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Antigen Recognition during Progression from Acute to Chronic Infection with a *cagA*-Positive Strain of *Helicobacter pylori*

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We have previously published two reports on acute infection with *Helicobacter pylori*, one of an adult male and one of a family of four. In the present study, we have isolated *H. pylori* from each of twin boys in the family and compared these by use of random amplified polymorphic DNA PCR. In addition, we have monitored the antibody response over time of the family and the adult male by Western blotting (immunoblotting) with two different strains of *H. pylori* as the antigen and by use of a commercial kit. The acutely infected twin boys were infected by an identical strain of *H. pylori*. The twin boys responded to antigens of 19, 26.5, and 29 kDa 30 days after the initial diagnosis, with recognition of 43- to 49-, 66-, 69-, and 87-kDa antigens by day 63. One twin responded to the CagA antigen on day 63, whereas the other responded on day 857. Antibody to the CagA antigen was not detected by use of the infecting strain, UNSW-RU1. Investigation of UNSW-RU1 revealed the presence of *cagA*. In two acutely infected adults (one, the father of the boys), the initial response to a 45-kDa antigen was later followed by responses to 19-, 29-, 49-, 60-, 77-, and 84-kDa antigens. Sera from the twins' younger sister, born 17 months after the twins acute episode, indicated that she also had become infected. This report supports intrafamilial transmission of *H. pylori*. Initial antibody responses in the children were to small-molecular-size antigens; in the adults, the initial responses were to larger-molecular-size antigens. The pattern of the serological response differs according to the antigen used. This has implications in regard to international data comparisons.

A number of studies have examined the occurrence of antibodies to specific *Helicobacter pylori* antigens in sera collected from patients attending for endoscopic examination (2, 9, 13, 25). There have been few reports, however, regarding the development of antibodies to specific *H. pylori* antigens following acute infection. This has been due to the limited number of identifiable cases of natural acute *H. pylori* infection. In the present literature, there are seven such reports, two of which have originated from our group (5, 10, 11, 19, 21–23).

Our first report described acute *H. pylori* infection in a 46-year-old man presenting with profuse vomiting and midepigastric pain, culminating in an episode of hematemesis (10). The second documented acute *H. pylori* infection in three members of the same family (19). This latter study involved acute infection in a 21-month-old boy who presented with a bleeding gastric ulcer. *H. pylori* was cultured from biopsy specimens obtained from this child. Immunoglobulin G (IgG) seroconversion did not occur until 63 days later. Serological investigation of this child's family showed that his nonidentical twin brother (twin 2), who had suffered an episode of vomiting at a similar time, was also acutely infected with *H. pylori*, this being evidenced by an IgG seroconversion 30 days after twin 1 presented. Follow-up studies indicated that the father became infected with *H. pylori* 7 months after the first child's (twin 1's) episode, again evidenced by an IgG seroconversion. The children's mother had an established asymptomatic infection of unknown duration. We postulated that the source of *H. pylori* infection was the mother, who transmitted the organism to one or both of the twin boys, with any of these three potentially infecting the father (19). This scenario is consistent with recent epidemiological studies showing that early childhood may be the critical period for the acquisition of *H. pylori* (15, 16, 18). We were not able to compare the *H. pylori* strains of the family members and thereby substantiate that the spread of infection had been from one family member to another.

Subsequent to the publication of the report described above, the second twin required an endoscopic examination. At this time, diagnostic biopsy specimens were collected and *H. pylori* was cultured. Throughout this period, we monitored the family by collecting periodic blood samples, including one sample from a third child born during this time.

With the availability of cultures from both twins and periodic serum samples from the family, we have been able to compare, by use of random amplified polymorphic DNA PCR (RAPD-PCR), the isolates of *H. pylori* obtained from both twin boys. In addition, we have monitored the development of the serological response to specific *H. pylori* antigens in the family over time. For comparison, we also examined the development of antibodies to specific antigens in periodic blood samples collected from the adult male subject of the first acute case reported by our group (10).

MATERIALS AND METHODS

Culture. (i) Biopsy specimens. Biopsy specimens from the second of the twin boys (twin 2) presenting for endoscopic examination were plated directly onto 5% horse blood agar (blood agar base no. 2; Oxoid, Basingstoke, United Kingdom) containing Skirrow's antibiotic supplements (Oxoid). The plates were incubated at 37°C for 7 days in a Stericult CO_2 incubator (Forma Scientific) set at 10% CO_2 in air and 95% relative humidity. Isolates were identified as *H. pylori* by phase microscopy and their urease, catalase, and oxidase reactions. Isolates were stored frozen in brain heart infusion broth (Oxoid) plus 20% (wt/vol) glycerol (BHIG) in liquid nitrogen.

(ii) Bacterial cultures. *H. pylori* NCTC 11637, NCTC 11639, 10 clinical isolates, and the strain isolated from the first twin (UNSW-RU1) were removed from liquid nitrogen, thawed, and inoculated onto horse blood agar as required.

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Inoculated plates were incubated as described above. The isolate from the second of the twin boys was given the designation UNSW-RU2.

RAPD fingerprinting. Fingerprints of the *H. pylori* isolates obtained from the body and antrum of each of the twin boys (UNSW-RU1 and UNSW-RU2) were compared with the fingerprints from the clinical isolates and the two NCTC strains.

(i) DNA extraction. A chloroform-phenol extraction method was used to obtain DNA from the strains described above. In brief, a loopful of a 48-h culture of *H. pylori* was suspended in 500 μl of 50% ethanol. This suspension was then vortexed for 30 s, and 500 μl of phenol-chloroform (50:50) was added. The DNA was extracted by centrifugation at 12,879 × *g* for 3 min, after which the upper aqueous phase containing the DNA was removed for immediate use or stored at −20°C.

(ii) Primers. Two 10-nucleotide primer sequences were selected on the basis of work by Akopyanz et al. (1). These were primer 1, AACGCGCAAC, and primer 2, GTGGATGCGA. Primers were titrated against DNA extracted from reference strains of *H. pylori.*

(iii) PCR. The PCR was performed with a 50-μl volume which contained 3 mM $MgCl_2$ (Western Biotech, Perth, Australia), 1× reaction buffer (Western Biotech), 0.2 mM each of the four free deoxynucleotides (Boehringer GmbH, Mannheim, Germany), and 1 U of *Thermus thermophilus* plus DNA polymerase (Western Biotech). Ten microliters of undiluted primer 1 or 2 was used in each reaction. The PCR was carried out with a FTS-320 thermal cycler (Corbett Research, Sydney, Australia) with the following program: five cycles of 94°C for 5 min, 40°C for 5 min, and 72°C for 5 min, followed by 15 cycles of 94°C for 1 min, 40°C for 1 min, and 72°C for 2 min, followed by 15 cycles of 94°C for 1 min, 36°C for 1 min, and 72°C for 2 min, followed by 1 cycle of 72°C for 10 min and 5°C for 1 min.

Serology. Blood samples were collected from twin 1 on the day of his initial endoscopic examination (day 1) and on the following occasions: days 30, 63, 209 (29 weeks), 259 (37 weeks), 875 (2 years 5 months), 1001 (2 years 9 months), and 1027 (2 years 10 months). Blood was collected from twin 2 on days 1, 30, 63, 209, 259, 1001, and 1027 and from the twins' parents on days 1, 30, 63, 209, 259, and 1027. On day 1027, blood was collected from the then-16-month-old sister of the twin boys. The age of the twin boys on days 1, 30, 63, 209, 259, 875, 1001, and 1027 was 21 months, 22 months, 23 months, 28.5 months, 30 months, 4 years 2 months, 4 years 6 months, and 4 years 7 months, respectively.

Blood samples were also collected from a 46-year-old male patient (designated patient X), the first acutely infected patient studied by our group (10). Samples were collected on the first day of presentation and then 3 and 14 weeks, 15 and 21 months, and 5 years later.

Sera were stored at −20°C prior to analysis.

Western blot (immunoblot) analysis. (i) IgG response. Two strains of *H. pylori* were used as antigens for the Western blot analysis, these being UNSW-RU1 and NCTC 11637 (*cagA*$^+$ *vacA*$^+$). These strains were grown on horse blood agar for 48 to 72 h, and the cells were harvested in 0.05 M Tris HCl (pH 6.8; Tris). The cells were then washed three times with Tris, after which a protein estimation was carried out (bicinchoninic acid method; Pierce Chemicals, Rockford, Ill.), and the cells were resuspended to a protein concentration of 1 mg/ml. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (4% stacking gel and 12% running gel [1.5 mm thick]) with a Mini-Protean II system (Bio-Rad, Richmond, Calif.). Antigen (1 mg/ml) was diluted 50:50 in reducing buffer and heated at 95°C for 5 min. The equivalent of 5 μg per lane was loaded onto the gel. The electrophoresis was carried out at 30 mA.

After electrophoresis, the proteins were transferred onto an Immobilon P membrane (Millipore) by means of a MilliBlot-SDE system (Millipore). After completion of the transfer, the Immobilon P membrane was placed in a 24-lane Milliblot M apparatus (Millipore), and immunoprobing was carried out with the reagents outlined in the Immunoblot Assay Kit (Bio-Rad). In brief, nonspecific binding sites were blocked with 3% gelatin for 1 h at 37°C. The lanes were then washed twice in wash buffer at 37°C for 5 min, after which 100 μl of a 1/100 dilution of each serum sample was added to the individual lanes. The lanes were incubated at 37°C for 1 h. After incubation, each lane was washed in wash buffer three times for 5 min at 37°C. One hundred microliters of a 1/1,000 dilution of a biotin-labelled goat anti-human IgG was then added to each lane, and the lanes were incubated at 37°C for 1 h. After incubation, each lane was washed three times in wash buffer and three times with distilled H_2O. A second incubation with 100 μl of avidin alkaline phosphatase was then carried out at 37°C for 1 h. After washing, the membrane was removed and placed in 10 ml of development solution (Bio-Rad) until bands developed (approximately 10 min). The reaction was stopped by washing with distilled H_2O.

(ii) IgM response. The procedure described above was repeated for the determination of the IgM antibody response. However, in this case, sera were diluted 1/10 and biotin-labelled goat anti-human IgM conjugate diluted 1/3,000 was used.

(iii) Image scanning of Western blots. To aid in the determination of the molecular size of the antigens detected by Western blotting, each blot was scanned with a Scanmaster 3+ (Howtek, Hudson, N.H.) and the images were analyzed by the Bioimage Whole Band Analysis program (Millipore).

Commercial *H. pylori* antibody immunoblot detection method. Serum samples collected from twin 1 and twin 2 on days 1, 30, 63, 209, 259, 1001, and 1027, serum samples collected from all of their family members on days 1, 30, 63, 209, 259, and 1027, and serum samples from patient X collected on day 1 and at 3 weeks, 15 months, 21 months, and 5 years were tested with a commercially available immunoblotting kit, Helico Blot 2.0 (Genelabs Diagnostics, Singapore), to compare the detection of the antibody response by use of a standardized system available to most *H. pylori* research groups. In addition, the serum sample collected from the 16-month-old sister of the twin boys was examined with this system.

Prepared membrane strips were run as described in the manufacturer's directions. Sera were diluted 1/100.

Dot blotting of *H. pylori* for *cagA*. (i) *cagA* probes. DNA fragments containing a region of *cagA* were generated by PCR and verified by cycle sequencing. The *cagA* PCR primers were designed as described by Tummuru et al. (24). PCRs were performed with 50-μl volumes containing 1× reaction buffer (Western Biotech), 0.2 mM each of the four free deoxynucleotides (Boehringer), 0.2 ml of *T. thermophilus* polymerase (Western Biotech), 2 mM $MgCl_2$, and 25 pmol of each primer and run in a Corbett FTS320 thermal cycler at the following settings: 2 min at 94°C, 2 min at 54°C, and 2 min at 72°C. This cycle was repeated 25 times.

The PCR products were ethanol precipitated and labelled with dioxigenin (DIG)-dUTP by use of the Boehringer DIG DNA labelling kit.

(ii) Preparation of bacterial DNA. Bacteria were harvested in 1 ml of brain heart infusion broth and centrifuged at 12,879 × *g* for 5 min. DNA was isolated from the pellet by the miniprep bacterial genomic DNA method described in *Current Protocols in Molecular Biology* (3). The concentration of DNA was determined with a UV spectrometer.

(iii) Dot blots. All DNA samples were adjusted to a concentration of approximately 1,400 ng/ml. DNA samples obtained from *Campylobacter jejuni*, *Campylobacter coli*, *Campylobacter fetus*, *Helicobacter cinaedi*, and *Helicobacter fenelliae* as well as herring sperm DNA (Boehringer) at a concentration of 1,800 ng/ml were used as negative controls. The NCTC 11637 strain was included as a positive control. DNA samples were boiled for 10 min and then chilled on ice. One microliter of the denatured DNA samples was spotted onto a positively charged nylon membrane (Boehringer) and UV cross-linked in a Hybaid crosslinker for 3 min.

Prehybridization and hybridization with the *cagA* probe were conducted as described in the Boehringer DIG system user's guide for filter hybridization. Detection of hybridized signals was performed with the Boehringer DIG luminescent detection kit.

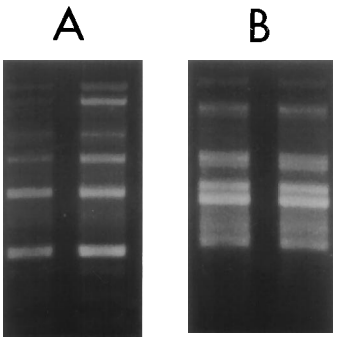

FIG. 1. RAPD fingerprint patterns obtained from the two *H. pylori* strains (UNSW-RU1 and UNSW-RU2) isolated from the antra of twins 1 and 2 (left and right lanes, respectively). (A) Patterns from twins 1 and 2 by use of primer 1; (B) patterns from twins 1 and 2 by use of primer 2.

RESULTS

RAPD fingerprinting. The DNA (RAPD) fingerprinting patterns of the gastric body and antrum isolates from both twin boys gave identical patterns with each of the random primers (Fig. 1). RAPD fingerprinting of the two NCTC strains and selected clinical isolates gave unique fingerprints distinct from those obtained for the isolates from the twin boys.

Western blot analysis. (i) Twin 1. (a) IgM response to the UNSW-RU1 strain. With the sera collected on day 1, antibody recognizing an antigen at 55 kDa was detected (considered to be a cross-reactive antigen). By day 30, additional antigens at 18, 28, 31, 55, and 60 kDa were detected; however, on day 63, only antibodies recognizing antigens at 55 and 60 kDa were detected. Sera collected on day 1001 recognized antigens at 16,

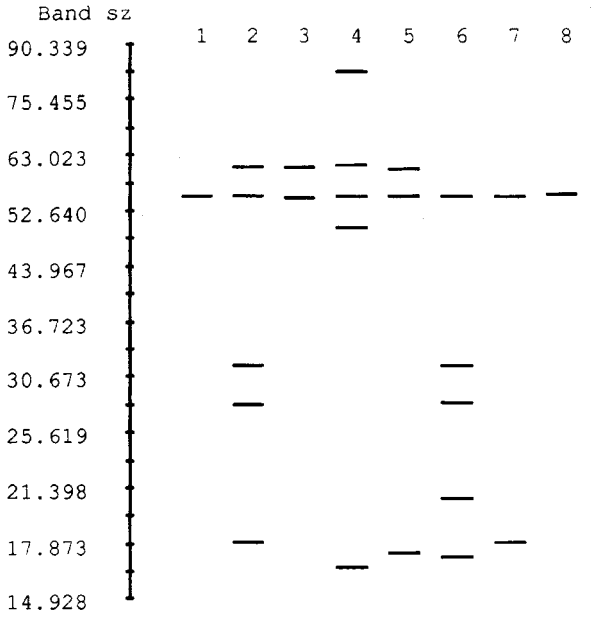

FIG. 2. Scanned image of the blot showing the IgM immune response to UNSW-RU1 for twin 1 (lanes 1 to 5, days 1, 30, 63, 875, and 1001, respectively) and twin 2 (lanes 6 to 8, days 1, 30, and 63, respectively). Band sz, band size (in kilodaltons).

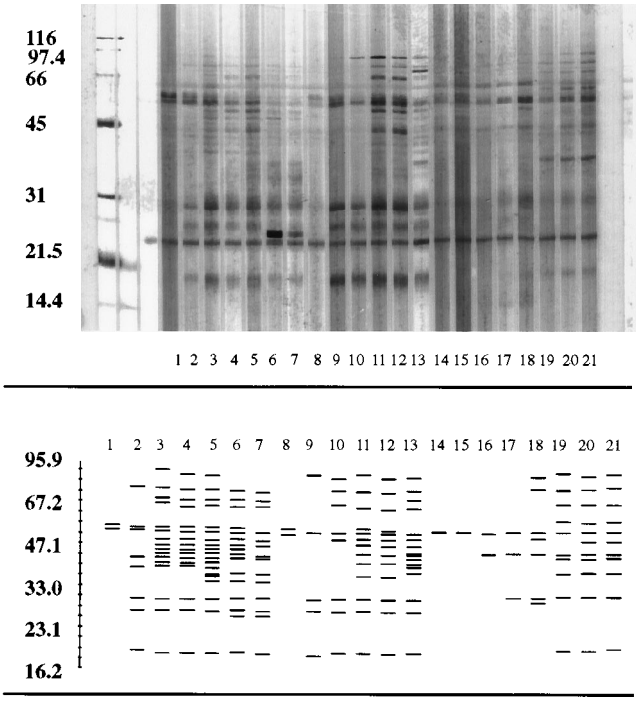

FIG. 3. Immunoblot (top) and scanned image of the blot (bottom) showing the IgG immune response to UNSW-RU1 for twin 1 (lanes 1 to 7, days 1, 30, 63, 209, 259, 875, and 1001, respectively), twin 2 (lanes 8 to 13, days 1, 30, 63, 209, 259, and 1001, respectively), the father of the twins (lanes 14 to 18, days 1, 30, 63, 209, and 259, respectively), and the mother of the twins (lanes 19 to 21, days 1, 30, and 63, respectively). With both the UNSW-RU1 and NCTC 11637 strains, a band was detected at 24 kDa in all samples as well as in wells containing no serum. This band is due to the biotinylated carboxylase of *H. pylori* and was removed from the scanned images. Values on the left indicate molecular sizes in kilodaltons.

50, 55, and 82 kDa, but by day 1027, recognition of antigens at 17, 55, and 60 kDa only was apparent (Fig. 2).

(b) IgG response to the UNSW-RU1 strain. The development of the antibody response to UNSW-RU1 over time is shown in Fig. 3. On the day twin 1 underwent endoscopic examination, antibodies recognizing antigens of 52.5 and 54 kDa were detected (considered to be cross-reactive antigens). Thirty days later, additional antibodies recognizing antigens of 19, 26.5, and 29 kDa were noted and faint bands appeared at 38, 41.5 and 75 kDa. Over time, antibodies to an increasing number of antigens were recognized and faint bands at 39, 43 to 49, 66, 69, and 87 kDa were apparent on day 63. On day 209, bands at 38 and 48 kDa intensified and additional faint bands at 64, 68, and 83.5 kDa were detected. However, bands of 66 and 69 kDa were no longer recognized. On day 259, antibodies recognizing additional antigens at 34, 35, and 36 kDa were apparent, and on day 875, a particularly prominent band was present at 25 to 26 kDa, with bands at 34 and 36 kDa increasing in intensity. The 84-kDa band was no longer apparent on this day, and recognition of the 54-kDa antigen was very weak. On day 1001, bands at 47 and 54 kDa were not detected and the intensity of the 25-kDa band decreased.

(c) IgG response to the NCTC 11637 strain. The development of the antibody response to NCTC 11637 over time is shown in Fig. 4. As observed with the homologous strain (UNSW-RU1), prominent bands to the 53- and 55 kDa protein antigens were detected in the response of serum sample collected on day 1. On day 30, additional bands at 19, 26, and 29 kDa were detected. On day 63, additional faint bands at 45 and 48 kDa were also observed. On day 209, further faint bands at 37, 40 to 47, and 56 kDa were apparent, and this pattern remained on day 259. On days 857 and 1001, additional bands were detected at 33 and 36 kDa and a faint band was present at 128 kDa. At this time, the band at 43 kDa was no longer detectable.

(d) IgG response with a commercial *H. pylori* Western blot kit (Helico Blot 2.0). On day 1, antibody recognizing an antigen at 60 kDa was detected (considered to be a cross-reactive antigen). On day 30, strong reactions against antigens of 26.5 and 60 kDa as well as faint reactions against antigens of 19.5 and 30 kDa were observed. On day 63, strong reactions against antigens at 19.5, 26.5, 30, and 60 kDa were detected. On days 209 and 259, strong reactions against antigens at 19.5, 26.5, and 30 kDa as well as faint reactions against antigens at 45, 54, 60, and 68 kDa were detected. A similar response was observed on day 1001; however, a strong reaction with a band running at 116 kDa (CagA antigen) was observed. The response on day 1027 was similar to that on 1001; however, additional reactions with 35- and 89-kDa antigens were also detected.

(ii) Twin 2. (a) IgM response to the UNSW-RU1 strain. When the serum sample collected from twin 2 on day 1 was tested, antigens were detected at 17, 21, 28, 31, and 55 kDa. However, by day 30, only two antigens were detected, those at 18 and 55 kDa. By day 63, only the 55-kDa antigen was detected (Fig. 2).

(b) IgG response to the UNSW-RU1 strain. The development of the antibody response to the UNSW-RU1 strain over time is shown in Fig. 3. Sera collected from twin 2 on day 1 contained antibodies recognizing antigens of 50 and 53 kDa. Thirty days later, antibodies recognizing antigens at 18, 27, and 29 kDa appeared, with a weak response to antigens at 38 and 84 kDa. Thirty-three days later (day 63), an additional band at 83 kDa was detected and faint bands were also detected at 49, 67, and 76 kDa. On day 209, additional bands at 37, 40 to 47, and 56 kDa were detected. The antigen recognition pattern found on day 259 was identical to that found on day 209. On

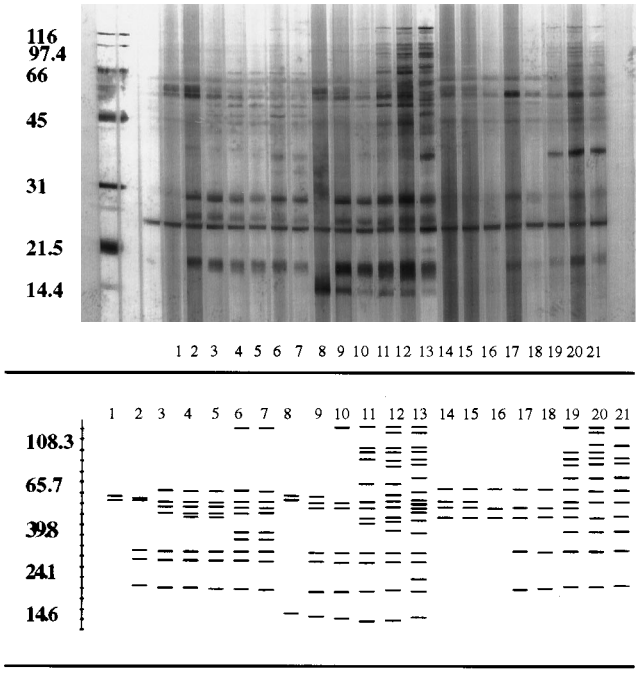

FIG. 4. Immunoblot (top) and scanned image of the blot (bottom) showing the IgG immune response to NCTC 11637 for twin 1 (lanes 1 to 7, days 1, 30, 63, 209, 259, 875, and 1001, respectively), twin 2 (lanes 8 to 13, days 1, 30, 63, 209, 259, and 1001, respectively), the father of the twins (lanes 14 to 18, days 1, 30, 63, 209, and 259, respectively), and the mother of the twins (lanes 19 to 21, days 1, 30, and 63, respectively). With both the UNSW-RU1 and NCTC 11637 strains, a band was detected at 24 kDa in all samples as well as in wells containing no serum. This band is due to the biotinylated carboxylase of *H. pylori* and was removed from the scanned images. Values on the left indicate molecular sizes in kilodaltons.

day 1001, additional bands to antigens at 39 and 70 kDa were detected, while there was a loss of band recognition at 37 and 54 kDa.

(c) IgG response to the NCTC 11637 strain. The development of the antibody response to NCTC 11637 over time is shown in Fig. 4. Sera collected from twin 2 on day 1 contained antibodies recognizing antigens of 14, 52, and 54 kDa. The response on day 30 was similar to that on day 1, but additional prominent bands at 18, 25, and 28 kDa and a fainter band at 51 kDa were detected. On day 63, an additional faint band appeared at 128 kDa but the band at 54 kDa was no longer detected. Sera collected on day 209 showed additional bands at 42, 44.5, 63.5, 89, 96, and 102 kDa, and the intensity of the 128-kDa band increased. On day 259, additional bands at 40, 54, 81, and 115 kDa appeared, and again the intensity of the 128-kDa band increased. Sera from day 1001 showed additional bands at 48, 49, and 67 kDa and a faint band at 21 kDa. In this serum sample, the 63-kDa band was not detected.

(d) IgG response with the Helico Blot 2.0. When the commercial immunoblotting method was used, the antibody response of twin 2 was almost identical to that of twin 1. However, the timing of the response to certain antigens was slightly different. In twin 2's initial serum sample (day 1), strong reactions to antigens of 26.5 and 60 kDa were observed. In addition, the initial reaction with the 19.5-kDa antigen occurred on day 30, and on day 63, a faint reaction with the 116-kDa (CagA antigen) was first noted. A strong antibody response to the CagA antigen (116 kDa) was present in all subsequent serum samples. In twin 2, a response to the 35-kDa antigen was first observed on day 1001 and was still present on day 1027.

(iii) Father. (a) IgG response to the UNSW-RU1 strain. The development of the antibody response to UNSW-RU1 over time is shown in Fig. 3. On days 1 and 30, serum samples from the father of the twin boys showed a single band at 53 kDa. However, on day 63, an additional band at 45 kDa was visible. By day 209, another band was detected at 29 kDa, and by day 259, bands at 27, 51, 76, and 84 kDa were also detected.

(b) IgG response to the NCTC 11637 strain. The development of the antibody response to NCTC 11637 over time is shown in Fig. 4. On days 1 and 30, serum samples from the father of the twin boys showed identical antibody responses, giving faint bands at 43, 51, 54, and 58 kDa. On day 63, the 54-kDa band was not detected. On day 209, additional bands at 18 and 28 kDa were recognized, and this pattern persisted to day 259.

(c) IgG response with the Helico Blot 2.0. No antibody response was detected in the serum samples collected from the father of the twin boys on days 1, 30, and 63. However, on day 209, a strong reaction with the 26.5-kDa antigen as well as a faint reaction with the 30-kDa antigen was noted. A similar reaction was seen on day 259; however, an additional band in response to the 19.5 kDa antigen was noted. On day 1027, further antibody reactions with antigens at 30, 45, and 60 kDa and a weak reaction with a 72-kDa antigen were observed.

(iv) Mother. (a) IgG response to the UNSW-RU1 strain. Serum samples collected from the mother of the twin boys on days 1, 30, 63, 209, 259, 1001, and 1027 recognized antigens occurring at 17, 29, 36, 43, 44, 52, 59, 66, 76, and 86 kDa. (Results for days 1, 30, and 63 are illustrated in Fig. 3.)

(b) IgG response to the NCTC 11637 strain. The antibody response to NCTC 11637 over time is shown in Fig. 3. With the NCTC 11637 strain of *H. pylori* as the antigen, the serum samples collected from the mother of the twin boys on days 1, 30, and 63 showed recognition of antigens at 18, 28, 36, 43.4, 51, 54, 66, 74, 87, 96, 114, 120, and 126 kDa. Antibody against a 36-kDa antigen appeared to be the most prominent throughout this series. (Results for days 1, 30, and 63 are illustrated in Fig. 4.)

(c) IgG response with the Helico Blot 2.0. In all serum samples collected from the mother of the twin boys and tested by the commercial immunoblotting method, antibody responses to antigens at 19.5, 26.5, 30, 35, 45, 60, and 116 kDa were observed; however, a response to the 35-kDa antigen did not appear until day 63.

(v) Sixteen-month-old sister: IgG response with the Helico Blot 2.0. Sera collected from the 16-month-old sister of the twin boys showed strong reactions to antigens at 19.5, 26.5, 30, and 60 kDa.

(vi) Acutely infected patient X. (a) IgG response to the UNSW-RU1 strain. The development of the antibody response to UNSW-RU1 over time is shown in Fig. 5. Sera collected from patient X on day 1 and at 3 weeks showed clear recognition of antigens with molecular sizes of 45, 49, and 51 kDa and a weak recognition of a 77-kDa antigen on day 1 which increased by 3 weeks. By week 14, recognition of 10 more antigens had occurred, the strongest reaction being against antigens with molecular sizes of 30.5, 45, 49, 51, 64, and 77 kDa. Weaker reactions were detected against antigens of 14, 19, 20.5, 35, 41, and 69 kDa, with slight reactivity against antigens of 22 and 87 kDa. At 15 and 21 months, no extra bands were detected. However, at 15 months, there was a marked increase in antibody to the 87-kDa band. At 21 months, a further increase in the intensity of the 87-kDa band and a marked fall in antibody binding to the 30.5-kDa antigen occurred. The increase in antibody to the 87-kDa antigen at these times did not relate to symptomatology in this patient.

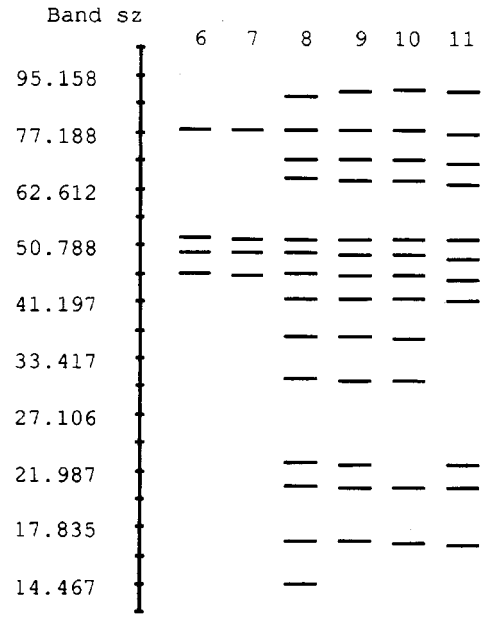

FIG. 5. Scanned image of the blot showing the IgG immune response to UNSW-RU1 for patient X (lanes 6 to 11, day 1 and 3 weeks, 14 weeks, 15 months, 21 months, and 5 years later, respectively). Band sz, band size (in kilodaltons).

Four years after the initial infection, antibody responses to the 30.5- and 35-kDa antigens had disappeared.

(b) IgG response with the Helico Blot 2.0. Sera collected from patient X on day 1 showed clear recognition of antigens with molecular sizes of 55 and 60 kDa and much weaker reactions to antigens at 26.5, 45, and 68 kDa. A similar pattern was observed at 3 weeks. In serum samples collected at 15 months, 21 months, and 4 years, bands to antigens of 19.5, 21, 26.5, 30, 45, 55, 60, 68, 89, and 116 kDa were detected.

Dot blotting of *H. pylori* for *cagA*:*cagA* probes. By use of the dot blotting technique, *cagA* signals were shown to be present in the UNSW-RU1, UNSW-RU2, and NCTC 11637 strains and absent in the herring sperm DNA control (Fig. 6). *cagA* signals were also shown to be absent in *C. jejuni*, *C. coli*, *C. fetus*, *H. cinaedi*, and *H. fenelliae.*

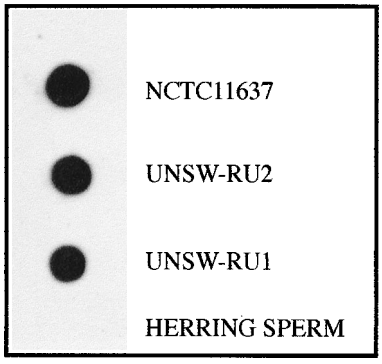

FIG. 6. *cagA* signals observed by use of the dot blotting technique in UNSW-RU1, UNSW-RU2, and NCTC 11637. This signal was absent in the herring sperm DNA control.

DISCUSSION

Regardless of whether the route of transmission of *H. pylori* is fecal-oral, oral-oral, or both, there is good evidence supporting intrafamilial transmission as a primary factor in the dissemination of this organism (8, 12, 17, 19). Given that early childhood may be the critical period for the acquisition of *H. pylori* in both developing and developed countries, the issue of intrafamilial transmission is of particular importance (15, 16, 18).

The RAPD fingerprints in the present study showed that both twin boys were infected with indistinguishable strains of *H. pylori*. The staging of the specific serological response together with the RAPD data indicates that these children were infected from a common source, which we consider to be their mother (19). Although an isolate of *H. pylori* from the mother was not available for comparison by RAPD analysis, such an interpretation is supported by the pattern of antigens recognized by the mother's sera when used to probe Western blots of NCTC 11637 and UNSW-RU1, which is almost identical to that detected with the sera from the twin boys. In contrast, the pattern of antigen recognition in the unrelated acutely infected patient (patient X) was distinct. Additional evidence that the transmission of *H. pylori* is familial was the finding that the 16-month-old sister of the twin boys was also infected with *H. pylori.*

Our findings showing indistinguishable strains in two members of this family are consistent with the findings of Nwokolo et al., who found by DNA fingerprinting that three of eight members of one family were infected with indistinguishable strains of *H. pylori* (20). Similarly, Bamford et al., by use of ribotyping, reported indistinguishable strains of *H. pylori* to be present in some family members in three of four families (4).

Consistent with our earlier report (19), the results of the IgM immunoblot studies showed that both twin boys mounted a transitory IgM response to *H. pylori*. In the serum samples collected from twin 1, on day 1, only a cross-reacting 55-kDa band was detected (14); however, by day 30, recognition of antigens at 18, 28, 31, 55, and 60 kDa was apparent. By day 63, the majority of these bands had disappeared. The second IgM response in twin 1, on day 875, was unexpected and remains unexplained (see later discussion). In twin 2, the major IgM response to *H. pylori* occurred on day 1, suggesting that infection with *H. pylori* may have occurred in this child just before that in twin 1.

Over the first 259 days, the antigen recognition patterns found in the twins were very similar. On day 875, antibodies to the 36-, 38-, and 84-kDa antigens were no longer detected in twin 1; however, an enhanced reaction to the 26-kDa antigen was detected. These changes coincided with the appearance of the second IgM response detected in this child. In the 2 weeks prior to day 875, twin 1 had suffered a period of severe vomiting and as a result underwent another endoscopic examination. At this time, twin 1 was *H. pylori* positive but had no ulcers present (no biopsies for culture were taken at this time).

Given that an antibody response to the 26-kDa antigen was one of the first to appear after infection with *H. pylori*, this finding may indicate that there has been either a gastric disturbance affecting colonization, infection with an organism with cross-reactive antigens, or alternatively, infection with a second strain of *H. pylori*. The disappearance of the IgG antibody response and the transient IgM recognition of the 84-kDa antigen (possibly the vacuolating cytotoxin) at this time may indicate that an altered form of this toxin, to which twin 1 mounted an altered serologic response, had been induced. Alternatively, idiopathic factors may have altered the host re-

sponse to this protein given that twin 1 was now over 4 years old. The observations described above may be of clinical significance; we may ask, Are periodic bouts of vomiting and other gastrointestinal disturbances marked by changing antibody responses and are immunological disturbances linked to the development of such symptoms?

The development of the antibody response in the two adult patients with *H. pylori* infection was slightly different from that found in the twin boys. In the father of the twins, the earliest specific antibody response appeared to be to an antigen at 45 kDa which was detected on day 63. Although this may be a cross-reactive antibody, response to this antigen was also found in the early samples from patient X. On day 209, sera from the father recognized another antigen at 29 kDa, followed by antigens at 27, 51, 76, and 84 kDa on day 259. The appearance of these responses coincided with the rise in IgG found on day 209 in our previous study (19) and the subsequent IgG seroconversion on day 259. Sera collected from patient X on day 1 and at 3 weeks recognized specific antigens at 45, 49, and 77 kDa. This was followed at 14 weeks by the recognition of antigens with molecular sizes of 14, 19, 20.5, 30.5, 35, 41, 69, 45, 49, 51, 64, and 68 kDa. Although, because of the small number of subjects, few conclusions regarding differences in antibody response between adults and children over time can be drawn from this study, our results suggest that the development of the antibody response to lower-molecular-weight antigens may occur slightly later in adults.

In this study, we compared the antibody response to the indigenous strain with that of a strain not directly related to that causing the infection. When the antigens of NCTC 11637 and the commercial Western blot strips were probed, antibodies to additional antigens were detected. Of particular interest was the strong antibody reaction to 120- to 128-kDa antigen (CagA) (6, 7) in both twin boys and their mother that was not detected with the UNSW-RU1 strain.

The lack of antibody recognition of the 120- to 128-kDa antigen in the members of this family by the indigenous strain (UNSW-RU1) is interesting. SDS-PAGE analysis of the UNSW-RU1 and NCTC 11637 strains showed that both strains of *H. pylori* produce a protein band approximately 120 kDa in size (unpublished data). Although a protein band of this size would be consistent with the CagA protein, further examination of this protein is required before definitive identification can be made. Why a response to the 120-kDa antigen is absent when the indigenous *cagA*$^+$ strain is used is unknown; however, this may represent an interesting biological phenomenon and certainly requires further detailed exploration.

In the only other report of an acute case of *H. pylori* infection where the development of the antibody response to specific *H. pylori* antigens was examined over time, Sobala et al. (23) reported "only a slight increase in serum IgM immunoblot positivity to occur on day 14 of infection and recognition of the 120 kDa antigen only on days 74 and 138." Sobala et al. also noted that no systemic IgG antibodies occurred until 74 days after infection, with antibody only to a 120-kDa antigen being present at this time. It was not until day 198 that serum IgG recognition of antigens of 40 to 50, 70 to 85, and 90 kDa was detected (23). The late response to these latter antigens is inconsistent with our IgG data, with NCTC 11637 as the antigen, where these antigens were recognized much earlier in the infection. The difference in the pattern of antigen recognition found in the study of Sobala et al. may relate to the antigen used in their immunoblot studies. In the present study, the response differed depending upon the antigen preparation used. It would appear that immunoblotting is more sensitive for the detection of acute *H. pylori* infection than the enzyme-linked immunosorbent assay (ELISA). If Western blotting is to be used as a diagnostic or epidemiological tool, standardized antigen preparations may be of value.

The commercial immunoblotting system used in part of the present study, although failing to recognize some antibody responses detected by the in-house Western blotting technique, identified responses to major antigens diagnostic of *H. pylori*. Examination of the antigen recognition pattern found in this study suggests that a positive antibody response to at least two of the 19-, 26-, 29-, 45-, 60-, 76- to 77-, and 120- to 128-kDa antigens would diagnose *H. pylori* infection in both acute and chronic cases in adults and children. Faulde et al. (9) considered that antibody responses to any combination of at least two antigens with molecular sizes of 180, 120, 90, 75, 67, 29.5, and 19 kDa to be diagnostic of current *H. pylori* infection. Again, standardized antigen preparations would be of value.

In conclusion, the present study has enhanced the evidence for intrafamilial spread of *H. pylori* and has further emphasized the importance of childhood acquisition of this organism. Analysis of the antigen recognition pattern during the period of acute to chronic infection has shown that in children, the initial antibody responses in *H. pylori* infection are to small-molecular-size antigens and that in adults, responses to these lower-molecular-weight antigens may occur slightly later. The use of different strains of *H. pylori* as antigen in the Western blot technique may result in recognition of different antigens. Application of a standardized system such as the commercially available Helico Blot 2.0 immunoblotting kit may overcome this and may allow identification of acute cases of *H. pylori* infection as well as confirmation of *H. pylori* status in sera which fall in the "gray zone" in many commercial ELISA kits. It is also possible that gastrointestinal disturbances may be marked by changes in antibody response to specific antigens.

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