Contact-independent cytotoxicity of *Trichomonas vaginalis*

F F Pindak, M Mora de Pindak, W A Gardner Jr

Abstract

***Objective*—To test the dependency of haemolytic and cytocidal manifestations of pathogenicity of *Trichomonas vaginalis* on direct contact between the target cells and the organism.**

***Test organism*—*T vaginalis* strain Baltimore 42.**

***Design*—Haemolysis in the presence of live *T vaginalis* and of its filter-sterilised metabolic products was compared. The dependence of haemolytic and cytocidal effects on retention of low pH of metabolic products of the organism was demonstrated by parallel titrations of sterile filtrates in normal saline and in phosphate buffered saline (PBS) pH 7·0.**

***Results*—Near complete lysis was obtained when erythrocytes mixed with *T vaginalis* were incubated for 1 h at 37°C in saline containing 1% glucose. The same degree of haemolysis was present in *filter-sterilised* glucose-saline in which the organism was incubated (1 h/37°C) before erythrocytes were added and incubated under the same conditions as in the mixture with the organism. The degree of haemolysis in filtrates was dependent on retention of low pH (below 5·0) of the suspending fluid in which the organism alone was incubated. Dilution of filtrates in PBS, as opposed to normal saline, abolished or diminished the haemolytic effect. Presence of glucose (energy source) in the saline during incubation of the organism had a pronounced enhancing effect. The production of haemolytic metabolites was temperature dependent, whereas the haemolytic process per se was not. The effect was not an exclusive property of *T vaginalis* since it was also demonstrated with other trichomonads. The same filtrates applied to tissue culture exerted cytocidal effect strikingly similar to that observed in the haemolysis experiments.**

***Conclusion*—Neither haemolytic nor cytocidal effect of *T vaginalis* was contact-dependent.**

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Department of Pathology
F F Pindak
W A Gardner Jr

Department of Comparative Medicine
M Mora de Pindak

University of South Alabama College of Medicine, Mobile, Alabama 36617, USA

Address correspondence to: Frank F Pindak, Department of Pathology, University of South Alabama, College of Medicine, 2451 Fillingim Street, Mobile, AL 36617, USA.

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Introduction

According to some epidemiology sources *Trichomonas vaginalis* may infect three million individuals in the United States, and perhaps over one hundred million worldwide.[1–3] The validity of these estimates is difficult to verify since trichomoniasis is not included among regularly reported diseases. It seems clear, however, that the wide range in the presentation of symptoms suggests differences in pathogenicity among the strains of the organism. Considering the apparent number of infected individuals, fundamental knowledge of pathogenic mechanism(s) is necessary for development of control and preventive measures.

Honigberg's mouse assay of pathogenicity has been used to correlate the severity of symptoms with size of lesion induced by subcutaneous inoculation of various isolates.[4] The squirrel monkey (*Saimiri sciureus*) was reported to present signs of infection after intravaginal inoculation,[5] but this approach to the study of the organism is not in wide use, primarily owing to the scarcity and cost of this animal.

Attempts to demonstrate pathogenic mechanisms of *T vaginalis* by in vitro methods have focused on the cell-detaching factor (TvCDF),[6–11] tissue culture cytotoxicity,[12–15] haemolytic activity,[16] and "contact-dependent" cytotoxicity assays by which cytocidal or haemolytic effects were demonstrated, but only when the target cells were in contact with the organism.[17 18] It is difficult to interpret the results of reports dealing with contact-dependent effects. Contact-dependent effectiveness has not been defined and no known or postulated mechanism has been presented by which the reported results may have been produced. Thus, their relevancy to pathogenicity is not established.

The resolution of pathogenicity of *T vaginalis* by in vitro technology is by no means simple. *T vaginalis* is a complex organism, as suggested by the list of known factors which may contribute to its pathogenicity. A number of proteinases and other enzymes which may affect tissue cultures have been described;[3 11 19–22] lactic acid, acetic acid, CO_2 and H_2 are among major metabolic end products.[23] All of these may have a deleterious effect on tissue cultures. It is not clear whether such effects stem from a combination of these entities or are due to a single component.

During studies of interactions between erythrocytes and metabolic products of various trichomonads, including *T vaginalis*, at times we encountered haemolysis (unpublished). This feature was always associated with low pH (< 5·0). Recently Garber and Bowie suggested that very low pH associated with metabolically active *T vaginalis* may be an important factor in "contact-dependent" killing of mammalian

cells.[24] Studies presented here were designed to address the controversy of "contact-dependent" cytotoxicity. We examined the conditions under which haemolytic and cytocidal effects of *T vaginalis* can be demonstrated. Our data show that both effects can be elicited without the presence of the organism. By inference, the contact-dependent manifestations may not necessarily be indicators of pathogenicity of the organism.

Materials and methods

Test organism

T vaginalis strain Baltimore 42 was received through the courtesy of BM Honigberg. For routine purposes it was propagated in GMP medium in co-culture with McCoy or RK-13 cell monolayers.[10] In specific instances the organism was grown in TYM medium supplemented with 10% inactivated (56°C/30 min) fetal bovine serum (FBS).

Culture components for cytotoxicity assays

Three basic culture products were used: (a) late stationary culture (3 to 4-day old) supernatant filtered through 0·45 μm Acrodisc membrane; (b) whole organism harvested from 2-day culture, washed in normal saline (0·9% NaCl in H_2O), adjusted to density of 6·5 × 10^6/ml in saline containing 1% glucose (glucose-saline) and 1% washed human erythrocytes type O; (c) filtrates of normal saline, glucose-saline and phosphate buffered saline (PBS) pH 7·0 in which the organism of same age and density as in (b) was incubated for 2 h at 37°C, at room temperature (~25°C) or at 4°C. pH values of all filtered products were determined before addition of erythrocytes or application to tissue culture.

Haemolysis assay

For quantitative determinations of haemolysis fresh human erythrocytes washed three times in normal saline and adjusted to a 10% suspension were added to the various preparations in volumes required to obtain 1% final concentration. After 2 h incubation at 37°C intact erythrocytes were counted in a haemocytometer chamber using 400 × magnification. The degree of haemolysis (%) was determined from the deficit of erythrocytes relative to the erythrocyte count (100%) in appropriate control preparations.

Semiquantitative determinations of haemolytic titre were performed in 48-well flat bottom tissue culture plates (No. 3548, Costar, Cambridge, MA). One tenth ml of 5% suspension of erythrocytes was added to test preparations (0·8 ml) serially diluted in normal saline and PBS pH 7·0. The plates were kept in flat position and evaluated after incubation for 2 h. Presence of haemolysis was shown by the transparency of well contents as seen in a bottom-view mirror. The ease of reading was enhanced by covering the plate with a lid with a fine square grid which became clearly visible where the haemolysis was complete but remained obscured in the presence of unlysed erythrocytes. With this aid incomplete lysis in the approximate range of 50% to 25% could be recognised. The readings were verified microscopically under 200 × magnification.

Tissue culture cytotoxicity assay

The same preparations as those used in the haemolysis assays were added to washed monolayers of HeLa cells grown in 48-well plates. After 2 h incubation at 37°C the cells were washed 3 times with saline, stained with trypan blue, washed again and examined for the presence of dye uptake.

Statistical treatment of data

The assessment of haemolytic effect was based on three or more repetitions of each experiment. In each run intact erythrocytes were counted in at least eight one-millimeter squares of a standard haemocytometer (0·1 mm^3). Higher numbers of squares were counted when the individual square scores were less than ten. When initial counts appeared to be more than 100 per square the samples were appropriately diluted in normal saline and the resulting counts were arithmetically adjusted to 0·1 mm^3 volume. From the individual counts the mean values per 0·1 mm^3 were determined. These values were then assessed by the Student's t test (2-tailed) for significance of difference from appropriate controls. The values of percent haemolysis were based on the mean count values of each experimental group;

$$\text{per cent lysis} = 100 - \frac{(\text{count in test sample}) \times 100}{\text{count in control}}.$$

Some experiments required special modification of methods; for better clarity those are presented with results.

Results

pH-dependent haemolysis in sterile T vaginalis culture supernatant

The importance of retention of the original pH of filtered supernatant from 4-day old culture of *T vaginalis* (pH 4·4) serially diluted in saline, as opposed to the neutralizing effect of PBS, is shown in table 1. Three matched sets of dilutions in the two diluents were dispensed in 48-well plates. The haemolysis scores were recorded after addition of erythrocytes and incubation for 2 h at 37°C and at room temperature respectively. The third set was

Table 1 Effect of pH and temperature on haemolysis in sterile culture filtrate

Diluent	*Incubation temperature and haemolytic titre*		
	37°C	*~25°C*	*4°C*
Saline	1:16	1:16	1:16
PBS	<1:2	<1:2	<1:2

Filter-sterilised supernatant of late stationary culture of *T vaginalis* (pH 4·4) was diluted in normal saline and in PBS pH 7·0. The initial 1:2 dilution in saline retained its pH 4·4 while in PBS it increased to 5·1. Upon addition of erythrocytes, matched sets of dilutions were incubated at different temperatures, as shown. After 2 h at 37°C or at room temperature, as well as after 18 h at 4°C, the same haemolysis titre was obtained in saline. There was no haemolysis when samples were diluted in PBS.

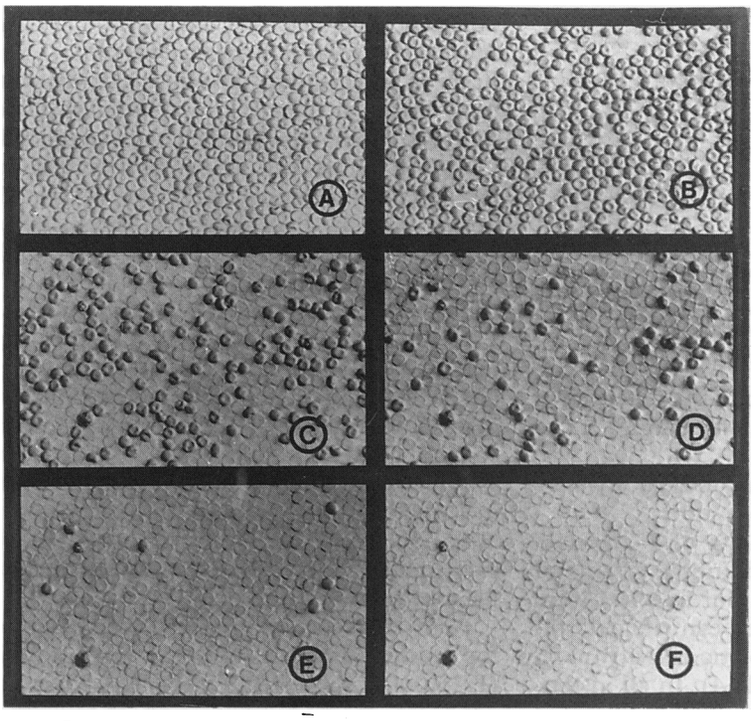

Figure 1 Progressive lysis of erythrocytes in filtrate of glucose-saline (pH 4·3) in which T vaginalis was incubated at 37°C for 2 h. The progress of haemolysis, as seen in the same field, was observed and photographed during 3 h at room temperature. A: "monolayer" of settled erythrocytes at 20 min; B–F: increasing haemolysis at 30 min intervals. After 2½ h the lysis was complete and only empty vesicles remained. The two cells in frame F still apparently intact were presumed to be leukocytes, as judged from their appearance. (Inverted microscope, Hoffman optics, 200 × original magnification).

incubated at 4°C and evaluated after 18 h because at 2 h it was difficult to discern a clear endpoint of haemolysis. In saline the haemolytic titre was 1:16, regardless the temperature of incubation. No haemolysis was present at any temperature when PBS was used as diluent. Very similar results were obtained with supernatant from a 4-day old culture (pH 4·5) grown in TYM (data not shown).

Progression of contact-independent haemolysis at room temperature

Filtered glucose-saline in which the organism was incubated for 2 h at 37°C was dispensed in 24-well flat bottom tissues culture plate. After addition of erythrocytes (empirically diluted to form a single cell monolayer upon settling) the plate was positioned on the stage of an inverted microscope and left undisturbed during periodic observations. A series of photographs of the same field was taken at 30 min intervals (fig 1). Few foci of lysed cells developed in less than one h. These areas became progressively larger, and all erythrocytes were lysed after 2½ h. The same end result was seen in seven matched wells. There was no haemolysis in erythrocyte control wells containing uninoculated glucose-saline.

Lack of discrimination between contact-dependent and contact-independent haemolysis

T vaginalis harvested from 2-day old culture was resuspended in glucose-saline and split into two aliquots of 4·5 ml. Five tenths ml of erythrocytes (10% in saline) were added to one (A), and 0·5 ml of normal saline to the other (B). Control consisted of glucose-saline and erythrocytes mixed in the same proportions as in (A). The samples were incubated for 2 h at 37°C, with mixing by inversion at 20 min intervals.

Unlysed erythrocytes in sample A were counted immediately after incubation. Sample B was centrifuged, and filter-sterilised. Erythrocytes were added to the filtrate as before to sample A. These were counted after incubation for 2 h at 37°C.

The viability of *T vaginalis* at the end of incubation was in excess of 90%, as indicated by activity of organelles of motility observed at 400 × magnification. The erythrocyte count in control samples was virtually unchanged. In samples A and B there was nearly complete lysis (table 2). The difference between them was not significant.

Conditions affecting contact-independent haemolysis

Table 3 shows results of a comparative study of haemolytic effect in filtrates prepared after incubation of *T vaginalis* of the same concentration in PBS pH 7·0, normal saline and glucose-saline. The suspensions of the organism were incubated for 2 h at 37°C. Additional two matched samples in glucose-saline were incubated at room temperature and at 4°C respectively. Erythrocytes added to the filtrates were incubated for 2 h at 37°C. Unlysed erythrocytes were counted as in the preceding experiments. Mean count values per 0·1 mm^3 were based on 8 replicate determinations except for experiment D in which 24 1-mm^2 fields were counted. There was significant haemolysis in PBS filtrate (B) which, however, was not as pronounced as in normal saline filtrate (C). The greatest haemolytic effect by far was obtained in glucose-saline (D); in a matched sample kept at room temperature (E) the haemolytic effect was decreased, and no appreciable haemolysis occurred in one maintained at 4°C (F). Thus the degree of haemolysis was influenced by three factors: a)

Table 2 Lack of difference between contact-dependent and contact-independent haemolysis

			Dependent haemolysis		*Independent haemolysis*	
Experiment	*Value*		*Sample A**	*Control*	*Sample B†*	*Control*
1	n=20	x̄‡	37·3	1087	15·5	1045
		Lysis	96·6%	0%	98·5%	0%
2	n=35	x̄	20·1	1336	26·3	1272
		Lysis	98·5%	0%	97·9	0%

*Unlysed erythrocytes incubated with *T vaginalis* for 2 h at 37°C.
†Unlysed erythrocytes similarly incubated in filtered glucose-saline in which *T vaginalis* was incubated for 2 h at 37°C.
‡Mean count of erythrocytes per 0·1 mm^3 (n = number of replicate counts). In experiment no. 1 the pH of samples A and B was 4·4 and 4·3 respectively. In experiment no. 2 and three others (not shown) these measurements were within 0·2 units

Table 3 Effect of incubation conditions on contact-independent haemolysis

	Incubation Condition	Mean Count per 0·1 mm³	SD	Difference from Control	Lysis
A.	Control erythrocytes in glucose-saline	600·2	58·2	—	0%
B	In PBS pH 7·0, 37°C	495·0	49·2	<0·01	17·5%
C.	In Saline, 37°C	383·7	23·9	<0·001	36·1%
D.	In Glu-sal, 37°C	0·2	0·07	<0·001	99·97%
E.	In Glu-sal, ~25°C (RT)	247·5	16·7	<0·001	58·8%
F.	In Glu-sal, 4°C	589·4	29·1	NS	2·6%

Suspensions of *T vaginalis* were incubated for 2 h in PBS pH 7·0, normal saline, and in glucose-saline. Unlysed erythrocytes were counted after incubation (2 h/37°C) in filtrates prepared from the suspensions. Values for difference from control were based on Student's *t* test. The degree of lysis was calculated from count differences between test samples and control.

modulation of pH by buffer; b) the presence of energy source (glucose); c) temperature.

Contact-independent haemolytic activity of other trichomonads

In order to test the specificity of *T vaginalis* haemolytic property five additional strains of trichomonad (representing four species) were incubated in glucose-saline, centrifuged, filtered, and titred for haemolytic activity, as in preceding experiments (table 4). In all instances the pH values of the filtrates approximated those of *T vaginalis* and its filtered culture supernatant (included for control). Haemolysis was demonstrated with each strain. The actual haemolytic titres suggested a relation to the pH value of individual preparations.

Table 4 Haemolytic property of various trichomonads

Organism and pH of filtrate	Haemolytic titre: In Saline	Haemolytic titre: In PBS
T mobilensis M776 (pH 4·3)	1:16	Neg.
T mobilensis FP4295 (pH 4·9)	1:8	Neg.
T foetus ATCC 30003 (pH 4·4)	1:32	1:2
T augusta ATCC 30077 (pH 4·4)	1:32	1:2
P hominis ATCC 30000 (pH 4·7)	1:8	Neg.
T vaginalis (pH 3·9)	1:32	1:2
T. vag. supernatant (pH 4·3)	1:32	1:2
Cell control	0	0

Washed trichomonads were incubated for 2 h at 37°C in glucose-saline. Filtered supernatants (pH indicated in parentheses) were diluted 1:2 to 1:64 in saline and in PBS pH 7·0. Haemolysis endpoints were determined microscopically after the cells were incubated with the filtrates for 2 h at 37°C.

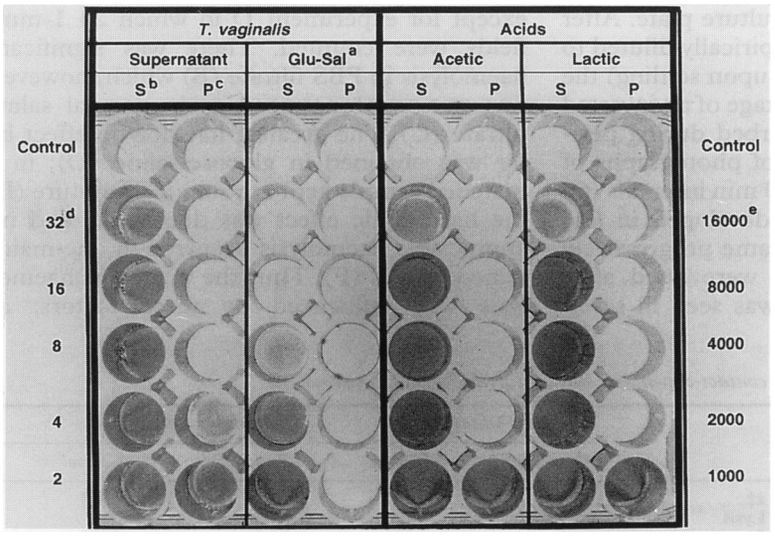

Figure 2 Staining of HeLa monolayers with trypan blue after exposure to T vaginalis products and free acids. [a]Filtered glucose-saline after incubation (2 h/37°C) with T vaginalis. [b]Dilutions in saline. [c]Dilutions in PBS pH 7·0. [d]Dilution reciprocals of T vaginalis products. [e]Dilution reciprocals of acids. Dark wells show retention of trypan blue after 2 h/37°C contact with test material; blank wells present unstained cells.

Contact-independent and nonspecific cytocidal effect of T vaginalis in tissue culture

Filtered supernatant of late stationary culture of *T vaginalis* (pH 4·3) and filtered glucose-saline in which the organism was incubated for 2 h at 37°C, as well as controls consisting of free lactic and acetic acid, were diluted in saline and in PBS pH 7·0 (both diluents contained 1% FBS). These were added to HeLa monolayers which, after 2 h at 37°C, were washed and stained with trypan blue.

In saline, all cells exposed to culture supernatant were stained in dilutions 1:2 to 1:16; partial staining, verified by microscopic examination, was seen in 1:32 dilution. In contrast, staining of all cells (100%) did not occur above 1:2 dilution prepared in PBS; faint dye uptake in 1:4 dilution represented staining of less than 50% of cells (fig 2). Similar difference between saline and PBS as diluents were obtained with glucose-saline filtrate.

Both lactic and acetic acid were very toxic for tissue culture, as the end point dilutions (1:16,000) show. As with the *T vaginalis* products, marked suppression of toxicity was seen where the acids were diluted in PBS. These acids applied to erythrocytes under the same test conditions produced haemolysis in equal range of dilutions (data now shown).

Discussion

Cytocidal and haemolytic properties of *T vaginalis* have been cited as supportive of the concept of "contact-dependent" cytotoxicity. These features could be detected only when there was intimate contact between the organism and the target cells.[17 18 25] Supportive of this theory were experiments without this essential condition; *eg*, sterile culture filtrates were ineffective.

The objective of this study was to show that, with techniques other than those used by earlier investigators,[17 18 25] haemolytic and cytocidal effects of *T vaginalis* can take place *without* direct contact between the organism and the respective target cells. We did not intend to pursue all possible ramifications of the unexplained cytotoxic manifestations. Nevertheless, we were able to demonstrate that at least one factor, namely, production of acidic metabolites by the living organism, whether in direct contact with the target cells or not, can lead to haemolysis or death of cultured cells. It was essential, however, to test these products in unneutralized form. Thus, the assays were performed in normal saline. When this was substituted by PBS pH 7·0 the effect was greatly reduced or absent.

Our findings are in agreement with those of Garber and Bowie[24] who demonstrated that much of the cytotoxicity reported to be contact-dependent "is predominantly a pH effect which can be eliminated by rigidly controlling the pH of the media overlaying the monolayer".

Reports on contact-dependent haemolysis were based on the use of human erythrocytes; it was shown that erythrocytes from all human blood groups,[16] as well as those of several other

species,[16 25] were susceptible to lysis. In order to avoid unnecessary deviation from materials used in those studies we have chosen human erythrocytes type O for our haemolysis experiments.

In some instances where our tests conditions approximated those reported by the proponents of the "contact-dependent" cytotoxicity[17 18 25] our results were comparable, for example, incubation of washed *T vaginalis* with erythrocytes produced near complete haemolysis (table 2). At the end of incubation the pH of the organism-erythrocyte mixture was 4·4 (as opposed to 6·5 at the beginning). In parallel to this experiment a carefully measured aliquot of the organism suspension was first incubated alone, and after 2 h it was removed by centrifugation and filtration. The pH of the filtrate was 4·3. Erythrocytes incubated in it for 2 h/37°C showed the same degree of lysis as those incubated in the presence of the organism. Therefore, what in the first instance could be interpreted as a contact-dependent effect became unequivocally contact-*independent* in the second one.

Lack of haemolysis in trichomonal culture supernatants which were filtered, concentrated and dialysed against PBS (presumably in order to adjust their pH to 6·0) has been reported as evidence in support of the concept of contact-dependency effect.[25] We have shown that filtered unconcentrated supernatant from a late stationary culture (with usual pH less than 5·0) exerted haemolytic effect over the range of several serial dilutions, providing that the assay was done in normal saline instead of a buffer (table 1). The lytic factor was apparently of small molecular size as indicated by the results of stepwise ultrafiltration of culture supernatant, *i.e.*, effluent passed successively through 100 000, 50 000, 10 000 and 500 mol wt cut-off membranes had the same lytic titre as that of unfiltered supernatant (data not shown).

Unconcentrated filter-sterilised supernatant (pH 4·3) caused death of tissue cultures, again, providing the dilutions were prepared in normal saline, not in PBS pH 7·0 (fig 2). These results were also produced with free lactic acid and acetic acid tested under the same conditions. The supernatants and the free acids also lysed erythrocytes when these were used in place of tissue culture. Thus, most of the features attributed to the "contact-dependent" effects were duplicated with organism-free, filter-sterilised materials. The effect was not a specific attribute of *T vaginalis*, as basically the same results were obtained with filtered products of other trichomonads (table 4).

In agreement with Garber and Bowie[24] we propose that free acids produced by the organism play a key role in the so called "contact-dependent" cytotoxicity. Acids are generated by *T vaginalis* during incubation. Their production is dependent on energy source (glucose in our experiments), temperature (table 3), and on remaining unneutralised, as the differences in assays in PBS and normal saline showed. The haemolytic assay *per se* is not temperature-dependent (table 1). Haemolytic and cytocidal effects are clearly distinguishable from the activity of TvCDF. Recently Dailey, *et al*[25] misquoted our original report[10] on the properties of this substance, citing the effect of TvCDF as an example of contact-dependent cytotoxicity. On the contrary, in terms of activity, TvCDF is not related to contact-dependent cytotoxicity. It is a *filter-sterilised* product from culture of *T vaginalis*. Its effective pH range[7 8 10] is well above that shown in cytocidal experiments. It does not kill mammalian cells in culture and does not lyse erythrocytes.

We do not propose that all aspects of contact-dependent manifestations of cytotoxicity are due solely to low pH. The various proteinases of *T vaginalis*[23 26] requiring for their activity a pH in the range here reported cannot be a priori excluded. On the other hand, their participation in the cytocidal or haemolytic effect could not be tested directly either, since the pH ($\sim$4·0) necessary for their expression produces the very same effect to be measured.

It should be noted that none of the authors reporting the so called "contact-dependent" cytotoxicity specified the terminal pH values of their experiments. Judging from our experience it may have decreased to the range which we observed, with consequences here described.

Using adequate controls and appropriate techniques we have demonstrated haemolytic and cytocidal effects of sterile metabolic products of *T vaginalis*. Therefore, these two phenomena, proposed to be prime examples of "contact-dependent" cytotoxicity of *T vaginalis*, are not necessarily contact-dependent.

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