Immunoblot fingerprinting of *Campylobacter pylori*

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Summary. One hundred and fifty isolates of *Campylobacter pylori* were divided into nine groups by immunoblot fingerprinting. All isolates were typable and reproducibility between immunoblots was good provided both antigen and antibody preparation were standardised. Discrimination was a problem as Groups 1 and 2 accounted for 66% of isolates. Recurrence after bismuth treatment was due to indistinguishable strains in five patients and different strains in three patients.

Introduction

Recent work has identified *Campylobacter pylori* in the gastric mucosa of normal controls, patients with type-B chronic gastritis, and patients with peptic ulceration (Jones *et al.*, 1984; Langenberg *et al.*, 1984; McNulty and Watson 1984). There has been debate as to the role of the organism in upper gastrointestinal pathology (Rathbone *et al.*, 1986). In favour of an active role is the finding that deliberate ingestion of the organism results in active gastritis in man (Marshall *et al.*, 1985; Morris and Nicholson, 1986) and in gnotobiotic piglets (Lambert *et al.*, 1987). *C. pylori* is sensitive to bismuth which has been used to treat gastritis (McNulty *et al.*, 1986) and duodenal ulcers (Martin *et al.*, 1981). Relapse following the treatment of duodenal ulceration has been linked to the re-isolation of *C. pylori* from biopsy material (Coghlan *et al.*, 1987). However, *C. pylori* has been isolated from asymptomatic, healthy medical students (Langenberg *et al.*, 1984) and serological studies, with partially purified and extracted material from *C. pylori* as antigen, showed that there was no correlation between the extent of mucosal colonisation with *C. pylori* and the pattern of antibody response detected by ELISA or immunoblotting (Jones *et al.*, 1986; Steer *et al.*, 1987).

Serodiagnostic studies have concentrated on single isolates without regard to the heterogeneity between isolates (Perez-Perez and Blaser, 1987). The development of a serotyping scheme would allow clearer interpretation of the antibody response in infection. No such formal serotyping exists at present (Langenburg *et al.*, 1986).

The symptoms of many patients recur during therapeutic trials and it is important to differentiate between a re-infection with a different isolate and

recrudescence due to the same organism. A simple and sensitive typing system is required. Restriction endonuclease DNA analysis has been applied to the *C. pylori* isolates from 16 patients but the method is difficult and time consuming (Langenberg *et al.*, 1986).

Recent work has seen the development of immunoblot fingerprinting systems for *Candida albicans* (Lee *et al.*, 1986), *Staphylococcus aureus* (Krikler *et al.*, 1986; Lee and Burnie, 1988) and coagulase-negative staphylococci (Burnie and Matthews, 1987). The basis of these test systems is the production in a rabbit of hyperimmune antiserum against a single isolate of the pathogen. The pattern of cross-reactivity with other isolates by immunoblotting is used to produce "fingerprints" of the isolates. Differences in the presence, position or intensity of at least three antigenic bands is required to differentiate between two isolates (Burnie and Matthews, 1987). Immunoblotting is preferred to simple SDS-PAGE profiles because, as with *C. albicans* (Lee *et al.*, 1986) and staphylococci (Lee and Burnie, 1988), these were sufficiently similar to invalidate their use as a typing system (Pearson *et al.*, 1984; Megraud *et al.*, 1985).

This paper reports the immunoblot fingerprinting of 150 isolates of *C. pylori* from 142 patients, including eight patients from whom repeat isolates following treatment were available. The degree of conservation of each individual *C. pylori* antigenic band is important when developing serodiagnostic tests and this is discussed.

Materials and methods

C. pylori isolates

Isolates were obtained from 150 biopsy specimens taken at gastroscopy. These came from 142 separate

patients, including eight patients in whom a second endoscopy was performed after treatment. Specimens were inoculated on to blood agar and campylobacter selective medium (Skirrow, 1977). All plates were incubated in microaerobic conditions (O_2 5%, CO_2 10%; Oxoid Gas Pak) at 37°C and examined after 3, 5 and 7 days. Gram-negative spiral organisms with the characteristic colonial appearance and giving a positive result in a rapid urease test were called *C. pylori*. A single colony of each isolate was then subcultured on five blood-agar plates, and the harvested growth was stored in liquid nitrogen until required. It was then subcultured on to five blood-agar plates and incubated at 37°C for 3 days. Isolates were harvested in sterile distilled water, pelleted by centrifugation at 12 000 *g* for 10 min and frozen at -20°C. The pellets were melted and resuspended in 0.5 ml of lysis solution (1M Tris, pH 7.5, containing sodium dodecyl sulphate 10% and 100 mM phenyl methyl sulphonyl fluoride) in Eppendorf microtubes and vortex mixed in a rotary mixer five times (5×30 s) with 0.25 ml of tiny glass beads. The tubes were cooled in ice between each 30-s vortex. The suspensions were centrifuged for 5 s and the supernates were used in subsequent experiments.

Histology

Histological results were available from 136 of the biopsy specimens (table IV). These were graded as normal mucosa (grade 0), mild gastritis (grade 1), moderate gastritis (grade 2) and severe gastritis (grade 3) by the Warren Marshall classification (Coghlan *et al.*, 1987).

Rabbit hyperimmune antiserum

A *C. pylori* protein extract was prepared by growing *C. pylori* on blood-agar plates, harvesting the growth in distilled water, crushing in an Xpress at -20°C and centrifuging at 12 000 *g* for 10 min. The supernate (25 mg) was dissolved in 1 ml of sterile water and mixed with an equal volume of complete Freund's adjuvant. New Zealand White rabbits were given subcutaneous injections of this mixture on days 0, 14 and 28. The rabbits were bled out 10 days after the last injection and the hyperimmune antiserum was pooled and used as the antibody probe in all further experiments.

SDS-PAGE and immunoblotting

Immunoblots were prepared as described in detail by Burnie and Matthews (1987). Briefly, 100- μ l volumes of the supernates were boiled for 5 min in sodium dodecyl sulphate 2.5% and 2-mercaptoethanol 1.3% and 60 μ l of this preparation was loaded on to a 10% polyacrylamide gel. This was run for 4 h in a discontinuous buffer system (Laemmli, 1970). It was then either silver stained by the Amersham Silver Stain kit (Amersham Laboratories) or transferred on to a nitrocellulose membrane in an LKB Transblotter (LKB Laboratories). The buffer contained

methanol 20%, 25 mM Tris and 192 mM glycine at pH 8.3 and transfer was allowed to proceed at 25°C for 45 min. The nitrocellulose paper was blocked in bovine serum albumin 3% in buffered saline (sodium chloride 0.9% and 10 mM Tris, pH 7.4) at 4°C overnight. The nitrocellulose was then incubated at 25°C for 2 h with the rabbit hyperimmune serum diluted 1 in 5 in buffered saline containing bovine serum albumin 3% and Tween 20 0.05%. After washing five times for 30 min in saline 0.9% and Tween 20 0.05%, nitrocellulose strips were incubated for 1 h at 25°C with alkaline phosphatase-conjugated goat anti-rabbit conjugate (Sigma). Immediately before use, the conjugates were diluted 1 in 1000 in bovine serum albumin 3% in buffered saline. After washing again, as above, the membranes were incubated for 5–15 min at 25°C with 100 ml of buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) containing a mixture of 660 μ l of Nitro blue tetrazolium (NBT 50 mg/ml in N,N-dimethyl formamide 70%) and 330 μ l of 5-bromo-4-chloro-3-inosyl phosphate (BCIP 50 mg/ml in N,N-dimethyl formamide 70%). The reaction was stopped by washing in water. All the immunoblots were examined by eye to establish the presence of a set of 11 antigenic bands in at least one of the type isolates. The results for each of these bands for all isolates were then recorded as absent (-), trace or inconsistent (tr), always present (+) or double band (DB).

Results

Silver staining of the SDS-PAGE extracts of the *C. pylori* isolates demonstrated that these were very similar to each other. The gels of extracts from the isolates subsequently defined as the type isolates by immunoblotting are shown in fig. 1.

After immunoblotting it was possible to divide the 150 isolates examined into nine groups on the basis of the 11 antigenic bands with mol. wts in the range $(26-74) \times 10^3$ (table 1). All isolates were typable. One hundred of the original 150 isolates belonged to either Group 1 or Group 2. Other Groups contained 1–12 isolates. Immunoblots of 29 of the isolates are shown in figs. 2, 3 and 4, and the patterns of their antigenic bands are summarised in table II. Immunoblotting revealed highly variable bands at mol. wts (10^3) 29, 34, 37, 40, 44, 59 and 74, whereas eight of the nine groups had a band or double band at mol. wts (10^3) 26, 53, 56 and 62.

Two isolates were examined from each of eight patients. Examples are presented as isolates 10–19, in table II and fig. 3, and patients 1–5 in table III. In all cases the patients had been treated with bismuth between biopsies. Five of the relapses were due to infection with isolates of the same immunoblot group (table III).

The histology results are summarised in table IV. Overall, 33% of isolates came from patients with

Fig. 1. Tracks 1–9. Silver stained extracts of the nine type isolates of *C. pylori*. Mol. wt markers at 45 000 (ovalbumin), 66 200 (bovine serum albumin), 92 500 (phosphorylase B), 116 500 (beta-galactosidase) and 200 000 (myosin) are illustrated by the left hand column.

either a normal mucosa or mild gastritis. However in immunoblot Group-1 isolates, this percentage fell to 17.5%, compared with 40% from Group 2. This suggests that isolates in Group 1 were more likely to have come from a patient with histologically-proven moderate or severe gastritis than isolates in Group 2. The low number of isolates in

Groups 3–9 invalidates any comment on an association between those groups and histological disease.

Discussion

This work confirms the similarity between *C. pylori* isolates on SDS-PAGE analysis as shown

Table I. Summary of the patterns of bands detected in immunoblots

Group no.	Number of isolates	Presence of bands of mol. wt (10^3)										
		74	62	59	56	53	44	40	37	34	29	26
1	51	+ / tr	DB	–	+	tr	–	–	–	–	+ / tr	–
2	49	+	DB	tr	+	+ / tr	+	+	+	+	+	+
3	8	+	+ / DB	–	+	+ / tr	+	+	+ / tr	–	–	+
4	9	+	+	–	+	+ / tr	–	tr / –	+	–	+	+
5	4	+	+	tr / –	+	+	+	tr / –	+	–	–	+
6	6	+	+	tr / +	+	+	+	tr	+	–	–	+
7	10	+	+	tr / –	+	+	tr	+ / tr	–	tr / +	tr / +	+
8	12	tr	DB	–	+	+	–	–	+	–	–	+
9	1	tr	tr	tr	+	+	–	tr	–	tr	–	+

+, Present; tr, trace; –, absent; DB, double band.

Table II. Summary of the immunoblot results with isolates shown in figs. 2, 3 and 4

Isolate no.	Group no.	Presence of bands of mol. wt (10^3)										
		74	62	59	56	53	44	40	37	34	29	26
1	2	+	DB	tr	+	tr	+	+	+	+	+	+
2	1	+	DB	-	+	tr	-	-	-	-	+	+
3	1	+	DB	-	+	tr	-	-	-	-	+	+
4	2	+	DB	tr	+	++	+	+	+	+	+	+
5	2	+	DB	tr	+	tr	+	+	+	+	+	+
6	1	tr	DB	-	+	tr	-	-	-	-	+	+
7	1	+	DB	-	+	tr	-	-	-	-	+	+
8	3	+	+	-	+	tr	+	+	tr	-	-	+
9	1	+	DB	-	+	tr	-	-	-	-	+	+
10	7	+	+	tr	+	+	-	+	-	+	tr	+
11	9	tr	tr	tr	+	+	-	tr	-	tr	-	+
12	5	+	+	-	+	+	+	tr	-	-	-	+
13	5	+	+	-	+	+	+	tr	-	-	-	+
14	6	+	+	tr	+	+	+	tr	+	-	-	+
15	6	+	+	tr	+	+	+	tr	+	-	-	+
16	5	+	+	tr	+	+	+	tr	-	-	-	+
17	5	+	+	tr	+	+	+	tr	-	-	-	+
18	6	+	+	-	+	+	+	tr	tr	-	-	+
19	4	+	+	-	+	+	-	tr	+	-	+	+
20	7	+	+	tr	+	+	tr	tr	-	+	tr	+
21	2	tr	DB	tr	+	+	+	+	+	+	+	+
22	7	+	+	tr	+	+	tr	+	-	tr	+	+
23	1	tr	DB	-	+	tr	-	-	-	-	tr	+
24	3	+	DB	-	+	+	+	+	+	-	-	+
25	7	+	+	-	+	+	tr	+	-	tr	tr	+
26	7	+	+	-	+	+	tr	+	-	tr	tr	+
27	8	tr	DB	-	+	+	-	-	+	-	-	+
28	7	+	+	-	+	+	tr	+	-	tr	tr	+
29	1	tr	DB	-	+	tr	-	-	-	-	tr	+

Abbreviations as in table I.

previously (Pearson *et al.*, Megraud *et al.*, 1985). The immunoblot fingerprinting results revealed constant bands or double bands of mol. wts (10^3) 26, 53, 56 and 62. Perez-Perez and Blaser (1987), with six isolates of *C. pylori*, demonstrated constant bands at mol. wts (10^3) 26, 29, 56 and 62 by silver

staining. This paper extends their results and confirms the homogeneity of these bands after immunoblotting against a rabbit hyperimmune antiserum. The presence of the band at mol. wt 29×10^3 was variable but was constant for the two commonest groups. Perez-Perez and Blaser (1987) suggested that the outer-membrane preparations of *C. pylori* could be used as the basis of a typing system. They demonstrated heterogeneity in two antigenic bands between mol. wts (10^3) 52 and 57 and a band at mol. wt 44×10^3 . The present study demonstrated variable bands at mol. wts (10^3) 53 and 44. Jones *et al.* (1986) immunoblotted human antibody against six isolates of *C. pylori* and commented that the sera reacted in varying degrees with antigenic bands of mol. wt $> 33 \times 10^3$. This suggested some degree of antigenic heterogeneity between isolates which we have now confirmed.

The immunoblot results explain some of the equivocal results obtained in serodiagnostic tests with whole-cell preparations (Rathbone *et al.*, 1986;

Table III. Immunoblot fingerprint results of repeated isolates from the same patients

Patient no.	First isolate Group no.	Second isolate Group no.
1	7	9
2	5	5
3	6	6
4	5	5
5	6	4
6	1	1
7	2	2
8	1	3

Fig. 2. Tracks 1-9. Immunoblot fingerprints of isolates from table II.

Fig. 3. Track 10, patient 1, 1st isolate; track 11, patient 1, 2nd isolate; track 12, patient 2, 1st isolate; track 13, patient 2, 2nd isolate; track 14, patient 3, 1st isolate; track 15, patient 3, 2nd isolate; track 16, patient 4, 1st isolate; track 17, patient 4, 2nd isolate; track 18, patient 5, 1st isolate and track 19, patient 5, 2nd isolate.

Fig. 4. Immunoblot fingerprints of isolates 20–29 from table II.

Steer *et al.*, 1987). The existence of nine serotypes means that antibody detection with a single isolate will only be adequate if the host's antibody response is aimed primarily against the conserved antigenic bands. Immunoblotting with sera from infected patients has demonstrated antibodies against a number of antigenic bands of mol. wts (10^3) 61, 56, 54, 48 and 31 (Newell, 1987). The present immunoblotting results have shown that only the bands at mol. wts (10^3) 62 and 56 are conserved. If the key immunodominant antigens are not conserved, this

would explain the lack of correlation between mucosal colonisation by *C. pylori* and the antibody response measured with one isolate (Steer *et al.*, 1987).
The antigenically variable bands at mol. wts (10^3) 29, 34, 37, 40, 44, 59 and 74 form the basis of the immunoblot fingerprinting system. All isolates were typable and the degree of reproducibility was high provided that the preparation of antigen was standardised, the same batch of antibody was the probe, and trace antigenic bands were ignored.

Table IV. Classification of the histological results of 150 biopsies

Immunoblot Group no.	Classification of histological result					Total number of isolates
	Normal	Mild gastritis	Moderate gastritis	Severe gastritis	Unknown	
1	0	7	13	20	11	51
2	6	13	18	10	2	49
3	0	1	5	2	0	8
4	0	3	3	3	0	9
5	0	2	1	1	0	4
6	2	3	1	0	0	6
7	0	4	4	2	0	10
8	0	3	5	3	1	12
9	0	1	0	0	0	1
Total	8	37	50	41	14	150

Changing either of the first two criteria could be compensated by comparing isolates on the same gel (Burnie and Matthews, 1987). The typing method had poor discrimination because Groups 1 and 2 accounted for 66% of the isolates. This lack of discrimination reflected the use of only a single antibody probe and the strict criteria adhered to when describing two isolates as definitely different (Burnie and Matthews, 1987). When sequential isolates from the same patient were examined (fig. 3), strains were either identical or had multiple antigenic band differences. This suggests that a relaxation to one or two antigenic band differences could be justified. In the case of staphylococci (Burnie and Matthews, 1987) the pooling of results obtained with several rabbit hyperimmune antisera greatly enhanced discrimination. This could be performed for *C. pylori* by preparing hyperimmune antisera against each of the Group strains.

Previous typing has been performed by restriction endonuclease DNA analysis, but the difficulty

in performing this technique limited its application (Langenberg *et al.*, 1986). However, both this technique and the immunoblot fingerprinting demonstrated that relapses of peptic ulcers after bismuth treatment are most likely due to the original isolate. This was true of the four patients in the study of Langenberg *et al.* (1986) and five of the eight patients in the current study. The finding of three cases in which the recurrence of symptoms was due to a different isolate type could be explained by a new infection or by the presence of multiple types in the original infection. The isolation of a different type in the second biopsy does not preclude its presence in the original specimen because only one colony was sampled. The association of Group-1 isolates with more severe histological changes is interesting in that it implies that not all isolates are equally virulent. This hypothesis could explain the isolation of strains from volunteers with apparently normal gastric mucosa (Langenberg *et al.*, 1984) and will be investigated in further work.

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