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Effects of Antimicrobial Agents on Growth and Chemotaxis of *Trichomonas vaginalis*

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The motility of viable *Trichomonas vaginalis* organisms is readily demonstrable in a clinical wet mount or cultured specimen. We attempted to determine whether migration is a dynamic process such that the organisms move to avoid exposure to toxic antimicrobial agents. With the use of axenic cultures of *T. vaginalis* that were radiolabeled and assayed for chemotaxis in plastic multiwelled plates with a membrane filter inserted to trap organisms, the response of clinical isolates to various antimicrobial agents was studied. Chemotaxis was readily demonstrable and dependent upon factors including time of incubation, media used, and viscosity of media. Nitroimidazoles (e.g., metronidazole) which readily inhibited the growth of these organisms also caused significant chemorepulsion after minutes of exposure. The antifungal imidazoles ketoconazole and miconazole inhibited growth nearly as readily and caused chemorepulsion, but to a lesser degree. The spermicide Nonoxynol-9 also inhibited growth and caused significant chemorepulsion. The minimal concentrations of many compounds which inhibited growth were very similar to those which caused significant chemorepulsion. Imidazole and antibiotics (e.g., penicillin) which did not inhibit growth did not induce any chemotactic effects. Chemotaxis of *T. vaginalis* is an active and dynamic process, and the organisms display chemorepulsion shortly after exposure to toxic antimicrobial agents, well before toxicity can be demonstrated.

Numerous imidazole compounds have activity against *Trichomonas vaginalis*, and occasional reports of resistance to these compounds (e.g., the 5-nitroimidazole metronidazole) explain some cases of clinical failure (6, 10, 11). Because treatment failures seem to be far more common than the presence of resistant organisms (5, 8, 11), the motility of these organisms as a possible virulence factor was postulated by using the precedent of motility of *Vibrio cholerae* and the pathogenesis of cholera (4, 16).

A recent report on the effect of hormones on *T. vaginalis* described a new assay system for the chemotaxis of this organism and noted again how motile they are (15). An attempt was made to better define the chemotaxis of these organisms and to study the effects of antimicrobial agents on this process.

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MATERIALS AND METHODS

T. vaginalis. Isolates were cultivated from women with positive saline wet mounts examined for *T. vaginalis* by light microscopy. Sterile cotton swabs were used to inoculate secretions into borosilicate glass screw-cap tubes (8 by 100 mm) containing 6 ml of Diamond DL8 defined medium, pH 7.2, supplemented with 8% human serum, 40 μg of gentamicin per ml, and 2.5 μg of amphotericin B (3, 7) per ml. Cultures were maintained in DL8 with 4% heat-inactivated equine serum at 36°C and were subcultured every 3 to 4 days (after reaching the stationary phase). Antibiotics were removed from the medium after several passages. Two isolates with vastly differing (i) abilities to adhere to mammalian cells (15) and (ii) activities of acid hydrolases were selected for these experiments.

Antimicrobial agents. Nonoxynol-9 was graciously provided by Advanced Care Products, Ortho Diagnostics, Inc., Raritan, N.J. Others were purchased from Janssen, Piscataway, N.J. (ketoconazole and miconazole); E. R. Squibb & Sons, Princeton, N.J. (amphotericin B solubilized with sodium deoxycholate); and Sigma Chemical Co., St. Louis, Mo. (all others). They were individually prepared for each concentration, dissolved in pH 7.3 phosphate-buffered saline (PBS), and stored in ×100 final concentration at −70°C until use. 4-Nitroimidazole was first dissolved in 0.1 N NaOH, and ketoconazole and miconazole were dissolved in 0.1 N HCl and then diluted in PBS. This changed the pH at most by 0.2 U, and when diluted in medium for assays, there was no change in pH of the medium (pH 7.2).

Growth assay. Stock cultures of *T. vaginalis* were centrifuged at 250 × *g* for 5 min and suspended in 1.0 ml of warmed medium. After tubes were vortexed, 0.1 ml (generally, about 2.5×10^3 organisms) was added to each tube containing 6 ml of DL8. Tubes were incubated for up to 3 days. Tubes were centrifuged, the supernatant was removed, and the trichomonads were suspended in 1.0 ml of warmed PBS before motile organisms were counted with a hemacytometer.

Radiolabeling. Exponential-phase *T. vaginalis* isolates were radiolabeled with 5 μCi of [^{3}H]thymidine (78.3-Ci/mmol specific activity; Dupont, NEN Research Products, Boston, Mass.) per ml for 18 h at 36°C (1). Organisms were washed three times by centrifugation in PBS before use in further assays.

Chemotaxis assay. Radiolabeled *T. vaginalis* were washed in PBS and suspended in media to a concentration of 10^5/ml. A 0.5-ml portion of the organisms was placed in the bottom of a 24-well Transwell plate (Costar, Cambridge, Mass.). An 0.45-μm-pore size polycarbonate membrane filter insert (small enough to trap organisms, but large enough for passage of medium and chemotactic agents) was placed on top of the *T. vaginalis* suspension, and 0.5 ml of medium was placed on top of the membrane. Concentrated (×100 final

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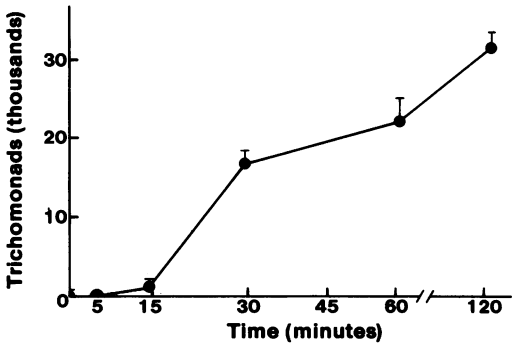

FIG. 1. Dependence of time on random migration of *T. vaginalis* into membrane filters. Horizontal bars indicate 1 standard deviation.

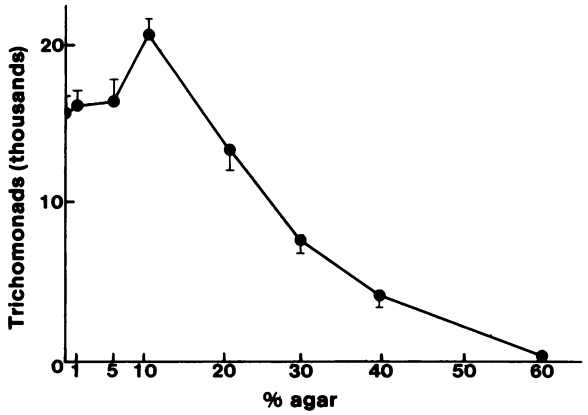

FIG. 2. Effect of medium viscosity on migration of *T. vaginalis* into membrane filters. Horizontal bars indicate 1 standard deviation.

concentration) supplemental antimicrobial agents or PBS control (all of equal volume) was added to the bottom or the top medium to yield the final concentrations mentioned. After migrating, *T. vaginalis* were trapped in the filter during incubation at 36°C for 45 min; the membrane insert was removed and inverted, and any excess fluid was removed by blotting with filter paper. The membranes were removed from the inserts and solubilized with 0.5 ml of 0.5 M Protosol (Dupont, NEN Research Products), scintillation fluid was added, and vials were counted in a liquid scintillation counter.

Data conversion and statistical analysis. Duplicate experiments were performed with known quantities of *T. vaginalis* counted in a liquid scintillation counter to allow conversion of all counts per minute to number of trichomonads. All assays were performed with controls at least six times, and data were analyzed with (nonparametric) Wilcoxon signed rank tests. Numerical data are presented as mean ± standard deviation of the mean.

RESULTS

The approximate doubling time of these clinical isolates was 10 to 14 h, and they reached the stationary phase of growth after about 3 days of incubation with this in vitro system. The chemotaxis assay showed no random migration into filters at zero time and increasing migration over minutes (Fig. 1).

Addition of agar to the medium to make it more viscous resulted in an increase in motility at around 10% agar (100% = 15 g of dry agar per liter) and then continued decline at concentrations of >20% such that there was no motility at about 60% agar (Fig. 2).

Chemokinesis of trichomonads. DL8 nutrient medium is a chemotactic agent (chemoattractant) compared with PBS (15). To assay chemokinesis separately from chemotaxis, the assay was performed eight times with nutrient medium or PBS on both sides of the filter. The chemotactic agent did not affect chemokinesis, as membrane trapping was the same, with a <3% difference whether in PBS or nutrient medium. Further, light microscopy revealed no overall changes in random movement when isolates were in PBS, nutrient medium, or metronidazole.

Effect of antimicrobial agents. The 5-nitroimidazole metronidazole, which is routinely used for the treatment of clinical *T. vaginalis* infections, was incubated with several strains of organisms up to the stationary phase of growth. For the first day there was no significant effect on random motility or growth of the organisms. However, by the end of 3 days of incubation, significant inhibition of growth and killing of organisms occurred with at least 0.05 μg of metronidazole per ml (Table 1). Placement of the same concentrations of metronidazole on the top or bottom of the wells (with the trichomonads on the bottom of the well) was used to assay chemotaxis. Metronidazole acted as a chemorepellent when placed on the bottom of the well; more organisms migrated away from this highest-concentration area and went toward the upper compartment of the well, so that more organisms were trapped in the filter. Conversely, when placed on the top of the well, this chemorepellent prevented randomly migrating organisms from going toward the top of the well with its higher concentration. Table 2 shows that, after 45 min of incubation, the organisms were repelled from approximately the same concentrations (0.1 μg/ml or greater) which inhibited or killed the organisms over a 3-day period, and there was a dose-response effect. Use of 4-nitroimidazole resulted in similar findings, but with minimal concentrations of 0.05 to 0.1 μg/ml necessary to inhibit growth of the same two isolates and 0.25 μg/ml necessary to cause significant chemorepulsion. Higher concentrations again produced more significant effects. The use of two other imidazole antimicrobial agents commonly used for fungal infections (ketoconazole and miconazole) revealed a similar effect on growth compared with metronidazole, but chemorepulsion occurred with concentrations of at least 0.5 μg/ml, depending on the antimicrobial agent and the isolate of *T. vaginalis*. The spermicide Nonoxynol-9 also both inhibited the growth of *T. vaginalis* and caused significant chemorepulsion of the organisms at similar concentrations of 1 to 5% (Table 3).

Conversely, antibiotics which did not alter the growth of trichomonads had no significant effect on their chemotaxis.

TABLE 1. Growth of trichomonads in metronidazole after 72 h of incubation

Metronidazole concn (μg/ml)	No. of trichomonads (10^3)	
	Isolate 1	Isolate 2
None (control)	264 ± 43	231 ± 38
0.025	252 ± 38	221 ± 24
0.05	150 ± 50[a]	143 ± 29[a]
0.1	92 ± 2[a]	86 ± 2[a]
0.25	4 ± 3[a]	3 ± 1[a]
0.5	1 ± 1[a]	1 ± 1[a]
1.0	0 ± 0[a]	0 ± 0[a]

[a] $P < 0.05$ compared with control.

TABLE 2. Movement of trichomonads away from metronidazole after 45 min of incubation with organisms in bottom well and metronidazole in bottom or top side of well

Metronidazole concn (μg/ml)	Trichomonads (10^3) per filter			
	Isolate 1		Isolate 2	
	Top	Bottom	Top	Bottom
None (control)	13.6 ± 0.6	13.6 ± 0.6	13.4 ± 0.5	13.4 ± 0.5
0.01	13.4 ± 0.5	13.2 ± 0.3	13.5 ± 0.6	13.3 ± 0.5
0.05	13.4 ± 0.5	13.2 ± 0.9	13.4 ± 0.5	13.3 ± 0.6
0.10	13.2 ± 0.6	16.7 ± 1.3[a]	13.1 ± 0.7	15.9 ± 1.1[a]
0.25	11.6 ± 1.3	17.6 ± 0.6[a]	11.3 ± 1.0[a]	16.8 ± 0.9[a]
0.50	10.4 ± 0.4[a]	19.6 ± 1.8[a]	10.0 ± 0.6[a]	19.1 ± 1.2[a]
1.0	9.9 ± 0.7[a]	20.7 ± 1.8[a]	9.6 ± 0.5[a]	20.4 ± 1.3[a]

[a] $P < 0.05$ compared with control.

Incubation in this culture system with rather high concentrations of amphotericin B (2.5 μg/ml) penicillin G (100 U/ml), and gentamicin (40 μg/ml) resulted in no significant effect on the growth of the trichomonads (data not shown). These drugs, when assayed for chemotaxis, were not consistent chemorepellants or chemoattractants and altered chemotaxis by <10% (Table 4). Furthermore, imidazole by itself had no effect on the growth or chemotaxis of the trichomonads (Table 5).

DISCUSSION

Trichomonads have the ability to actively move away from toxic compounds after minutes of exposure. The same compounds take hours to days longer to effect a demonstrable toxic action upon the organisms. This was noted with numerous imidazole derivatives. Increased chemotactic activity was not associated with a generalized increase in random movement, but rather with specific movement away from chemorepellants. The active compounds did not merely alter adherence characteristics to the filters, since the same chemorepellants increased trapping of organisms in the filter when placed below it, but decreased filter trapping when placed above (and can readily pass through the 0.45-μm pores). The marked similarity of results obtained with two different *T. vaginalis* isolates is quite surprising and most likely coincidental, as preliminary observations with other trichomonads reveal the same trend but quite different results.

TABLE 4. Lack of movement of trichomonads (isolate 1) away from nontoxic antimicrobial agents after 45 min of incubation with organisms in bottom well and antimicrobial agents in bottom or top side of well

Antimicrobial agent	Organisms (10^3) per well	
	Top	Bottom
None (control)	14.9 ± 1.6	14.9 ± 1.6
Amphotericin B (2.5 μg/ml)	15.9 ± 1.7	16.6 ± 0.8
Penicillin G (100 U/ml)	15.9 ± 1.6	16.3 ± 1.2
Gentamicin (40 μg/ml)	16.3 ± 2.0	16.9 ± 0.8

The activities of the antifungal imidazoles ketoconazole and miconazole against numerous nonfungal pathogens have been described before (9, 12, 14). A recent study, however, using a similar sensitivity assay (but a larger inoculum), noted negligible susceptibility in vitro of five *T. vaginalis* isolates to miconazole and ketoconazole (13). The two isolates we tested were susceptible to these drugs in vitro, but no in vivo data support their use for *T. vaginalis* infections. Also, the spermicide Nonoxynol-9 inhibits and repels trichomonads and has been shown in the past to have the ability to inhibit the growth of several other sexually transmitted pathogens (2). Active motility of the organisms could be related to the response to treatment of *T. vaginalis* infections.

At about the same concentration that affects growth, there is significant chemorepulsion of the organisms by numerous antimicrobial compounds. We find intriguing the dichotomy between the time necessary for antimicrobial agents to inhibit the growth of trichomonads (longer than a day) and the ability of the organisms to move rapidly away from an area with these toxic compounds present after only minutes of incubation. Since treatment failures for trichomoniasis seem to be far more common than the presence of resistant organisms (5, 8, 11), it is theoretically possible that some treatment failures are due to the sequestering of sensitive organisms via chemotaxis into areas with no, or significantly less, active drug (e.g., deep in a mucus layer). The ability of trichomonads to move as freely in 20% agar as in control media could enhance such sequestration. Studies with *V. cholerae* suggest a role of motility in the development of cholera (4, 16). A chemotactic gradient appears to attract organisms to the surface mucus gel of intestinal mucosa, and nonmotile mutants do not associate with intestinal wall

TABLE 3. Effect of Nonoxynol-9 on growth and chemotaxis of trichomonads

Nonoxynol-9 concn (%, vol/vol)	Growth (trichomonads, 10^3)[a]		Chemotaxis (trichomonads, 10^3, per filter)[b]			
	Isolate 1	Isolate 2	Isolate 1		Isolate 2	
			Top	Bottom	Top	Bottom
None	255 ± 29	268 ± 26	13.8 ± 0.8	13.8 ± 0.8	13.1 ± 0.9	13.1 ± 0.9
0.01	246 ± 33	259 ± 28	13.7 ± 1.0	14.4 ± 0.9	13.0 ± 0.8	13.8 ± 0.9
0.05	—[c]	—	14.2 ± 1.2	14.9 ± 0.8	13.1 ± 1.0	14.0 ± 0.9
0.10	—	—	14.7 ± 1.7	16.3 ± 0.6[d]	13.2 ± 1.3	15.1 ± 1.1
1.0	223 ± 20	240 ± 21	12.9 ± 1.7	17.2 ± 0.6[d]	12.4 ± 1.2	16.3 ± 0.8[d]
5.0	159 ± 27[d]	162 ± 18[d]	10.9 ± 2.7[d]	18.9 ± 0.8[d]	10.6 ± 2.0[d]	17.9 ± 1.0[d]
10.0	145 ± 21[d]	149 ± 15[d]	9.9 ± 1.3[d]	20.7 ± 0.9[d]	9.7 ± 1.4[d]	19.3 ± 1.0[d]
20.0	120 ± 13[d]	121 ± 17[d]	—	—	—	—
30.0	108 ± 14[d]	105 ± 16[d]	9.4 ± 0.9[d]	21.3 ± 1.6[d]	9.3 ± 0.7[d]	20.8 ± 1.1[d]

[a] After 72 h of incubation.
[b] After 45 min of incubation with organisms in bottom of well and Nonoxynol-9 in bottom or top side of well.
[c] —, Not done.
[d] $P < 0.05$ compared with control.

TABLE 5. Effect of imidazole on growth and chemotaxis of trichomonads

Imidazole concn (μg/ml)	Growth (trichomonads, 10^3)[a]		Chemotaxis (trichomonads, 10^3, per filter)[b]			
			Isolate 1		Isolate 2	
	Isolate 1	Isolate 2	Top	Bottom	Top	Bottom
None	1,790 ± 707	2,186 ± 713	13.9 ± 0.5	13.9 ± 0.5	13.6 ± 0.6	13.6 ± 0.6
0.01	—[c]	—	13.8 ± 0.6	14.0 ± 0.4	13.5 ± 0.7	13.8 ± 0.7
0.025	1,865 ± 652	2,094 ± 612	—	—	—	—
0.05	1,781 ± 437	2,177 ± 680	14.0 ± 0.6	13.9 ± 0.4	13.7 ± 0.6	13.9 ± 0.4
0.1	1,804 ± 727	2,189 ± 704	13.9 ± 0.5	13.8 ± 0.6	13.7 ± 0.6	13.8 ± 0.6
0.25	1,794 ± 546	2,136 ± 605	14.2 ± 1.0	13.8 ± 0.9	13.5 ± 0.5	13.5 ± 0.6
0.5	1,858 ± 597	2,190 ± 582	14.0 ± 0.6	14.1 ± 0.7	13.8 ± 1.0	13.4 ± 0.7
1.0	1,914 ± 733	2,163 ± 609	14.1 ± 0.8	14.2 ± 1.0	13.7 ± 0.7	13.6 ± 0.8

[a] After 72 h of incubation.
[b] After 45 min of incubation, with organisms in bottom of well and imidazole in bottom or top side of well.
[c] —, Not done.

nearly as readily as motile organisms. The chemotaxis of *T. vaginalis* as a virulence factor and cause for treatment failure warrants further studies.

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