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Expression of *Haemophilus influenzae* type b idiotype 1 on naturally acquired antibodies

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SUMMARY

The Chinese population in Hong Kong has a low incidence of invasive *Haemophilus influenzae* type b (Hib) disease, as well as carriage of the microorganism. Likely stimuli for the natural antibodies to Hib, which might protect against Hib infection, are cross-reactive antigens of bacteria like *Escherichia coli* K100. Our aim was to determine the isotype and idiotype distribution and cross-reactivity of natural antibodies against Hib capsular polysaccharide (CP) in healthy Hong Kong Chinese. Titration of 20 sera by ELISA showed IgG antibodies reacting with Hib CP in all individuals. The antibodies were mainly IgG2, and their avidity index ranged widely. Isoelectric focusing (IEF) combined with immunoblotting showed patterns of IgG2 antibody clones against the CP of Hib and *E. coli* K100 which were similar in 10 cases. Absorption with Hib CP only eliminated some bands in two sera. Absorption with K100 CP did not remove any anti-Hib CP bands. In three sera additional clones of antibodies reacting to K100 CP only, disappeared after absorption with this CP. Spectrotypic analyses of IgG antibodies reacting with anti-Hib idiotype 1 (Id-1) revealed stronger IEF patterns with bands in differing locations compared with anti-Hib CP antibodies. The strong reactivity of serum IgG, IgA and IgM antibodies with monoclonal anti-Hib Id-1 was confirmed by ELISA. This reactivity was not abolished after absorption of the sera with either Hib CP, or K100 CP. The data indicate a high prevalence of Id-1 among Hong Kong Chinese. However, only one individual had Id-1 antibodies specific for Hib CP, judging from absorption experiments. Others had much lower activity of Id-1 anti-Hib CP antibodies compared with the total IgG Id-1, suggesting that Hong Kong subjects have Id-1-positive antibodies in their serum which are not specific for Hib CP. This is consistent with the nature of Id-1, which is a marker of $A2V_L$ region usage rather than a marker of a Hib CP paratope. We suggest that natural antibodies reacting with Hib CP in healthy Hong Kong Chinese are the product of exposure to some cross-reactive antigen(s), different from both Hib and *E. coli* K100 CP.

Keywords natural antibody idiotype cross-reactive antigens isoelectric focusing *Haemophilus influenzae* type b

INTRODUCTION

Haemophilus influenzae type b (Hib) is an important bacterial pathogen causing meningitis, septicaemia, pneumonia, epiglottitis and other invasive diseases in young children in different parts of the world [1,2]. In Northern Europe and Australia the annual incidence of invasive Hib infections varied between 25 and 80 per 100 000 children under 5 [3–5] and in the USA 60–130 per 100 000, before the introduction of Hib conjugate vaccines. Genetic factors have been implicated in susceptibility to Hib infections, since some native American populations have much higher prevalence of the infections as well as an impaired antibody response to Hib polysaccharide vaccines [7,8].

The carriage rate of Hib in populations of healthy infants and children was found to be 2–5% [9–11]. However, Stephenson *et al.* [12] found the pharyngeal carriage rate of Hib among healthy day-care children under 14 years of age in Central Massachusetts to be 15·1%.

The Chinese population in Hong Kong has a low incidence of invasive Hib infection. The annual incidence of Hib invasive disease for children under 5 years of age is 2·7 per 100 000 [13]. The carriage of the microorganism in healthy individuals is also uncommon in this area [14]. Lau *et al.* [15] isolated no Hib from the nasopharynx of 1812 healthy children in Hong Kong. Although there is no vaccination against Hib in Hong Kong, a significant

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level of IgG antibody against Hib capsular polysaccharide (CP) was found in healthy children and adults by ELISA [15]. Serum antibodies against the CP of Hib have been shown to protect against invasive forms of the disease caused by this microorganism [16].

There is evidence that cross-reactive antigens on other encapsulated bacteria may be responsible for the acquisition of 'natural' immunity against Hib. Among these bacterial species are *Staphylococcus aureus, Streptococcus pneumoniae, Bacillus subtilis, B. pumilis, Lactobacillus plantarum,* group D streptococci, *Escherichia coli* [17]. In particular, the CP of *E. coli* K100 is structurally similar to the Hib CP [18]. The induction of anti-Hib polysaccharide antibody in serum by gastrointestinal colonization with these bacteria was described by Schneerson & Robbins [19].

Recently Lucas *et al.* demonstrated that most vaccine-induced human antibody to Hib CP expresses Hib idiotype 1 (Id-1), an idiotope located in the κII-A2V$_L$ region [20]. This idiotype seems to ensure optimal protection against Hib disease [20]. However, natural exposure in contrast to immunization may selectively stimulate Id-1$^-$ B cell clones [21]. It was also suggested that antibodies to Hib CP cross-reactive with *E. coli* K100 CP do not express Id-1 since they have either Vλ, VκI, or VκIII chains, but not VκII [22]. Since the Chinese population in Hong Kong seems to be protected against invasive Hib disease, we were interested in the antigenic specificity and idiotypic expression of the naturally acquired antibodies to Hib CP in this population.

The aim was to determine the isotype and idiotype distribution and cross-reactivity of natural antibodies to Hib CP in healthy Hong Kong Chinese.

MATERIALS AND METHODS

Sera from 20 healthy Hong Kong Chinese (19 adults and one 4-year-old child) without any record of Hib disease or Hib carriage were studied. The project was approved by the Ethics Committee of Hong Kong University.

Isoelectric focusing/affinity immunoblotting analysis

An isoelectric focusing (IEF)-affinity immunoblot analysis using our modified rapid micro system for separation of serum samples was applied. Briefly, 1% agarose gel containing 15% D-sorbitol (Merck, Darmstadt, Germany), 5% ampholytes 3–10 and 1·25% ampholytes 8–10·5 (Pharmacia, Uppsala, Sweden) was made in a plexiglass matrix (48 × 50 × 0·6 mm) with GelBond (Pharmacia). IEF was performed in the PhastSystem apparatus (Pharmacia). The gels were prefocused (30 min, 350 V, 73 Vh) before the application of the samples (sample volume 4 μl), then focusing was performed (total run of ≈60 min, 1000 V, 300 Vh). The serum samples and the pI protein standard (Pharmacia) were applied to the gel surface with Phast gel sample applicators (Pharmacia).

Agarose gel strips containing the pI protein standard were fixed with 20% trichloracetic acid, treated with 30% methanol and stained with coomassie blue.

For immunoblotting, nitrocellulose (NC) membranes (0·45 mm pore size; BioRad, Richmond, CA) were soaked in PBS for 15 min and then coated with 10 μg/ml Hib CP (Pasteur Merieux, Lyon, France), or with 10 μg/ml *E. coli* K100 CP (kindly provided by Dr R. Schneerson, NIH, Bethesda, MD), or 5 μg/ml of a MoAb to Hib Id-1 (LuC 9) [20] overnight at room temperature on a horizontal shaker. After washing with PBS the membranes were blocked with 4% fish gelatin (Sigma Chemical Co., St Louis, MO) for 1 h at room temperature. The NC membranes were then placed on the focused gels with separated serum samples, covered with few layers of absorbant paper, a glass plate and a weight during 1 h for blotting.

To detect the clonotypes of total IgG antibodies against the two bacterial antigens, the blotted NC membranes were incubated for 1 h at room temperature with alkaline phosphatase (AP)-conjugated mouse anti-human IgG (Jackson Immuno- Research Labs, West Grove, PA) for 1 h at room temperature.

To detect the clonotypes of IgG subclass antibodies, the blotted NC membranes were incubated with mouse anti-human IgG2 MoAbs (clone HP 6014; Sigma) or anti-human IgG1 (clone HP 6069; Caltag, San Francisco, CA) and after washing with PBS–0·05% Tween (PBS–T) incubated for 1 h at room temperature with AP-conjugated rabbit anti-mouse immunoglobulins (Dakopatts, Glostrup, Denmark).

Antibodies against Hib CP expressing Id-1 were detected using the anti-Id-1 MoAb LuC 9 [20] instead of monoclonals to human IgG subclasses. NC membranes coated with LuC 9 were blocked, separated sera were blotted onto the membranes, and the membranes were incubated with AP-conjugated mouse anti-human IgG (Jackson ImmunoResearch Labs) for 1 h at room temperature.

After washing with PBS–T and Tris-buffered saline (TBS) the substrate containing 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine and *p*-nitroblue tetrazolium chloride (BioRad) was added and the staining was stopped after 10–20 min by washing the NC membranes with distilled water.

The isolated IgG anti-Hib CP antibody of one vaccinated individual (US FDA, Bethesda, MD) referred to as the standard preparation was used in a 1:4 dilution.

ELISA for anti-Hib CP antibodies

Antibodies of the IgG, IgA, IgM isotypes and of IgG subclasses to Hib CP were analysed using ELISA according to [23] with some modifications. Microplates (Costar Corp., Cambridge, MA) were precoated with 5 μg/ml of avidin (Sigma) diluted in PBS and left overnight at room temperature. After washing with PBS the plates were coated with biotinylated Hib CP (kindly provided by Dr R. Schneerson) diluted in PBS in a concentration of 2 μg/ml for 3 h at room temperature. After washing with PBS the plates were blocked with 4% fish gelatin for 1 h at room temperature, washed with PBS–T and incubated with serum samples in serial dilutions (1:50–1:1500) with 0·1% fish gelatin in PBS–T in duplicates and left overnight at room temperature. The plates were washed and incubated with AP-conjugated mouse anti-human IgG (Jackson), goat anti-human IgA (Sigma), or goat anti-human IgM (Sigma) for 5 h at room temperature.

To study IgG subclass distribution of Hib CP antibodies the mouse monoclonal anti-human IgG1 (clone HP 6069; Caltag), anti-human IgG2 (HP 6014; Sigma), anti-human IgG3 (HP 6050; Sigma), or anti-human IgG4 (HP 6025; Sigma) were incubated in the plates for 3 h at room temperature. After washing, AP-conjugated rabbit anti-mouse immunoglobulins (Dakopatts) were incubated in the plates for 2 h at room temperature. All dilutions were made with 0·1% fish gelatin in PBS–T.

The plates were washed and the substrate, 1 mg/ml *o*-nitrophenyl-b-D-galactopyranoside (Sigma) in 1 M diethanolamine buffer pH 9·8, was added. The enzyme–substrate reaction was read in a Titertek Multiscan (Flow Labs, Irvine, UK) as the optical density (OD) at 405 nm after 30 min of incubation. Antibody levels were expressed as OD of serum samples in the chosen dilution

1:100 ($OD_{1:100}$). For quantification of antibody levels the standard preparation was used.

Antibodies against Hib CP expressing Hib Id-1 were studied in the same system except that monoclonal LuC 9 was used instead of isotype-specific antibodies. AP-conjugated rabbit anti-mouse immunoglobulins (Dakopatts) were applied as well.

ELISA for Id-1 antibodies

The plates were coated with LuC 9 at 5 μg/ml overnight at room temperature. After blocking of the plates with 4% fish gelatin (1 h at 37°C) serially diluted serum samples were added and incubated for 2 h at 37°C. The plates were developed with AP-conjugated mouse anti-human IgG (Jackson), AP-conjugated goat anti-human IgA, or IgM (Sigma) overnight at room temperature. The possible anti-mouse activity of all sera was checked using mouse immunoglobulins for coating instead of LuC 9.

Absorption of the specific antibodies

Absorption of the sera with Hib and *E. coli* K100 CP was performed according to [24] by adding the polysaccharide in concentrations of 25–5000 μg/ml to the same volume of the undiluted serum samples (for IEF) or of the samples diluted 1:50 (for ELISA) followed by incubation for 1 h at 37°C on a shaker and then overnight at 4°C.

Determination of antibody avidity

Avidity of IgG2 antibodies to Hib CP was estimated by potassium thiocyanate elution in ELISA, where the molarity of thiocyanate required to elute 50% of bound antibody under conditions of antigen excess was used as a measure of relative avidity [25,26].

Each serum sample was incubated in 18 replicate wells of the antigen-coated plate in an appropriate dilution. The dilution of serum used for each sample was chosen to ensure optimal conditions of antigen–antibody reaction as determined by previous titration in ELISA. After three washes of the plates with PBS–T, bound antibodies were eluted with varying molarities of KSCN in PBS during 30 min incubation at room temperature. Two wells for each sample were incubated with PBS alone, without KSCN. The wells were then aspirated and washed six times with PBS–T. The amount of residual bound antibody was determined using monoclonal anti-human IgG2 and AP-conjugated anti-mouse immunoglobulins.

The mean absorbance values for the duplicate wells at the various molarities of KSCN were plotted after subtraction of sample-free background values. The interpolated point at which the absorbance was 50% of that without the thiocyanate for the same serum sample was determined. The relative avidity index was expressed as the molarity of KSCN at the 50% elution point.

Statistical analysis

Results were expressed as mean ± s.e.m.

RESULTS

Isotypes of antibodies binding to Hib CP in ELISA

In all 20 sera we found IgG antibodies reacting with solid-phase Hib CP. The most prevalent antibody isotype was IgG2 (Fig. 1a). Low levels of IgG1 antibodies ($OD_{1:100} < 0{\cdot}3$) were found in all samples except that of the 4-year-old child ($OD_{1:100} = 1{\cdot}28$). IgG4 antibodies were detected in several samples as well. High levels of IgA antibodies were found in seven samples and high levels of both IgA and IgM in two (Fig. 1b).

Influence of absorption of serum samples with CP of Hib and E. coli *K100 on antibody levels in Hib CP ELISA*

Neither IgG nor IgG2 antibody levels decreased after absorption with Hib or *E. coli* K100 CP in most Hong Kong sera. Only one sample (N16) showed a 40% decrease in IgG2 antibodies after absorption with K100 CP. N16 also decreased in total IgG and IgG2 levels (up to 38% and 56%, respectively) after absorption with Hib CP.

In another serum (N18) the level of both total IgG and IgG2 antibodies diminished significantly (up to 88% and 76%, respectively) after absorption with Hib CP.

However, in the standard preparation antibody levels significantly decreased in a dose-dependent manner after absorption with Hib CP: IgG1 was almost completely absorbed and IgG2 decreased up to 50%. This also proves the adequacy of the method for absorption of specific antibodies.

Avidity of IgG2 antibodies in Hib CP ELISA

The avidity index of the tested sera ranged widely from 0·4 to 5·0 M (on average 3·0 ± 0·4).

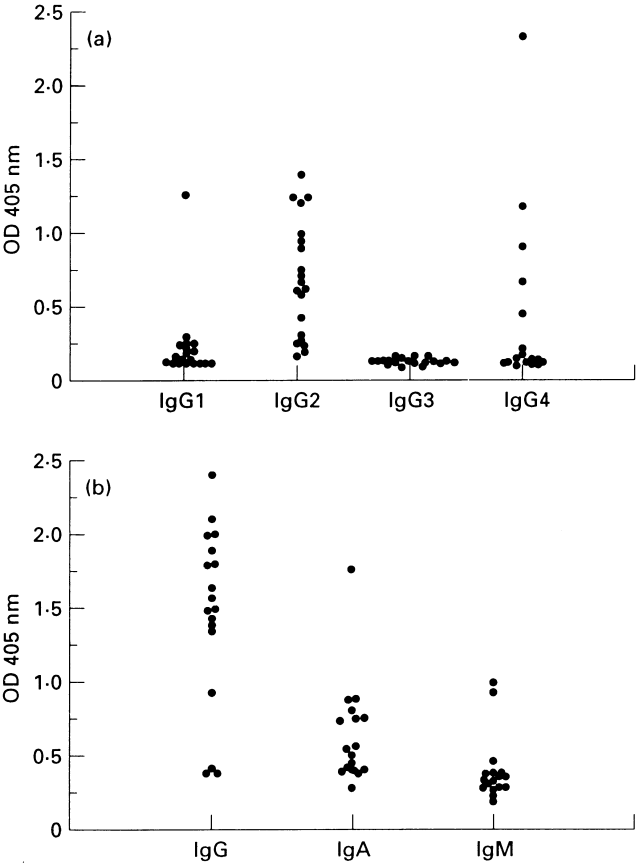

Fig. 1. Determination of antibodies to *Haemophilus influenzae* (Hib) capsular polysaccharide (CP) of different isotypes in sera of 20 healthy Hong Kong residents by ELISA. (a) IgG subclass antibodies. (b) IgG, IgA, IgM antibodies.

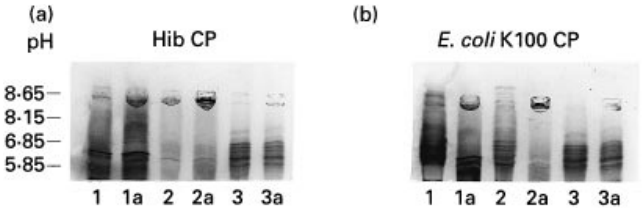

Fig. 2. Isoelectric focusing (IEF)/immunoblotting patterns in three sera of healthy Hong Kong residents before and after (a) absorption with 5 mg/ml of *Escherichia coli* K100 capsular polysaccharide (CP). (a) Nitrocellulose (NC) membrane coated with *Haemophilus influenzae* (Hib) CP. (b) NC membrane coated with *E. coli* K100 CP. Sera 1,2, additional clones of IgG2 antibodies to K100 disappeared after absorption; serum 3, identical clones of IgG2 antibodies to Hib and K100.

Clonotypes of antibodies reacting with the CP of Hib and E. coli *K100*

IEF/immunoblotting patterns of IgG2 antibody clones binding to the CPs of Hib and *E. coli* K100 were very similar in 10 of 20 cases. The bands were located within the pI range from 5·6 to 9·0. In nine samples additional antibody clones reacted only with K100 CP. In three samples (N3, N10, N16) they disappeared after absorption with the K100 CP.

In two samples (N16, N18) additional antibody clones reacted with Hib CP, and in both cases they disappeared after absorption with Hib CP. Absorption of the standard preparation with Hib CP completely abolished IgG2 antibody bands. On the other hand, absorption of Hong Kong sera with K100 CP did not remove any IgG2 anti-Hib CP bands (Fig. 2).

IgG1 antibodies in four serum samples studied formed very weak and similar bands with both Hib and *E. coli* K100 CP, which could not be absorbed with K100 CP.

In order to detect Id-1 antibodies reacting with Hib CP, the membranes were incubated with LuC 9 after the transfer of separated sera. In all 17 Hong Kong sera studied no bands were detected, while the standard preparation showed very strong bands of IgG Id-1 anti-Hib CP antibodies (Fig. 3).

Clonotypes of total serum IgG antibodies expressing Id-1

IEF separation of the sera followed by transfer onto the NC membrane coated with LuC 9 showed very clear clonotypes of IgG antibodies expressing Id-1. In 12 serum samples out of 20 the bands were much stronger and differently located than those of IgG antibodies reacting with membrane-bound Hib CP (in Fig. 4 only six are shown). In general the clones of Id-1 IgG antibodies were found in a more basic region than those of IgG and IgG2 anti-Hib CP (pI from 6·55 to 9·30 and higher) (Fig. 4).

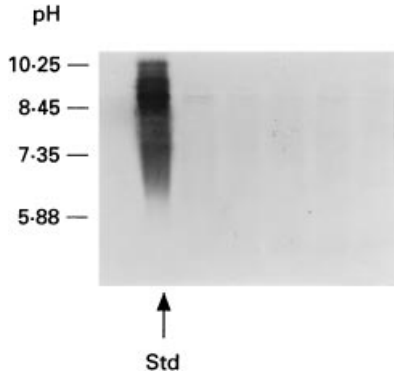

Fig. 3. Isoelectric focusing (IEF)/immunoblotting patterns of anti-*Haemophilus influenzae* (Hib) Id-1 antibodies in six sera of healthy Hong Kong residents and standard preparation (Std). (Nitrocellulose membrane coated with Hib capsular polysaccharide (CP), developed with monoclonal antibody LuC 9.)

Serum IgG, IgA and IgM expressing Id-1 determined by ELISA

The presence of IgG Id-1^+ antibodies in all Hong Kong sera was confirmed by ELISA with the use of monoclonal LuC 9 for coating (Fig. 5). In four samples IgA and IgM antibodies were present at significant levels as well. The specificity of the reaction was confirmed using mouse immunoglobulins instead of LuC 9 for the coating. There was no anti-mouse activity in the sera.

Influence of absorption of serum samples with CP of Hib and E. coli *K100 on the levels of total IgG antibodies expressing Id-1*

Absorption of the sera with Hib and K100 CP did not diminish the activity of IgG antibody binding to the anti-Id-1 in ELISA.

Antibodies expressing Id-1 and binding to Hib CP

IgG antibodies reacting with Hib CP and expressing Id-1 were detected in Hib CP ELISA using the monoclonal LuC 9 as secondary antibody (Fig. 5). It revealed much lower level (with $OD_{1:100}$ not higher than 0·10 in 19 out of 20 samples) compared with both Hib CP ELISA (Fig. 1) and ELISA with LuC 9 used for coating. N18 was the only sample with higher levels of Id-1 antibodies against Hib CP ($OD_{1:100} = 0{\cdot}67$).

The standard preparation contained high levels of anti-Hib CP antibodies expressing Id-1, and absorption with Hib CP in this system completely removed Id-1 activity.

DISCUSSION

Our study showed that all sera of Hong Kong Chinese had natural antibodies binding to Hib CP on the solid phase, but most of them did not express Id-1 anti-Hib CP. Id-1 antibodies found in these sera were not specific for either Hib or *E. coli* K100 CPs.

We wanted to investigate these natural antibodies binding to Hib CP in Hong Kong Chinese. A previous study reported protective levels of serum antibodies against Hib CP in healthy Hong Kong individuals, but did not elucidate the origin of these antibodies [15]. Since the carriage of Hib in this population is reported to be uncommon [14,15], exposure to Hib may not be a significant factor in the development of protective immunity. It is possible that other common bacteria containing cross-reactive antigens may affect the development of natural immunity against Hib in this population [17,18].

IgG antibodies binding to the Hib CP were found in all Hong Kong sera studied by ELISA and were mostly IgG2, but in several cases also IgG1 and IgG4. Some individuals had IgA and IgM antibodies as well. Thus, the isotype distribution of antibodies binding to Hib CP in healthy Chinese is similar to those in unvaccinated Caucasians. Natural antibodies against Hib CP found in adult Caucasians were also mainly IgG2 [27]. However, Lau *et al.* reported that the Chinese population had remarkably high levels of serum IgG2, which may influence the occurrence of natural anti-Hib antibodies [28].

Using the combination of IEF and immunoblotting we found similar spectrotypes of IgG2 antibodies binding to the Hib and K100 polysaccharides in 10 of 20 serum samples, indicating the

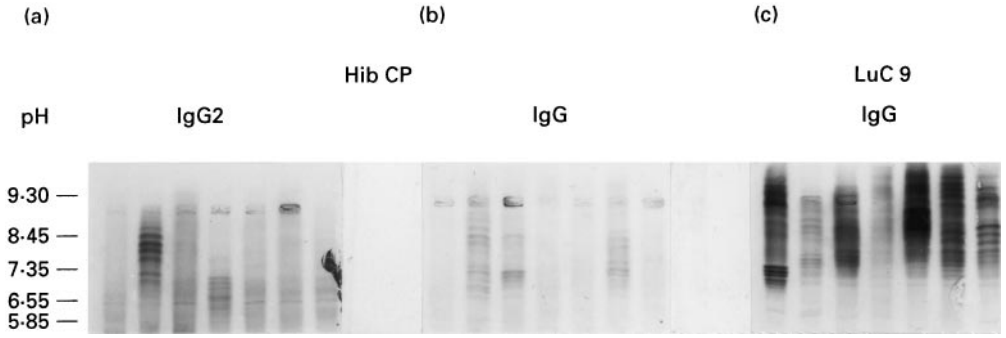

Fig. 4. Isoelectric focusing (IEF)/immunoblotting patterns in seven sera of healthy Hong Kong residents. (a) Nitrocellulose (NC) membrane coated with *Haemophilus influenzae* (Hib) capsular polysaccharide (CP), developed with mouse anti-human IgG2 antibody, alkaline phosphatase (AP)-conjugated rabbit anti-mouse immunoglobulins. (b) NC membrane coated with Hib CP, developed with AP-conjugated mouse anti-human IgG. (c) NC membrane coated with monoclonal LuC 9, developed with AP-conjugated mouse anti-human IgG.

occurrence of cross-reactive antibodies among the Hong Kong Chinese. However, the antibody bands common to Hib and K100 could not be absorbed by K100 CP. Some additional antibody clones binding only to K100 CP could be absorbed in three samples of nine.

In only two sera showing additional clones of antibodies binding to Hib CP was it possible to remove the corresponding IgG2 clones in IEF/immunoblotting, as well as to decrease significantly the activity in ELISA of both IgG and IgG2 antibodies by absorption with Hib CP. In most of the samples the antibodies binding to Hib CP were not absorbed by either Hib or K100 CPs.

Although we found antibodies binding to Hib CP both in ELISA and IEF/immunoblotting in all the sera, only one sample showed the expression of the Id-1 among these antibodies. That serum differed from the others, since it had an additional clone of IgG2 antibodies against Hib CP that could be absorbed with the homologous antigen. At the same time the high expression of Id-1 anti-Hib CP antibodies was detected in the standard preparation. Moreover, absorption of the standard preparation with Hib CP completely abolished the binding in Hib CP ELISA with LuC 9 as the secondary antibody.

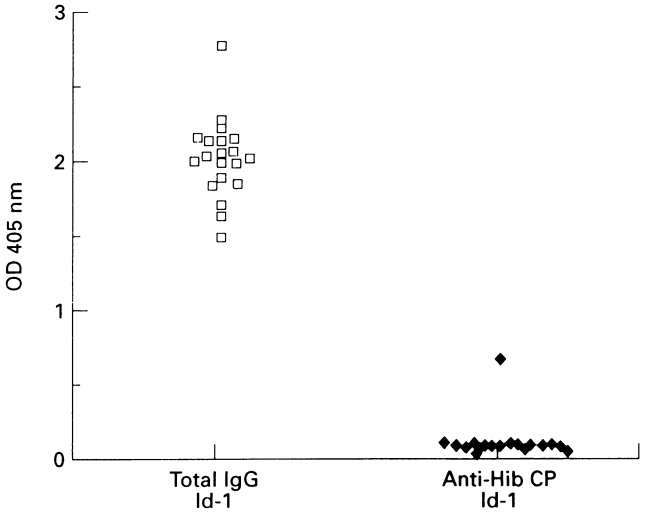

Fig. 5. Determination of total IgG antibodies belonging to Id-1 and anti-*Haemophilus influenzae* (Hib) capsular polysaccharide (CP) antibodies belonging to Id-1 in sera of healthy Hong Kong residents by ELISA.

The peculiarities in behaviour of antibodies from Hong Kong individuals could be related to a low avidity of the natural antibodies [29], but we found a wide range of avidity indices in different samples. Thus antibodies of both high and low avidity bound to the solid phase-immobilized Hib CP. Presumably they consisted of a mixture of different cross-reactive antibodies.

Natural antibodies may have properties to bind to multiple, structurally unrelated antigens [30]. Another striking feature of natural antibodies is an increased affinity for immobilized antigens [31], which may explain why we failed to inhibit antibody binding to the solid phase-immobilized Hib CP in ELISA with the corresponding antigen in the liquid phase. It has also been pointed out that ELISA overestimates low anti-Hib CP antibody levels induced by natural exposure compared with radioimmunoassay (RIA), while the ELISA and RIA results strongly correlate for post-vaccination antibodies [32].

Since the cross-reactive antibodies against Hib CP have either Vλ, VκI, or VκIII, but not VκII chains [22], it seems logical that they do not express Id-1. However, studying idiotype expression in the sera of Hong Kong Chinese we had some unexpected results. All samples showed clear clonotypes of IgG antibodies expressing Id-1, and in 12 sera they were stronger than those of anti-Hib CP antibodies with bands in different location. The high reactivity of serum IgG, IgA and IgM to the monoclonal anti-Id-1 was also confirmed by ELISA. This reactivity was not abolished by absorption of the sera either with Hib, or K100 CPs.

The data indicate the high prevalence of Id-1-expressing antibodies in the Hong Kong population. However, the IgG Id-1^+ antibodies were not Hib CP-specific in most cases. This is consistent with the nature of Id-1 being a marker for $A2V_L$ region usage, rather than a marker of a Hib CP paratope.

The Hib Id-1 parallel set, i.e. antibodies using VκII-A2, but not specific for Hib CP, may exist [21]. We suggest that these antibodies are the product of exposure to some antigens of bacteria different from Hib and *E. coli* K100. It is known that cross-reactivity between different common bacteria is rather wide. The molecular basis for cross-reactivity between bacterial antigens was indicated by recent studies. It has been shown that the same V genes are used in antibody production against different bacterial and even autoantigens [33].

Effective protection against different pathogenic bacteria may be achieved by natural exposure to cross-reactive antigenic components of the normal human microflora [17,19]. Although we found high levels of antibodies binding to Hib CP *in vitro*, it remains to be determined if they are protective against Hib *in vivo*. The possible origin and biological role of the Id-1-expressing cross-reacting antibodies in the Hong Kong Chinese population needs to be analysed further. Our data also raise a question about possible genetic and/or environmental differences between Chinese living in Hong Kong and in other parts of the world and between Chinese and Caucasian populations, the latter highly susceptible to Hib infection.

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