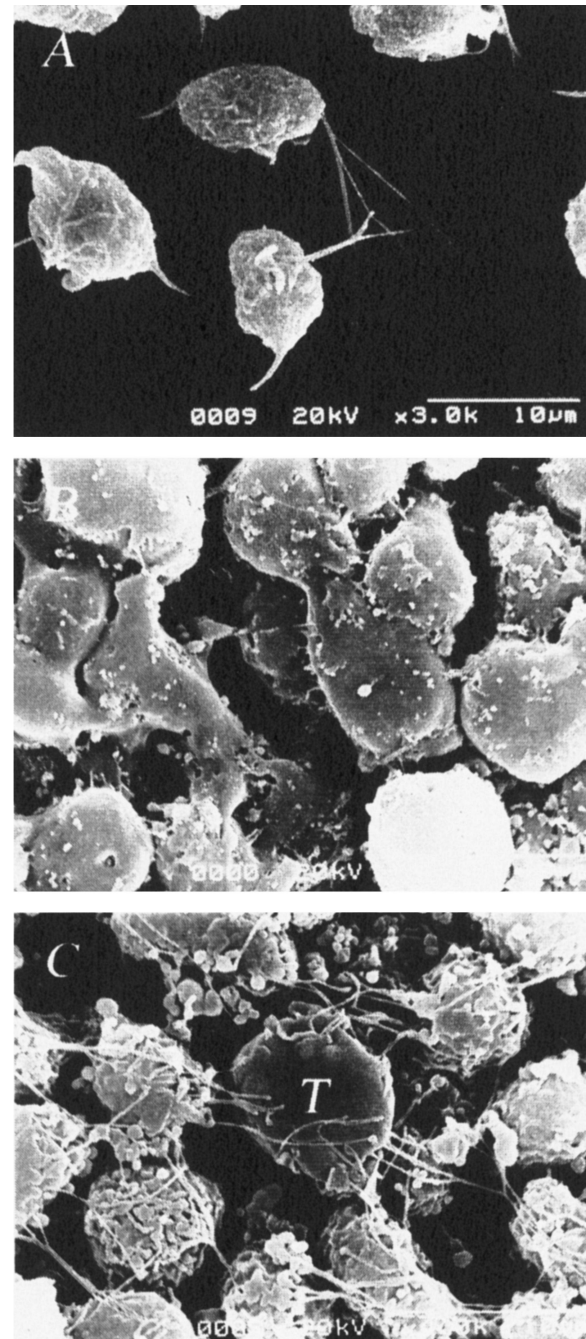


FIG. 7. Scanning electron micrographs of neutrophils. Neutrophils (2×10^6) and *T. vaginalis* (2×10^5) were coincubated for 24 h at 37°C. (A) Normal *T. vaginalis* had flagella, and each trophozoite contacted other trichomonads with flagella. (B) The few microvilli present were small spherical extensions of the cell membrane; some were on short processes or stalks. (C) When the neutrophils were incubated with *T. vaginalis* at a ratio of 10:1, several activated neutrophils surrounded one trophozoite, and many filopodia extended toward the trichomonad (T). The plasma membrane of the stimulated neutrophils contained irregular ridges and small ruffles, in contrast to the smooth plasma membrane of normal neutrophils.

the present study, IL-8 produced by stimulation with live *T. vaginalis* was found to be much higher than the amount produced by stimulation with ESP or *T. vaginalis* lysate. These results were in agreement with the results of *Toxoplasma gondii* infection of fibroblasts, in which intact, viable tachyzoites are a primary factor in inducing increased IL-8 production (6). In contrast, the membrane component of *T. vaginalis* induced much larger amounts of IL-8 produced by human monocytes than live trichomonads or ESP (33). It is quite likely that *T. vaginalis* stimulates different cellular types, which involve distinct molecular triggers.

A certain number of live trichomonads is required for IL-8 production by neutrophils, because we found that small numbers of trichomonads (1×10^4) did not induce IL-8 production, and the optimal ratio of neutrophils (2×10^5) to trichomonads (2×10^4 to 4×10^4) was 10:1 to 5:1 in this study.

To determine the effect of the virulence of *T. vaginalis* on IL-8 production, we used five isolates of *T. vaginalis*, including a highly virulent isolate, KT4, and trichomonads of low virulence, such as KT9, KT-Kim, KT-12, and CDC85, whose virulence levels had been previously determined (29). The results of the present study showed that there was no significant difference in IL-8 production by neutrophils caused by the virulence of the *T. vaginalis* isolate used. This finding was in agreement with that found for the IL-8 production of monocytes stimulated with *T. vaginalis* (three symptomatic patients and two asymptomatic patients) (31). However, the vaginal discharges of symptomatic trichomoniasis patients had larger amounts of IL-8 than those of asymptomatic patients (32). This difference might have been due to the differences between in vivo and in vitro cultivation.

Previous studies on the specificity of the adherence of *T. vaginalis* to VECs demonstrated that adherence is a multifactorial process, in which microtubules, microfilaments, four adhesins, and cysteine proteinases participate (1, 17, 21), and evidence in the present study indicated that these factors were required for the induction of IL-8 production by neutrophils, because various proteinase, microtubule, microfilament, and adhesin inhibitors significantly reduced IL-8 production. Also, we investigated the importance of contact between neutrophils and *T. vaginalis* for IL-8 production by using Transwell chambers (Costar). When the Transwell chamber was used to prevent adhesion, the amount of IL-8 (709.4 pg/ml) was similar to that of untreated control neutrophils (415.5 pg/ml). These results indicate that adherence or contact between neutrophils and *T. vaginalis* is essential for IL-8 production. Adherence was seen in vaginal smears; trichomonads were found fused with polymorphonuclear leukocytes from 17 of 20 trichomoniasis patients (5). However, further studies are necessary to elucidate the mechanism of contact, including the molecules involved in contact between neutrophils and *T. vaginalis* and the molecules involved in IL-8 production.

To examine the morphological change in neutrophils after *T. vaginalis* stimulation, we incubated neutrophils with trophozoites at a ratio of 10:1 for 24 h under the same conditions used for IL-8 induction. We observed many filopodia and also saw ridges and ruffles on the surfaces of neutrophils with the scanning electron microscope. This activation and the morphological changes of neutrophils are very similar to the morphological changes of neutrophils observed when neutrophils and

Naegleria fowleri were cocultured following the addition of tumor necrosis factor (22).

Since NF- κ B plays a central role in regulating the transcription of cytokines, adhesion molecules, and other mediators involved in acute inflammatory response and MAP kinase contributes to complex regulatory events, such as mitogenesis, differentiation, survival, and migration (15, 19), we examined the involvement of the NF- κ B and MAP kinase signaling pathways in this study. The results of this study showed that a NF- κ B inhibitor (PDTC), MAP kinase (MEK) inhibitor (PD98059), or p38 MAP kinase inhibitor (SB203580) significantly suppressed IL-8 synthesis by neutrophils, thus indicating the involvement of NF- κ B and the MAP kinase pathways in the up-regulation of IL-8 production in neutrophils activated by *T. vaginalis*.

GRO- α is another CXC chemokine secreted by human neutrophils. GRO- α has powerful chemotactic and activation effects on PMNs, including degranulation, increased expression of adhesion molecules, and in vivo recruitment of neutrophils to sites of infection (3, 25). Therefore, we measured GRO- α production under the same conditions as those used for IL-8 induction and found that GRO- α was also produced by neutrophils in response to *T. vaginalis* activation, although the amount of GRO- α (<1,000 pg/ml) was relatively low compared with the amount of IL-8.

Although neutrophils were confirmed to strongly induce IL-8 production after neutrophils were stimulated with *T. vaginalis* in the present study, the involvement of many cell types, including VECs, was expected. It is possible that IL-8 production is induced in VECs early in infection when the VECs are activated with *T. vaginalis*, because *Candida albicans* and *Neisseria gonorrhoeae* have been reported to induce IL-8 production by VECs early in the infection (8, 35).

On the basis of the present study together with several earlier studies (10, 20, 26), we hypothesize that many trichomonads in the vagina after acute *T. vaginalis* infection secrete proteins, including ESP, which have a chemotactic effect on neutrophils. These neutrophils can be further stimulated by *T. vaginalis* to produce chemokines, such as IL-8 and GRO- α , and these chemokines may subsequently induce more infiltration and the recruitment of neutrophils by chemotaxis at the reaction site. The involvement of VECs early in the infection might also play a role in IL-8 production. Finally, the accumulated neutrophils are thought to cause continued inflammation and/or aggravated vaginal inflammation.

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REFERENCES

1. Alderete, J. F., M. W. Lehker, and R. Arroyo. 1995. The mechanism and molecules involved in cytoadherence and pathogenesis of *Trichomonas vaginalis*. *Parasitol. Today* 11:70–74.
2. Arroyo, R., A. Gonzalez-Robles, A. Martinez-Palomo, and J. F. Alderete. 1993. Signalling of *Trichomonas vaginalis* for amoeboid transformation and adhesin synthesis follows cytoadherence. *Mol. Microbiol.* 7:299–309.
3. Baggolini, M., B. Dewald, and B. Moser. 1994. Interleukin-8 and related chemotactic cytokines—CXC and CC chemokines. *Adv. Immunol.* 5:97–179.
4. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156–159.

5. Demirezen, S., Z. Safi, and S. Beksac. 2000. The interaction of *Trichomonas vaginalis* with epithelial cells, polymorphonuclear leucocytes and erythrocytes on vaginal smears: light microscopic observation. *Cytopathology* **11**: 326–332.
6. Denny, C. F., L. Eckmann, and S. L. Reed. 1999. Chemokine secretion of human cells in response to *Toxoplasma gondii* infection. *Infect. Immun.* **67**:1547–1552.
7. Diamond, L. S. 1957. The establishment of various trichomonads of animals and man in axenic cultures. *J. Parasitol.* **43**:488–490.
8. Fichorova, R. N., P. J. Desai, F. C. Gibson III, and C. A. Genco. 2001. Distinct proinflammatory host responses to *Neisseria gonorrhoeae* infection in immortalized human cervical and vaginal epithelial cells. *Infect. Immun.* **69**:5840–5848.
9. Fouts, A. C., and S. J. Kraus. 1993. *Trichomonas vaginalis*: reevaluation of its clinical presentation and laboratory diagnosis. *J. Infect. Dis.* **141**:137–143.
10. Graves, A., and W. A. Gardner. 1993. Pathogenicity of *Trichomonas vaginalis*. *Clin. Obstet. Gynecol.* **36**:145–152.
11. Juffrie, M., G. M. van der Meer, C. E. Hack, K. Haasnoot, Sutaryo, A. J. P. Meerman, and L. G. Thijs. 2000. Inflammatory mediators in dengue virus infection in children: interleukin-8 and its relationship to neutrophil degranulation. *Infect. Immun.* **68**:702–707.
12. Jung, H. C., L. Eckmann, S. K. Yang, A. Panja, J. Fierer, E. Morzycka-Wroblewska, and M. F. Kagnoff. 1995. A distinct array of proinflammatory cytokines is expressed in human colon epithelial cells in response to bacterial invasion. *J. Clin. Invest.* **95**:55–65.
13. Kent, H. L. 1991. Epidemiology of vaginitis. *Am. J. Obstet. Gynecol.* **165**: 1168–1176.
14. Kim, J. S., H. C. Jung, J. M. Kim, I. S. Song, and C. Y. Kim. 1998. Interleukin-8 expression by human neutrophils activated by *Helicobacter pylori* soluble proteins. *Scand. J. Gastroenterol.* **33**:1249–1255.
15. Kim, J. S., J. M. Kim, H. C. Jung, and I. S. Song. 2001. Expression of cyclooxygenase-2 in human neutrophils activated by *Helicobacter pylori* water-soluble proteins. *Dig. Dis. Sci.* **46**:2277–2284.
16. Kim, S. R., and J. S. Ryu. 2001. Scanning electron microscopic observation of *Trichomonas vaginalis* contacted with human vaginal epithelial cells. *Korean J. Electron Microsc.* **31**:235–244.
17. Krieger, J. N., J. I. Ravdin, and M. F. Rein. 1985. Contact-dependent cytopathogenic mechanism of *Trichomonas vaginalis*. *Infect. Immun.* **50**:778–786.
18. Laga, M., A. Manoka, M. Kivuvu, B. Malele, M. Tuliza, N. Nzila, J. Goeman, F. Behets, V. Batter, M. Alary, et al. 1993. Non nucleated sexually transmitted diseases as risk factors for HIV-1 transmission in women: results from a cohort study. *AIDS* **7**:95–102.
19. Marie, C., S. Roman-Roman, and G. Rawadi. 1999. Involvement of mitogen-activated protein kinase pathways in interleukin-8 production by human monocytes and polymorphonuclear cells stimulated with lipopolysaccharide or *Mycoplasma fermentans* membrane lipoproteins. *Infect. Immun.* **67**:688–693.
20. Mason, P. R., and L. Forman. 1982. Polymorphonuclear cell chemotaxis to secretions of pathogenic and nonpathogenic *Trichomonas vaginalis*. *J. Parasitol.* **68**:457–462.
21. Mendoza-Lopez, M. R., C. Becerril-Garcia, L. V. Fattel-Facenda, L. Avila-Gonzalez, M. E. Ruiz-Tachiquin, J. Ortega-Lopez, and R. Arroyo. 2000. CP30, a cysteine proteinase involved in *Trichomonas vaginalis* cytoadherence. *Infect. Immun.* **68**:4907–4912.
22. Michelson, M. K., W. R. Henderson, E. Y. Chi, T. R. Fritsche, and S. J. Klebanoff. 1990. Ultrastructural studies on the effect of tumor necrosis factor on the interaction of neutrophils and *Naegleria fowleri*. *Am. J. Trop. Med. Hyg.* **42**:225–233.
23. Min, D. Y., J. S. Ryu, S. Y. Park, M. H. Shin, and W. Y. Cho. 1997. Degradation of human immunoglobulins and cytotoxicity on HeLa cells by live *Trichomonas vaginalis*. *Korean J. Parasitol.* **35**:39–46.
24. Minkoff, H., A. N. Grunebaum, R. H. Schwarz, J. Feldman, M. Cummings, W. Crombleholme, L. Clark, G. Pringle, and W. M. McCormack. 1984. Risk factors for prematurity and premature rupture of membranes: a prospective study of the vaginal flora in pregnancy. *Am. J. Obstet. Gynecol.* **150**:965–972.
25. Oppenheim, J. J., C. O. C. Zachariae, N. Mukaide, and K. Matsushima. 1991. Properties of the novel proinflammatory supergene “intercrine” cytokine family. *Annu. Rev. Immunol.* **9**:617–648.
26. Park, K. J., J. S. Ryu, D. Y. Min, and K. T. Lee. 1984. Leukocyte chemotaxis to *Trichomonas vaginalis*. *Yonsei J. Med. Sci.* **17**:77–88.
27. Rein, M. F., J. A. Sullivan, and G. L. Mandell. 1980. Trichomonad activity of human polymorphonuclear neutrophils: killing by disruption and fragmentation. *J. Infect. Dis.* **142**:575–585.
28. Ryu, J. S., H. L. Chung, D. Y. Min, Y. H. Cho, Y. S. Ro, and S. R. Kim. 1999. Diagnosis of trichomoniasis by polymerase chain reaction. *Yonsei Med. J.* **40**:56–60.
29. Ryu, J. S., H. K. Choi, D. Y. Min, S. E. Ha, and M. H. Ahn. 2001. Effect of iron on the virulence of *Trichomonas vaginalis*. *J. Parasitol.* **87**:457–460.
30. Scapini, P., J. A. Lapinet-Vera, S. Gasperini, F. Calzetti, F. Bazzoni, and M. A. Cassatella. 2000. The neutrophils as a cellular source of chemokines. *Immunol. Rev.* **177**:195–203.
31. Shaio, M. F., P. R. Lin, J. Y. Liu, and K. D. Yang. 1994. Monocyte-derived interleukin-8 involved in the recruitment of neutrophils induced by *Trichomonas vaginalis* infection. *J. Infect. Dis.* **170**:1638–1640.
32. Shaio, M. F., and P. R. Lin. 1995. Leukotriene B₄ levels in the vaginal discharges from cases of trichomoniasis. *Ann. Trop. Med. Parasitol.* **89**:85–88.
33. Shaio, M. F., P. R. Lin, J. Y. Liu, and K. D. Yang. 1995. Generation of interleukin-8 from human monocytes in response to *Trichomonas vaginalis* stimulation. *Infect. Immun.* **63**:3864–3870.
34. Soper, D. E., R. C. Bump, and W. G. Hurt. 1990. Bacterial vaginosis and trichomonas vaginitis are risk factors for cuff cellulitis after abdominal hysterectomy. *Am. J. Obstet. Gynecol.* **163**:1016–1023.
35. Steele, C., and P. L. Fidel, Jr. 2002. Cytokine and chemokine production by human oral and vaginal epithelial cells in response to *Candida albicans*. *Infect. Immun.* **70**:577–583.
36. Strieter, R. M., K. Kasahara, R. M. Allen, T. J. Standiford, M. W. Rolfe, F. S. Becker, S. W. Chensue, and S. L. Kunkel. 1992. Cytokine-induced neutrophil-derived interleukin-8. *Am. J. Pathol.* **141**:397–407.
37. Westwick, J., S. W. Li, and R. D. Camp. 1989. Novel neutrophil-stimulating peptides. *Immunol. Today* **10**:146–147.