Immunoblot analysis of immune response to *Campylobacter pylori* and its clinical associations

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SUMMARY Systemic immune response to *Campylobacter pylori* was detected by the immunoblot technique in serum samples from 200 patients, 129 blood donors, and 96 children. The results of the IgG immunoblot test showed excellent correlation with the detection of *C pylori* by culture and also with histopathological examination of the antrum, as well as with peptic ulcer disease. An IgA response also occurred and gave results comparable with those of the IgG immunoblot test, although on a quantitatively lower scale. The IgM immunoblots were of no help in the serodiagnosis of *C pylori* infection. The protein bands that seemed to be the most specific for *C pylori* and which were consistently observed in patients positive for *C pylori* were a 110 kilodalton and a 63 kilodalton band on the IgG immunoblot and an 89 kilodalton band on the IgA immunoblot. A 94 kilodalton and a 28 kilodalton band were also included in the evaluation.

While immunoblot analysis may be used effectively for the serodiagnosis of *C pylori* infection and can distinguish between patients with normal antrum mucosa and those with gastritis, the test does not help to distinguish between those patients with antrum gastritis who subsequently develop peptic ulcers and those who do not.

A correlation between finding *Campylobacter pylori* in the antrum mucosa and gastritis in addition to peptic lesions was first described by Warren and Marshall^{1,2} and has since been confirmed in numerous studies throughout the world.³⁻⁷ Several authors have also shown that patients positive for *C pylori* develop a systemic as well as local immune response. Different methods have been used, including complement fixation tests,^{3,7} agglutination tests,⁵ passive haemagglutination assays,⁸ enzyme linked immunosorbent assays,^{6,9,10} and immunoblot techniques.^{7,11}

As we have previously shown⁷ immunoblots of seropositive sera display various band patterns which make interpretation rather difficult. Some bands appear to be quite consistent while others are only rarely found or are not specific. In this study we screened a larger number of sera from patients, blood donors, and children using the immunoblot technique to assess its value in serodiagnosis and to define patterns characteristic of *C pylori* infection. At the same time we wanted to restrict the number of bands evaluated to facilitate interpretation of the blots.

Material and methods

Serum samples from 200 patients who had been referred to the medical or surgical endoscopy units of the Universitätskrankenhaus Eppendorf or the medical endoscopy unit of the Israelitisches Krankenhaus Hamburg for upper gastrointestinal endoscopy were evaluated. Antrum biopsy specimens were taken for culture and Gram stain of *C pylori* and processed as previously described.⁷ In 167 the antrum mucosa was examined histologically. Serum samples from 129 blood donors and 96 children with suspected viral hepatitis (mean age 6.7 years, range 6 months to 15 years) were also included in the study.

SEROLOGY

Antigen

Three different *C pylori* isolates which we have described earlier¹² were used separately as antigen for comparative purposes: CLO 162; CLO 185, which contains a plasmid; and CLO 232, which differs from the other two strains and all our other isolates in that it does not exhibit any detectable urease activity. Strains were maintained at -80°C in tryptic soy broth

supplemented with 10% horse serum and 10% glycerol and were recultured on supplemented blood agar plates for four days as described previously.⁷

SODIUM DODECYL SULPHATE-POLYACRYLAMIDE GEL ELECTROPHORESIS

Cells were harvested from agar plates by scraping and washed twice in phosphate buffered saline (PBS). Preparation of total cell lysates and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli¹³ was performed as described previously.⁷ Samples containing about 250 µg of protein¹⁴ were subjected to SDS-PAGE using a 0.15 cm thick slab gel of 12% acrylamide as running gel and 3.5% acrylamide as stacking gel. Marker proteins were run alongside.

Immunoblot analysis

Protein transfer to nitrocellulose was accomplished according to a modification described earlier⁷ of a vacuum blotting procedure.¹⁵ After the blotting procedure the liberated gel was stained with Coomassie blue¹⁶ to ensure that the proteins had banded evenly throughout the gel. The nitrocellulose was then washed three times for 10 minutes in a blocking buffer (0.01 M Na₂HPO₄/NaH₂PO₄, 0.15 M NaCl, pH 7.5, 0.5% Tween 20, 0.05% sodium azide) and was then cut into strips about 3 mm wide. One strip and the part of the nitrocellulose containing the marker proteins were stained overnight with India ink diluted 1/1000 in blocking buffer. The other strips were each incubated with 2 ml of patient serum diluted 1/100 in blocking buffer overnight with rotation at room temperature. After washing three times for 10 minutes in blocking buffer antibody-antigen reaction was detected as previously described⁷ using alkaline phosphatase conjugated rabbit antihuman IgG, IgA, or IgM (Dianova GmbH, Hamburg, West Germany), each diluted 1/2000 in blocking buffer.

Statistical analysis was done by the χ^2 test.¹⁷

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Results

Fig 1 shows typical examples of IgG and IgA immunoblots that were obtained using *C. pylori* strain CLO 185 as antigen. The blot strips were grouped according to the histological appearance of the patients' antrum mucosa; blots from patients with either severe or moderate antrum gastritis displayed a much denser set of reactive protein bands than any of those from patients with normal antrum mucosa.

Correlation of reactivity of individual protein bands with serum samples from patients with antrum gastritis on the one hand and non-reactivity with sera from patients with normal antrum mucosa on the other resulted in the selection of four protein bands for the IgG immunoblot: 110 kD, 94 kD, 63 kD, and 28 kD. For evaluation of the IgA immunoblots, the 28 kD band was omitted and an 89 kD band was included instead. IgM immunoblots are not shown because all 40 sera tested showed only a single band around 60 kD. Longer substrate incubation or dilution of sera 1/50 instead of 1/100 increased the reactivity of protein bands sharply, but no differences could be observed between serum samples from patients with normal antrum mucosa and those from patients with antrum gastritis. Thus this IgM reactivity was regarded as non-specific and further probing for IgM was stopped.

With a set of 40 patient samples the different *C. pylori* strains CLO 162, CLO 185, and CLO 232 were used as antigen and tested in parallel for comparison of the IgG, IgA, and IgM reactivity patterns. CLO 162 and CLO 185 gave identical results; CLO 232 was discarded as it failed to show a protein band of about 110 kD that was found to be quite characteristic for patients positive for *C. pylori*. In respect of the other bands studied CLO 232 did not differ from the other two strains. As reported elsewhere,¹² CLO 232 differs from all our other isolates in that it does not hydrolyse urea. CLO 185 was the antigen used in all tests finally evaluated.

Table 1 shows the percentages of reactivity of some

Table 1 Immunoblot reactivity of some characteristic *C. pylori* protein bands, detection of *C. pylori* on culture or Gram stain, and percentages of positive IgG as well as IgG + IgA scores

Group	(n =)	Percentage 110 kD IgG positive	Percentage 63 kD IgG positive	Percentage 89 kD IgA positive	Percentage IgG score ≥ 1.0	Percentage IgG + IgA score ≥ 1.5	Percentage <i>C. pylori</i> positive
Normal antrum mucosa	37	11	25	8	30	27	8
Mild antrum gastritis	61	47	74	52	83	72	57
Moderate antrum gastritis	41	47	81	59	86	83	68
Severe antrum gastritis	29	81	88	88	100	100	88
Duodenal ulcer	35	59	91	71	94	94	97
Gastric ulcer	36	67	81	58	86	86	64
Gastrectomy	12	42	67	58	75	67	64
Gastric cancer	13	46	62	38	69	62	40
Blood donors	129	21	22	22	32	32	
Children	96	15	18	7	21	19

Fig 1 *C. pylori* immunoblots using whole cell lysate of the *C. pylori* strain (CLO 185) as antigen. Blots were incubated with patient sera diluted 1/100 and developed with alkaline phosphatase conjugated anti-human IgG (a) or IgA (b). M = marker proteins.

of the different protein bands for the different categories of antrum gastritis and groups of patients and also gives the corresponding results of detection of *C. pylori* on culture or Gram stain, or both. The total number of patients adds up to more than the 200 sera that were evaluated as any one patient may fit into more than one category—for example, severe antrum gastritis and duodenal ulcer. The percentages of reactivity for the other protein bands evaluated were much lower. This is also true for the 110 kD and 63 kD bands on the IgA immunoblot and for the 89 kD protein band which was not even evaluated for the IgG immunoblot due to its poor reactivity.

Although the 63 kD band alone was already positive for IgG in 88% of all sera from patients with severe antrum gastritis, an evaluation that took into account the reactivities for the other bands as well yielded much better results. A semiquantitative grading system was therefore introduced, in which one point was assigned to each reactive band and half a point to a weakly reactive band. This resulted in a maximum score of 4 for each tested serum for IgG or IgA, or a maximum combined score of 8. Table 1 also gives the results achieved for the different groups of patients

Table 2 Contingency table showing correlation between immunoblot results and detection of *C. pylori* on culture or Gram stain in 200 patients

Immunoblot	<i>C. pylori</i> state		
	Positive	Negative	
Positive	95	47	142
Negative	14	44	58
Total	109	91	200

} $p < 0.001$

using either an IgG score of ≥ 1.0 or a combined IgG + IgA score of ≥ 1.5 as cut off values.

Table 2 shows the correlation between detection of *C. pylori* on culture or Gram stain, or both with the *C. pylori* immunoblot using a combined IgG + IgA score of 1.5 as the cut off. Using the χ^2 test, the results correlated positively ($p < 0.001$). Table 3 gives the median scores for the different groups of patients and the corresponding ranges, in addition to the mean ages and male:female ratios.

Figs 2 and 3 show the different cumulative percentages of the IgG and IgA scores, both for the different

Fig 2 Cumulative percentages of IgG and IgA immunoblot scores for different gastritis groups. □—□ Normal antrum mucosa; ×—× mild antrum gastritis; ○—○ moderate antrum gastritis; ■—■ severe antrum gastritis.

Table 3 Male:female ratio, mean ages, median IgG + IgA scores and corresponding ranges for various groups of patients, blood donors, and children

Group	(n =)	Male:female ratio	Mean age	Median IgG + IgA score	Range
Normal antrum mucosa	37	14:23	50.3	0	0-5
Mild antrum gastritis	61	25:36	57.6	2.5	0-6
Moderate antrum gastritis	41	20:21	60.7	3.0	0.5-6.5
Severe antrum gastritis	29	19:10	57.2	3.0	1.5-8.0
Duodenal ulcer	35	25:10	51.4	3.0	0-6
Gastric ulcer	36	17:19	53.9	4.0	0-7.5
Gastrectomy	12	9: 3	63.3	2.5	0-4.5
Gastric cancer	13	8: 5	68.1	2.0	0-5
Blood donors	129	100:29	39.3	0	0-5.5
Children	96	57:39	6.7	0	0-4

Fig 3 Cumulative percentages of IgG and IgA immunoblot scores for ulcer patients, blood donors, and children. ●—● Duodenal ulcer; △—△ gastric ulcer; *—* blood donors; ○—○ children.

gastritis groups and different patient groups. The distributions of the IgG and IgA scores in the various groups were shown by χ^2 test to differ significantly ($p < 0.001$). As judged from the curves in fig 2, this difference is accounted for by the low scores in the normal antrum mucosa group as opposed to the much higher scores in the gastritis groups. Where the curves for the different groups of mild, moderate, and severe gastritis run closely parallel and even intertwine,

suggests that the distribution of scores within these groups does not vary significantly (fig 2). The same applies to the curves for patients with gastric ulcer and duodenal ulcer which are clearly distinct from those of the blood donors and children (fig 3). Again, there is no significant difference in the distribution of the scores between patients with gastric and duodenal ulcers. Fig 3 also clearly shows that sera from children displayed very little IgA reactivity.

Discussion

The IgG immunoblot test for *C. pylori* showed a very good correlation with the detection of *C. pylori* and even more so with the histopathological examination of tissue from the antrum. We also detected an IgA response that gave results comparable with those of the IgG immunoblot test, although somewhat on a quantitatively lower scale. The IgA immunoblot test only rarely yielded information supplementary to that already supplied by the IgG immunoblot. The IgM immunoblots were of no help in the serodiagnosis of *C. pylori* infection. This is not really surprising in a chronic mucosal infection where one would normally expect the immune response to be chiefly of the IgG and IgA type. Other authors have also predominantly observed an IgG response and, to a lesser degree, an IgA response.^{6,9} They also did not find testing for IgM to be useful in serodiagnosis. Wyatt *et al.*, investigating the local immune response to *C. pylori*, did, however, ascribe an important role both to the IgA and the IgM response.¹⁸ Goodwin *et al.* detected only IgG and no IgA or IgM by ELISA.¹⁰

Protein bands which we found to be highly specific for *C. pylori* were a 110 kD and a 63 kD band for the IgG immunoblot and an 89 kD band for the IgA immunoblot. Kaldor *et al.* also considered a band around 100 kD to be of special interest in their *C. pylori* immunoblot study.¹¹ Apel *et al.* also described a band around 120 kD to be highly specific for *C. pylori* (Abstract presented at International Workshop on Campylobacter infections, Göteborg, 1987). Both authors probably were referring to the same protein which we have termed 110 kD. The protein band that we called 63 kD probably corresponds to the 60–62 kD protein that Newell described as being specific for *C. pylori*.¹⁹ She also observed that sera from patients positive for *C. pylori* regularly reacted with this protein.

Other protein bands seem to be less specific or less immunoreactive. Serological cross reactivities of *C. pylori* with other Campylobacter species as well as with other bacteria have been described by some authors.^{5,9,10,11,19} These cross reactivities, however, apparently do not have an important role in the serodiagnosis of *C. pylori* with the serum dilutions generally used in serological tests.

The results for patients with normal antrum mucosa have to allow for the fact that the inflammatory changes may not always be distributed evenly throughout the antrum, and we also saw some patients with normal antrum mucosa who showed both superficial gastritis and *C. pylori* in the body of the stomach.

The results for the blood donors agree with the findings of Langenberg *et al.*, who detected *C. pylori* and antrum gastritis in six of 24 healthy medical

students.³ These results also correspond to published reports²⁰ on the prevalence of chronic antrum gastritis and peptic ulcers in the general population. The results we obtained with the sera from children show that some of these children may actually have had active *C. pylori* infection, and that the hepatitis serology was just part of the laboratory assessment of their abdominal pain. Some authors have recently pointed out that antrum gastritis and even peptic ulcers are not the rare childhood diseases that they are generally considered to be.^{21–23}

The results we achieved with the sera from patients with gastric cancer should be viewed with caution as the number of patients was quite small. This is particularly pertinent in the case of those with intestinal gastric cancer, which has been associated with long standing type B gastritis.^{24–27} The two phenomena, however, might also be coincidental and not casually related.²⁸

While our immunoblot test may be used quite effectively to distinguish between patients with normal antrum mucosa and those with antrum gastritis, neither the reactivity of specific bands nor the number of reactive bands were significantly correlated with the degree of severity of the antrum gastritis. Nor did the test help to distinguish between those patients with antrum gastritis who develop peptic ulcers and those who do not. Jones *et al.* also did not observe an association between antibody titre and the severity of the antrum gastritis when they used a complement fixation test for *C. pylori*.⁵

What, now, might be the possible clinical applications for such a *C. pylori* serology? Firstly, it might serve to complement or confirm other non-invasive procedures such as the recently described urea breath test,²⁹ as a negative *C. pylori* serology makes any association with a possible *C. pylori* aetiology rather improbable. It may also aid diagnosis in patients in whom endoscopy is not feasible or difficult to perform—for example, in children. Furthermore, it might turn out to be a valuable tool in the long term monitoring of therapeutic trials with antimicrobial drugs or bismuth salts.

A further application might be the screening of certain high risk patients. In a preliminary retrospective study we looked at sera from patients before and after renal transplantation.³⁰ Some patients who were clearly positive for *C. pylori* on immunoblot analysis (both IgG and IgA) two years before transplantation but had not presented with a previous history of gastritis or peptic ulcers, developed peptic ulcers and even gastrointestinal haemorrhage soon after renal transplantation. Their *C. pylori* immunoblot patterns had not changed. One might speculate, therefore, that chronic *C. pylori* infection predisposed these patients to peptic ulcer disease that did not become overt, however, until further injury to their mucosal defence mechanisms occurred—renal transplantation and

subsequent immunosuppressive treatment. Early diagnosis and appropriate treatment might have prevented these patients from developing peptic ulcers and gastrointestinal haemorrhage.

Part of this work was presented at the IVth International Workshop on Campylobacter Infections in Göteborg, 1987.

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