Interaction of human Tamm–Horsfall glycoprotein with *Bordetella pertussis* toxin

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Tamm–Horsfall glycoprotein (THP), which is synthesized by renal tubular cells, is the most abundant protein in normal human urine. Although its physiological function remains unclear, it has been proposed that THP may act as a defence factor against urinary tract infections by inhibiting the binding of S- and P-fimbriated *Escherichia coli* to renal epithelial cells. Because THP-related proteins are also found in the superficial layers of the oral mucosa, the authors investigated the ability of THP to interfere with the cytoadherence of pathogenic bacteria that colonize mucosal surfaces other than those of the urogenital tract. In this report, it is shown that THP binds to virulent *Bordetella pertussis* and reduces its adherence to both renal and pulmonary epithelial cells. This cytoadherence inhibitory effect was not observed with a *B. pertussis* mutant lacking the pertussis toxin (PTX) operon, and was dependent on the direct interaction of THP with the S2 subunit within the PTX B oligomer. The authors also show that the glycosylation moiety of THP is crucial for its binding to PTX. The THP–PTX interaction was exploited to develop an affinity chromatography method that allows a one-step purification of active PTX. These observations suggest that besides its anti-adherence activity, THP may also trap toxins produced by pathogenic bacteria that colonize mucosal surfaces.

Keywords: cytoadherence, purification, virulence

INTRODUCTION

Tamm–Horsfall glycoprotein (THP; 90 kDa) is the most abundant protein found in normal human urine and its daily excretion ranges from 20 to 200 mg (Hunt *et al.*, 1985; Tamm & Horsfall, 1952). The carbohydrate moiety of THP is essentially composed of N-linked sugars, and accounts for 30% of the protein by weight (Easton *et al.*, 2000; Hard *et al.*, 1992). THP is synthesized by the renal tubular cells of the thick ascending limb of the loop of Henle (Bachmann *et al.*, 1990), and it has been postulated that THP might be responsible for the water impermeability of this segment of the nephron (Kumar & Muchmore, 1990). THP displays a marked tendency to form reticulated aggregates (Bjugn & Flood, 1988), which has obvious

implications for urinary-cast formation (Worcester *et al.*, 1988).

Although the physiological function of THP remains uncertain, several observations suggest that it could be involved in urothelial defences against infection; for example Orskov *et al.* (1980) showed that *Escherichia coli* with type I fimbriae are trapped by THP. The binding of THP to *E. coli* type I pili depends on the mannose content of THP (Kuriyama & Silverblatt, 1986); more recently, *in vitro* cytoadherence assays showed that THP also inhibits the binding of S- and P-fimbriated *E. coli* to human renal tubular epithelial cells (Lecker *et al.*, 1997). Additionally, THP may stimulate polymorphonuclear leukocytes in the course of an inflammatory response (Horton *et al.*, 1990). Although THP distribution in humans is limited to the kidney, immunoreactive THP-related proteins have been detected at other mucosal surfaces of several vertebrates (Howie *et al.*, 1993). These observations suggest that THP-like proteins could also be involved in the defence(s) against infectious agents at other mucosal sites,

Abbreviations: CHO, chinese hamster ovary; O-glycosidase, glycopeptide α -N-acetylgalactosaminidase; PTX, pertussis toxin; FHA, filamentous haem-agglutinin; THP, Tamm–Horsfall glycoprotein.

by impeding their cytoadherence. To test the capability of THP to reduce the epithelial adherence of non-uropathogenic bacteria, we investigated the interaction of THP with *Bordetella pertussis*, the aetiological agent of whooping cough, which colonizes the human upper-respiratory tract. In this study, we show that besides its anti-adherence activity, THP is also able to trap pertussis toxin (PTX).

METHODS

Bacterial strains and growth conditions. *B. pertussis* strains (Table 1) were grown on Bordet–Gengou agar (Bordet & Gengou, 1906) supplemented with 20% (v/v) defibrinated sheep blood and containing either 100 µg streptomycin ml⁻¹ (Sigma) for strains BPSM, BPRA, BPGR4 and BPLOW, or 20 µg ampicillin ml⁻¹ (Sigma) for strains ΔS2 and ΔS3. Liquid cultures were performed as described previously (Menozzi *et al.*, 1991), using modified Stainer & Scholte (SS) medium containing 1 g (2,6-*O*-dimethyl)-β-cyclodextrin l⁻¹ (Imaizumi *et al.*, 1983) as well as the relevant antibiotics. For cytoadherence assays, *B. pertussis* cells grown for 48 h (37 °C) on Bordet–Gengou agar were suspended to an OD₆₀₀ of 0.15 in test tubes containing 2.5 ml SS medium supplemented with 65 µCi (2.40 MBq) L-[³⁵S]methionine + cysteine ml⁻¹ (Express³⁵S Protein Labelling Mix; 1175 Ci mmol⁻¹; NEN). After 24 h growth at 37 °C under agitation, the bacteria were washed three times with 10 ml PBS (20 mM Na₂HPO₄; 3.5 mM KH₂PO₄; 140 mM NaCl; 5 mM KCl; pH 7.4) and resuspended in RPMI 1640 (Gibco) without serum to an OD₅₄₀ of 0.12, corresponding to approximately 1.2 × 10⁸ c.f.u. ml⁻¹.

Protein purification. For the purification of human THP, a urine sample was collected over a 24 h period from a healthy adult male and frozen at -30 °C. After thawing at room temperature, the precipitate was collected by centrifugation at 20000 g for 20 min at 4 °C. The pellet was resuspended in 40 ml distilled water adjusted to pH 8.8 with 1 M NaOH and dialysed three times for 3 h against 500 ml TBS⁺⁺ (20 mM Tris/HCl, pH 7.5; 1 mM MgCl₂; 1 mM CaCl₂; 1 mM MnCl₂). The solution was then chromatographed at room temperature, using a 1 cm diameter glass column packed with 10 ml Concanavalin A Sepharose (Pharmacia) equilibrated in TBS⁺⁺. After sample loading and washing with TBS⁺⁺, THP was eluted with TBS⁺⁺ containing 500 mM methyl α-D-mannopyranoside (Sigma). Purified THP was dialysed three times against 900 ml PBS and then stored at -30 °C until further use.

PTX was purified from *B. pertussis* BPSM culture supernatant using Affi-Gel Blue chromatography (Bio-Rad) followed by fetuin-Sepharose (Sigma) affinity chromatography, as described by Sekura *et al.* (1983). Purified PTX was dialysed against PBS and stored in 500 µl aliquots at -30 °C.

Production of antisera. Five hundred microlitres of purified PTX or THP antigen [100 µg (ml PBS)⁻¹] were mixed with 500 µl of a monophosphoryl lipid A solution (MPL + TDM Adjuvant System; Sigma) prepared according to the manufacturer's recommendations. BALB/c mice were immunized with 300 µl of the antigen suspension in the following way. For each animal, 200 µl of the antigen suspension was given intraperitoneally, followed by two subcutaneous injections of 50 µl each. The animals were boosted the same way with the same amounts of antigen 4 and 6 weeks later; the antisera were collected 3 weeks after the last boost.

Enzymic deglycosylation of THP. Purified human THP at 5 mg ml⁻¹ in PBS was treated for up to 15 h at 37 °C with 0.5 U N-glycosidase F (EC 3.5.1.52; Roche) per mg THP or with 1 mU glycopeptide α-N-acetylgalactosaminidase [O-glycosidase; EC 3.2.1.97; Roche] per mg THP.

Covalent coupling of THP to Sepharose. Native or deglycosylated human THP was covalently coupled to CNBr-activated Sepharose 4B (Pharmacia) as follows. Three grams of CNBr-activated Sepharose 4B was first washed with 50 ml ice-cold 1 mM HCl followed by 150 ml PBS. The gel was then mixed with 10 ml of THP (5 mg ml⁻¹) in PBS and incubated for 5 h at room temperature. After blocking of the remaining active groups by incubation for 1 h at room temperature in 25 ml 0.2 M glycine, pH 8.0, the THP-Sepharose gel was stored at 4 °C in PBS containing 20% (v/v) ethanol. For both the native and the deglycosylated forms of human THP, coupling efficiencies were between 95 and 100%.

Chromatography of *B. pertussis* culture supernatants onto THP-Sepharose. Late-exponential phase supernatants from 800 ml *B. pertussis* cultures were collected by centrifugation at 10000 g for 15 min at 4 °C and were then loaded at 2 ml min⁻¹ onto 5 ml PBS-equilibrated THP-Sepharose packed in a 1 cm diameter column. After extensive washing, retained material was step-wise eluted using successively PBS + 3 M NaCl, PBS + 0.5 M methyl α-D-mannopyranoside, PBS + 3 M urea and PBS + 6 M urea. Eluted material was collected in 1 ml fractions and stored at -30 °C until further use.

***B. pertussis* cytoadherence assays.** Human pulmonary epithelial cells (A549; ATCC CCL-185) and canine kidney epithelial cells (MDCK; ATCC CCL-34) were cultured in 75 cm² Roux flasks (Costar) using RPMI medium sup-

Table 1. *B. pertussis* strains used in this study

Strain	Relevant features	Reference
BPSM	Streptomycin-resistant <i>B. pertussis</i> Tohama I derivative	Menozzi <i>et al.</i> (1994a)
BPRA	BPSM lacking the PTX structural gene	Antoine & Loch (1990)
BPGR4	BPSM lacking <i>fhaB</i>	Locht <i>et al.</i> (1992)
BPLOW	BPSM carrying a chromosomal deletion of <i>bvgAS</i>	Antoine <i>et al.</i> (2000)
ΔS2	Derivative of BPRA with an integrated <i>ptx</i> operon lacking the S2 subunit gene without frame shift	D. Raze and others (unpublished)
ΔS3	Derivative of BPRA with an integrated <i>ptx</i> operon lacking the S3 subunit gene without frame shift	D. Raze and others (unpublished)

plemented with 50 µg streptomycin ml⁻¹ (Sigma), 2 mM L-glutamine (Gibco) and 10% (v/v) heat-inactivated fetal bovine serum (Gibco). The day before the adherence assay, the wells of 24-well tissue culture trays (Nunc) were seeded with 2×10^5 exponentially growing epithelial cells resuspended in 2 ml of serum-supplemented RPMI medium. Before the addition to each well of 500 µl of RPMI medium containing 4×10^6 ³⁵S-labelled *B. pertussis* cells, corresponding to an m.o.i. of 10, the epithelial cells were washed twice with 1 ml PBS. At the end of 2 h incubation at 37 °C, non-adherent bacteria were removed by three washes with 1 ml PBS and the cells were lysed by adding 500 µl distilled water containing 0.1% (w/v) SDS. The radioactivity associated with the cellular lysates was quantified using a liquid scintillation counter (model LS 6000SC; Beckman). Bacterial adherence was expressed as the percentage of radioactivity associated with the cell lysates relative to that present in the inoculum. The adherence assays were performed in quadruplicate and the results are expressed as the mean $\pm$ SD.

Flow cytometry analysis of the *B. pertussis*–THP interaction.

Approximately 10^8 exponentially growing *B. pertussis* cells were harvested by low-speed centrifugation and washed twice in 2 ml PBS. Bacteria were then resuspended in 1 ml PBS containing 100 µg purified human THP and incubated for 30 min at room temperature. After three washes with PBS, the cells were incubated for 1 h at room temperature with mouse-anti-human THP antiserum diluted 500-fold in PBS. The bacteria were then washed three times with PBS and incubated for 30 min at room temperature in 1 ml of FITC-conjugated donkey-anti-mouse IgG (Jackson ImmunoResearch Laboratories) diluted 500-fold in PBS. After three final washes with PBS, the bacteria were fixed with 500 µl of 4% paraformaldehyde. Cell-associated fluorescence (expressed as the percentage of FITC-labelled *B. pertussis*) was quantified by flow cytometry using an EPICS Elite cytometer (Coulter); fluorescence associated with bacteria that were not incubated with THP was used for background determination.

Scanning electron microscopy. *B. pertussis* BPSM grown for 48 h on Bordet–Gengou agar plates was scraped using an inoculating loop and carefully resuspended at a density of 10^7 cells ml⁻¹ in PBS or in PBS supplemented with 100 µg THP ml⁻¹. The bacteria were then directly fixed for 5 h at room temperature in a 1.25% (v/v) glutaraldehyde solution prepared in 100 mM sodium cacodylate buffer, pH 7.0. Aliquots containing approximately 10^6 cells were filtered through a 25 mm diameter/0.2 µm porosity Anodisc (Whatman); the filters were then rinsed five times for 10 min in 25 ml cacodylate buffer. Post-fixation was performed for 3 h in a 1% OsO₄ solution prepared in cacodylate buffer, followed by five washes in ultrapure water. The samples underwent progressive dehydration by successive soaking in 50, 70, 95 and 100% ethanol. Soaking in isopentyl acetate was performed before critical-point drying in CO₂, using an EMSDCOPE CPD 750 apparatus. The filters were then attached to large scanning-electron-microscopy stubs and coated with gold/palladium by cathodic spreading in a Polaron E 5100 coater. Sample observation and microphotographs were done in a JEOL JSM35CF scanning electron microscope, operating at a voltage of 10 kV.

Other analytical procedures. Chinese hamster ovary (CHO) cells (CHO-K1; ATCC CRL-9618) were cultured in monolayers using RPMI 1640 medium containing 10% (v/v) fetal bovine serum. The clustering effect of PTX on CHO-K1 cells was monitored in flat-bottom microtitre plates (Dynatech Laboratories) as described by Hewlett *et al.* (1983), using

purified PTX at concentrations ranging from 2 µg ml⁻¹ to 1 ng ml⁻¹.

SDS-PAGE was performed according to Laemmli (1970) by using a 4% stacking gel and a 15% separating gel. After electrophoresis, the gels were stained with Coomassie brilliant blue R-250 (Merck).

The transfer of proteins from SDS-polyacrylamide gels to nitrocellulose sheets (BA 85; Schleicher & Schuell) was performed as described by Towbin *et al.* (1979). Immobilized PTX was probed with a murine-anti-PTX antiserum diluted 1000-fold and developed with alkaline phosphatase-linked goat-anti-mouse immunoglobulin G (ProtoBlot System; Promega).

Protein concentrations were determined with the bicinchoninic acid protein assay (Pierce), using BSA as a standard.

RESULTS

THP inhibits the adherence of *B. pertussis* BPSM to MDCK cells

To determine whether THP is able to inhibit the cytoadherence of pathogenic bacteria other than those infecting the urogenital tract, we studied the interaction of virulent *B. pertussis* BPSM with renal epithelial MDCK cells in the presence of THP at concentrations ranging from 0 to 20 µg ml⁻¹. Using an m.o.i. of 10 bacteria per MDCK cell, as little as 10 µg THP ml⁻¹ reduced the binding of radiolabelled *B. pertussis* BPSM to MDCK cells by approximately 80% (data not shown). No further decrease in adherence was observed with THP concentrations of up to 100 µg ml⁻¹ (data not shown). The observation that the THP-mediated inhibition of adherence was saturable suggests the presence of a specific THP ligand on the surface of *B. pertussis* BPSM.

THP binding to virulent and avirulent *B. pertussis*

To investigate whether the THP ligand is part of the general virulence regulon under the control of the *BvgAS* two-component system (Uhl & Miller, 1995), we compared the binding of THP to virulent *B. pertussis* BPSM and to avirulent *B. pertussis* BPLOW, which carries a chromosomal deletion of the *bvgAS* operon. The binding of THP to *B. pertussis* BPLOW was reduced by approximately 40% compared to that of virulent *B. pertussis* BPSM, as evidenced by flow cytometry analysis (data not shown). This indicates that *bvgAS*-regulated virulence factors are involved in the interaction with THP.

Involvement of PTX in the THP-mediated inhibition of *B. pertussis* adherence

To determine whether any of the known virulence factors, such as filamentous haemagglutinin (FHA) and/or PTX, are involved in the THP-mediated inhibition of *B. pertussis* adherence, the adherence assay was carried out using BPSM derivatives lacking FHA or

Fig. 1. Effect of increasing concentrations of THP on the adherence of *B. pertussis* BPSM (◆), *B. pertussis* BPRA (■) and *B. pertussis* BPGR4 (▲) to A549 human type II pneumocytes. Metabolically labelled *B. pertussis* was incubated for 2 h with A549 cells using an m.o.i. of 10. For each strain, the radioactivity associated with the eukaryotic cells was determined, and residual adherence was expressed as the percentage of radioactivity associated with the cell lysates (c.p.m. +THP) relative to that present in the inoculum (c.p.m. -THP). The data represent the means of quadruplicate experiments $\pm$ SD.

PTX. As observed with MDCK cells, THP also induced a saturable and dose-dependent inhibition of *B. pertussis* BPSM adherence onto A549 pneumocytes (Fig. 1). However, this inhibition was less pronounced than that observed with MDCK cells, since it was limited to approximately 50% in the presence of 10 μ g THP ml⁻¹. Although the adherence of *B. pertussis* BPGR4 (a strain lacking FHA) was reduced by approximately 70% compared to its parental *B. pertussis* BPSM strain, even in the absence of THP, 10 μ g THP ml⁻¹ further reduced *B. pertussis* BPGR4 adherence by an additional 50%. This finding suggests that FHA is not involved in the THP-mediated inhibition of adherence. In contrast, the adherence of *B. pertussis* BPRA (a strain deficient for PTX), which is comparable to that of *B. pertussis* BPSM in the absence of THP, was less sensitive to THP, since 10 μ g THP ml⁻¹ induced only a 15% reduction of adherence, suggesting that PTX is implicated in the *B. pertussis*-THP interaction.

Direct interaction of THP with PTX

THP-Sepharose 4B was used as an affinity chromatography matrix to analyse a *B. pertussis* BPSM culture supernatant containing secreted PTX, to determine whether PTX was able to interact directly with THP. After sample application followed by washing, material retained on the matrix was successively eluted with PBS containing 3 M NaCl, 0.5 M methyl α -D-mannopyranoside, 3 M urea and finally 6 M urea. As shown in Fig. 2(a), only the PBS + 3 M urea elution step yielded an elution peak, as monitored at A₂₈₀. SDS-PAGE analysis of the 1 ml fractions of this peak revealed that it contained five protein bands, displaying an electrophoretic pattern typical of PTX (Sekura *et al.*, 1983). Immunoblot analysis using anti-PTX antibodies confirmed that these bands corresponded to PTX (Fig. 3). Similar analyses using anti-FHA monoclonal antibodies failed to demonstrate the binding of FHA to THP-

Fig. 2. (a) Chromatogram of 800 ml *B. pertussis* BPSM culture supernatant on 5 ml THP-Sepharose 4B equilibrated with PBS. After sample application followed by washing with PBS (arrow 1), the gel was eluted step-wise with PBS + 3 M NaCl (arrow 2), PBS + 0.5 M methyl α -D-mannopyranoside (arrow 3), PBS + 3 M urea (arrow 4) and PBS + 6 M urea (arrow 5). (b) SDS-PAGE analysis of the *B. pertussis* BPSM culture supernatant prior to chromatography (S), column flow-through (F) and 1 ml fractions from the 3 M urea elution peak presented in (a). S1-S5 represent the various subunits of the B oligomer of PTX.

Sepharose (data not shown). In the experimental conditions used, no PTX was detected by immunoblotting in the column flow-through, indicating that immobilized THP is a powerful ligand for PTX. Moreover, the THP-PTX interaction was very strong, since up to 5 M NaCl did not elute PTX. Elution of PTX required the presence of 3 M urea or 0.1% SDS (data not shown). The THP-Sepharose was also quite stable, since no decrease in PTX purification yield was observed during repeated use of the affinity chromatographic matrix.

PTX binds to THP via the S2 subunit of the B oligomer

SDS-PAGE analysis of the tail fraction of the 3 M urea elution peak showed the presence of, essentially, the S2-S5 subunits of the B oligomer of PTX with no detectable S1 subunit (Fig. 2b), suggesting that the THP-PTX interaction is mediated by this oligomer. Since the highly similar S2 and S3 subunits of the B oligomer are involved in the various binding activities of

Fig. 3. Comparative analysis of the interaction of PTX, PTX Δ S3 and PTX Δ S2 with THP or with *N*-deglycosylated THP covalently bound to Sepharose. Samples (800 ml) of the following culture supernatants were chromatographed on THP-Sepharose or on *N*-deglycosylated THP-Sepharose and were then eluted. (A) *B. pertussis* BPSM (PTX) with THP-Sepharose; (B) *B. pertussis* Δ S3 (PTX Δ S3) with THP-Sepharose; (C) *B. pertussis* Δ S2 (PTX Δ S2) with THP-Sepharose; (D) *B. pertussis* BPSM with *N*-deglycosylated THP-Sepharose. The elution peaks with PBS + 3 M urea are shown (top of each panel); protein content is shown as A_{280} , as well as their contents as analysed by SDS-PAGE (middle of each panel) and by immunoblotting with anti-PTX antibodies (bottom of each panel).

PTX (Lobet *et al.*, 1993), we tested the THP–PTX interactions using PTX produced by *B. pertussis* mutants which were unable to produce the S2 or S3 subunit (Δ S2 or Δ S3 mutant, respectively). [These mutants do, however, produce a correctly assembled holotoxin, through replacement of the missing subunit by a second copy of the S3 or S2 subunit (D. Raze, F. D. Menozzi, R. Antoine, Y. Sato and C. Loch, unpublished)]. As shown in Fig. 3, after chromatography on THP-Sepharose, the elution peak of the culture supernatant of *B. pertussis* Δ S3 was identical to that obtained with *B. pertussis* BPSM, suggesting that the absence of the S3 subunit has no significant effect on the toxin interaction with THP. In contrast, the deletion of the S2 subunit in *B. pertussis* Δ S2 resulted in a 35-fold decrease of PTX binding to THP, as determined by protein quantitation of the material contained in the 3 M urea elution peak. This indicates that the S2 subunit is involved in the recognition of THP. The residual THP binding observed with the PTX Δ S2 mutant is likely due to the high amino

acid similarity displayed by the S2 and S3 subunits (Lobet *et al.*, 1993).

Glycosylation of THP is required for PTX binding

Since carbohydrates account for approximately 30% of the THP weight (Fletcher *et al.*, 1970), we investigated the role of the carbohydrate moiety in the THP–PTX interaction. THP was enzymically deglycosylated using *N*-glycosidase F and *O*-glycosidase, which hydrolyse *N*-glycans and *O*-glycans, respectively. As shown in Fig. 4, *N*-glycosidase F treatment reduced the apparent molecular mass of THP from approximately 95 kDa to a doublet of approximately 70 and 66 kDa. In contrast, no apparent shift in molecular mass was observed when THP was treated with *O*-glycosidase, consistent with the low level of *O*-glycosylation of THP (Easton *et al.*, 2000). Since THP contains several disulfide bridges that could interfere with the enzymic deglycosylation, THP was also reduced and carboxymethylated prior to

Fig. 4. SDS-PAGE analysis of deglycosylated THP. Purified THP was incubated at 37 °C for the indicated time intervals in the presence of O-glycosidase or N-glycosidase F. Control THP, incubated for 0 and 15 h in the absence of glycosidase, respectively, is presented in the left part of the electropherogram.

deglycosylation (data not shown). When O- or N-deglycosylated THP was covalently coupled to Sepharose 4B and tested for its ability to bind to PTX produced by *B. pertussis* BPSM, the toxin was no longer retained by immobilized N-deglycosylated THP (Fig. 3). In contrast, the PTX-binding capacity of O-deglycosylated THP was similar to that of native THP (not shown). The same results were obtained using deglycosylated reduced THP. These findings indicate that the N-glycans borne by THP are required for PTX binding.

THP-purified PTX retains its activity

To determine whether PTX purified by THP-Sepharose chromatography retained its toxic activity, the activity of THP-purified PTX was compared in the CHO-cell-clustering assay to that of PTX purified by standard methods (Sekura *et al.*, 1983). As shown in Fig. 5, at 1 ng purified PTX ml⁻¹ the clustering effect induced by either

method was very similar. Because the cell clustering requires binding of the holotoxin to cell surface receptors (Brennan *et al.*, 1988), as well as the ADP-ribosylation of Gi proteins catalysed by the S1 subunit (Burns *et al.*, 1987), this result indicates that the THP-purified PTX has completely retained its conformational structure and its toxic activity. When investigated for its ability to interfere with PTX activity, THP was shown to be unable to inhibit the PTX-induced clustering of CHO cells.

THP reduces *B. pertussis* autoagglutination

Since FHA is the major *B. pertussis* adhesin involved in cytoadherence (Locht *et al.*, 1993), we wanted to know whether the THP-PTX interaction at the *B. pertussis* cell surface could interfere with FHA activities. We took advantage of the fact that surface-exposed FHA induces *B. pertussis* autoagglutination (Menozzi *et al.*, 1994b). Freshly grown *B. pertussis* BPSM were therefore suspended in PBS or in PBS containing 100 µg THP ml⁻¹ and observed by scanning electron microscopy. As shown in Fig. 6, THP drastically reduced the *B. pertussis* autoagglutination, indicating that its interaction with PTX may impede FHA activities.

DISCUSSION

Infectious diseases are multi-step processes that often start on mucosal surfaces by the attachment of the infectious micro-organism to target cells. This avoids rapid mechanical clearance. Once attached onto specific host tissues, the pathogen multiplies within an infectious focus and sometimes secretes toxins that may perturb the physiology of the host. Therefore, the microbial adhesins that mediate the initial mucosal adherence, as well as the toxins that weaken the host defences, are important virulence factors. Interference with these factors by host molecules may constitute an important mechanism for mucosal protection against infectious agents. In the lungs, the surfactant which contains mucoproteins is involved in the defence of the pulmonary mucosa by enhancing macrophage phagocytosis

Fig. 5. PTX-induced CHO-cell clustering. The micrographs represent CHO cells cultured in the absence of PTX (a), CHO cells cultured for 12 h at 37 °C in the presence of 1 ng ml⁻¹ of PTX purified by the two-step standard method, including Affi-Gel Blue chromatography and fetuin-Sepharose chromatography (b), or in the presence of 1 ng ml⁻¹ of PTX purified by THP-Sepharose (c). Bar, 100 µm.

Fig. 6. Scanning electron micrographs of *B. pertussis*. *B. pertussis* BPSM grown on Bordet–Gengou agar plates was resuspended in PBS (a) or PBS containing 100 µg THP ml⁻¹ (b). Bar, 10 µm.

and by limiting microbial adherence to the underlying epithelium (Pison *et al.*, 1994). In the urinary tract, no similar wide-spectrum defence mechanism has been described so far. The mucoprotein THP is the most abundant urinary protein and virtually covers the urothelium from the thick ascending limb of the loop of Henle to the urethra (Kumar & Muchmore, 1990). The physiological function of THP is still unknown, but several studies have shown that it may inhibit the urothelial adherence of uropathogenic *E. coli* (Kuriyama & Silverblatt, 1986; Leeker *et al.*, 1997; Orskov *et al.*, 1980; Pak *et al.*, 2001). To investigate whether a similar mechanism may apply to respiratory pathogens, and since immunoreactive THP-related proteins have been detected in a number of different mucosal surfaces of various vertebrates (Howie *et al.*, 1993), we investigated the anti-adherence properties of THP towards *B. pertussis*. We found that THP is able to inhibit the adherence of *B. pertussis* to both renal and pulmonary epithelial cells, suggesting that THP expresses a wide-spectrum anti-adherence activity. This finding also suggests that the immunoreactive THP-related proteins found on other vertebrate mucosae may play a role against infection. However, these proteins have not been

characterized as yet. Surprisingly, the ligand of THP on *B. pertussis* is not one of the known adhesins, such as those described for uropathogenic *E. coli*, but is instead a toxin (PTX). Since the epithelial adherence of *B. pertussis* is essentially mediated by FHA and not by PTX, we postulate that the inhibitory effect observed here may be due to the capacity of THP to form large multimers (Friedmann & Johnson, 1966), therefore allowing it to mask other surface components, like FHA. This assumption was confirmed by demonstrating that the binding of THP to *B. pertussis* inhibits FHA-mediated autoagglutination.

The interaction of PTX with immobilized THP has been shown to be particularly strong, since it requires 3 M urea to be completely abolished. This feature allowed us to develop a one-step chromatographic procedure to purify PTX to homogeneity from *B. pertussis* culture supernatant. This new purification procedure is cheap and may thus constitute an attractive alternative to conventional methods that generally combine two purification steps (Sekura *et al.*, 1983). Moreover, the THP-purified PTX totally recovers its conformational structure and toxic activity, as demonstrated by the

CHO-cell-clustering assay. The present study has also demonstrated that the THP-PTX interaction is essentially mediated by the S2 subunit of the PTX B oligomer and that it depends on the glycosylation of THP. From enzymic deglycosylation experiments, it appears that the N-linked polysaccharides of THP are crucial for the interaction with PTX, whereas the O-linked sugars do not appear to be involved in this interaction. This finding may reflect the predominant and complex N-glycosylation content of THP (Hard *et al.*, 1992) and its very low O-glycosylation, which is limited to short and simple chains terminated with either sialic acid or fucose (Kumar & Muchmore, 1990).

Although the receptors for PTX on eukaryotic cells have not yet been identified, it has been shown that CHO cells express a 165 kDa surface glycoprotein that is recognized by PTX or by purified B oligomer (Brennan *et al.*, 1988). The sialic acid moiety on the N-linked oligosaccharide of this protein is an important motif of the receptor, since treatment of CHO cells with sialidase abolishes PTX binding. Moreover, some serum sialoglycoproteins, such as haptoglobin and fetuin, have been shown to bind to PTX via their N-glycosylated moieties (Witvliet *et al.*, 1989). Similar to THP, which also bears sialylated carbohydrate chains (Hard *et al.*, 1992), the binding of PTX to haptoglobin is mediated by the S2 subunit of the B oligomer (Francotte *et al.*, 1989). This suggests that the same binding mechanism may mediate the interaction of PTX with THP as mediates binding of PTX with haptoglobin.

The observations reported in the present study certainly open new research perspectives for the elucidation of the physiological role of THP within the urinary tract. We show that the anti-adherence activity of THP is not limited to fimbriated uropathogenic *E. coli*. Moreover, we have demonstrated that THP may directly interact with a bacterial toxin, PTX. Even if this interaction has no physiological relevance, it can be taken as a model to investigate the interactions of THP with bacterial virulence factors. Because of the large repertoire of sugar motifs borne by THP, it is possible that it may also bind to toxins like the haemolysins secreted by uropathogenic bacteria, such as *E. coli* or *Proteus mirabilis*. Investigations are currently in progress in our laboratory to clarify these potential antimicrobial activities of THP.

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