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Immunochemical properties of a 60 kDa cell surface-associated heat shock-protein (Hsp60) from *Helicobacter pylori*

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Abstract

Western blot analysis (immunoblotting) of cell surface-associated proteins from *Helicobacter pylori* confirmed our previous findings that binding of human IgG is a common property (among *H. pylori* strains). Purification of the IgG-binding proteins (IGBP) was achieved by two purification steps, affinity chromatography on IgG-Sepharose and nickel chelate affinity chromatography. SDS-PAGE and immunoblotting analysis revealed a 60 kDa protein with affinity for peroxidase labeled human IgG. Solid phase binding assays showed that IgG binds to an immobilized protein (IGBP). The 60 kDa IGBP binds human IgG_1, IgG_3 and IgM. Binding could be inhibited by the kappa chain of the human IgG, but not with its Fc fragment, nor with IgA or IgM. In addition, rabbit polyclonal antibodies raised against the 60 kDa IGBP blocked IgG binding. Monoclonal antibodies, specific to the Hsp60 heat shock protein of *H. pylori* recognized the 60 kDa IGBP as revealed by immunoblotting analysis, both in crude preparations and in the purified fractions.

Keywords: IgG-binding protein; *Helicobacter pylori*; Heat shock protein; Chaperonin

1. Introduction

Helicobacter pylori is the causative agent of type B antral gastritis, associated with peptic ulcer disease, gastric carcinoma and lymphoma [1–4]. *H. pylori* colonize the mucus layer of the human stomach and attach to the surface of gastric epithelial cells [5]. A variety of virulence factors in this pathogen have been recognized such as flagella, cytotoxins, urease, haemagglutinins and mucinase [5,6]. Urease co-purifies with a 60 kDa heat shock protein (Hsp60), a member of a chaperonin family [7,8].

Heat shock proteins (Hsps) are highly conserved proteins in all prokaryotic and eukaryotic cells [9] and Hsp60 proteins act together with other heat-shock proteins (the Hsp10, or GroES homologue protein) in facilitating protein folding and assembly of polypeptide chains, without becoming a part of the final structure [10]. Synthesis of Hsps is increased when cells are exposed to various environmental stress factors such as rapid temperature changes and tissue inflammation [9]. Nevertheless, the majority of Hsps are constitutively and abundantly expressed in the absence of stress. These proteins seem to be essential

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for cell viability under normal growth conditions [11,12].

The biochemical characteristics of a 60 kDa protein of *H. pylori* has been studied in detail [7,8]. *H. pylori* cells undergo spontaneous autolysis during culture and urease, HspB (i.e. a homologue of the GroEL family of heat shock proteins) probably become surface associated to *H. pylori* cells [13].

In the present study, we focused on the immunochemical properties of a 60 kDa protein from *H. pylori* towards the characterization of its high non-immune affinity for human IgG and its remarkable immune reactivity to monoclonal antibodies raised against the 60 kDa cell-associated heat shock protein from *H. pylori*.

2. Materials and methods

2.1. Chemicals

Human IgG was purchased from Zymed (San Francisco) and human IgG-Fc fragments from Calbiochem (La Jolla). Monoclonal antibodies recognizing the heat-shock protein (Hsp60) from *H. pylori* were kindly supplied by Lars Engstrand, Department of Medical Microbiology, University of Uppsala and monoclonal antibodies (T15 D5) recognizing a heat-shock protein of Group A *Streptococcus* was a gift from Claes Schalen, Department of Medical Microbiology, University of Lund. CH-Sepharose 4B was purchased from Pharmacia-Biotec Uppsala (Sweden). Peroxidase-conjugated immunoglobulins and 1,2-*o*-phenylene diamine (OPD) tablets were purchased from Dakopatts A/S (Glostrup). Gelatin and all electrophoresis reagents for SDS-PAGE were purchased from Bio-Rad Laboratories (Richmond) and 1-ethyl-3-3-dimethylaminopropyl carbodiimide hydrochloride (EDAC) was obtained from Sigma Chemical (St. Louis). Nitrocellulose membranes were purchased from Millipore (Bedford). All other chemicals were purchased from different commercial sources and were of analytical grade.

2.2. Bacterial strains and culture media

H. pylori strains CCUG 17875, 25, 133, 95, 66, 12225, 1139, 52 were obtained from the Culture Collection of the University of Gothenburg, or isolated by gastric biopsy at the Lund Hospital, Sweden. Strains were stored at −80°C in trypticase soy broth (TSB) containing 15% glycerol and were cultured on GAB-Camp media [14] at 37°C under microaerobic conditions (5% O_2, 10% CO_2, 85% N_2) for 2–3 days. *Staphylococcus aureus*, strain Cowan 1, was cultured on blood agar and incubated for 18 h at 37°C. *Campylobacter jejuni* strain 128 was grown on blood agar under microaerophilic conditions for 2 days at 37°C.

2.3. Protein extraction

Cell-surface associated proteins from *H. pylori* strain CCUG 17875 were released by washing the cells with distilled water. Briefly, *H. pylori* cells were harvested from agar plates and washed once with 0.015 M sodium phosphate buffer (pH 7.2) containing 0.15 M NaCl (PBS). Cells (1 g w/w) were suspended with 20 ml of distilled water and incubated at 4°C for 1 h followed by centrifugation (12,000 × *g*/20 min/4°C). Cell extracts were dialyzed overnight at 4°C against 10 mM Tris–HCl buffer (pH 7.5) containing 0.15 M NaCl (Tris-buffer).

Cell surface proteins of *S. aureus* strain Cowan-1 were extracted with 2 M lithium chloride as described [15]. Cell surface proteins of *C. jejuni* strain 128 were extracted with 0.2 M glycine–HCl pH 2.2 as described [16]. Protein concentrations of the bacterial cell extracts were determined using the Bio-Rad protein dye with bovine serum albumin as a reference protein to construct a standard curve.

2.4. Purification of the IgG-binding protein (Hsp60)

Human IgG was immobilized on CH-Sepharose 4B according to the instructions of the manufacturer. The gel was packed in a chromatographic column (5 ml bed volume) and equilibrated with 50 mM Tris buffer (pH 7.5) and *H. pylori* samples were applied. The column was washed with the same buffer and the bound proteins were eluted with 0.5 M NaCl in 50 mM Tris buffer (pH 7.5), followed by elution with 0.1 M glycine–HCl (pH 2.2) and a final elution with 3 M guanidine–HCl in 0.1 M phosphate buffer (pH 6.0). The pH of all fractions was neutralized with NaOH and dialyzed against 0.01 M ammonium

bicarbonate (pH 7.5). The proteins eluted with 0.5 M NaCl were dialyzed against 20 mM Na_2HPO_4, pooled and passed through a nickel chelate affinity column previously washed with 20 mM Na_2HPO_4 containing 0.5 M NaCl which includes 0.5 M NaCl pH 7.0 (equilibration buffer). The bound proteins were eluted with 0.1 M NaH_2PO_4 containing 0.5 M NaCl (pH 3.8) and column equilibration buffer containing 0.2 M immidazol (pH 7.0), each fraction was dialyzed overnight against 0.01 M ammonium bicarbonate (pH 7.5).

2.5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Separation of proteins was performed using the discontinuous buffer system of Laemmli in a Protean II Xi apparatus from Bio Rad [17]. Protein extracts were stacked in a 4% (w/v) acrylamide gel and separated in a 12% (w/v) polyacrylamide gel. Proteins were electrophoresed initially at 20 mA (constant current), and later at 30 mA when the tracking dye entered the separation gel. Molecular weights were determined from a standard plot of low molecular weight standards (Bio-Rad).

2.6. Western blotting

The separated proteins as described above were electrophoretically transferred to a nitrocellulose membrane (0.45 μm) in a semi-dry Trans-blot cell for 2 h at 100 mA in 25 mM Tris–20% methanol. The membranes were soaked in 10 mM Tris-buffer containing 1% gelatin for 2 h at 20°C to saturate remaining active binding sites. The membranes were washed three times with Tris-buffer containing 0.05% Tween-20 (TNT buffer) and incubated overnight at 4°C with 20 μg ml^{-1} human IgG or Fc fragment of human IgG. Membranes were washed three times with the TNT buffer and incubated at 22°C for 90 min with horseradish peroxidase (POD)-labelled rabbit, anti-human IgG. After washing the membranes four times with the TNT buffer, protein bands were visualized by incubating the membranes for 10 min in 50 ml of 50 mM sodium-acetate buffer (pH 5.0) containing 2 ml of 1% (w/w) 3-amino-9-ethyl carbazol in acetone and 25 μl of 30% H_2O_2. The reaction was stopped with 0.1 M sodium metabisulfite.

2.7. Immunization

2-month-old New Zealand white rabbits were immunized with the purified 60 kDa IgG-binding protein (200 μg ml^{-1}) emulsified in an equal volume of Freund complete adjuvant (Difco, Detroit). Booster doses of 180 μg of this protein in Freund incomplete adjuvant were injected subcutaneously 15 and 30 days after the first immunization. Rabbits were bled on day 40 and the serum were stored at −70°C. Preimmune sera were collected before the first immunization. The antibody titer was determined by ELISA and the specificity and purity was evidenced by immunoblot analysis.

2.8. Solid phase IgG-binding inhibition assay

ELISA plates (Nunc, Roskilde) were coated with 100 μl of a solution containing the 60 kDa IgG-binding protein in 0.01 M sodium carbonate buffer, pH 8.6 (10 μg of protein/well) and incubated overnight at 4°C. Plates were washed 3 times with PBS and the remaining active sites were blocked by incubating the plate with 1% BSA in PBS (2 h at 22°C). Wells were washed three times with PBS containing 0.05% Tween-20 (PBS-Tween). 100 μl of the rabbit polyclonal antibodies raised against the 60 kDa *H. pylori* IgG-protein were added and diluted with 0.05% PBS–BSA in a two-fold dilution series, followed by 90 min incubation at 37°C. The wells were washed with PBS-Tween, and 100 μl of either human IgG or IgG-Fc fragments in PBS-Tween (20 μg ml^{-1}) were added to each well. Plates were incubated at 37°C for 90 min followed by three washes with PBS-Tween. 100 μl of POD-labelled anti-human IgG in PBS-Tween were added to each well and incubated for 90 min at 37°C. Wells were washed as above, and a POD-substrate solution (containing 1 mg ml^{-1} OPD in 50 mM sodium citrate buffer pH 5.0) was added to each well, followed by an additional 30 min incubation at 22°C. The reaction was stopped by adding 50 μl of 1 M H_2SO_4, and the absorbance at 450 nm was measured. Other inhibition experiments were carried out by using (i) various immunoglobulin classes, (ii) human IgG subclasses, (iii) IgG L chains (integral part of the Fab region of the IgG molecule) and (iv) Fc fragment of IgG at a final concentration of 10 μg protein in 100 μl/well, as described above.

2.9. Biotinylation of H. pylori IGBP

Purified IGBP was dialyzed overnight against 0.1 M sodium borate buffer pH 8.8; 750 μg of *N*-hydroxysuccinimide biotin stock (10 mg ml^{-1} in dimethyl sulfoxide) were added to 3 mg ml^{-1} of protein. The protein was mixed and incubated at 22°C for 4 h, the reaction was stopped by adding 60 μl of 1 M NH_4Cl for 10 min. The biotin–IGBP conjugate was dialyzed extensively against PBS (pH 7.2).

2.10. Dot blot assay

Human IgG (025H-4821, IgG_1 λ I-4014, IgG_2 λ 1 I-4264, IgG_3 λ I-4514), IgA I-2636, IgM I-8260 and HSA from Sigma, were applied in a range of 10 μg to 0.015 μg/100 μl per well onto a nitrocellulose membrane using a dot blot apparatus. After saturation for 2 h with 3% BSA, membranes were incubated for 2 h at 20°C with 3 μg ml^{-1} IGBP Biotin-labelled in PBS. Membranes were washed four times with PBS-Tween, and 10 μl ml^{-1} streptavidin-POD were added for 2 h at 20°C and washed extensively with PBS-Tween and visualized in 2.5 μg aminobenzidine in 10 ml 0.1 M sodium acetate pH 5.0 and 2.5 μl H_2O_2, the reaction was stopped with 0.1 M sodium metabisulfite.

3. Results

3.1. Occurrence of immunoglobulin binding components among H. pylori strains

Previously, we found that IgG-binding activity is a common property among *H. pylori* strains as revealed by a particle agglutination assay, since cell-surface associated proteins from different *H. pylori* strains extracted with distilled water yielded a fraction rich in IgG-binding (IGBP) as revealed by a particle agglutination assay [18] and by Western blot analysis (Fig. 1).

3.2. Purification of cell associated IGBP from H. pylori strain 17875

Seven ml of a *H. pylori* water extract, containing approximately 11 mg of protein (previously dialyzed against 50 mM Tris-buffer, pH 7.5) were loaded in a IgG-Sepharose column equilibrated with 50 mM Tris–HCl (pH 7.5). The column was washed with the same buffer (until absorbancy at 280 nm reached the base line values), followed by elution with 0.5 M NaCl in Tris buffer (pH 7.5) and 0.1 M glycine–HCl buffer (pH 2.2). From the original amount of protein applied into the column, the yield was 1.75 mg of protein. Elution with 0.5 M NaCl in 50 mM Tris

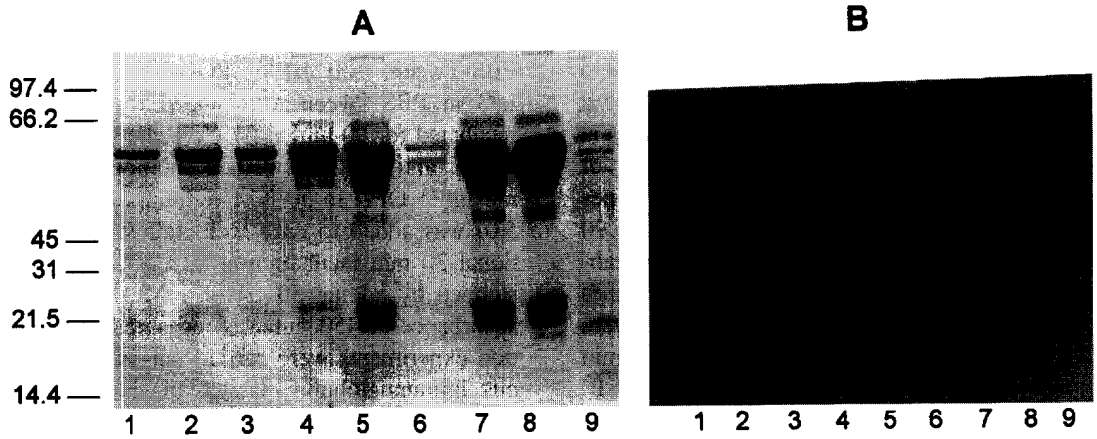

Fig. 1. Occurrence of IGBP among *H. pylori* strains. Panel A: SDS-PAGE. Lanes 1–9 *H. pylori* strains 75, 25, 133, 95, 253, 66, 12225, 1139 and 52 respectively. 100 μg of water extract material was electrophoresed on a 12% SDS-PAGE gels and then blotted onto a nitrocellulose membrane (panel B). The IGBP were developed using POD-labelled human IgG as a probe.

buffer pH 7.5 gave a peak containing 1.18 mg ml^{-1} of IGBP, while the second elution step with 0.1 M glycine–HCl (pH 2.2) yielded a second protein peak with IgG-binding activity of approximately 0.57 mg ml^{-1}. IGBP fraction eluted with NaCl were pooled and loaded into the nickel chelate chromatography column, the IGBP was eluted with 0.1 M Na_2HPO_4 containing 0.5 M NaCl (pH 3.8) and the same buffer containing 0.2 M immidazol, pH 7.0. SDS-PAGE analysis of the chromatographic fractions showed one major protein band of 60 kDa (Fig. 2).

3.3. Characterization of the immunglobulin binding properties of the H. pylori Hsp60, i.e. a putative immunoglobulin binding protein (IGBP)

We first studied the binding of human IgG to the chromatographically purified 60 kDa IGBP from water extracts. The binding of IgG was tested over the concentration range of 2 to 500 $\mu g\ ml^{-1}$. IgG binds to immobilized Hsp60 in a ligand concentration fashion (Fig. 3). The reactivity of the *H. pylori* 60 kDa IGBP with six immunoglobulin preparations from

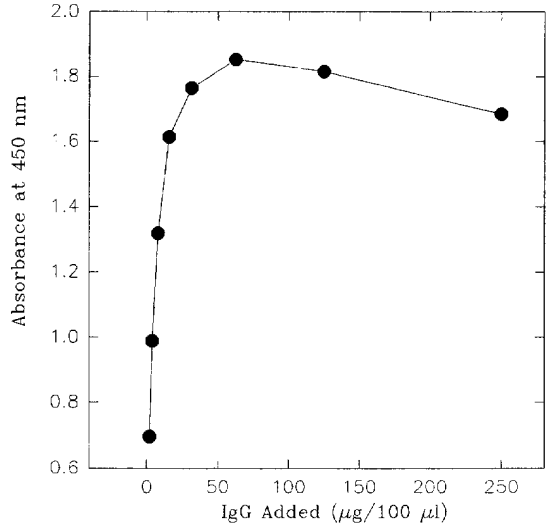

Fig. 3. Dose–response analysis of the binding of human IgG to *H. pylori* 60 kDa IGBP. Plates coated with 60 kDa IGBP (10 μg/well) were incubated for 1 h at 37°C with increased amounts of human IgG, followed by 1 h incubation at 37°C with POD-labelled rabbit IgG to human IgG. Results are expressed as the mean absorbance of determinations made in triplicates. Standard deviation was less than 5%.

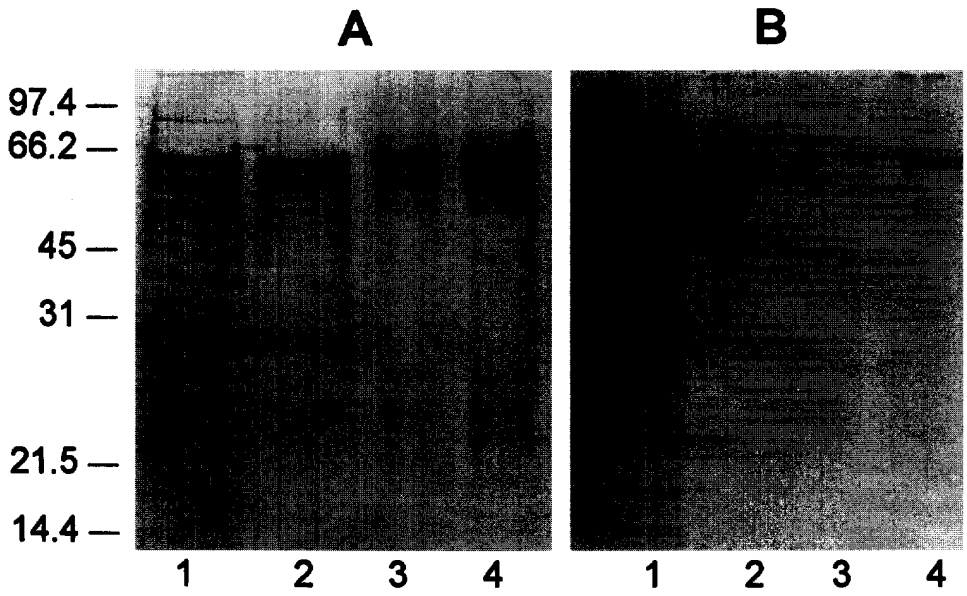

Fig. 2. SDS-PAGE (panel A) and Western blot analysis (panel B) of the chelate affinity chromatographic fractions. Lane 1: *H. pylori* IGBP eluted with 0.5 M NaCl from IgG-sepharose affinity chromatographic column; lane 2: *H. pylori* IGBP eluted with 20 mM NaH_2PO_4 containing 0.5 M NaCl, pH 7.0 from the chelating chromatography column; lane 3: *H. pylori* IGBP eluted with 0.1 M NaH_2PO_4 containing 0.5 M NaCl, pH 3.8, from the chelating chromatography column and lane 4: *H. pylori* IGBP eluted with 0.1 M NaH_2PO_4 containing 0.5 M NaCl and 0.2 M imidazole, pH 7.0, from the chelating chromatography column.

human IgG (monoclonal IgG_1, IgG_2, IgG_3, IgA, IgM, and HSA) was tested by a dot blot technique. It was found that biotin-labelled 60 kDa IGBP interact with human IgG_1, IgG_3 and IgM, but not with other proteins tested (Fig. 4).

To explore if 60 kDa IGBP specifically binds human IgG, we investigated whether binding was influenced by the presence of various immunoglobulin preparations. Plates were coated with 10 μg/well of 60 kDa IGBP and subsequently incubated for 1 h at 37°C with various immunoglobulin preparations (10 μg/well) prior to incubation for 1 h at 37°C with human IgG. The results indicated that binding could be inhibited only with IgG, especially with its kappa chain, but not with IgA or IgM (Table 1). In another experiment, we evaluated whether the 60 kDa IGBP bound rabbit or mouse IgG. For this experiment, plates coated as described above were preincubated for 1 h at 37°C with 10 mg/well of rabbit or mouse IgG prior to incubation with human IgG. Our findings indicate that binding of human IgG was not influenced by a preincubation with rabbit or mouse IgG (Table 1).

Table 1
Inhibition of IgG-binding to the 60 kDa IGBP of *H. pylori*. Data are expressed as percentage of binding inhibition in comparison with the control, mean values of triplicates, and a SD less than 5%

Inhibitor	Percentage of binding inhibition
Control (without inhibitor)	0
IgA	6.1
IgG	
Lambda chain	16.0
Kappa chain	35.9
Fc fragment	6.0
Rabbit IgG	2.4
Mouse IgG	6.6
Preimmune rabbit sera	2

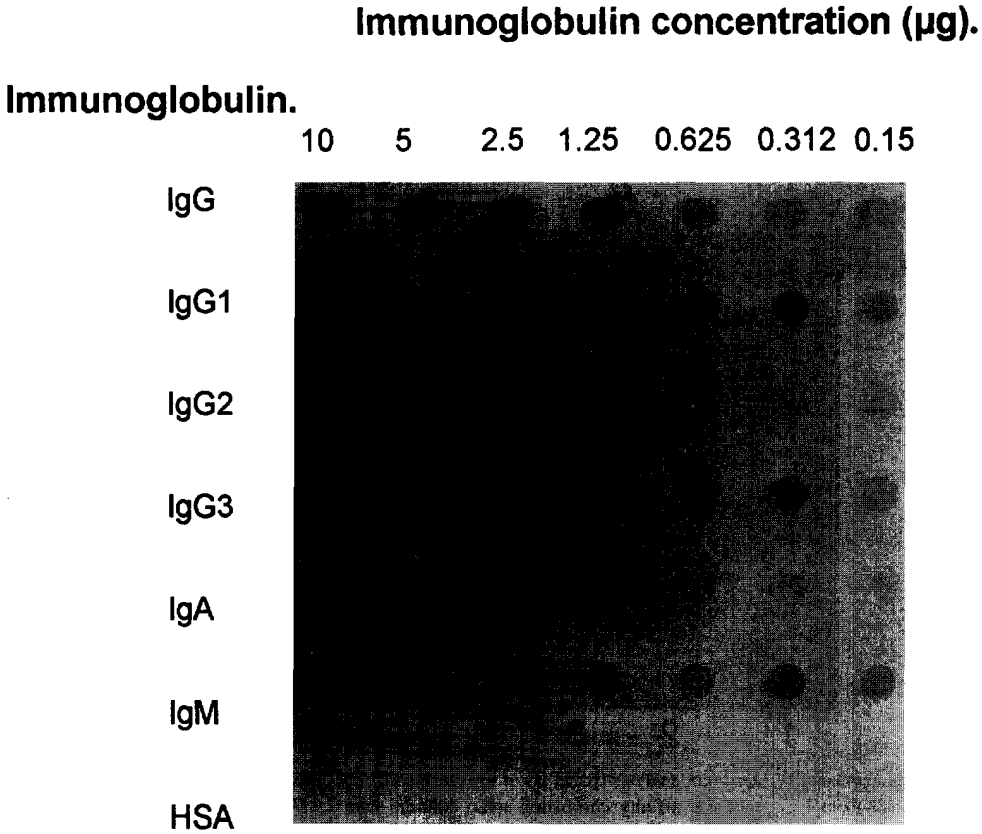

Fig. 4. Binding of biotinylated *H. pylori* 60 kDa IGBP to different immunoglobulin classes and subclasses. Lane 1: Human IgG, lane 2: IgG1, lane 3: IgG2, lane 4: IgG3, lane 5: IgA, lane 6: IgM and lane 7: HSA (human serum albumin).

3.4. Blocking of IgG binding to a H. pylori 60 kDa IGBP by antisera

Rabbit serum from immunization with the 60 kDa IGBP purified by affinity chromatography was tested for the presence of antibodies which were able to block the binding of IgG to *H. pylori* IGBP (Fig. 5). Our results indicate that blocking of human IgG binding to immobilized IGBP in the presence of sera from rabbits immunized with IGBP in a dose dependent manner. Preimmune rabbit serum was unable to block the binding of human IgG to the 60 kDa IGBP immobilized in the microtiter plates (Table 1).

3.5. Antigenic cross-reactivity of the 60 kDa IGBP with Hsp60 from H. pylori

Rabbit antiserum to the 60 kDa IGBP recognized two bands at 66 and 60 kDa in *C. jejuni* extracts, several protein bands in *S. aureus* extracts and a single band of 60 kDa in the *H. pylori* chomatographically purified IGBP (data not shown). Mouse monoclonal antibody, specific for the 60 kDa heat shock protein (Hsp60) from *H. pylori* [8], recognized the 60 kDa IGBP from *H. pylori*, both in crude preparations and in the chromatographically purified fractions (Fig. 6). It was also found that the later antibody also recognized a *H. pylori* protein migrating at 35 kDa (Fig. 6A). However, monoclonal antibody T15 D5, specific for the *Streptococci* group A heat shock proteins did not recognize the *H. pylori* IGBP (Fig. 6B).

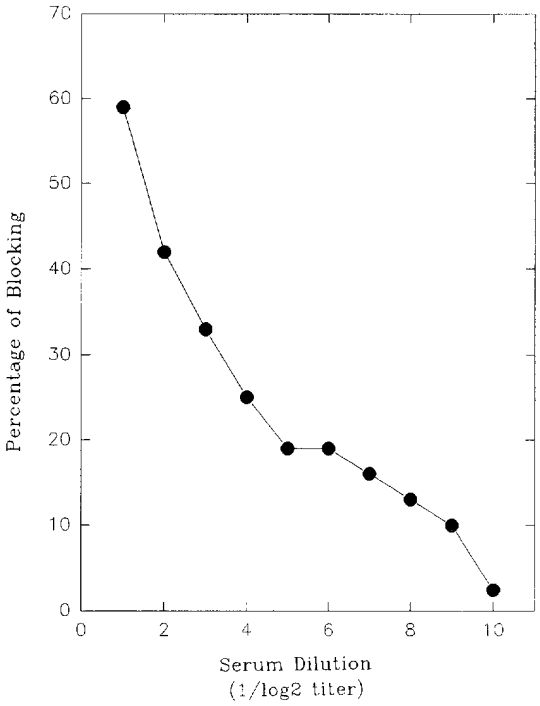

Fig. 5. Solid phase IgG-binding inhibition assay to *H. pylori* 60 kDa IGBP. See Section 2 for details. Results are expressed as the mean absorbance of determinations made in triplicates. Standard deviation was less than 5%.

4. Discussion

A number of putative cell surface adhesins have been identified in *H. pylori* [5,19,20], and various cell surface molecules allow the microbe to adhere, colonize, and persist in gastric tissues [5,20,21]. This pathogen is able to establish itself and maintain a persistent infection despite the production of antibodies to a number of *H. pylori* proteins [1,5,22] and suggests that the microbe has the ability to evade the host immune system.

We present evidence that the binding of a nonimmune IgG to *H. pylori* cells is mediated by a 60 kDa cell surface protein. IgG binding protein (IGBP) is easily solubilized by washing *H. pylori* cells with water and in significant quantities purified on IgG-Sepharose and nickel chelate affinity chromatography. Hsp60 copurified with a large urease complex that was produced by this organism [8]. Assuming that the 60 kDa IGBP is related to the Hsp60 [8], the purification procedure described is simple and yields relatively large amounts of purified IGBP in only two purification steps.

Mouse, rabbit and human IgG did not inhibit the apparent binding of IgG to *H. pylori* cells, but it could be achieved with the kappa chains of the human IgG. Similarly, monoclonal antibodies raised against *H. pylori* Hsp60 protein could effectively inhibit binding. On the other hand, the characteristics of the IgG-binding activity of the 60 kDa IgG-binding protein from *H. pylori* seem to differ from the well defined IgG-binding proteins of *Staphylococcus* and *Streptococcus* species [23], since it binds IgG_1

and IgG_3 myeloma globulin as revealed by a dot blot analysis (Fig. 4). It has been reported for a number of other pathogens that soluble Fc fragments of the IgG molecule do not interfere with the expression of IgG-binding activity [24,25]. Our studies with *H. pylori* indicate similar findings, where IgG binding to *H. pylori* did not inhibit the Fc fragment of IgG as the IgG light chains did. This suggests that binding is through the Fab region of the IgG molecule as reported for *Peptococccus magnus* [26]. It is interesting that the IgG-binding characteristics of the 60 kDa IGBP of *H. pylori* show a remarkable similarity to the Protein L of *P. magnus* in the sense that both proteins interact with isolated light chains, specially the kappa chain, which is the integral part of Fab fragment and restricted to the V region [27]. However, further studies need to be done to define the molecular basis of the interaction between the 60 kDa IGBP of *H. pylori* and the V_k region of IgG, and to determine whether the IGBP of *H. pylori* have the Fab-binding specificity produced for a B-cell superantigen, as well as the ability to influence the formation of human immune repertories as proposed for staphylococcal protein A [28].

The uptake of host immunoglobulins by bacterial strains expressing IgG-binding proteins might theoretically interfere with defense mechanisms. In this respect, it has been demonstrated that the binding of human plasma proteins to *S. aureus* dramatically changes its cell surface properties [29]. This would suggest that binding of immunoglobulins to the cell surface of the pathogen could influence the coexistence between the host and the parasite. We believe that *H. pylori* cells may probably be coated with

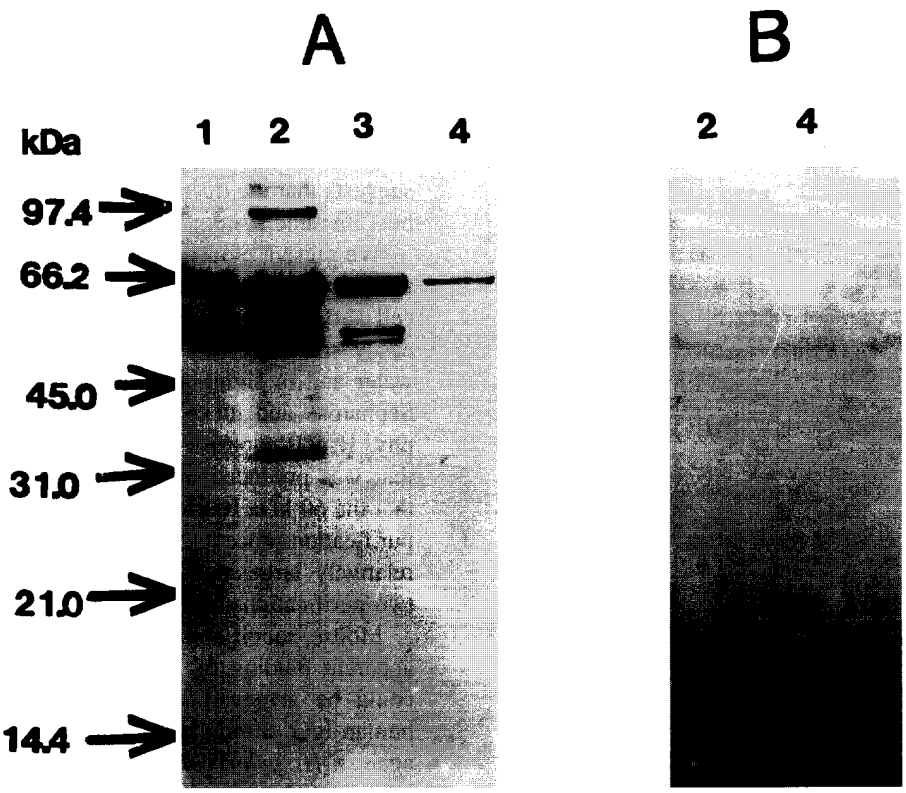

Fig. 6. Cross-reactivity of the *H. pylori* 60 kDa IgG-binding protein (IGBP) with monoclonal antibodies specific for *H. pylori* Hsp60. Panel A: Immunoblotting using monoclonal antibody specific for *H. pylori* Hsp60 as a probe. Lane 1: Acid glycine extracts of Campylobacter jejuni strain 128, lane 2: *H. pylori* strain 17875 cell surface water protein extracts, lane 3: Partial purified *H. pylori* IGBP from IgG-Sepharose, lane 4: Purified *H. pylori* IGBP from chelate affinity chromatography. Panel B: Immunoblot using monoclonal antibody T15 D5 (specific for Streptococci group A heat shock proteins) as a probe. Lane 2 and 4 as described.

host proteins such as IgG and albumin and may help to evade the host defense by a mechanism of molecular mimicry [30]. However, further studies are needed to determine whether immunoglobulin binding proteins are implicated in the pathogenesis of human ulcer gastroduodenal diseases.

Since the Hsp60 family of heat shock proteins are immunodominant antigens in many microbial pathogens [31,32] and can induce immune tolerance [33]. Recent studies have been carried out to demonstrate whether Hsp60 proteins can be included in a vaccine to provide immunity against pathogenic bacteria. It has been shown that guinea pigs vaccinated with *Legionella pneumophila* Hsp60 protein exhibited a high humoral immune response i.e. high antibody titers to Hsp60, but a low cellular immune response [34]. However, immunized animals with *L. pneumophila* Hsp60 protein did not survive a lethal challenge [34]. It has been recently shown that heat shock proteins of *H. pylori* induce protective immunity and have been assessed as a potential protective antigen in a murine model of gastric *Helicobacter* infection [35].

In evaluating proteins for potential vaccine efficacy in humans, survival must take precedence over protection measured in terms of bacterial clearance [36]. Since *H. pylori* Hsp60 proteins have IgG-binding activity in a nonimmune manner, one may hypothesize that the apparent high antibody titers raised against Hsp60 proteins are actually influenced by a nonimmune IgG-binding activity. However, further studies are needed to determine whether IgG-binding activity is a common property among the members of the Hsp60 protein family or is an exclusive property of the *H. pylori* Hsp60 protein.

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