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Characterisation of a monoclonal antibody and its use to purify the cytotoxin of *Helicobacter pylori*

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Abstract

The vacuolating cytotoxin (VacA) is a major virulence factor of *Helicobacter pylori* which is not yet well characterised and is difficult to obtain in large quantities. Here we describe the production of a monoclonal antibody that recognises the native but not the denatured form of VacA. The antibody can be efficiently used in affinity chromatography for one-step purification of large quantities of VacA from culture supernatants. Elution at acidic pH dissociates the oligomeric molecule into monomers that reanneal in a time-dependent fashion. The purified cytotoxin is able to bind, and to intoxicate HeLa cells.

Keywords: Immunoaffinity; Cytotoxin; *Helicobacter pylori*

1. Introduction

Helicobacter pylori infection is now recognised as the cause of most peptic ulcer diseases and is strongly associated with gastric cancer [1,2]. Pathogenic strains produce a potent cytotoxin (VacA) which causes massive vacuolation and death in a number of cell lines in vitro and which is believed to play a key role in *H. pylori* associated gastric disease [3–5].

The toxin is synthesised as a 140-kDa precursor polypeptide which is processed during release from the cell, by removal of the 45-kDa carboxy-terminal exporter domain. After release from the bacteria, the 95-kDa toxin polypeptide is further processed by specific cleavage at an exposed loop to produce the 37-kDa and 58-kDa subunits [3]. High quality electron microscopy images of the toxin reveal that the native toxin is a regular oligomer with either a six-fold or a seven-fold radial symmetry. The structure of the monomer is clearly formed of two distinct

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subunits which are likely to be the 37-kDa and 58-kDa processed products [6,7].

de Bernard et al. have demonstrated that treatment of VacA at pH below 5.0 results in an increase in vacuolating activity which is associated with a conformational change in the molecule [8]. The activation was reported to be stable after neutralisation of the protein. Recently, Cover et al. showed that the oligomeric structure was disrupted by low pH treatment giving place to a monomeric molecule that reassembles into the hexameric structure upon neutralisation [9]. It has been proposed that the conformational change induced by low pH facilitates cell association and entry of the molecule into the cell cytoplasm where the biological activity results in a defective fluid phase endocytosis at the late endosome stage [10,11]. Moreover, it has recently been shown that the vacuoles induced by the *H. pylori* cytotoxin contain both endosomal and lysosomal markers, suggesting that VacA induces the accumulation of a post-endosomal compartment [12].

Here we report a simple procedure to purify the cytotoxin which is based on immunoaffinity chromatography. The procedure involves elution at low pH resulting in a monomeric form of the molecule that re-anneals in a time-dependent manner. The purified cytotoxin is capable of entering the cytoplasm of HeLa cells and is fully active in vacuolation.

2. Materials and methods

2.1. Bacterial strain and toxin production

The toxin producing strain *H. pylori* CCUG 17874 (tox+ strain, NCTC 11638) was used as the source for VacA purification. Colonies of *H. pylori* grown on a blood agar plate (Brucella agar with 5% horse blood) were inoculated into Brucella broth containing 0.2% of β-cyclodextrin and were cultured for 3–4 days at 150 rpm in a controlled micro-aerophilic atmosphere at 8% O_2 and 10% CO_2.

2.2. Production of the monoclonal antibody C1G9

The monoclonal antibody (mAb) C1G9 was obtained from a BALB/c mouse immunised with partially purified VacA (5 μg per dose, six doses) according to the standard protocols. Positive hybridomas were selected by ELISA using purified oligomeric VacA and cloned by limiting dilution. Specific mAb producing clones were expanded and injected in prystane sensitised BALB/c mice to produce ascitic fluid from which purified IgG was prepared by protein G affinity chromatography (MAb trap G2, Pharmacia, Uppsala, Sweden). The isotype of the mAb was determined as IgG1 using a commercial isotyping kit.

2.3. Preparation of the immunoaffinity resin

15 mg of mAb C1G9 were immobilised on 2.5 ml of CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) according to the manufacturer. This resin was used to pour a column of 1×5 cm.

2.4. Immunopurification of VacA

Supernatant from *H. pylori* cultures was subjected to a 50% saturation $(NH_4)_2SO_4$ precipitation, and the pellet containing VacA was resuspended in 10 ml of PBS buffer, dialysed against PBS buffer and loaded onto the immunoaffinity column equilibrated with PBS buffer. After 5 column volumes wash with PBS, bound VacA was eluted with 50 mM glycine-HCl pH 2.5. Fractions containing VacA were pooled, neutralised by dropwise addition of 250 mM Na_2HPO_4 pH 10.3 and stored at −80°C.

2.5. Assessment of cytotoxin internalisation by confocal microscopy

HeLa cells were incubated with 3.5 μg ml^{-1} of immunopurified VacA and samples were processed as previously described [11]. The anti-native VacA antibody was diluted 1:500 in the permeabilisation buffer (0.2% (w/v) saponin, 0.5% (w/v) BSA in PBS) and incubated with cells for 1 h. After several washes, fluoresceinated antibodies diluted 1:2000 in the same buffer were added. After 30 min of incubation samples were washed and mounted on 90% (v/v) glycerol, 0.2% (w/v) *N*-propylgallate in PBS and observed with a fluorescence microscope (Zeiss Axioplan).

2.6. Cytotoxicity test on HeLa cells and neutral red uptake

Vacuolating activity was assessed on HeLa cells by light microscopy and quantified by neutral red uptake according to [13].

2.7. Quick-freeze deep-etching and electron microscopy

VacA was prepared for microscopy by a procedure of absorption to mica followed by quick-freeze deep-etching described by [14]. The samples were processed according to [6].

3. Results

3.1. Characterisation of the C1G9 antibody

mAb C1G9 was produced by immunising mice with partially purified, active VacA. Screening was performed by ELISA using highly purified oligomeric VacA. Following extensive characterisation we found that C1G9 recognised VacA in ELISA but did not recognise VacA in immunoblots after denaturation on SDS-PAGE, and failed to react with full length recombinant (P95) VacA produced in *Escherichia coli*. These data suggest that C1G9 recognises a conformational epitope in the molecule which is dependent on the correct structural organisation of the protein. The mAb did, however, recognise a partially refolded recombinant form of the p58 subunit indicating that the epitope is located in the P58 moiety of the cytotoxin (Table 1).

3.2. Immunopurification of VacA

15 mg of purified C1G9 mAb was conjugated with 2.5 ml of CNBR-activated Sepharose 4B slurry and poured into a 1×5 cm column. In these conditions, we were able to purify in one step the *H. pylori* cytotoxin. We obtained a yield of 1–2 mg of purified cytotoxin from 1 l of culture supernatant. This rapid and simple method gave VacA of similar purity (approximately 90%) as the previously published method [15] as judged by Coomassie staining after SDS-PAGE (Fig. 1). Alternative neutral elution methods such as using 4 M $MgCl_2$ pH 7, 2 M NH_4SCN pH 7, or 2 M urea pH 7 gave very poor yields of toxin and thus were not considered for routine purposes (data not shown).

Table 1
Biological characteristics of the C1G9 monoclonal antibody

	VacA	rP95	rP37	rP58
Western blot	−	−	−	−
ELISA	+	−	−	+

VacA, native molecule; rP95, recombinant P95 molecule; rP37, refolded recombinant P37 subunit; rP58 refolded recombinant P58 subunit.

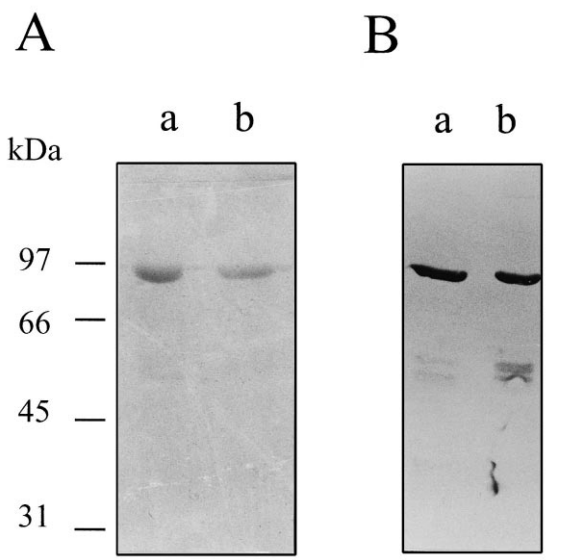

Fig. 1. SDS-PAGE and immunoblot of purified cytotoxin. A: Coomassie blue staining of purified cytotoxin after SDS-PAGE. 3 μg of immunopurified (lane a) or conventionally purified VacA (lane b) were run on a 9% polyacrylamide gel. B: Immunoblot of conventionally purified VacA (lane a) or immunopurified VacA (lane b). VacA was purified as described in Section 2 and was immunoblotted with a 1:5000 dilution of rabbit antiserum raised against the native molecule.

3.3. Electron microscopy analysis of immunopurified VacA

Cover et al. have demonstrated that low pH treatment of VacA results in disruption of the oligomeric structure of VacA which is reformed on neutralisation [9]. Electron microscopy analysis of immunopurified VacA was performed in order to check for

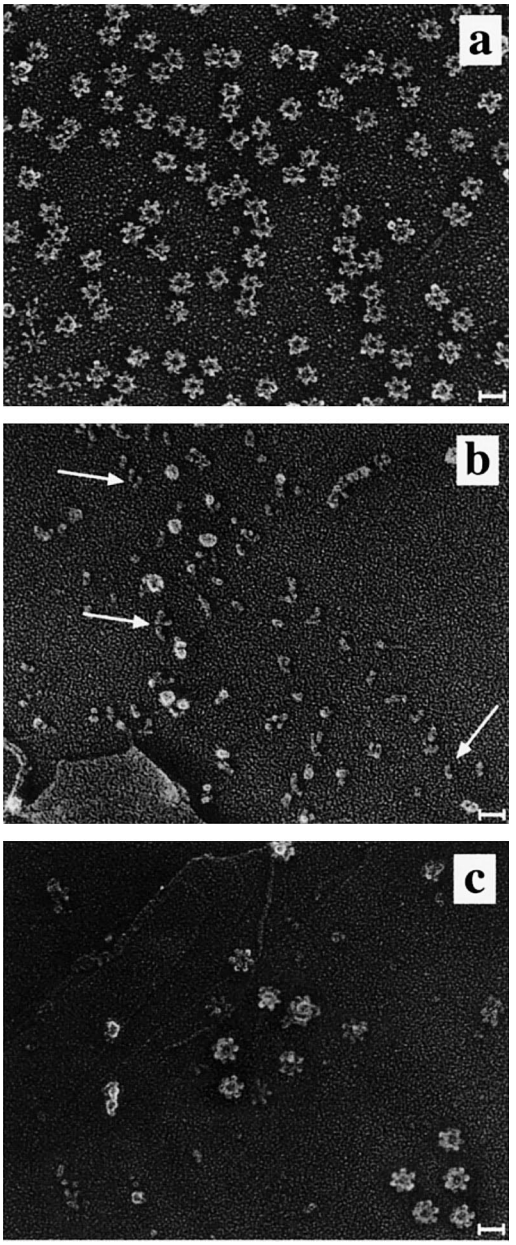

Fig. 2. Transmission electron microscope visualisation of purified toxin after quick-freeze deep-etching. A: Conventionally purified cytotoxin. B: Immunopurified cytotoxin after elution at acidic pH. Arrows indicates monomers. C: Immunopurified cytotoxin after 72 h of neutralisation. Bar, 30 nm.

the structural integrity of the molecule. As shown in Fig. 2, most of the molecules were found to be in a monomeric form after elution with acidic pH. However, after 72 h at 4°C we observed a reannealing of the monomer into the oligomeric structure.

3.4. Internalisation and activity of the monomeric VacA cytotoxin in HeLa cells

To test functional integrity, immunopurified VacA was added to HeLa cells for 4 h at 37°C. The HeLa cells were then fixed, permeabilised and processed for indirect immunofluorescence using anti-VacA polyclonal antibodies. Confocal microscopy revealed multiple focal intracellular fluorescence in the cytoplasm of the cells (Fig. 3), as previously described by Garner and Cover [16]. Control cells incubated with the cytotoxin and reacted with preimmune rabbit serum showed no fluorescence (data not shown). This result demonstrates that VacA purified by immunoaffinity was capable of interacting with and being internalised by the cell.

Since low pH treatment is required to reveal VacA activity, we expected that the immunoaffinity purified protein which was eluted at pH 2.5 would be fully active [8]. Identical amounts of affinity purified and conventionally purified VacA toxin were added to HeLa cells and the extent of vacuolisation was measured using the neutral red uptake assay. As shown in Fig. 4, in the absence of low pH treatment the conventionally purified VacA was almost completely inactive, but low pH treatment revealed full vacuolation. In contrast, the immunopurified VacA was fully active as such and did not require activation. This result demonstrates that the elution of VacA from the monoclonal antibody by acidic treatment is perfectly compatible with the preservation of biological activity. The protein is activated during elution and moreover retained full activity after storage for some weeks at −80°C.

4. Discussion

Here we describe a monoclonal antibody recognising the conformational structure of VacA and the development of a rapid and simple method to purify the cytotoxin of *H. pylori* directly from a culture supernatant. In terms of yield and purity this method gives similar results to those already published [15]. Elution by low pH treatment was shown to yield an

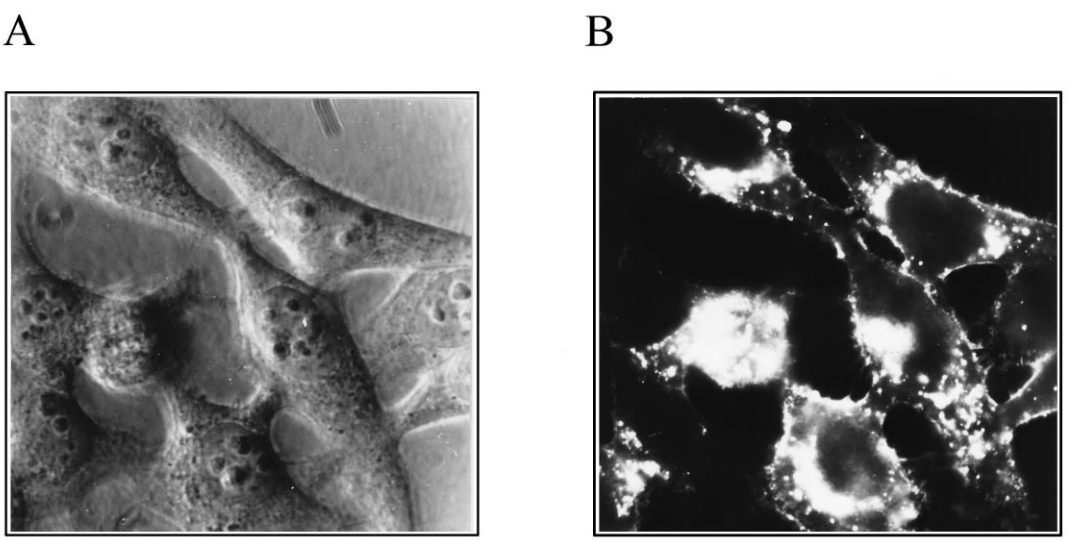

Fig. 3. Internalisation of the immunopurified VacA by HeLa cells. A: Phase contrast microscopy. B: Immunofluorescence micoscopy. HeLa cells were incubated for 4 h at 37°C at a concentration of 3.5 μg ml^{-1}. The localisation of VacA was determined by indirect fluorescence microscopy by using an anti-native VacA antibody diluted 1:500, the secondary antibody (Texas red labelled) was diluted 1:2000 (Jakson Immunoresearch Laboratories). Magnification is 350×.

active toxin, capable of being internalised by HeLa cells. This preparation can be stored at −80°C for weeks without significant loss of cytotoxic activity even if the low pH used for the elution from the column led to preactivation as described [8]. Interestingly, the immunopurified active VacA was shown to be in a stable monomeric form during elution by acidic treatment. However, after neutralisation the molecule was shown to reanneal into the oligomeric structure as observed by electron microscopy. We did not observe the random reassociation of the monomers into an approximative oligomeric structure as described [9]. The VacA cytotoxin was, however, still recognised by the mAb after elution from the column, suggesting that the conformational epitope recognised by C1G9 is not destroyed by the low pH treatment (data not shown).

In conclusion, this new method of purification should facilitate the production of pure cytotoxin and help in deciphering the role of the monomeric versus oligomeric structure in the biological activity of this toxin. Moreover, compilation of the pictures of the monomeric form of VacA should allow the 3-D reconstruction of the monomeric structure and thus give insight into the architecture and the function of the assembled molecule.

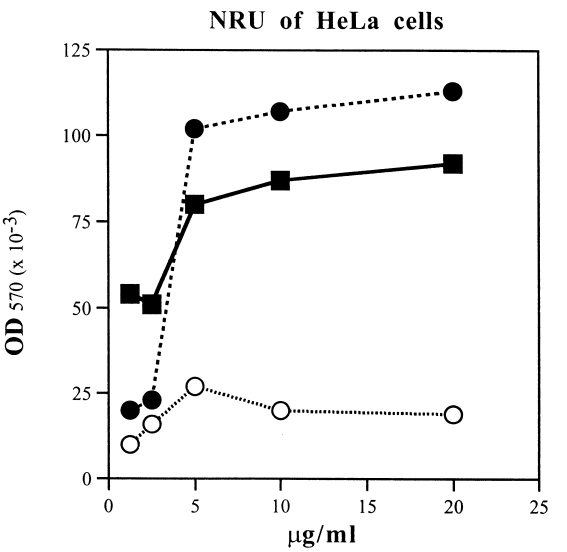

Fig. 4. Induction of HeLa cell neutral red uptake by immunopurified VacA. HeLa cells were incubated with VacA and the extent of cell vacuolisation was assayed by measuring neutral red uptake. Non-activated conventionally purified VacA (open circle), activated conventionally purified VacA (filled square), immunopurified VacA (filled circle).

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