Functional similarity between the haemolysins of *Escherichia coli* and *Morganella morganii*

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Summary. Haemolysin produced by a clinical isolate of *Morganella morganii* was examined for antigenic relatedness to the haemolysin of *Escherichia coli* and for similarities in mode of action. The *M. morganii* haemolysin migrated in SDS-PAGE as a single protein band with a slightly higher molecular weight than that of *E. coli* haemolysin. Several murine monoclonal antibodies against *E. coli* haemolysin cross-reacted with the *M. morganii* haemolysin in Western blots. Diminished haemolysis in the presence of osmotically-stabilising solutes indicated the formation of a pore by *M. morganii* haemolysin with an effective diameter of 1.5–3 nm. Results from dose-response experiments indicated that a single “hit” was sufficient for lysis of an erythrocyte. Detergent solubilisation of toxin-treated membranes led to recovery of bound toxin exclusively in monomeric form. *M. morganii* haemolysin was a potent leucocidin, that caused rapid leakage of ATP and death of human polymorphonuclear leucocytes. Under in-vitro conditions *M. morganii* haemolysin displayed similar leucocidal and haemolytic efficiency. The data demonstrate that *M. morganii* haemolysin shows functional properties virtually identical with those of *E. coli* haemolysin.

Introduction

In a recent study, 56% of clinical isolates of *Morganella morganii* were haemolytic and produced a filterable haemolysin.¹ Production of this haemolytic toxin appeared to be a virulence factor because intranasal application of haemolytic strains in mice caused death within a short time and haemorrhagic lung oedema was found at autopsy.² Moreover, in an intraperitoneal virulence assay, mice receiving haemolytic strains developed haemoglobinuria, and the LD50 was 10 times lower than that of non-haemolytic strains. Because of their similar biological effects, Emödy *et al.*² predicted that *M. morganii* and *Escherichia coli* haemolysins might be structurally related to each other.

Recently, clear, albeit incomplete, homology between the regions encoding production of haemolysins in *M. morganii*, *E. coli* and *Proteus spp.* and of leucotoxins of *Pasteurella haemolytica* and *Actinobacillus actinomycetemcomitans* were found by Southern hybridisation experiments.^{1, 3–7} These cytotoxins thus form a newly recognised family of toxins that may have a similar mode of action,

because of similarities in their predicted protein structure. Functional studies have demonstrated that the cytotoxins of *E. coli* and *P. haemolytica* exert their cytotoxic action by generating discrete transmembrane pores in the plasma membranes of target cells.^{8,9} In vitro, *E. coli* haemolysin acts on erythrocytes of many species,¹⁰ and on renal tubular cells;¹¹ it has recently been identified as a powerful leucocidin.¹² In contrast, the *P. haemolytica* cytotoxin has minimal haemolytic activity and attacks only leucocytes of ruminants.¹³

The purpose of the present study was to obtain information on the mode of action of *M. morganii* haemolysin and compare it with that of *E. coli* haemolysin.

Materials and methods

Preparation of haemolysins

The haemolytic *M. morganii* strain used for production of haemolysin was isolated in our laboratory from a wound swab. It was identified to species level by means of the API 20E test kit (Bio Merieux, Nürtingen, FRG). From an overnight culture, 100 µl were added to 20 ml of Todd-Hewitt Broth (Difco Laboratories, Detroit, MI, USA) containing 10 mM CaCl₂. After incubation in a waterbath

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at 37°C with agitation for 3.5 h, the bacteria were pelleted by centrifugation (5 min, 20 000 g) and the supernatant fluid was filtered (Millex GV 4, 0.22 µm, low protein binding; Millipore, Eschborn, FRG). In some experiments these crude supernates (haemolytic titres usually 32–64 HU/ml) were used without further processing; in others, the haemolysin was concentrated by precipitation with polyethylene glycol 4000 20% as described previously for *E. coli* haemolysin.⁸ The precipitates, obtained from 20 ml of supernate and dissolved in 200 µl of distilled water, yielded titres of 500–2000 HU/ml. *E. coli* haemolysin was prepared as described previously.⁸

Titration for determination of haemolytic activity were performed as described previously:¹⁴ a haemolysin dilution lysing 60% of a rabbit erythrocyte suspension, 10% in phosphate-buffered saline, within 1 h at 37°C was defined as containing 1 HU of toxin/ml.

Cross-reaction of E. coli haemolysin-antibodies with M. morganii haemolysin

Western blots of *M. morganii* haemolysin were performed according to a protocol already described¹⁵ and developed with murine monoclonal antibodies raised against *E. coli* haemolysin.¹⁶

Sucrose density centrifugation of haemolysin-treated erythrocyte membranes

Sheep erythrocytes were treated with *M. morganii* haemolysin (final concentration 80 HU/ml) for 1 h at 37°C. The membranes were washed, solubilised with 250 mM deoxycholate detergent and centrifuged in a 10–50% w/v sucrose density gradient (150 000 g, 16 h, 4°C). Ten equal fractions were collected from each gradient and analysed by immunoblotting.⁸

Effect of erythrocyte concentration on number of lysed cells

The experiment followed the protocol of Jorgensen *et al.*¹⁷—200-µl samples of cell suspensions containing $(0.5–28) \times 10^8$ cells/ml were incubated with 200 µl of toxin (final toxin concentration 0.1 or 0.05 HU/ml), or with 200 µl of PBS. After 60 min at 37°C, haemolysis was determined spectrophotometrically. For each erythrocyte concentration, the average reading of three tubes minus the average reading of three blank tubes was determined. The number of lysed erythrocytes was calculated by comparing the absorbance with that of samples completely lysed by detergent.

Osmotic protection experiments

Rabbit erythrocytes (5×10^8 cells/ml) were suspended in buffer (90 mM NaCl, 45 mM KCl, 12.5 mM phosphate, pH 7.2) containing one of the following substances (60 mM): erythrol, arabinose, sucrose, raffinose (Merck AG, Darmstadt, FRG), dextran FP1 (M_r c. 900–1200)

and dextran 4 (M_r c. 4000–6000) (Serva, Heidelberg, FRG). Haemolysin was serially diluted in carbohydrate-free buffer and one volume of rabbit erythrocyte suspension was added to each sample. Haemolysis was read after 60 min at 37°C by measurement of haemoglobin absorbance at 412 nm in the supernates. The dilution of toxin causing 60% haemolysis was determined with each protective substance. The haemolytic titre recorded was compared with the control titre obtained in the absence of the carbohydrates. In the presence of dextran 4 the erythrocytes did not lyse. They were then washed thrice in dextran-containing buffer, resuspended in dextran-free buffer, and the haemolytic titre was determined after incubation for 60 min at 37°C.

Toxin-induced transmembrane K^+ - and $^{45}Ca^{2+}$ -fluxes were detected by measuring changes in the concentrations of the ions in the supernates of dextran-protected erythrocytes.⁸

ATP-release from polymorphonuclear leucocytes (PMNL)

Human PMNL were isolated as described previously.¹² ATP-efflux from the cells was detected by the firefly assay and continuously recorded by means of a Whole-blood Aggrometer (model 500 VS, Chrono Log Corporation, Haverton, USA) equipped with a 2210 2-channel Recorder (Pharmacia LKB, Bromma, Sweden): 170 µl of PBS was pre-warmed to 37°C and 170 µl of firefly reagent (ATP Bioluminescence CLS, Boehringer, Mannheim, FRG) and 40 µl of PMNL suspension (5×10^7 cells/ml) were added. After 60 s, 10 µl of haemolysin were added to give a final concentration of haemolysin in the range 0.005–10 HU/ml.

In a second assay, haemolysin was serially diluted in 100 µl of Hanks's Balanced Salts Solution (HBSS) and 15 µl of a cell suspension (5×10^7 cells/ml) were added to each sample. After 1 h at 37°C, the residual ATP-content of the cells was determined in the following manner: 30 µl of a cell sample were pipetted into 300 µl of a pre-warmed (37°C) solution of firefly reagent containing 3 mM Triton X-100 (Serva, Heidelberg, FRG). The detergent caused instant disruption of the plasma cell membrane, and liberated cellular ATP could be quantified by bioluminescence. The ATP-content of the cells was expressed as the percentage of that of the controls. Haemolytic assays were performed in parallel with the same haemolysin preparations and employing similar target-cell concentrations (5×10^7 erythrocytes/ml). Additionally the same experiments were conducted in the presence of autologous serum instead of buffer.

Results

Electrophoretic behaviour of M. morganii haemolysin

Culture supernates harvested in the late log-phase displayed titres of 64–128 HU/ml. Haemolytic activity was lost on standing (at 37°C within

hours, at 0°C within 1 day). Concentrated haemolysin samples obtained by PEG-precipitation yielded titres of 500–2000 HU/ml. These haemolysin preparations exhibited a single protein band in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with an apparent molecular weight slightly above that of *E. coli* haemolysin (Mr 107 000). Polyclonal rabbit antiserum against *E. coli* haemolysin cross-reacted strongly with the *M. morganii* haemolysin in Western blots. A panel of monoclonal murine antibodies raised against *E. coli* haemolysin¹⁶ also cross-reacted with the *M. morganii* haemolysin (fig. 1).

Osmotic protection experiments

The presence of a sugar or a dextran reduced the haemolytic titre to an extent that depended on the size of the added substance and on the length of incubation (fig. 2). Erythrol (molecular radius 0.28 nm) caused no significant reduction in titre, whereas molecules with radii of 0.3–0.8 nm reduced the speed of lysis. Dextran 4 (molecular radius $\approx$ 1.5 nm) protected erythrocytes and, after incubation for 60 min, no haemolysis was found even at high haemolysin doses. In the latter case, unbound toxin was removed by washing the cells in buffer containing dextran 4. Subsequent incubation of these cells in dextran-free buffer resulted in haemolysis, showing that protection of erythrocytes by dextran 4 was not due to an inhibition of haemolysin binding to the cells.

The existence of pores in membranes of dextran-protected cells was confirmed by measurements of ion fluxes. As shown in fig. 3, a very rapid efflux of K^+ and a parallel influx of $^{45}Ca^{2+}$ into the cells

occurred that was complete within 1–2 min following haemolysin application.

Effect of erythrocyte concentration on number of lysed erythrocytes

When a constant amount of *M. morganii* haemolysin was added to erythrocyte suspensions containing increasing numbers of cells, the amount of released haemoglobin increased with increasing cell concentrations up to a maximum value (fig. 4). Doubling of haemolysin concentration doubled the number of lysed cells. This dose-response behaviour indicates a “single hit” mechanism of cell lysis.²⁰

Properties of membrane-bound toxin

Membranes of haemoglobin-treated erythrocytes were solubilised with deoxycholate and centrifuged through a sucrose density gradient. Subsequent analysis of the density gradient fractions by SDS-PAGE and Western blotting showed that *M. morganii* haemolysin was present exclusively in monomeric form in detergent. The results (not shown) were identical with those previously obtained with *E. coli* haemolysin.⁸

Effect of *M. morganii* haemolysin on PMNL

Application of *M. morganii* haemolysin to human PMNL evoked rapid release of ATP, lag periods of seconds being noted at moderate haemolysin doses. With decreasing haemolysin doses, the lag phase between toxin application and commencement of ATP-release increased (fig. 5A). In a second type

Fig. 1. Western blots of *M. morganii* haemolysin (left lanes) and *E. coli* haemolysin (right lanes) developed with a polyclonal rabbit antiserum against *E. coli* haemolysin (a) and with different murine monoclonal antibodies against *E. coli* haemolysin: b, clone h2A; c, clone h11A; d, clone h11D; e, clone f11F; f, clone k12B; g, clone 11C¹⁶.

Fig. 2. (A) Parallel titrations (60 min, 37°C) of *M. morganii* haemolysin in presence of different carbohydrates (30 mM final concentration): ○, arabinose (M_r 150); ▲, sucrose (M_r 342); △, raffinose (M_r 504); ■, dextran FPI (M_r 900–1200); □, dextran 4 (M_r 4000–6000); ●, control (buffer). The dotted line indicates 60% haemolysis. (B) Titres in the presence of the carbohydrates are expressed as a percentage of the control titre measured in buffer. Average values of five independent experiments are shown. Molecular radii (nm) of sugars¹⁸ and dextrans¹⁹ are: erythrol 0.28 (◆); arabinose 0.31 (○); sucrose 0.46 (▲); raffinose 0.57 (△); dextran FPI c. 0.8 (■); dextran 4 c. 1.5 (□).

of assay, PMNL were incubated with various haemolysin doses for 60 min. Cells were solubilised with Triton to liberate cellular ATP. The relative amount of cellular ATP were determined at each applied haemolysin dose taking the ATP-content of control (untreated) cells as 100%. No residual ATP in the cells could be detected with haemolysin doses higher than 0.06–0.3 HU/ml and this corresponded with positive trypan blue staining of all cells. Parallel experiments with erythrocytes from the same donors were performed and the degree of haemolysis with each haemolysin dose was determined. As shown in fig. 5B, concentrations of

haemolysin that evoked ATP-depletion corresponded exactly with haemolytic activity, which indicates that the leucocidal potency of *M. morganii* haemolysin matches its haemolytic capacity in protein-free buffer. When cells were suspended in autologous serum, erythrocytes were found to be protected from lysis by up to 50 HU/ml of the *M. morganii* haemolysin. In contrast, PMNL succumbed to attack by only 10 HU/ml of the haemolysin in the same conditions.

Discussion

The properties of native *M. morganii* haemolysin resembled those of *E. coli* haemolysin. In SDS-PAGE the *M. morganii* haemolysin displayed a slightly higher molecular weight than *E. coli* haemolysin. Cross-reaction of monoclonal antibodies with both haemolysins indicated the presence of common epitopes. Like that of other members of this family of toxins, the activity of *M. morganii* haemolysin seems to be calcium-dependent. Haemolysin produced by bacteria grown in medium without calcium supplementation showed virtually

Fig. 3. Efflux of K^+ (A) and influx of $^{45}Ca^{2+}$ (B) in dextran-protected rabbit erythrocytes (3×10^9 cells/ml) induced by *M. morganii* haemolysin 2 HU/ml.

Fig. 4. Effect of increasing erythrocyte concentration on number of cells lysed by haemolysins (●, 0.1 HU/ml; ■, 0.05 HU/ml) after 60 min at 37°C. Each point represents the average reading from three samples minus the average of three control samples. Numbers of lysed erythrocytes were calculated on the basis of absorbance measurements at 412 nm of released haemoglobin in cell supernates.

no haemolytic activity, but could be "activated" by addition of 10 mM CaCl_2 . After standing, the haemolysin of *M. morganii* lost activity at a rate similar to that of *E. coli* haemolysin. The nature of this inactivation process remains unknown, but does not appear to be due to proteolysis.⁸

The *M. morganii* haemolysin causes haemolysis by generating a pore in the erythrocyte membrane. Addition of 30 mM dextran 4 to the incubation buffer prevented haemolysin-induced lysis of erythrocytes, whilst rapid leakage of K^+ and influx of $^{45}\text{Ca}^{2+}$ could be measured in these cells. Dextran 4 is obviously too large to pass the haemolysin pore and prevents rupture of the erythrocytes by counterbalancing the intracellular colloid-osmotic pressure exerted by haemoglobin. On the basis of the osmotic protection experiments, we estimate a functional pore diameter of 1.5–3 nm, equivalent to the estimated size of the *E. coli* haemolysin pore.^{8,21}

That the haemolytic efficacy of a given amount of *M. morganii* haemolysin was not diminished by the addition of large numbers of potential target cells indicated that a single haemolysin "hit" was sufficient for the lysis of an erythrocyte. After detergent-solubilisation of haemolysin-treated membranes, bound haemolysin was found exclusively in monomeric form. Analogous experiments have yielded similar results with *E. coli* haemolysin,^{8,17} which leads to the conclusion that *E. coli* haemolysin generates pores by insertion as a monomer into the membrane.

Because *E. coli* haemolysin has recently been demonstrated to exhibit great leucocidal potency,¹² we tested the possibility that the *M. morganii* haemolysin might also attack human granulocytes. *M. morganii* haemolysin caused leakage of ATP and death of attacked cells. In our assay, the leucocidal efficiency of *M. morganii* haemolysin was as high as its haemolytic efficiency when cells were

Fig. 5. (A) ATP-release from human PMNL (5×10^6 cells/ml of PBS) induced by *M. morganii* haemolysin. Haemolysin was applied (I) at the final concentrations stated and released ATP measured by bioluminescence. Time scale: one column represents 1 min. (B) Comparison of leucocidal and haemolytic efficiency of *M. morganii* haemolysin. Upper panel: relative amounts of cellular ATP in human PMNL (7.5×10^6 cells/ml) after incubation with haemolysin for 60 min at 37°C. Lower panel: parallel titration (60 min, 37°C) with erythrocytes (7.5×10^6 cells/ml) of the same donor. In both experiments cells were suspended in HBSS. The haemolysin preparation exhibited an activity of 200 HU/ml.

suspended in protein-free buffer, but if titrations were done in autologous serum, the leucocidal effect clearly surpassed haemolytic capacity. Similar findings have been reported for *E. coli* haemolysin.¹²

In conclusion, all the data have shown complete parallelism between the action of *M. morganii* haemolysin and *E. coli* haemolysin on human erythrocytes and PMNL. Within the family of

homologous toxins from gram-negative organisms, the cytolytins of *E. coli*, *M. morganii* and, presumably, *Proteus spp.* appear to comprise a sub-group exhibiting very similar functional properties that distinguish them from *P. haemolytica* cytolytin, which displays marked species and cell-target specificity. This divergence in functional properties may in part account for the differences in pathogenicity of the respective bacterial species.

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