Investigation of the structure and localization of the urease of *Helicobacter pylori* using monoclonal antibodies

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The urease of *Helicobacter pylori* (formerly *Campylobacter pylori*) has been partly purified by fast protein liquid chromatography. This material contained 10 nm doughnut-like structures when examined by electron microscopy and comprised three major polypeptides (61 kDa, 56 kDa and 28 kDa). Only two of these polypeptides (61 kDa and 28 kDa) were observed in urease-containing material isolated by preparative non-denatured PAGE. Monoclonal antibodies (mAbs) were produced which were directed against two of these polypeptides (56 kDa and 28 kDa). Only mAbs directed against the 28 kDa polypeptide inhibited or captured urease activity. These results suggest that the 56 kDa polypeptide is not essential for enzyme activity. Anti-urease mAbs were used in an indirect immunogold technique to localize the enzyme at the ultrastructural level. In both prefixed bacteria and ultrathin cryosectioned bacteria the enzyme was located on the cell surface and in material apparently shed from that surface.

Introduction

Helicobacter pylori (previously *Campylobacter pylori*) is a Gram-negative spiral organism which colonizes the gastric mucosa of humans, non-human primates and pigs. A close correlation between the presence of this bacterium, gastritis and peptic ulceration is now well established (Blaser, 1987). Although, the pathogenic role of this organism in gastritis is now accepted, the mechanisms of pathogenicity are undefined.

A significant host immune response to *H. pylori* has been demonstrated by various methods. This serological response has been exploited to develop assays for the non-invasive diagnosis of *H. pylori* infections and for epidemiological surveys (Newell & Rathbone, 1989). As gastritis is essentially the recruitment of immuno-competent cells into the gastric mucosa, apparently in response to foreign antigen, it is important, therefore, to identify those antigens which induce dominant and specific reactions by the host.

The production of a urease of high activity is a characteristic of *H. pylori* and antibodies are produced against urease-containing material during infection (Newell & Rathbone, 1989). The pathogenic role of this enzyme is unknown, but Hazell & Lee (1986) have suggested a complex back diffusion model involving

ammonia production by the urease, while Marshall *et al.* (1988) ascribed its role to protecting the bacteria in the acid environment of the stomach.

In this study a panel of monoclonal antibodies (mAbs) was produced to allow the investigation of the structure and antigenicity of the enzyme using Western blotting, enzyme capture and inhibition of the urease activity. In addition, the location of the urease in *H. pylori* was determined by immunolabelling techniques at the ultra-structural level.

Methods

Bacterial strains. *H. pylori* strain NCTC 11638 was isolated by Dr B. Marshall and kindly supplied by Dr M. B. Skirrow. All other strains were isolated from gastric biopsies by direct culture on blood agar [2% (v/v) blood agar base no. 2] containing 5% (v/v) defibrinated, lysed horse blood with Skirrow's antibiotics (Skirrow, 1977), incubated at 37 °C in microaerobic conditions. Strains were stored in 10% (v/v) glycerol in 1% (w/v) proteose peptone in liquid nitrogen.

Sonicated antigen preparation. Bacteria were harvested, from 48 h lawned agar plate cultures, into sterile distilled water and subjected to three 30 s bursts of sonication (10 µm amplitude: MSE Soniprep) interrupted by 30 s cooling periods. This crude sonicate was then centrifuged for 5 min at 11 000 g and the supernatant fraction passed through a 0.2 µm filter to give a sonicated antigen preparation.

Acid-extracted antigen preparation. Acid extracts of whole organisms were prepared by incubation in 0.2 M-glycine/HCl buffer, pH 2.2, as previously described (Newell *et al.*, 1984).

Abbreviations: FPLC, fast protein liquid chromatography; mAb, monoclonal antibody.

Outer membrane preparation. Outer membranes of *H. pylori* were prepared by solubilization of crude membranes preparations with sodium *N*-lauroyl sarcosinate as previously described (Newell *et al.*, 1984).

Fast protein liquid chromatography (FPLC). *H. pylori* proteins were separated by size exclusion gel filtration. Whole-cell sonicate (200 µl of 20 mg protein ml⁻¹) was applied to a Superose 6 FPLC column (Pharmacia) equilibrated in phosphate-buffered saline (PBS; 140 mM-NaCl, 2.7 mM-KCl, 8 mM-Na₂HPO₄ and 1.5 mM-KH₂PO₄, pH 7.2). Fractions (1 ml) were collected at a rate of 0.5 ml min⁻¹. Fractions were screened for urease activity by adding 10 µl of the fraction to 10 µl 100 mM-urea with 0.2% (w/v) phenol red as described by Mobley *et al.* (1988). Urease-positive fractions produced an intense red coloration. The titre of the enzyme in each fraction was measured by double diluting, in PBS, 100 µl of the fraction starting at 1:10 dilution and incubating at room temperature with 100 µl 100 mM-urea with 0.2% (w/v) phenol red. The end point was taken as the highest dilution showing colour change from yellow to red within 1 min. The column was calibrated for molecular mass using high molecular mass standards (Pharmacia).

SDS-PAGE. Linear gradient (10–25%, w/v) SDS-PAGE was done as described by Lambden *et al.* (1979) and the protein profiles visualized by Coomassie brilliant blue staining. Relative molecular masses were estimated from standards (Sigma) containing α-lactalbumin, trypsin inhibitor, trypsinogen, carbonic anhydrase, glyceraldehyde-3-phosphate dehydrogenase, egg albumin and bovine serum albumin.

Non-denaturing PAGE. Linear (5%, w/v) acrylamide gels were prepared as described by Johnstone & Thorpe (1987). Samples were prepared in 50% (w/v) sucrose/0.1% (w/v) bromophenol blue and electrophoresed at 100 V for 19 h at 4 °C. The urease band was detected by a urea overlay technique and phenol red staining (Mobley *et al.*, 1988). The urease-containing band was cut out, homogenized in 100 µl dissociation buffer and denatured for SDS-PAGE.

mAb production. F1 (BALB/c × CBA) mice were primed subcutaneously with outer membrane preparation, acid extract or the urease fraction (20 µg protein in Freund's complete adjuvant), then boosted twice (20 µg protein in incomplete adjuvant) at two weekly intervals. A final boost (20 µg aqueous protein) was administered, intravenously, 3 d before the fusion.

Immune spleen cells were fused with NS1 myeloma cells using PEG (Newell *et al.*, 1988). Hybridoma culture supernatant fluids were screened by ELISA, initially against sonicated antigen preparation then against the urease fraction (see below). Positive hybridomas were cloned twice. Ascitic fluid was obtained by introducing hybridoma cells, via intraperitoneal inoculation, into mice pretreated with pristane. Purification of ascitic fluid was by ion-exchange chromatography (Newell *et al.*, 1988). The mAbs produced were isotyped using rabbit anti-mouse immunoglobulin class and subclass antibodies coupled to biotin (Amersham) in an indirect ELISA.

ELISA. Whole cell sonicate (100 µl at 10 µg ml⁻¹) or fraction 14 antigen (100 µl at 5 µg ml⁻¹) in 0.1 M-potassium carbonate buffer, pH 9.6, was adsorbed onto 96-well microELISA plates (Dynatech) overnight at 20 °C. The plates were then washed three times with ELISA wash solution [8.5 g NaCl l⁻¹/0.05% (v/v) Tween 20]. Hybridoma supernatant fluid (diluted 1 in 2) or ascitic fluid (diluted 1 in 100) diluted in 0.05 M-Tris/acetate buffer, pH 7.2, containing 1% (w/v) bovine serum albumin (ELISA diluent) was added to the wells and incubated for 2 h at 37 °C. After washing the wells sheep anti-mouse IgG F(ab')₂ conjugate (1:2000 in ELISA diluent; Amersham) was added and incubated at 37 °C for 30 min. Bound peroxidase was detected with the substrate tetramethyl benzidine (TMB) and hydrogen peroxide. The absorbance was measured at 450 nm after stopping the reaction with 50 µl 2 M-sulphuric acid.

Urease-capture ELISA. Anti-urease mAbs (100 µl at 10 µg ml⁻¹), or an irrelevant control mAb directed against guinea-pig T lymphocytes (GPA) (McBride & Newell, 1989), were adsorbed onto microELISA plates as described above. Excess antibody was removed by washing. Sonicated antigen preparation (5 µg protein ml⁻¹) in ELISA diluent was added and incubated for 2 h at 37 °C. Bound urease was detected using urea as substrate, with addition of Berthelot reagents, and the A₆₂₀ was measured (Precious *et al.*, 1987).

Urease inhibition assay. Sonicated antigen preparation (10 µl at 8 mg ml⁻¹) was incubated with 10 µl mAb ascitic fluid (1:100 dilution) for 1 h at 37 °C. An irrelevant mAb (GPA) was used as control. The mixture (10 µl) was then added to 1 ml 5 mM-urea in 3 mM-sodium phosphate buffer, pH 6.8, containing 0.02% (w/v) phenol red. The rate of colour development was measured at 560 nm over a 5 min incubation period.

Electroimmunoblotting. SDS-PAGE gels were blotted as previously described (Newell, 1987a). The nitrocellulose blots were blocked in 3% (w/v) gelatine in 200 mM-Tris/HCl buffer, pH 7.5, containing 500 mM-NaCl and 0.05% (v/v) Tween 20 (TTBS) and incubated with hybridoma supernatant fluids (1:2 dilution) or ascitic fluid (1:100 dilution) diluted in TTBS containing 1% (w/v) gelatine. The blots were washed with TTBS and incubated with peroxidase-conjugated goat anti-mouse IgG F(ab')₂ (diluted 1:2000 in TTBS with 1% (w/v) gelatine; Amersham) for 1 h at room temperature with constant agitation. The blots were washed in TTBS with a final wash in TTBS without the Tween 20. Bound conjugate was detected by incubation with amino ethyl carbazole as the substrate (Newell *et al.*, 1988).

Immunogold-labelling. Bacteria were prefixed in 0.1% glutaraldehyde, in 0.1 M-sodium phosphate buffer, pH 7.2, for 10 min at room temperature, washed three times in PBS and incubated with mAb (1:100 dilution of ascitic fluid in PBS) for 2 h at 37 °C with constant agitation. After washing three times, by cycles of suspension and centrifugation, the organisms were adsorbed onto Formvar-carbon-coated copper grids before incubation, by flotation, on 100 µl droplets of goat anti-mouse IgG coupled to 10 nm gold particles (Biocell, Cardiff) [1:100 dilution in PBS containing 1% (w/v) bovine serum] in a humidified chamber. Unbound gold was removed by washing with PBS. The grids were negatively stained with 0.1% (w/v) phosphotungstic acid.

For cryostat sections pellets of bacteria were infused with 2.2 M-sucrose in PBS at room temperature for 15 min. The material was then mounted onto a microtome stub and snap-frozen in liquid nitrogen. The frozen block was then cut (500 nm sections) with a glass knife at -100 °C and collected on Formvar-carbon-coated copper grids. Three sections were then pre-incubated on droplets of 1% (w/v) ovalbumin (Sigma) in PBS (PBSO) for 1 h at room temperature. The sections were then incubated with mAb (1:100 dilution in PBSO) for 1 h at room temperature. After washing in PBSO for 1 h, the sections were incubated with goat anti-mouse IgG coupled to 10 nm gold particles for 1 h at room temperature. The sections were washed in PBSO three times, once in PBS and rinsed in glass distilled water. The sections were immersed in 2% (w/v) aqueous methylcellulose as a mountant and stained with 0.3% (w/v) aqueous uranyl acetate.

All specimens were examined using an Hitachi 7000 transmission electron microscope.

Results

Purification and characterization of urease

The urease activity of sonicated antigen preparation of *H. pylori*, when separated by FPLC gel filtration, was concentrated in fractions 14 and 15 (Fig. 1). By

Fig. 1. Fractionation of sonicated antigen preparation by FPLC. Column, Superose 6; elution buffer, PBS, pH 7.2; flow rate, 0.5 ml min⁻¹; detector absorption, 280 nm; sample, 4 mg protein sonicated antigen preparation. Fractions of 1 ml were collected and numbered as shown. The urease activity was determined from serial dilutions (1:2 dilution, starting from a 1:10 dilution, in PBS) of each fraction. Equal volumes of the diluted fraction and 100 mM-urea containing 0.2% (w/v) phenol red were incubated together and the red colour formation, indicating enzyme activity, was determined by eye. The highest dilution to give this coloration, in comparison with a PBS control, was taken as the urease titre (·▲·).

Fig. 2. Transmission electron micrograph of FPLC fraction 14, negatively stained with 0.1% phosphotungstic acid, pH 6.0. Note the doughnut-like structures. Bar, 0.1 µm.

comparison with molecular mass markers separated under identical conditions by the same chromatography column, the molecular mass of the urease was estimated to be 510 kDa. Fractions 14 and 15 only contained 10 nm doughnut-like structures when examined by electron microscopy (Fig. 2). Approximately 5.5% of the protein loaded onto the column was recovered in fraction 14. By

Fig. 3. SDS-PAGE of the sonicated antigen preparation, FPLC-fractionated urease and non-denaturing PAGE-prepared urease of *H. pylori*. Lanes: 1, molecular mass markers; 2, sonicated antigen preparation; 3, FPLC fraction 14; 4, the urease-containing band separated from the sonicated antigen preparation by non-denaturing PAGE, cut out of the gel, homogenized and reduced for SDS-PAGE.

SDS-PAGE the sonicated antigen preparation comprised many polypeptides with major bands of 61 kDa, 56 kDa and 54 kDa (Fig. 3, lane 2). In comparison, fraction 14 comprised three major polypeptides of 61 kDa, 56 kDa and 28 kDa (Fig. 3, lane 3). None of these subunits showed independent enzyme activity.

When the sonicated antigen preparation was separated by non-denaturing PAGE a single band of urease activity was detected by using the substrate overlay technique. This urease band, cut from the non-denaturing gel, when separated by SDS-PAGE comprised only two polypeptides of 61 kDa and 28 kDa (Fig. 3, lane 4).

Production and characterization of mAbs

A panel of monoclonal antibodies was produced that reacted with the urease-containing fractions 14 and 15 by ELISA, but reacted poorly with other FPLC fractions. These mAbs also reacted with outer-membrane preparation and acid-extracted antigen. Ten hybridomas (CP11 to CP20) were selected for further characterization (Table 1). In Western blots of SDS-PAGE of fraction 14, three mAbs immunolabelled the 56 kDa polypeptide, namely CP12, CP20 and CP19 (weak reaction). Each of the remaining mAbs immunolabelled the 28 kDa polypeptide (Fig. 4), although mAbs CP17 and CP18 reacted

Fig. 4. Western blot of SDS-PAGE of FPLC fraction 14 with a panel of mAbs. Lanes: 1, CP11; 2, CP12; 3, CP20; 4, CP14; 5, CP13; 6, CP16; 7, CP15; 8, CP17; 9, CP18; 10, CP19; 11, polyclonal mouse antiserum.

Table 1. The isotype and specificity of mAb directed against FPLC fraction 14

mAb	Isotype	Subunit recognized by mAb (kDa)*
CP11	IgG1	28
CP12	ND	56
CP13	IgG1	28
CP14	IgG2a	28
CP15	IgG1	28
CP16	IgG1	28
CP17	IgG1	28 (weak), 18
CP18	IgG2b	28 (weak)
CP19	ND	56 (weak)
CP20	ND	56

ND, Not determined.

*. Molecular mass of polypeptide subunit recognized by the mAb in Western blots of SDS-PAGE of fraction 14.

very weakly with this polypeptide. All mAbs directed against this polypeptide reacted with the 10 strains of *H. pylori* tested. In Western blots of sonicated antigen preparation separated by non-denaturing PAGE, the mAbs raised against the 28 kDa polypeptide reacted with a single protein band which also demonstrated urease activity detectable on the blot.

Fig. 5. The capture of urease activity by a panel of mAbs. mAbs were adsorbed onto microELISA plate wells. The bound antibodies were incubated with sonicated antigen preparation. Bound urease activity was detected by incubation with a urea substrate. Liberated ammonia was detected by addition of Berthelot reagents. The A_{620} was measured. Hatched and solid bars show the results of two separate experiments. Each mAb tested is shown and the control was the irrelevant mAb GPA.

The urease activity of the sonicated antigen preparation was reduced by 73% in the presence of mAb, CP11, when compared to the control mAb. CP11 was the only mAb which inhibited this enzyme activity. This same mAb, and to a lesser extent mAb CP18, also captured urease activity (Fig. 5).

None of the mAbs, that recognized the 56 kDa polypeptide captured or inhibited urease activity. No mAbs were produced that immunolabelled the 61 kDa polypeptide.

Electron microscopy

The mAb CP11 was used, in an indirect immunogold technique, to locate the urease on prefixed *H. pylori*. The enzyme was identified on the surface-exposed material which appeared to be loosely bound and possibly shed from the organism (Fig. 6a). To determine if there was any intracellular localization of the urease, ultrathin cryostat sections of unfixed bacteria were also immunolabelled. This technique requires the mounting of ultrathin, cryosectioned material in methylcellulose which, unfortunately, does not allow the sharp definition of bacterial morphology. Nevertheless, the clarity was sufficient to determine that once again the antigen was

Fig. 6. Indirect immunogold-labelling of urease antigen at the ultrastructural level with mAb CP11. (a) Bacteria were prefixed with glutaraldehyde and incubated with CP11. Bound antibody was detected by incubation with goat anti-mouse IgG coupled to 10 nm gold particles. Labelled bacteria were negatively stained with phosphotungstic acid. Note the location of the urease antigen on the surface of the bacteria, particularly in the loose surface-associated material. Bar, 0.5 nm. (b) Unfixed bacteria were infused with sucrose solution then frozen in liquid nitrogen for ultrathin cryosections (500 nm). The sections were incubated with mAb CP11 and bound antibodies were detected as in (a). The sections were then mounted in methylcellulose and stained with uranyl acetate. Note that the use of unfixed bacteria and methylcellulose mountant in this procedure did not allow the morphological clarity normally observed with fixed, plastic-embedded bacteria. However, intracellular antigenicity is preserved and detectable. The urease antigen in these sections was located on the extracellular surface of the bacterial membrane. Bar, 0.5 μ m.

surface-located, closely adjacent to the outer membrane (Fig. 6b). No labelling was observed with the control antibody in either technique.

Discussion

The urease of *H. pylori* has been partly purified by gel filtration chromatography using FPLC which yielded active enzyme in a native conformational state suitable for immunization. A number of mAbs have been produced against this urease-containing material and used to investigate the structure and localization of the enzyme.

Although the urease-containing FPLC fraction comprised three major polypeptides (61 kDa, 56 kDa and 28 kDa), the evidence from the non-denaturing gels suggested that only two of these polypeptides (61 kDa and 28 kDa) were essential to the enzyme activity. These results are in marked contrast to the published evidence of Dunn *et al.* (1989) who considered, on the basis of two dimensional gel electrophoresis, that 66 kDa and 63 kDa (probably equivalent to our 61 kDa and 56 kDa) polypeptides were subunit constituents of partly purified urease and that a 31 kDa polypeptide (probably equivalent to our 28 kDa polypeptide) was inconsistently present. We conclude that this anomaly is, as Dunn *et al.* (1989) suggest, the result of differences in electrophoretic technique. Our evidence suggests that the 61 kDa and the 28 kDa, but not the 56 kDa, polypeptides were closely linked in the native protein. Of several mAbs directed against the 28 kDa polypeptide, one, designated CP11, both inhibited and captured the active enzyme, indicating that this polypeptide is an integral part of the urease. None of the mAbs directed against the 56 kDa

protein, however, demonstrated anti-urease activity. Unfortunately, no antibodies directed against the 61 kDa polypeptide were produced, although this component appears to be immunogenic in the hyper-immunized rabbit (Newell, 1987b) and humans during infection (Newell, 1987a).

Our results are consistent with recent molecular biological evidence from Clayton *et al.* (1989a) and Labigne-Roussel & Courcoux (1989). Both groups have apparently cloned the urease gene of *H. pylori*. The former group achieved expression in *E. coli* of inactive urease which was found to comprise the 61 kDa and 28 kDa polypeptides. Moreover, mAb CP11 immunolabelled the *E. coli*-expressed 28 kDa polypeptide (Clayton *et al.*, 1989b). The latter workers have obtained active urease expression in *Campylobacter jejuni*. These molecular biological studies once again suggest that the 56 kDa polypeptide is irrelevant to enzyme activity, and is probably merely co-purifying with the urease when separated by size exclusion gel filtration. One possibility was that the 56 kDa polypeptide was equivalent to the 51 kDa flagellin polypeptide identified by Geis *et al.* (1989). However, preliminary investigations comparing the urease-containing fractions, from the flagellated wild-type strain, and an aflagellate variant, indicate that the 56 kDa polypeptide is still present despite the absence of flagella. Moreover, the 56 kDa polypeptide and purified flagella (kindly provided by Dr Geis, Ruhr University, Bochum, FRG) do not have the same electrophoretic mobility (D. G. Newell, unpublished observations).

The identity and role of the doughnut structures found in fraction 14 is unknown. These structures are considered to be an integral component of the outer membrane (Newell, 1987a; Jones *et al.*, 1985) and the

possibility remains that they are either associated with the urease or the 56 kDa polypeptide.

The mAb CP11 has been used to locate the enzyme on the organism. For the enzyme to be effective in protecting *H. pylori* from the acid microenvironment of the stomach, it would be expected to be expressed on the bacterial surface. The ultrastructural localization of the enzyme by immunogold-labelling demonstrated that the urease is, indeed, surface-exposed, which would allow the urea substrate to be hydrolysed extracellularly. The urease-containing material appears to be loosely bound to the surface of *H. pylori* (Fig. 6a) and may, therefore, be continually shed, thereby presenting an antigenic challenge to the gastric mucosa. The relationship of this antigenic insult to *H. pylori*-associated gastritis remains to be investigated.

Addendum

In a recent publication Hu & Mobley (1990) have purified urease by chromatographic techniques and confirmed that the enzyme comprises two polypeptide subunits of 66 kDa and 29.5 kDa.

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