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Identification and Partial Characterization of a Cytolytic Toxin Produced by *Gardnerella vaginalis*

GIANDOMENICO ROTTINI,[1]* ALDO DOBRINA,[1] ORNELLA FORGIARINI,[1] ERMANNO NARDON,[1] GIANNI A. AMIRANTE,[2] AND PIERLUIGI PATRIARCA[1]

Institute of General Pathology[1] and Department of Biology,[2] University of Trieste, Via A. Fleming 22, Trieste, Italy I-34127

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Generation and release into the culture medium of a cytolytic toxin by *Gardnerella vaginalis* has been demonstrated. Addition of starch and of the nonionic detergent Tween 80 to the culture medium was essential to recover cytolytic activity. A protein with an apparent molecular mass of 61 to 63 kDa was purified from the culture supernatants showing lytic activity towards erythrocytes and nucleated cells, such as human endothelial cells and human neutrophils. The protein had marked selectivity for human erythrocytes, while erythrocytes from other species were not lysed or were lysed at much higher concentrations of the protein than those needed for human erythrocytes. The cytolytic activity was remarkably unstable in polar media, but was stabilized by nonionic detergents, by binding, or by insertion into the target cell membrane, suggesting its amphiphilic nature.

Gardnerella vaginalis, an opportunistic pathogen harbored by the vaginal mucosa of healthy subjects (9, 30), is closely associated with bacterial vaginitis (10, 17). Occasionally it is also isolated from the urethra of healthy males (11, 14). Recently, *G. vaginalis* has been suggested as a possible etiological agent of severe infections, e.g., endometritis (25, 34), cystitis (24, 33), amnionitis (15, 18, 21), neonatal septicemia (26), and meningitis (2). However, the components of *G. vaginalis* pathogenicity are poorly characterized apart from its ability to adhere to cells (23, 32) and to interact with neutrophils in the presence of fresh human serum (8, 13). The ability of *G. vaginalis* to produce zones of hemolysis around colonies on human blood agar plates suggests that *G. vaginalis* may produce a cytolytic toxin. In this report, we describe the conditions needed to obtain optimal recovery of cytolysin from *G. vaginalis* cultures, methods for its preparation, and its biological properties.

MATERIALS AND METHODS

Bacterial culture and cytolysin production. The *G. vaginalis* strain used was a clinical isolate meeting the biological criteria for *G. vaginalis* reported by Greenwood and Pickett (19) and by Piot et al. (31) and having biochemical markers identical to those of the reference strain ATCC 14018. Bacteria were seeded in trypto-casein-soya broth (Diagnostic Pasteur, Marnes, France) supplemented with 2% human plasma and 0.4% *G. vaginalis* selective supplement (TSB/HP) (Oxoid Limited, Basingstoke, United Kingdom). Cultures were maintained at 37°C in an anaerobic jar (BBL, Cockeysville, Md.) equipped with Anaerocult reagent (Merck, Darmstadt, Federal Republic of Germany) and routinely subcultured every 36 h. For the production of cytolysin, 10^7 bacteria were inoculated in 150-ml flasks containing 125 ml of TSB/HP, 0.1% starch (Merck), and 0.3% nonionic detergent (Tween 80) (BDH, Poole, United Kingdom), unless otherwise stated. The inoculated medium was then incubated anaerobically for 36 h at 37°C before harvesting. The number of bacteria was evaluated by the dilution method and by counting the CFU in Columbia blood agar base (Oxoid) containing 2.5% human defibrinated blood and 0.4% *G. vaginalis* selective supplement. Cultures were incubated at 37°C in anaerobic conditions, as described above.

Hemolytic assay. The assay mixture contained (1 ml final volume) Krebs-Ringer phosphate buffer (pH 7.4) (KRP), 50 μl of a 1% suspension of group O human erythrocytes, and the required volume of sample. Tubes were incubated at 37°C for 30 min. After centrifugation (1,200 × *g* for 3 min), the A_{415} of the supernatant fluid containing the hemoglobin released from lysed erythrocytes was measured by a Lambda 5 spectrometer (Perkin Elmer, Uberlingen, Federal Republic of Germany). In each experiment, the concentration of erythrocytes was adjusted to give an A_{415} of 0.90 upon complete hemolysis. This was accomplished by diluting the same amount of erythrocytes in distilled water instead of KRP. One hemolytic unit (HU) was defined as the sample volume required to cause 50% hemolysis.

Purification of cytolysin. The whole procedure of cytolysin purification was carried out at 4°C. The culture supernatant (crude preparation) was obtained by centrifugation of *G. vaginalis* cultures at 10,000 × *g* for 15 min. The crude preparation was diluted with two volumes of distilled water in order to lower its ionic strength, and the pH was adjusted to 5.0 with 0.1 M NaOH. The preparation was applied to a column (4.0 by 20.0 cm) of SP-Sephadex C50 (Pharmacia, Uppsala, Sweden) equilibrated with 10 mM sodium acetate buffer and 0.1 M $(NH_4)_2SO_4$, pH 5. After washing with four bed volumes of the same buffer, the cytolysin was eluted with 10 mM sodium phosphate buffer, pH 7.4, containing 0.3 M ammonium sulfate and 10 mM octylglucoside (*n*-octyl-β-D-glucopyranoside; Sigma Chemical Co., St. Louis, Mo.), a detergent that allowed both dialysis and concentration of the hemolysin by ultrafiltration. Fractions of 4 ml were collected. The fractions with hemolytic activity were pooled and concentrated by ultrafiltration in an Amicon chamber (Amicon, Lexington, Mass.) equipped with a YM-30 Diaflo membrane (Amicon). The concentrated material was then chromatographed in an Ultrogel AcA-44 gel filtration column

* Corresponding author.

(1.5 by 95 cm; separation range, 10 to 150 kDa) (LKB, Bromma, Sweden) equilibrated and eluted with 10 mM sodium phosphate, pH 7.4, containing 0.3 M ammonium sulfate and 10 mM octylglucoside. Fractions (4.1 ml) were collected and assayed for hemolytic activity and protein concentration (A_{280}). Peak hemolytic activity fractions were pooled and concentrated by ultrafiltration as described above. Calibration of the AcA-44 column was obtained by using three protein standards, carbonic anhydrase (29 kDa; Sigma), bovine serum albumin (66 kDa; Sigma), and hexokinase (102 kDa; Sigma), under identical conditions of equilibrium and elution. Portions (15 μg) of the purified cytolysin were subsequently analyzed by 8.5% polyacrylamide gel electrophoresis (PAGE) under denaturing conditions. The initial current intensity was 4 mA until the bromophenol blue marker dye entered the separating gel. The amperage was then increased to 8 mA and run for 18 h. Gels were then blotted onto cellulose nitrate (37) and stained either with 2% amido black in a methanol-acetic acid solution or immunochemically, as described below. The Rainbow protein marker (Amersham Int., Buckinghamshire, United Kingdom) or the proteins myosin (205 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), and egg albumin (45 kDa) (Sigma) were used as standards for molecular mass determination.

Immunostaining. Monoclonal antibodies (MAb) were generated by immunizing BALB/c mice with gel filtration-purified cytolysin. Splenocytes were fused with the NS-1 nonsecretory murine myeloma line, as described by Galfrè et al. (16). The supernatants giving positive results in the dot spot assay were then tested for inhibition of hemolytic activity. This was achieved by incubating 30 HU of toxin with different amounts of each MAb, previously concentrated by 50% ammonium sulfate precipitation, in a final volume of 150 μl of KRP containing 10 mM octylglucoside and 0.3 M ammonium sulfate for 30 and 120 min at room temperature. 10 μl (2 HU) of this mixture was used to test hemolytic activity as described above. The most active supernatant, in terms of both binding to the cytolysin and inhibition of hemolytic activity, contained immunoglobulin G2a, determined by using the mouse MAb typing kit (ICN Immuno Biologicals, Lesle, Ill.) and was used in subsequent experiments. This clone remained positive after subcloning five times. Western blots of sodium dodecyl sulfate (SDS)-PAGE gels of the post-ion-exchange chromatography material or the post-gel filtration chromatography material were incubated with this MAb, named 5/1, which was obtained by 50% ammonium sulfate precipitation from the whole supernatant. The nitrocellulose strips were reacted with a biotinylated rabbit anti-mouse antibody (Zymed Laboratories, San Francisco, Calif.) and probed with streptavidin-conjugated alkaline phosphatase complex (Jackson Immunoresearch, Baltimore, Md.) by using 5-bromo-4-chloro-3-indolyl phosphate (Sigma) and Nitro Blue Tetrazolium chloride (Merck) as substrates. Controls were performed with all reagents but by omitting MAb 5/1 or using an unrelated mouse immunoglobulin G2a.

Detection of biotinylated toxin by [^{35}S]streptavidin. Portions of purified cytolysin were conjugated to biotin following the procedure described by Updyke and Nicolson (38). Buffers used in the biotinylation and dialysis procedures were added with 0.3 M ammonium sulfate and 10 mM octylglucoside. The biotinylated material was then incubated in KRP or KRP containing 0.3 M ammonium sulfate and 10 mM octylglucoside for 1 h at room temperature, chromatographed in an Ultrogel AcA-34 gel filtration column (1.5 by 42 cm) with a separation range between 350 and 20 kDa, equilibrated, and eluted with KRP containing 0.3 M ammonium sulfate and 10 mM octylglucoside. Samples eluted from the column were grouped in five 10- to 15-ml pools on the basis of molecular mass. Each pool was then passed through a cellulose nitrate disk mounted in place of the filter disk on a 25-mm glass microanalysis support (Millipore, Bedford, Mass.). Cellulose nitrate disks with biotinylated material were then treated with phosphate-buffered saline (PBS) containing 2% bovine serum albumin and washed three times with 15 ml of PBS containing 0.1% Tween 20 (Sigma). Disks were then incubated for 30 min at 37°C in a 24-well plate (Costar, Cambridge, Mass.) with 200 μl of PBS containing 5 μCi of [^{35}S]streptavidin (The Radiochemical Centre, Amersham, United Kingdom) per ml. After three 15-min washings with PBS, disks were dried and transferred to vials containing 10 ml of Insta gel II (Packard, Zürich, Switzerland) and counted in a liquid-phase 1215 Rock beta II counter (LKB).

Assay of phospholipase activity. Phospholipase C activity of the cytolysin (10 to 200 ng) was assayed spectrophotometrically by using *p*-nitrophenylphosphoryl-choline (Sigma) as described by Berka et al. (3) or 2-(*N*-hexadecanoylamino)-4-nitrophenyl-phosphocholine (Sigma) as described by Bernheimer and Bey (4) as the chromogenic substrate. Purified phospholipase C (Sigma) and sphingomyelinase (Sigma) were used in control assays.

Protein determination. Proteins were assayed by using the protein assay reagent bicinchoninic acid (Pierce Eurochemie, Oud-Beijerland, Netherlands). Egg white lysozyme (Sigma) was employed as the standard.

Isolation of PMN. Polymorphonuclear leukocytes (PMN) were isolated from peripheral blood obtained by venipuncture of healthy donors. The blood was collected into acid-citrate-dextrose, and the neutrophils were isolated by sedimentation in 4.5% Dextran T500 (Pharmacia, Uppsala, Sweden), ratio 1:5, followed by Ficoll-Paque (Pharmacia) gradient centrifugation and hypotonic saline lysis, as described previously (35).

Isolation of HUVEC. Human umbilical vein endothelial cells (HUVEC) were prepared by collagenase treatment of umbilical veins as described elsewhere (22) and maintained in endotoxin-free RPMI-1640 medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 20% fetal calf serum (Biochrom-Seromed, Berlin, Federal Republic of Germany), as previously described (12). Passaged HUVEC were maintained in RPMI-1640 supplemented with 20% fetal calf serum containing heparin (90 μg/ml; Sigma) and endothelial growth factor (50 μg/ml), as described by Thornton et al. (36). Endothelial cell growth factor was prepared from bovine hypothalamus according to the method of Maciag et al. (27). First- to fourth-passage HUVEC were harvested with 0.05% trypsin and 0.02% EDTA in balanced salt solution (GIBCO Laboratories). Cells were then plated in 11-mm-diameter wells (Costar Cluster) at 5×10^4 cells per well in RPMI-1640 supplemented with 20% fetal calf serum. Visually confluent monolayers (10^5 cells per cm^2) were formed after overnight incubation.

Cytolytic assay. Isolated peripheral blood PMN were suspended in KRP containing 5 mM glucose, at a final concentration of 10^7 cells per ml. Portions (0.2 ml) were then incubated for variable periods of time at 37°C with various amounts of hemolysin. Dye exclusion tests for cell viability were performed by mixing 50 μl of the PMN suspension with an equal volume of a 0.2% trypan blue solution in KRP and by enumerating under a light microscope the number of cells that had taken up the dye and that had excluded the dye.

TABLE 1. Generation of hemolytic activity in *G. vaginalis* cultures[a]

Additions to TSB/HP medium (concn)	No. of bacteria (CFU/ml)	Hemolytic activity (HU/ml)
None	0.9×10^{10}	57
Starch (0.1%)	10^{10}	102
Tween 80 (0.3%)	1.3×10^{10}	60
Octylglucoside (10 mM)	$<10^6$	0
Starch (0.1%) + Tween 80 (0.3%)	1.1×10^{10}	4,500
Starch (0.1%) + octylglucoside (10 mM)	$<10^6$	0

[a] Twelve 150-ml flasks, two for each experimental condition, each containing 125 ml of TSB/HP medium and the indicated supplements, were inoculated with 10^7 bacteria per flask and were incubated anaerobically for 36 h at 37°C. Portions from each culture were collected for bacterial count. Cultures were then centrifuged at 15,000 × *g*. Hemolytic activity was assayed in the culture supernatants. Values are means of duplicate flasks.

HUVEC lysis by cytolysin was evaluated in terms of release of ^{51}Cr by the cells essentially as described by Harlan et al. (20). Monolayers were incubated overnight with $Na^{51}CrO_4$ (1 μCi/ml in saline, 200 to 500 Ci/g) (Radiochemical Center) and then washed five times with RPMI-1640 to remove unincorporated radioactivity. Afterwards, 250 μl of RPMI-1640 with or without cytolysin was added to each well. Incubations were carried out at 37°C for variable periods of time. 50 μl of supernatant fluid was carefully withdrawn from each well and was then transferred into plastic tubes and counted in a gamma spectrometer. In each experiment, the maximum release of ^{51}Cr was determined from wells treated with 0.5% Triton X-100 in order to lyse the cells. Control (spontaneous) release was determined from wells incubated in RPMI-1640 alone. Results were expressed as percent specific ^{51}Cr release as follows: % release = (cpm test − cpm control)/(CPM Triton − cpm control).

Chemicals. Methyl-α-D-glucopyranoside, methyl-α-D-mannopyranoside, *n*-acetylglucosamine, *n*-acetyl-D-galactosamine, L-fucose, and *n*-acetylmuramic acid were purchased from Serva (Heidelberg, Federal Republic of Germany); phosphatidylserine, phosphatidylethanolamine, phosphatidylcholine, sphingomyelin, 2-mercaptoethanol, DL-dithiothreitol, collagenase type IV, and α-chymotrypsin type II were purchased from Sigma; and trypsin was purchased from Behringwerke (Marburg, Federal Republic of Germany).

RESULTS

Generation of hemolytic activity in *G. vaginalis* cultures. Table 1 shows that in TSB/HP medium alone hemolytic activity was always very low and sometimes hardly detectable. Addition of both starch (0.1%) and the nonionic detergent Tween 80 (0.3%) to the medium at the time of bacterial seeding increased the hemolytic activity by about two orders of magnitude (Table 1), whereas neither reagent alone had a relevant effect. In no instances was bacterial growth significantly influenced by starch and Tween 80. In contrast, bacterial growth was inhibited by the addition of the nonionic detergent octylglucoside to the medium. The addition of starch, Tween 80, or octylglucoside alone or starch in combination with either detergent after bacterial growth had ceased did not significantly influence the culture hemolytic activity, compared with that in TSB/HP alone. This indicates that the tremendous increase in hemolytic activity in the presence of starch and Tween 80 from the beginning of the culture cannot be attributed to bacterial lysis followed by release of the hemolytic activity. In all experimental conditions, more than 98% of hemolytic activity of whole cultures was recovered in the supernatant fluid after centrifugation at 10,000 × *g* for 15 min at 4°C. Figure 1 shows the time course of hemolytic activity generation during bacterial growth. Generation of hemolytic activity paralleled bacterial growth during the logarithmic and early stationary phases of growth (24 to 36 h after bacterial seeding, depending on the number of inoculated bacteria), but it was decreased at later time intervals, during the phase of bacterial death. Since the toxin sample used in the experiments described above contained either Tween 80 or octylglucoside, control experiments were carried out to test whether the detergents exerted a direct hemolytic effect or somehow sensitized erythrocytes to the toxin. Preincubation of erythrocytes for 30 min at 37°C in the presence of either detergent at the concentration present in the hemolytic assays did not produce hemolysis or increase the susceptibility of erythrocytes to the lytic action of the toxin preparations.

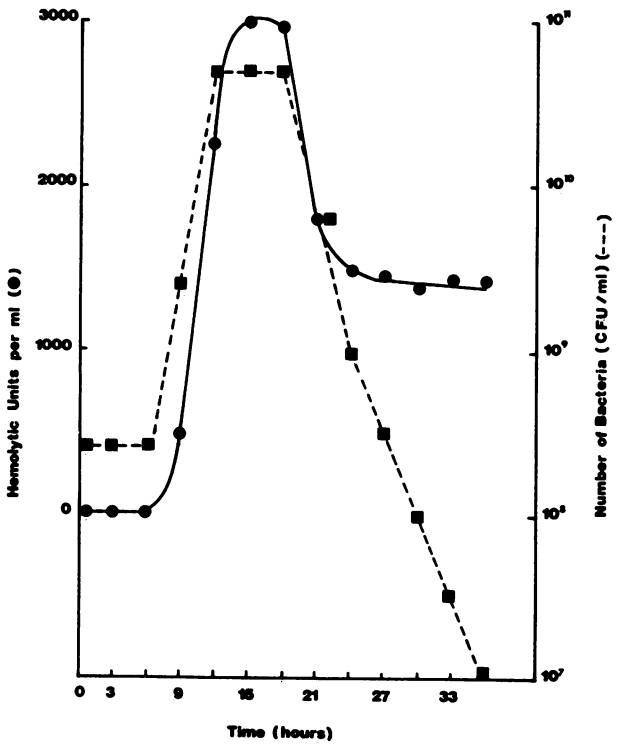

FIG. 1. Time course of generation of hemolytic activity during growth of *G. vaginalis*. A total of 5×10^{10} bacteria were seeded in 200 ml of growth medium containing 0.1% starch and 0.3% Tween 80 and were incubated at 37°C in anaerobic conditions. At selected time intervals, 1 ml of culture medium was tested for CFU (■). After centrifugation at 10,000 × *g*, hemolytic activity (●) was assayed in the supernatant.

Purification of cytolysin. Initial attempts of purification of hemolytic activity from culture supernatant by gel chromatography resulted in complete loss of activity. It was then found that partial protection of the activity was obtained by addition of Tween 80 and $(NH_4)_2SO_4$ to the buffers used for equilibration and elution of the column. However, Tween 80 did not permit concentration by ultrafiltration of the fractions collected from the column since a jelly material was formed. Therefore, Tween 80 was replaced with the nonionic, filterable detergent octylglucoside, which was as protective as Tween 80 for hemolytic activity. Results of ion-exchange chromatography of a crude preparation of *G.*

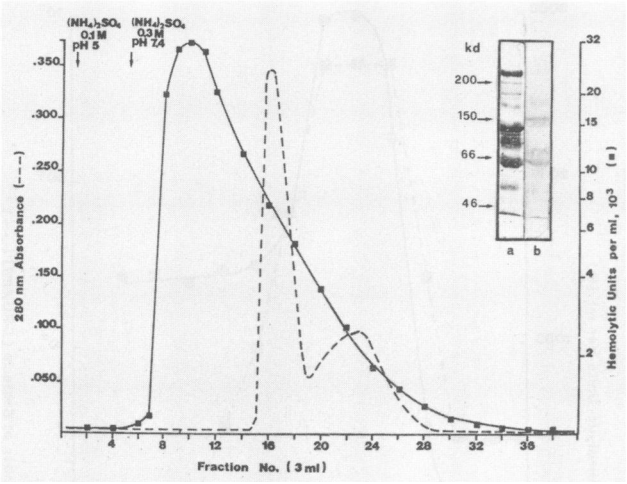

FIG. 2. Fractionation of a crude preparation of *G. vaginalis* toxin by SP-Sephadex C50 ion-exchange chromatography. About 1.53 g of protein was applied to a column (4 by 20 cm) equilibrated with 10 mM acetate buffer (pH 5) containing 0.1 M $(NH_4)_2SO_4$ and then washed with four beds of the same buffer. Elution was performed with 10 mM PBS buffer (pH 7.4) containing 0.3 M $(NH_4)_2SO_4$ and 10 mM octylglucoside. Fractions 8 through 24 (3 ml each) with hemolytic activity greater than 2,000 U/ml were pooled and concentrated by ultrafiltration. The inset shows the pattern of the concentrated material after SDS-PAGE and blotting onto cellulose nitrate, as revealed by amido black staining (a) or immunostaining with MAb 5/1 (b).

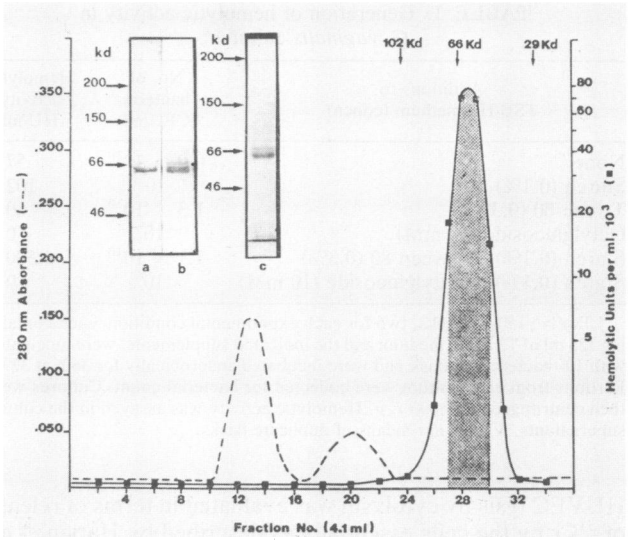

FIG. 3. AcA-44 Ultrogel filtration of pooled fractions obtained from ion-exchange chromatography. About 3 mg of protein was applied to a column (1.5 by 95 cm) equilibrated with 10 mM sodium phosphate buffer (pH 7.4) containing 0.3 M $(NH_4)_2SO_4$ and 10 mM octylglucoside and eluted with the same buffer at a flow rate of 6 ml/h. Fractions (4.1 ml) containing hemolytic activity (27 through 30, shaded area) were pooled and concentrated by ultrafiltration. Carbonic anhydrase (29 kDa), bovine serum albumin (66 kDa), and hexokinase (102 kDa) were used to calibrate the column. The inset shows the pattern of the purified toxin after SDS-PAGE and blotting onto cellulose nitrate. Blots were stained with amido black (a) or immunostained with MAb 5/1 (b), and gels were stained with Coomassie blue (c). The lower band on the gel indicates the migration front.

vaginalis hemolysin are shown in Fig. 2. Fractions with hemolytic activity higher than 2,000 U/ml were pooled and concentrated by ultrafiltration, and portions were electrophoresed on SDS-PAGE and blotted. At least nine major bands appeared after amido black staining, four of which (of 61, 74, 145, and 160 kDa relative molecular masses) reacted with the MAb 5/1 that inhibited the toxin activity, as described below. The remainder of the pooled fractions were then subjected to gel filtration chromatography on an AcA-44 Ultrogel column. The results are shown in Fig. 3. The hemolytic activity was eluted from the column in a single peak sharply separated from the two main peaks of protein. The molecular mass of the hemolysin was estimated to be about 63 kDa. Fractions 27 to 30 were pooled, concentrated by ultrafiltration, electrophoresed on SDS-PAGE, and blotted. A major band of 61 kDa relative molecular mass was seen both on gels stained with Coomassie blue and on blots stained with amido black or reacted with MAb 5/1 (Fig. 3, inset). This pattern was not modified by treatment of the sample with 2-mercaptoethanol (data not shown). This material (purified hemolysin) was used in subsequent experiments. The quantitative aspects of the purification procedure are shown in Table 2. An increase in specific activity of more than 1,000 times was obtained.

Effect of MAb 5/1 on the hemolytic activity of cytolysin. Preincubation of cytolysin with MAb 5/1 resulted in loss of its activity (Fig. 4). Inhibition of the hemolytic activity was dose dependent and increased by increasing the preincubation time from 30 to 120 min. In contrast, an unrelated mouse immunoglobulin G2a had no effect (data not shown).

Stability of cytolysin. When purified hemolysin was diluted in KRP (2.5 μg/ml, final concentration) and kept at room temperature, a 50% decay of activity was observed in 5 min and a 90% decay was observed in 30 min. Addition of 10 mM $(NH_4)_2SO_4$ partially protected hemolysin from decay, since 50 and 90% decay was observed after 15 min and 6 h, respectively. A better protection was obtained with 10 mM octylglucoside (50% decay at 3 h and 90% at 15 h). Combination of $(NH_4)_2SO_4$ and octylglucoside had an additive effect, since hemolytic activity was maintained for about 60 min and 50% activity was still present after 18 h. Decay of hemolytic activity in the absence of ammonium sulfate and octylglucoside was paralleled by a decrease in the amount of the elution products with molecular masses in the range of 40 and 80 kDa and by an increase of elution products with molecular masses higher than 80 kDa, suggesting that aggregation of toxin takes place in a polar medium and that this was accompanied by loss of activity (Fig. 5).

Hemolysin stability in the presence of ammonium sulfate and octylglucoside was temperature dependent: 20°C was the optimal temperature, whereas at both 4°C and 37°C a

TABLE 2. Purification of *G. vaginalis* toxin

Purification stage	Vol (ml)	Total protein (mg)	Total hemolytic activity (HU [10^3])	Sp act (HU [10^3]/ mg of protein)	Recovery (%)
Crude prepn	510	1,530	1,700	1.1	100
SP-Sephadex ion-exchange chromatography +YM-30 concn	10	3	558	186	33
AcA-44 Ultrogel filtration +YM-30 concn	10	0.254	370	1,457	22

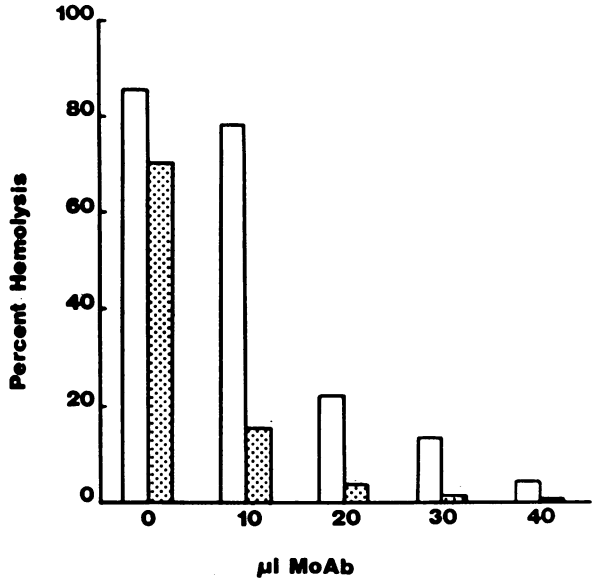

FIG. 4. Inhibition of the hemolytic activity of *G. vaginalis* toxin by MAb 5/1. A total of 30 HU of toxin was incubated with different amounts of MAb 5/1 in a final volume of 150 µl of KRP containing 10 mM octylglucoside and 0.3 M ammonium sulfate for 30 min (□) or 120 min (▨). A 10-µl sample (2 HU) from these mixtures was then used to test hemolytic activity.

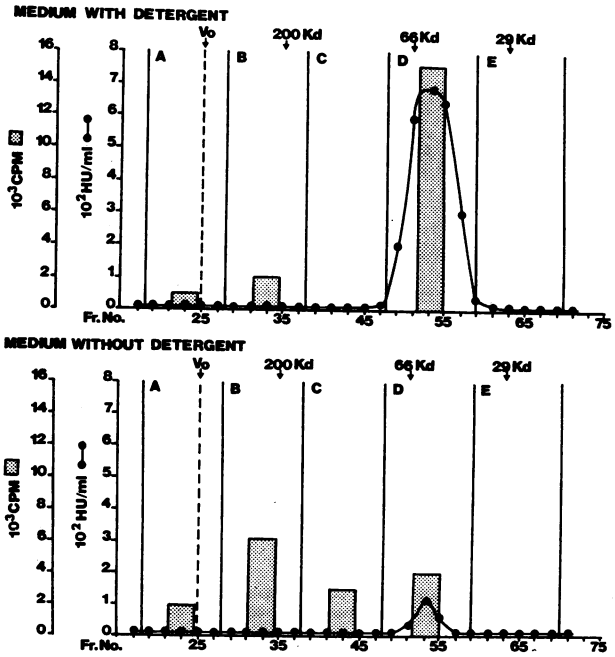

FIG. 5. Decay of *G. vaginalis* toxin and formation of high-molecular-mass products in the absence of detergents. Biotinylated *G. vaginalis* toxin (5 µg, equivalent to 7,300 HU) was maintained at room temperature for 60 min in 1 ml of KRP with or without 0.3 M ammonium sulfate and 10 mM octylglucoside, and each sample was then chromatographed in an Ultrogel AcA-34 gel filtration column. Carbonic anhydrase (29 kDa), bovine serum albumin (66 kDa), and beta-amylase (200 kDa) were used to calibrate the columns. Fractions (2.5 ml each) eluted from the columns were assayed for hemolytic activity. Elution fractions with relative molecular masses of about 350 (A), 350 to 170 (B), 170 to 80 (C), 80 to 40 (D), and 40 to 20 (E) kDa were pooled and filtered through cellulose nitrate disks. The biotinylated material retained by nitrate disks was then measured by radioactivity counting after incubation with [^{35}S] streptavidin. Vo, Void volume.

40% decay of the activity took place in 2 h. At 45, 60, and 80°C, a decay of activity of 50% was observed after 90, 2, and 0.5 min, respectively.

Binding of *G. vaginalis* toxin to erythrocytes. The need to use hemolysin immediately after dilution in KRP in order to reveal its full lytic activity suggests that it rapidly binds to the target and that binding results in its functional protection. This was tested in the following experiment. Hemolysin was diluted in KRP and incubated with erythrocytes at 4°C (at this temperature no lysis occurred, see below), and the erythrocytes were then exhaustively washed at 4°C and stored for various periods of time at 4°C. Portions of the purified toxin dilution in KRP or in KRP plus ammonium sulfate and octylglucoside were stored in parallel at 4°C for the same periods of time. At the end of each time interval, the hemolytic activity of the three samples was assayed. This was done by simply bringing the temperature of the erythrocyte suspension incubated with the toxin to 37°C or by adding suitable portions of the toxin incubated in the absence of erythrocytes to the usual hemolytic assay. The erythrocytes preincubated with the toxin were lysed at 37°C, indicating that binding of hemolysin had occurred (Fig. 6). The extent of lysis did not change appreciably, at least over 18 h, indicating that binding to the erythrocyte membrane stabilized hemolysin. In contrast, hemolysin rapidly lost activity when stored in KRP. Loss of activity was slower upon storage in KRP containing $(NH_4)_2SO_4$ and octylglucoside.

Effects of various substances on the hemolytic activity of *G. vaginalis* toxin. Trypsin and alpha-trypsin inactivated hemolysin at 37°C; papain and collagenase did not (Table 3). Inhibition was also obtained with phospholipids at micellar concentrations, by 2-mercaptoethanol, and by dithiothreitol. In contrast, carbohydrates were ineffective. None of the agents tested was hemolytic in the absence of toxin. Inhibition of hemolytic activity by phospholipids raised the question of whether the hemolysin had phospholipase activity. No phospholipase C activity was found in toxin preparations at doses up to 30 ng (corresponding to 500 HU) when tested with two substrates by the methods of Berka et al. (3) and Bernheimer and Bey (4).

Temperature dependence of hemolytic activity. A time course of hemolysis at various temperatures was obtained by adding 20 HU of hemolysin to 10 ml of a 0.05% human erythrocyte suspension and collecting samples (1 ml) at various times for spectrophotometric assay of hemolysis. The kinetics of hemolysis showed a sigmoidal curve and reached 100% hemolysis after 9 min at incubation temperatures of 30, 37, and 42°C. At 20°C, complete hemolysis was achieved in 15 min. In contrast, no hemolysis occurred at 4°C.

Target specificity. Erythrocytes from different species were incubated at 37°C for 30 min in the presence of various amounts of toxin. With human erythrocytes, 50% lysis was obtained with 0.75 ng of toxin. With rat erythrocytes, five times as much toxin was required to achieve the same extent of lysis. Horse, rabbit, sheep, and guinea pig erythrocytes were insensitive even at doses of toxin 100 times higher than those required for human erythrocytes.

Effect of cytolysin on cells other than erythrocytes. The influence of hemolysin on the integrity of human vascular endothelial cells and human neutrophilic leukocytes (PMN) was tested. Release of ^{51}Cr from endothelial cells was used

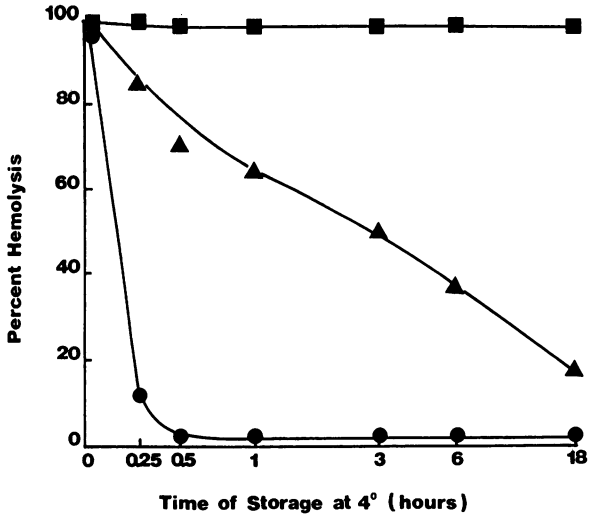

FIG. 6. Evidence that binding to erythrocytes at 4°C stabilizes the hemolytic activity of *G. vaginalis* toxin. A 1-ml portion of a 1% suspension of human erythrocytes was incubated with 40 HU of toxin at 4°C for 10 min, washed three times to remove the unbound toxin, and then stored at 4°C. Portions (50 μl) were withdrawn at various time intervals to test hemolysis at 37°C. As a control, toxin was kept at 4°C in KRP or in KRP plus 0.3 M $(NH_4)_2SO_4$ and 10 mM octylglucoside and was assayed for hemolytic activity at 37°C at the same time intervals. Symbols: ■, toxin preincubated with erythrocytes; ▲, toxin incubated without erythrocytes in KRP plus $(NH_4)_2SO_4$ and octylglucoside; ●, toxin incubated without erythrocytes in KRP.

TABLE 3. Effects of various substances on the hemolytic activity of *G. vaginalis* toxin[a]

Agent (concn)	Percent inhibition
Enzymes (U/ml)[b]	
Papain (2)	0
Trypsin (2)	87
α-Chymotrypsin (2)	91
Collagenase (2)	0
Phospholipids (μg/ml)	
Phosphatidylcholine (200)	90
Sphingomyelin (200)	80
Phosphatidylserine (200)	73
Phosphatidylethanolamine (200)	70
Carbohydrates (mM)	
Methyl-α-D-mannopyranoside (25)	0
Methyl-α-D-glucopyranoside (25)	0
n-Acetyl-D-galactosamine (25)	0
n-Acetyl-D-glucosamine (25)	0
n-Acetylmuramic acid (25)	0
L-Fucose (25)	0
Reducing agents (mM)	
2-Mercaptoethanol (75)	70
Dithiothreitol (10)	75

[a] Toxin (2 HU) was incubated with buffer alone (control) or with the indicated agents for 15 min at room temperature, and the hemolytic titer was then determined. Inhibition was calculated as follows: (hemolysis with substances/hemolysis in control samples) × 100.

[b] Enzyme units are defined as indicated by the manufacturer.

as a marker of cell injury (20), while the most commonly used vital dye exclusion test was employed with PMN. Hemolysin caused a dose-dependent ^{51}Cr release from endothelial cells in a 30-min incubation assay (Fig. 7). A time course experiment showed that ^{51}Cr release reached its maximum within 15 min of incubation. Figure 8 shows that hemolysin caused a dose-dependent uptake of trypan blue by PMN.

DISCUSSION

In the present study, we show that *G. vaginalis* produces and releases into the medium a protein with cytolytic activity towards erythrocytes and nucleated cells, such as endothelial cells and neutrophils.

Culture conditions for optimal recovery of hemolytic activity required incorporation of starch and the nonionic detergent Tween 80 into the medium at defined concentrations, as previously reported for group B *Streptococcus* hemolysin (28, 29). Explanation of this finding was not pursued in the present work, since our aim was to study cytolysin properties and its partial purification. In principle, starch and Tween 80 could facilitate release of hemolysin from the bacterial body into the medium, stimulate synthesis of hemolysin, or prevent its inactivation after release into the culture. The minimal hemolytic activity in the sonicate of *G. vaginalis* cultured in the absence of starch and Tween 80 rules out the first of the three possibilities. Some of the findings reported in this paper point to a protective effect of at least the detergent on hemolytic activity. These findings are essentially the following: (i) hemolytic activity soon disappeared when the crude hemolysin preparation (i.e., the culture supernatant) was diluted in buffer alone but was retained at least in part when Tween 80 was incorporated in the dilution buffer, (ii) another nonionic detergent, octylglucoside, could be substituted for Tween 80 in preventing decay of hemolytic activity, and (iii) even the purified preparation of the cytolysin required detergents in order to maintain stable hemolytic activity.

The mean specific activity of several preparations of our cytolysin, when tested on human erythrocytes, was about 1.5×10^6 HU per mg of protein. The relative molecular mass of purified cytolysin was estimated to be 63 kDa by AcA-44 Ultrogel filtration and 61 kDa by SDS-PAGE analysis fol-

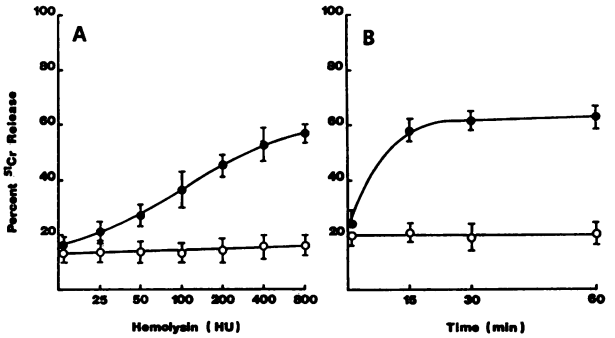

FIG. 7. *G. vaginalis* toxin-mediated endothelial cell injury. (A) Dose-response curve. Percent ^{51}Cr release was determined after a 30-min exposure of endothelial cells to various concentrations of *G. vaginalis* toxin or to equal amounts of the buffer used to dissolve the toxin (KRP plus 0.3 M $(NH_4)_2SO_4$ and 10 mM octylglucoside). (B) Time course. Percent ^{51}Cr release was determinated after exposure of endothelial cells to 200 HU of cytolysin for the indicated time periods. Symbols: ●, toxin; ○, control buffer. Values are means of 3 replicates ± standard error.

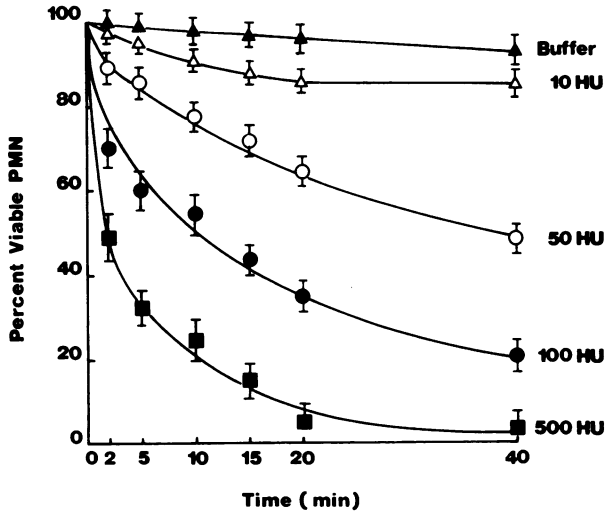

FIG. 8. Effect of *G. vaginalis* toxin on trypan blue uptake by human PMN. A total of 2×10^6 PMN in 0.2 ml of KRP containing 5 mM glucose was used in each assay. As a control, 13 μl of buffer [KRP plus 0.3 M $(NH_4)_2SO_4$ and 10 mM octylglucoside], that is, the maximum volume employed in the experiment with 500 HU of toxin, was used. Values are means of four replicates ± standard error.

lowed by blotting and immunostaining with a MAb that inhibited cytolysin hemolytic activity. Our results also indicate that the cytolysin tends to aggregate, particularly in polar environments, and that the aggregates are hemolytically inactive. In fact, decay of hemolytic activity in the absence of detergents was accompanied by an increased formation of products of higher molecular mass than that of the active cytolysin (Fig. 5, bottom). A small amount of such products was also present in the cytolysin preparations kept in the presence of detergents (Fig. 5, top). This is probably the reason why MAb 5/1 stained additional bands, besides the 61 kDa band of the presumably monomeric cytolysin, on SDS-PAGE nitrocellulose blots of material obtained by ion-exchange chromatography. We suggest that the 164-, 145-, and 160-kDa bands are aggregates of the cytolysin or of its degradation products. The functional inactivation and spontaneous aggregation of *G. vaginalis* cytolysin in a polar environment, which is prevented by ammonium sulfate and nonionic detergents, suggests that it has an amphiphilic nature. This is also supported by its affinity for phospholipids and by the finding that it maintains the activity for a long time when associated with the membrane of erythrocytes, where it probably finds a favorable hydrophobic environment.

The cytolysin was heat labile and was inactivated by proteases such as trypsin and α-chymotrypsin, which is suggestive of its protein nature. Furthermore, its inactivation by reducing agents, such as mercaptoethanol and dithiothreitol, suggests that disulfide bonds may be relevant to the molecule function. The latter finding clearly differentiates *G. vaginalis* hemolysin from the group of sulfydryl-activated hemolysins (1). Hemolysin showed a marked target specificity for human erythrocytes. In fact, erythrocytes from horses, rabbits, sheep, and guinea pigs were resistant even at cytolysin concentrations 100 times higher than those effective on human erythrocytes, and rat erythrocytes were lysed by a concentration 5 times higher than that required for human erythrocytes. In this respect, *G. vaginalis* cytolysin differs from the cytolytic toxins produced by other bacteria frequently harbored by vaginal mucosa, such as Group B streptococci (28, 29), *Escherichia coli* (7), and *Staphylococcus* sp. (6), that preferably lyse erythrocytes from species other than humans. Also, human neutrophilic granulocytes and human endothelial cells, two potential targets of *G. vaginalis* in vivo, were susceptible in vitro to lysis by *G. vaginalis* cytolysin, although at doses higher than those required for human erythrocytes. The lower susceptibility to lysis of nucleated cells, compared with that of erythrocytes, has already been reported for other known cytolysins and is usually attributed to the existence of repair mechanisms in the former cells (5). Studies on the mechanism of lysis, apparently not linked to phospholipase activity, are in progress.

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