JOURNAL OF CLINICAL MICROBIOLOGY, Dec. 1990, p. 2641–2646
0095-1137/90/122641-06$02.00/0

Vol. 28, No. 12

Serodiagnosis of *Helicobacter pylori* Infection in Childhood

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Received 27 April 1990/Accepted 11 September 1990

Sera from 100 children (ages, 6 to 16 years) presenting with upper gastrointestinal symptoms were examined for antibodies to *Helicobacter pylori* by enyzme-linked immunosorbent assay (ELISA) based on crude, loosely cell-associated antigens and a partially purified urease antigen preparation. All children underwent endoscopy, and 20 children were shown to have *H. pylori* infection by histology or direct culture. Serum anti-*H. pylori* immunoglobulin G (IgG) levels (crude antigen) were clearly raised in the infected group, particularly after preabsorption of sera against a *Campylobacter jejuni* antigen preparation, while IgM and IgA ELISA determinations did not discriminate between infected and *H. pylori*-negative patients. Only 14 children in the infected group had raised anti-urease IgG levels. Two patients in whom the organism was not demonstrated or cultured had raised specific IgG levels against both crude and urease antigens and pathological features consistent with *H. pylori* disease. Immunoblotting studies did not reveal any single protein antigen or simple combination of antigens that could be considered as a candidate for a more defined serodiagnostic reagent. Anti-*H. pylori* antibody determinations (crude antigen) performed on posttreatment samples from children in whom the organism could no longer be demonstrated suggested that sustained IgG levels may not be a reliable index of treatment failure. An IgG ELISA based on crude, loosely cell-associated antigens of *H. pylori* can be used for the serodiagnosis of *H. pylori* infection in childhood.

Helicobacter pylori was first isolated from a gastric mucosal biopsy specimen in 1983 (24). The organism causes gastritis in humans and has been associated with peptic ulceration in people in all age groups (1, 6).

Most cases of *H. pylori* infection are chronic and may be lifelong (12). Although presence of the organism on the gastric mucosa provokes specific serum antibodies in most individuals, neither this nor any other aspect of the immune response examined to date has been associated with clearance or protection. Indeed, a sustained systemic immune response, predominantly immunoglobulin G (IgG), appears to be characteristic of chronically infected adults (20).

Enzyme-linked immunosorbent assays (ELISAs) that use a variety of antigen preparations have been evaluated as serodiagnostic tests in adults (15, 22), and it has been reported that reliable serodiagnosis can be achieved by the detection of antibodies to urease or other high-molecular-weight antigens (4, 8, 9, 17). A specific IgG response at least as great as that found in adults can also be detected in infected children (5, 13; J. E. Thomas, E. J. Eastham, T. S. J. Elliott, C. M. Dobson, and D. M. Jones, Gut **29**:A707, 1988). However, the relative values of crude or purified and preabsorbed or unabsorbed sera have not been reported for this age group. There could be significant differences between adults' and children's sera, since the latter can be expected to represent the response to short-term infections and acute primary infections more frequently than sera obtained from adults do. Therefore, further investigation of the serological response to *H. pylori* infection in children is desirable.

Here we report the relationship between serum antibodies to *H. pylori* and *H. pylori* infections, in children and adolescents. The results obtained with crude, loosely cell-associated antigens and partially purified urease antigens in ELISA procedures were compared, and positive sera were examined to determine the immunoglobulin classes involved and the pattern of antigens recognized. The changes in antibody responses occurring after treatment aimed at eradicating infection are also reported.

MATERIALS AND METHODS

Patients. One hundred children (ages between 6 and 16 years) presenting consecutively to our unit during 1987 to 1989 with upper gastrointestinal symptoms requiring endoscopic evaluation were included in the study. Gastric antral mucosal biopsy specimens taken from within 5 cm of the pylorus were obtained from all patients. Twenty patients were shown to have *H. pylori* infection by culture or Giemsa staining (10). These are referred to as the infected group. The organism was not demonstrated by these methods in the remaining 80 subjects, who are referred to as the control group.

Blood samples were taken at the time of endoscopy, and further samples were taken at the start of treatment (for logistical reasons, treatment was often delayed until 2 weeks to 1 month after endoscopy) and at intervals for up to 18 months thereafter. In all cases, treatment consisted of 2 weeks of ampicillin (250 mg three times daily for children <12 years of age; 500 mg three times daily for children >12 years of age) and 2 months of tripotassium dicitratobismuthate (De-Nol; 120 mg twice daily for children <12 years of age; 240 mg twice daily for children >12 years of age) started simultaneously. Of the 20 infected individuals, 13 underwent repeat endoscopy after treatment, and *H. pylori* was not demonstrated in any of these posttreatment samples.

Urease and protein assay. Urease activity was detected by a phenol red assay (11) and quantitated by the modified Bertholet reaction (21) by using reagents prepared as described by Creno et al. (3). Protein concentrations were assayed by the method of Bradford (2) by using the Bio-Rad

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TABLE 1. Purification of urease from *H. pylori*

Purification step	Sp act (μmol of NH_3/min/mg of protein)	Purification (fold)	Protein concn (mg/ml)	% Protein recovered
Cell wash	10.1	1.0	1.850	100.0
Sephacryl S400	49.2	4.9	0.193	10.4
DEAE Sephacel	101.9	10.1	0.103	5.6

protein assay kit (Bio-Rad Ltd., Hemel Hempstead, United Kingdom).

Preparation of antigens. Separate assays performed by using antigens from six different *H. pylori* strains (four isolates and strains NCTC 11637 and NCTC 11916) or a pooled antigen preparation from eight different strains indicated that the strain used as the source of antigen had no significant influence on the antibody titer in any of the sera used in this study. This is consistent with previous reports (18). Therefore, a strongly urease-positive strain, CP END3, which was isolated from a child in this study and had a polypeptide profile on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) similar to those of strains described previously (17) (see Fig. 3), was used for the production of both the crude antigen and urease.

Crude antigen was prepared as follows. Organisms were harvested from blood agar plates (Oxoid Columbia base plus 7% [vol/vol] defibrinated horse blood) after 48 to 72 h of growth under microaerobic conditions, washed twice, and resuspended in phosphate-buffered saline (PBS) (Dulbecco A). Suspensions were incubated at 20°C for 20 min and then centrifuged at 7,000 × *g* for 10 min. The supernatant was recovered, incubated at 50°C for 1 h, and then stored at −70°C (3.6 mg of soluble protein per ml), ready for use.

Partially purified urease was obtained by harvesting cells after 72 h of growth from 120 blood agar plates in PBS. Cells were removed by centrifugation and discarded. Soluble protein was precipitated with 75% saturated ammonium sulfate; and the concentrated protein was resuspended in a small volume of 20 mM Tris hydrochloride buffer (pH 7.0) containing 100 mM NaCl, 1 mM EDTA, and 5% (vol/vol) glycerol. The protein was then chromatographed on a Sephacryl S400 column (400 by 16 mm) which was preequilibrated at 4°C with the buffer described above. Fractions showing maximum urease activity and the highest purity by SDS-PAGE were pooled and concentrated (Centricon 30 microconcentrator; Amicon). The concentrated material was then applied to a DEAE Sephacel column (200 by 10 mm), which was preequilibrated at 4°C with 20 mM Tris hydrochloride (pH 7.0) containing 1 mM EDTA and 50 mM NaCl. The bound protein was then eluted with a 50 to 400 mM NaCl gradient in the same buffer. Fractions showing the highest urease activity were examined by SDS-PAGE; and the purest fractions were pooled, concentrated, and stored at −70°C (0.25 mg of soluble protein per ml) in 50% (vol/vol) glycerol, ready for use. Urease purification results are shown in Table 1 and the final preparation is shown in Fig. 3, lanes A (Coomassie blue-stained SDS-polyacrylamide gel) and C (amido black-stained nitrocellulose filter blot). It was noted that after purification, the urease bands are slightly, but consistently, retarded on SDS-polyacrylamide gels compared with the crude antigen bands. The reason for this is not understood, but a similar observation has recently been made upon purification of an *H. pylori* flagellar protein with an M_r of 54,000 (C. J. Luke and C. W. Penn, personal communication). In light of observations published while the manuscript was in preparation (7), the purified urease described here appears to contain a major contaminant which others have also found to copurify with the two recognized urease subunits (18) (see Fig. 3, lane A). To avoid cumbersome terminology, reactivity with this preparation detected by ELISA is referred to as anti-urease rather than anti-partially purified urease.

Preabsorbing antigen was prepared by growing a recent clinical isolate of *Campylobacter jejuni* on blood agar at 42°C in a microaerobic atmosphere, harvesting the solid growth into PBS, and heating the resultant suspension to 50°C for 1 h. Particulate material was removed by centrifugation at 7,000 × *g* for 20 min, and the supernatant was stored at −70°C, ready for use. The stored material had a soluble protein concentration of 0.9 mg/ml.

ELISA. Both crude and partially purified urease test antigens were diluted in 0.1 M carbonate buffer (pH 9.6) and coated onto polyvinylchloride ELISA plates (Becton Dickinson) by incubation at 4°C overnight. Optimum antigen concentration (crude antigen, 36 μg/ml; partially purified urease, 0.25 μg/ml) and incubation times (see below) were determined by checkerboard titration. After coating, plates were washed three times in PBS-Tween (PBS plus 0.05% [vol/vol] Tween 20), flooded with blocking buffer (PBS-Tween plus 1% [wt/vol] bovine serum albumin), and incubated for 2 h at 20°C.

Sera were assayed at a final dilution of 1:100 in blocking buffer both with and without preabsorption against the crude *C. jejuni* antigen. Absorption was effected by diluting the stored *C. jejuni* antigen 1:100 in blocking buffer, preparing the final dilution of the test serum directly in this material, and incubating the mixture at 4°C overnight.

Aliquots of 50 μl of diluted sera were added to wells in coated plates in the following categories: up to 64 test wells (32 serum specimens assayed in duplicate), 16 wells for the generation of a standard curve (serial dilutions of a strongly reactive serum prepared by pooling sera from three known positive children, including one child from this study), and 10 control wells containing known sera with levels of anti-*H. pylori* reactivity covering the useful range of the assay (to assess within- and between-assay variabilities). A single reagent-blank well (no serum) was also included. In addition, testing of all positive sera (absorbed and unabsorbed) on control plates coated with bovine serum albumin failed to reveal any reactivity attributable to binding of immune complexes such as those present in the preabsorbed sera.

After incubation for 1 h at room temperature, plates were washed three times in PBS-Tween; and bound antibody was detected by the addition of 100 μl of 1 μg of horseradish peroxidase-conjugated rabbit anti-human globulin of the appropriate immunoglobulin class specificity per ml (1 h at room temperature, washed three times with PBS-Tween), followed by development for 5 min at room temperature with 50 μl of 0.012% (vol/vol) H_2O_2 in 25 mM phosphate–50 mM citrate buffer plus 0.4 mg of *o*-phenlylenediamine hydrochloride per ml. The reaction was stopped by the addition of 100 μl of molar H_2SO_4 (all reagents were obtained from Sigma). The optical density at 490 nm was determined by using a Dynatech MR700 microplate reader.

In establishing the assay, the standard pooled serum was titrated to the highest dilution at which reactivity could still be detected. For each immunoglobulin class detected, the serum was assigned an arbitrary ELISA unit value of 1 at this dilution. By this means the neat standard serum was determined to contain 600 ELISA units of IgG reactivity, 320 units of IgM reactivity, and 64 units of IgA reactivity. By

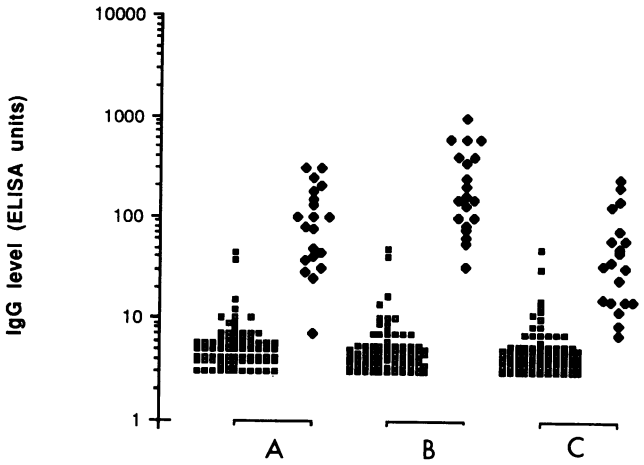

FIG. 1. Anti-*H. pylori* IgG levels measured by ELISA on blood taken at the time of endoscopy. In the control group, some of the results were offset by up to 1 ELISA unit so that all points can be clearly seen. (A) Crude antigen, no preabsorption of sera. (B) Crude antigen, sera preabsorbed against *C. jejuni* antigen. (C) Partially purified urease antigen, no preabsorption. ■, *H. pylori* not isolated or identified; ◆, *H. pylori* isolated or identified.

using these assigned values and the standard curve generated for each plate, it was possible to determine ELISA unit values for the control and test sera. The 95% confidence limits were established for five control sera by repeated assay on separate occasions; any test plate for which control values fell outside these limits was discarded and the assays were repeated. Comparison of values for the control sera revealed within- and between-assay coefficients of variation of under 6%.

SDS-PAGE and immunoblotting. SDS-PAGE and immunoblotting procedures were performed as described previously (14).

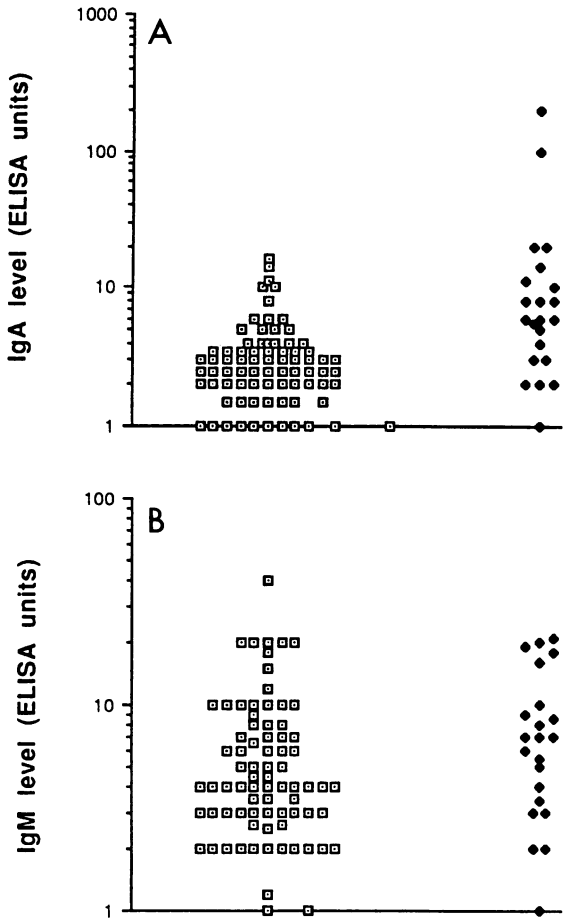

FIG. 2. Anti-*H. pylori* IgA (A) and IgM (B) levels measured by ELISA, after absorption, on blood taken at the time of endoscopy. ⊡, Anti-*H. pylori* IgG negative; ◆, anti-*H. pylori* IgG positive.

RESULTS

ELISA. The anti-*H. pylori* IgG levels obtained by ELISA are shown in Fig. 1. By using crude *H. pylori* antigen, 19 of the 20 infected subjects made up a discrete population with IgG levels in excess of 20 ELISA units. Two serum specimens from the control group also fell within this range (see Discussion). Absorption against *C. jejuni* antigen increased the ELISA unit values found in all sera from the infected group, including the one previously negative serum specimen from a child in this group (7 ELISA units preabsorption to 35 ELISA units postabsorption). This latter sample was obtained 27 days after initial presentation from patient 16, who was probably in the early phase of seroconversion (see below). Several other serum specimens from the infected group showed substantial enhancement of reactivity after absorption, while the remaining reactive serum specimens, including the two reactive control serum specimens, showed only minor increases. Minimal or no changes in ELISA unit levels were observed among the other control sera. As a result of these studies a cut off value of 20 ELISA units was assigned arbitrarily to differentiate *H. pylori*-reactive and nonreactive sera.

The distributions of the anti-urease IgG results (Fig. 1) obtained with our partially purified preparation were not as discrete as those found with the crude antigen. Only 14 of the 20 serum specimens from the infected group appeared to form a separate group with significantly raised IgG anti-urease levels. The two crude antigen-reactive serum specimens from the control group also gave urease-specific IgG levels within the range obtained for these 14 infected children.

With the exception of two serum specimens with high IgA levels, the distributions of IgA and IgM values did not distinguish between infected and control groups (Fig. 2). The patient with the highest IgM response had no other evidence of *H. pylori* infection and did not subsequently develop an IgG response.

Immunoblotting. Immunoblot analysis showed that the IgG response was directed against several different proteins in the crude antigen preparation (Fig. 3). No overall pattern of response was clearly discernible from our 22 positive serum specimens (including the two ELISA-positive serum specimens from children in the control group). Indeed, the most striking feature was that serum from each patient produced a unique pattern. Sera from the six children who did not have elevated anti-urease antibodies by ELISA had no detectable response to partially purified urease by immunoblotting (data not shown).

Sequential analysis of sera. The relationship between anti-*H. pylori* (crude antigen) IgG level determined at the time of presentation and the reported duration of antecedent clinical illness is shown in Fig. 4. Patient 16 presented 3 days

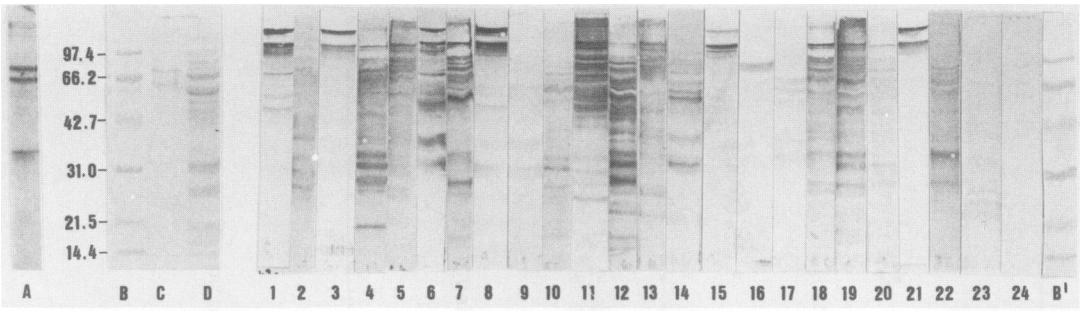

FIG. 3. IgG immunoblots. Lanes B, C, and D were stained with amido black and were part of a nitrocellulose filter blotted from one of the four gels, from which the immunoblots shown in lanes 1 through 24 were prepared. All sera (lanes 1 through 24) were reacted against crude antigen. The four gels were run concurrently under identical conditions, such that the mobilities of the same polypeptides on each of the four gels corresponded, as exemplified by lanes B and B'. Lanes B and B', Molecular size standards run on two of the four gels. M_r values (1,000) are shown to the left of lane B; lane C, partially purified urease antigen; lane D, crude antigen; lanes 1 through 20, patients' sera (infected group); lanes 21 and 22, the two IgG ELISA-positive sera from the control group; lanes 23 and 24, ELISA-negative sera; lane A; Coomassie blue-stained SDS-polyacrylamide gel of the partially purified urease antigen run in lane C, showing more clearly the bands that were poorly stained with amido black. (Note that although the same preparation was run in lanes A and C, lane A is from a gel that was run on a separate occasion and the molecular size standards in lanes B and B' are not applicable.)

after the onset of symptoms (27 days prior to endoscopy) and had an initial specific IgG level of 4 ELISA units (after absorption against *C. jejuni*). This rose to 35 ELISA units (absorbed serum) at the time of endoscopy, when he was shown to have *H. pylori*-related gastritis. All other patients in the infected group gave symptomatic histories of at least 1 month's duration. The highest IgG levels were detected in children who presented after 2 to 6 months of illness.

The relationship between serum IgG levels (crude antigen) and treatment is shown in Fig. 5 for 10 children. While a clear trend toward declining levels is apparent, this was not sustained in several instances. IgG immunoblots done on the same serum specimen (data not shown) revealed that while overall reactivity declined or fluctuated, the same pattern of response to proteins in our crude antigen preparation was maintained throughout the period of study for each child. Sequential ELISA for IgA and IgM levels (data not shown) did not reveal any clear relationship with either length of antecedent illness or treatment.

No evidence of continuing infection was found in any of these 10 children at follow-up endoscopy; indeed, all of them showed symptomatic improvement and resolution of gross and histological signs of gastritis.

One of the infected patients (patient 5, Fig. 3) showed a marked symptomatic improvement between initial presentation and endoscopy 4 weeks later. *H. pylori* was demonstrated on his gastric mucosa, but because his symptoms had resolved, he was not offered any treatment. Two months later he remained symptom-free, and a repeat endoscopy failed to demonstrate *H. pylori*. Four months later his IgG level had fallen to 4 ELISA units from its initial level of 55 ELISA units.

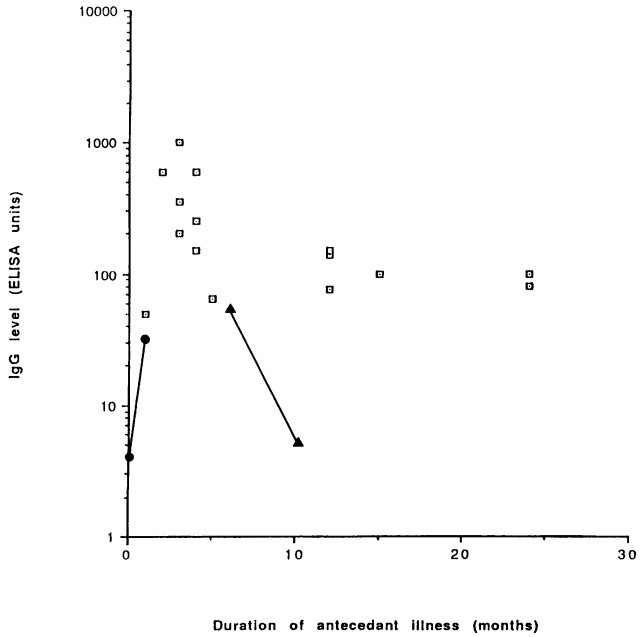

FIG. 4. Anti-*H. pylori* IgG levels related to duration of antecedent symptoms. Results are for sera from 17 infected patients. Two values are shown for patient 16 (●) (seroconversion) and patient 5 (▲) (spontaneous eradication of organism).

DISCUSSION

The results presented here demonstrate the inadequacy of using purified urease antigens for the diagnosis of *H. pylori* infection in childhood and provide further information on the evolution of the antibody response to *H. pylori*. With a

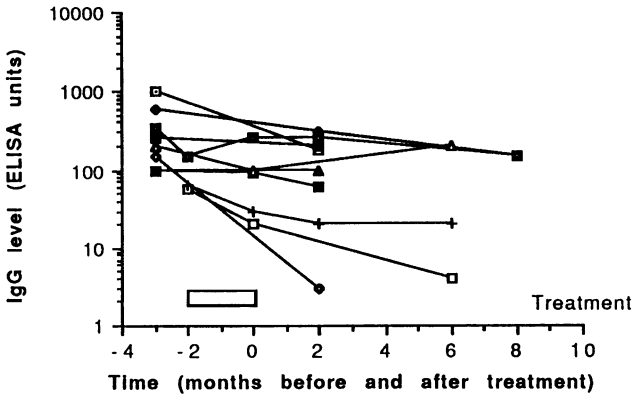

FIG. 5. Serial determinations of anti-*H. pylori* IgG related to treatment. The different symbols refer to separate patients.

crude, loosely cell-associated antigen, anti-*H. pylori* IgG levels were found to exceed the arbitrarily assigned cutoff point to 20 ELISA units in preabsorbed sera from all 20 infected patients and in two serum specimens from the control group. Sera from these two control patients also had raised anti-urease levels, and the patients had pathological features consistent with *H. pylori* disease in children (mucosal polymorph infiltration and nodular lymphoid hyperplasia; J. E. Thomas, unpublished results). This suggests that they had active infections that were not detected by endoscopic examination. Because there is no absolute reference method for establishing the diagnosis of *H. pylori* infection, sensitivity, specificity, and predictive values of the IgG ELISA cannot be calculated.

It is not clear why preabsorption with *C. jejuni* should have substantially enhanced the reactivity of some sera from the infected group. Nonetheless, absorption appears to be particularly useful for sera obtained early in the course of infection, since it was observed to raise IgG ELISA results (crude antigen) above the 20-ELISA-unit threshold both in patient 16 and several other children with short symptomatic histories examined more recently (J. E. Thomas, unpublished results).

The IgA and IgM results confirm the findings of Oderda et al. (19), being clearly correlated with neither the presence or absence of infection nor the duration of infection.

The immunoblot results demonstrate the diversity of serological responses to *H. pylori* that occur among children. Although several proteins in these preparations are recognized by antibodies from most patients' sera, no single protein or polypeptide band is clearly represented in all blots. Several workers have identified the urease antigens, an M_r-120,000 antigen, or pooled high-molecular-weight antigens as candidate preparations for serodiagnosis (4, 9, 18). On the basis of our results, six infected children would have been missed by using urease as the antigen for serodiagnosis, six would have been missed by using the M_r-120,000 antigen, and three would have been missed by using pooled high-molecular-weight antigens.

It is likely that the serum antibody response of one child (patient 16) was monitored very close to the onset of his acute *H. pylori* infection. Specific IgG seroconversion occurred at about day 30. This accords with the findings of Morris et al. (16) in an ingestion experiment.

The differences in IgG levels observed in relation to the length of antecedent clinical illness could be explained by children with high IgG titers presenting before children with low titers. We were, however, unable to correlate any clinical parameters with IgG level that would support this suggestion. The alternative explanation, that children with *H. pylori* infection have high IgG levels shortly after seroconversion (presumably, at about day 30) and that, as infection becomes established, these levels decline over the course of many months, seems more attractive.

It has been claimed that demonstration of a fall in the circulating IgG level following treatment is a reliable means of monitoring response to therapy in adults (23). Such a trend was apparent in several of our patients in the short term, but sustained responses in excess of the ELISA cutoff value were found in two patients who had made a good symptomatic response to chemotherapy and in whom no other evidence of continuing infection could be found. Whether these two children had persistent IgG responses in the absence of infection or whether the organism had not been eradicated remains to be established.

The infection in one of our patients (patient 5) apparently resolved without specific antimicrobial therapy after a 4-month illness. This boy had the lowest anti-*H. pylori* IgG level of any patient and had a duration of symptoms similar to those of the other children, but his serological response was otherwise unremarkable when compared with the responses of the remainder of the infected group.

In this study, clear distinction between infected and noninfected individuals was made by measuring anti-*H. pylori* IgG levels by using a simply prepared crude antigen and sera that were preabsorbed against *C. jejuni*. Adequate specificity was provided by ELISA done on unabsorbed sera, but this may not detect specific IgG in the early phase of the response. No clear candidate for a single protein or simple combined protein antigen for serodiagnostic use has emerged. A crude antigen-based ELISA appears to be sufficient for the serodiagnosis of *H. pylori* infection in childhood.

ACKNOWLEDGMENTS

This work was supported by a grant from Newcastle Health Authority Research Committee, and J.E.T. was supported by a Children Nationwide Paediatric Research Fellowship.

We thank M. Pinkney, D. M. Jones, and T. S. J. Elliott for helpful discussions.

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