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Evaluation of Antibodies Reactive with *Campylobacter jejuni* in Egyptian Diarrhea Patients

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Serum and stool samples were collected from 128 individuals: 96 diarrhea patients and 32 apparently healthy controls. Stool specimens were cultured for enteric bacterial pathogens, while sera were screened by enzyme-linked immunosorbent assay for *Campylobacter jejuni*-reactive antibodies. Of 28 diarrhea patients who demonstrated *C. jejuni*-reactive antibodies (titers, >100), 14 were culture positive for this organism. The 32 healthy controls showed significantly lower antibody titers ($P < 0.05$) with the exception of 10 subjects who were culture positive for *C. jejuni* and had reactive immunoglobulin M (IgM) (6 subjects) and IgG (7 subjects). IgA was not detected in those 10 individuals (asymptomatic). Avidity was expressed as the thiocyanate ion concentration required to inhibit 50% of the bound antibodies. The avidity was higher in symptomatic patients than asymptomatic healthy controls. IgG was less avid (0.92 M) compared to IgM (0.1 M) and IgA (1.1 M), with no correlation between antibody titer and avidity. However, the thiocyanate ion concentration required for the complete inhibition of IgG (5 M)-bound antibodies was higher than that of IgA (2 M) and IgM (3 M). This study also shows that *C. jejuni* antibodies were variably cross-reactive with *Escherichia coli*, *Shigella flexneri*, *Shigella sonnei*, and *Neisseria meningitidis* in addition to *Campylobacter coli* and *Campylobacter rectus*.

Campylobacter jejuni is among the most common bacterial causes of acute diarrheal disease in the world (4). Sequelae of the disease may include lymphadenitis, cystitis, cholecystitis, pancreatitis, hepatitis, hemolytic uremic syndrome, peritonitis, erythema nodosum, septic arthritis, osteomyelitis, septic abortion, and Guillain-Barré syndrome (20). Several lines of evidence suggest that protective immunity can be developed against this pathogen. Spreeuwel et al. (26) and Blaser and Duncan (5) reported the presence of specific serum immunoglobulin M (IgM), IgG, and IgA in acute *Campylobacter* intestinal disease. Both IgM and IgG persisted for 3 months or more, whereas IgA remained elevated for 1 month or less. Healthy individuals with occupational exposure to *C. jejuni* produced IgM without overt disease (28).

Although much information exists on the microbial characteristics of *C. jejuni* and disease transmission, little is known about the immune response to this infection. Blaser and coworkers (6) found IgG titers greater than 1:32 by the immunofluorescence technique in sera from convalescent subjects with positive stool cultures, whereas sera from healthy control individuals showed titers of 1:2 to 1:16. However, antibody elevation in a disease process may mediate a variety of effector mechanisms that are dependent on specificity, avidity, and affinity and may be determined largely by class or subclass and site of action (16). Many host defenses, including complement activation (25), immune elimination (2), and neutralization (3) are affected by the avidity of the participating antibody (8).

Since detection of antibody response to *C. jejuni* is critical to the study of disease vulnerability, pathogenesis, and protection, the purpose of this work was to evaluate *C. jejuni*-reactive antibodies in an area of endemicity in terms of avidity and specificity. Since cross-reactive antibodies may exist due to previous exposure to related antigens (*Campylobacter coli*, *Campylobacter rectus*, *Escherichia coli*, *Shigella flexneri*, *Shigella sonnei*, and *Neisseria meningitidis*), particularly in developing countries such as Egypt, we screened serum samples for specificity to *C. jejuni* through a series of absorption studies.

(This research fulfills some of the doctoral requirements for Tharwat F. Ismail.)

MATERIALS AND METHODS

Specimens. Stool and serum samples were collected from 96 Egyptian subjects suffering from diarrhea meriting treatment at the clinic of the Theodore Bilharz Institute, Ministry of Scientific Research, Cairo, Egypt. Subjects ranged from 12 to 60 years of age and had diarrhea resulting in three to five stools per day. For control purposes, samples were also collected from 32 age-matched individuals who were free from diarrhea symptoms in the preceding 3 months.

Stool culture. Fresh stool samples were cultured on Skirrow's medium and incubated at 42°C for 2 days. Recovered *Campylobacter* isolates were identified by standard microbiological methods (20). Stool samples were also used to inoculate MacConkey, Hektoen Enteric, and thiosulfate-citrate-bile salt sucrose agars to screen for other enteric bacterial pathogens.

Antigen preparation. To cover the complex, multivalent antigenic composition of *C. jejuni*, nine strains isolated from human stools (Lior serotypes 1, 2, 4, 9, 18, 28, 29, 36, and 55) (13) and ATCC strain 29428 were used for antigen preparation by acid-glycine extraction (14). Briefly, strains were grown on blood agar plates, and cells were washed twice in distilled water and suspended in 0.2 M glycine-HCl buffer (4% [wt/vol], pH 2.2). The cells were stirred and centrifuged (11,000 × *g*), and the supernatant was collected, neutralized, and dialyzed against distilled water. The concentration of protein in the soluble extract was determined by the method of Lowry et al. (17).

ELISA. Levels of antibody classes (IgA, IgM, and IgG) reactive with *C. jejuni* were evaluated by the enzyme-linked immunosorbent assay (ELISA) of Guruge et al. (10) with minor modifications. An acid-glycine extract of *C. jejuni* was diluted in carbonate buffer, pH 9.6, to yield a protein concentration of 5 mg/ml. Plastic microtiter plates (96 wells) (Dynatech, Chantilly, Va.) were coated with 100 μl of antigen/well by incubation at 4°C overnight. The wells were washed with PBS containing 0.05% Tween 20, and blocked with PBS containing 1% bovine

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TABLE 1. Mean ODs and dissociation analysis of *C. jejuni*-reactive IgA, IgM, and IgG in sera of symptomatic patients and asymptomatic healthy controls

Antibody[a]	Titer (log_{10}) ± SD	Mean OD ± SD	ID_{50} (M)	ID_{100} (M)
Symptomatic (n = 28)				
IgA (14)	2.23 ± 0.30	0.27 ± 0.19	1.10 ± 0.35	2
IgM (7)	2.25 ± 0.40	0.51 ± 0.21	0.92 ± 0.54	3
IgG (14)	2.40 ± 0.37	1.43 ± 0.27	0.70 ± 0.41	5
Asymptomatic (n = 10)				
IgA (0)	NA[b]	0.07 ± 0.02	NA	NA
IgM (6)	2.20 ± 0.24	0.43 ± 0.19	0.71 ± 0.10	3
IgG (7)	1.37 ± 0.25	1.49 ± 0.29	0.65 ± 0.13	5

[a] The number of reactive sera is indicated in parentheses. Of the 14 *C. jejuni* culture-positive patients, 7 had IgA only, 4 had IgA and IgM, and 3 had IgM and IgG. Of the 10 *C. jejuni* culture-positive healthy subjects (asymptomatic), 3 had IgM only, 3 had IgM and IgG, and 4 had IgG only.

[b] NA, not applicable.

serum albumin for 2 h at 37°C. Wells were washed three times, and then 100 μl of test serum diluted 1:100 in blocking buffer was added to three similar sets of coated plates. An uncoated well was included as a negative control for each sample. After incubation for 90 min, wells were washed three times, and 100 μl of goat anti-human IgA, IgM, or IgG labeled with alkaline phosphatase was added to each well (Sigma Chemical Co., St. Louis, Mo.). The plates were incubated for 60 min at 37°C and washed three times, and then 100 μl of substrate (*p*-nitrophenylphosphate; Sigma) was added. Absorbance was read at 405 nm.

Interpretation of readings. Optimal dilutions of all reagents were determined by checkerboard titration. The cutoff value for reactive sera was calculated as the mean optical density (OD) of sera from healthy, culture-negative subjects plus 2 standard errors of the mean (SEM) (5). Since the mean OD for normal controls in this study was 0.07 with an SEM of 0.025, a reading of 0.1 was used as the cutoff.

Dissociation analysis. Avidity was estimated by measuring the dissociation of antigen-antibody binding by increasing concentrations of ammonium thiocyanate (0 to 8 M in PBS-Tween) (19). Briefly, coated microtiter plates received a 1:100 dilution of each serum and were incubated for 45 min at 37°C. After a wash, 100-μl volumes of various concentrations of ammonium thiocyanate were added, and the samples were incubated for 1 h; then, the ELISA was performed as described above. For a particular serum sample, the percent antibody binding at each thiocyanate concentration was calculated relative to the original ELISA readings obtained before treatment. The thiocyanate dose required to inhibit 50% of the bound antibody (ID_{50}) was determined by regression analysis using the calculated percentages versus thiocyanate concentrations used.

Absorption of sera. Equal volumes of *C. jejuni*-reactive sera were mixed with individual suspensions (10% in PBS [vol/vol]) of *C. jejuni* (ATCC 29428), *C. coli* (ATCC 43474), *C. rectus* (ATCC 33238), *E. coli* serotype O119 (laboratory wild-type strain), *S. flexneri* (ATCC 29508), *S. sonnei* (ATCC 25931), and *N. meningitidis* (ATCC 13090). Mixtures were allowed to react for 1 h at 37°C in plastic microtiter plates and then were transferred to microcentrifuge tubes and centrifuged at 3,500 × *g* for 30 min. Supernatants were tested again by ELISA for reactivity with the acid-glycine extract of *C. jejuni*. Clean fine sand was used as an inert control for the absorption procedure. The percentage of remaining reactive antibodies was determined by dividing the ODs of the absorbed sera by the ODs of the unabsorbed sera.

Statistical analyses. Statistical relationships between antibody titers and ID_{50}s were evaluated by correlation (r) and chi-square analysis.

RESULTS

Cultural and serological analyses showed that 28 of 96 patients had significantly high titers of *C. jejuni*-reactive antibodies (>1/100 [$P < 0.05$]), of whom 14 (50%) demonstrated positive *C. jejuni* cultures. Of these, seven patients had IgA only, four had IgA and IgM, and three had IgM and IgG (Table 1). Of the healthy subjects (32 samples), 10 showed positive cultures for *C. jejuni*. IgA was not detected in these individuals, but three had IgM only, three had IgM and IgG, and four had IgG only. The remaining serum samples from the diseased (68 subjects of 96) or healthy (22 of 32) group had an average OD of 0.07 (SEM = 0.025) for the three classes of antibodies (Table 1). Differences in the OD values of culture-positive subjects (28 diseased and 10 asymptomatic) were significant ($P < 0.05$) compared to healthy, culture-negative individuals (22 of 32).

In the dissociation analysis, IgA that remained bound to the antigen ranged between 24 and 90% of the total reactive IgA at 1 M thiocyanate (data not shown). IgM and IgG that remained bound at the same thiocyanate concentration ranged between 33 and 55% and between 30 and 56%, respectively. The mean ODs of all *C. jejuni*-reactive antibodies (IgA, IgM, and IgG) and their respective thiocyanate concentrations are given in Fig. 1. The ID_{50}s for both IgG and IgM were lower than that for IgA (Table 1). Based on our cutoff value for the ELISA (OD = 0.1), 2, 3, and 5 M ammonium thiocyanate concentrations were enough for complete dissociation (ID_{100}) of IgA, IgM, and IgG, respectively (Table 1).

When IgM and IgG were analyzed, small differences were

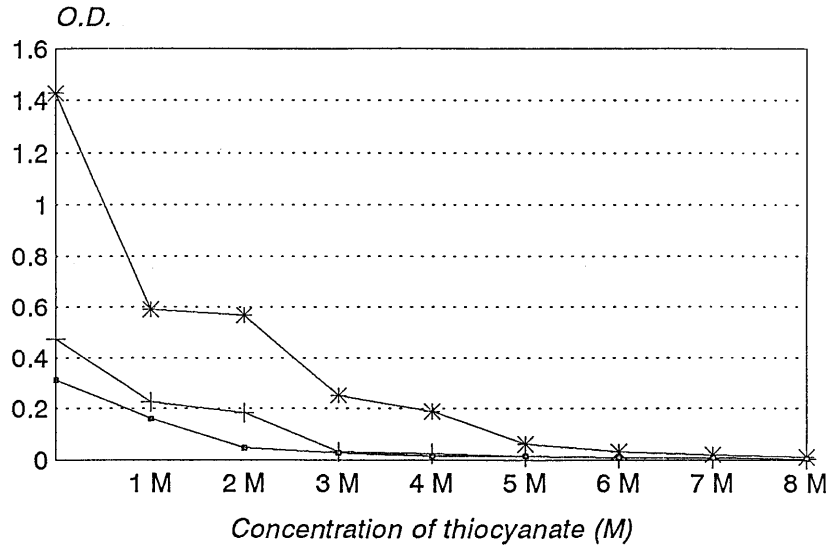

FIG. 1. Dissociation of the *C. jejuni* IgA (■), IgM (+), and IgG (✳) antibody classes as reflected by mean ODs of the symptomatic population.

TABLE 2. Immunoabsorption of anti-*C. jejuni* immunoglobulins by selected bacterial pathogens

Group (*n*)	Mean % OD in relation to *C. jejuni*[a]						
	C. jejuni	*C. coli*	*C. rectus*	*E. coli*	*S. flexneri*	*S. sonnei*	*N. meningitidis*
IgA[b], symptomatic (14/28)	31 ± 11	47 ± 28	52 ± 31	72 ± 26	76 ± 28	75 ± 28	91 ± 15
IgM							
Symptomatic (7/28)	40 ± 12	48 ± 17	45 ± 19	60 ± 20	65 ± 17	58 ± 27	69 ± 30
Asymptomatic (6/10)	45 ± 15	52 ± 19	40 ± 26	48 ± 17	57 ± 24	64 ± 26	45 ± 19
IgG							
Symptomatic (14/28)	33 ± 13	46 ± 17	46 ± 15	70 ± 23	83 ± 20	77 ± 22	60 ± 28
Asymptomatic (7/10)	41 ± 12	53 ± 24	42 ± 22	62 ± 18	74 ± 22	73 ± 23	51 ± 27

[a] As measured by ELISA of absorbed serum at a 1/100 dilution.
[b] No IgA-positive cases were found among the asymptomatic controls.

found between the mean ID_{50}s for sera of symptomatic patients and asymptomatic controls (Table 1). Differences in obtained ID_{50}s did not correlate with the antibody titers (log 10) of IgA, IgM, and IgG (r, 0.012, −0.12, and 0.34, for IgA, IgM, and IgG, respectively). ID_{50}s for reactive and nonreactive sera were not significantly different among the three groups of subjects (mean, 0.68 M; range, 0.324 to 1.468 M [$P > 0.05$]).

Considerable absorption of *C. jejuni*-reactive antibodies (IgA, IgM, and IgG) was observed in culture-positive subjects (diseased and asymptomatic) after the treatment of sera with whole cells of *C. jejuni*, *C. coli*, and *C. rectus* (Table 2). *E. coli*, *Shigella* spp., and *N. meningitidis* showed lower absorption effects. Table 2 also shows that sera from culture-positive healthy subjects demonstrated a higher cross-reactivity with *C. rectus*, *N. meningitidis*, *E. coli*, and *S. flexneri*, particularly in reactions involving IgM and IgG. No significant difference was observed between samples from patients and asymptomatic individuals ($P > 0.05$).

DISCUSSION

The host immune response to an etiologic agent is frequently evaluated as an antibody titer, without attention to the quality of the antibody or its immunologic role in complement activation, opsonization, toxin neutralization, or other defense mechanisms. Such biological functions were found to be more significant in highly avid antibodies (27). Avidity is the total strength of binding between an antigen, which may have multiple epitopes, and a multivalent antibody. Affinity, in contrast, is the strength of binding of a single antigen-binding site to a single epitope and constitutes an important criterion for judging the maturation of an immune response to antigenic stimulants (1, 16).

In this study, sera from patients positive for *C. jejuni* culture had significantly elevated *C. jejuni*-reactive antibodies ($P < 0.05$), in agreement with other studies on the humoral immune response to this pathogen (5, 28). Our results are also supported by the reports of Schwartz et al. (24) and Figueroa et al. (9). The detection of *C. jejuni* in 10 stool samples from apparently healthy individuals may reflect an asymptomatic form of the disease or a carrier state. None of the sera from asymptomatic patients showed detectable *C. jejuni*-reactive IgA. This agrees with the findings of Svedhem and colleagues (28), who reported that chronic carriers of *C. jejuni* may have elevated levels of IgG and IgM but not IgA. Sera from healthy control subjects (culture negative) showed no reactivity with *C. jejuni* antigen, which may conflict with previous reports on the presence of antibodies in healthy populations living in areas of hyperendemicity or developing countries (6).

Table 1 shows that the thiocyanate ion ID_{50} for IgA was 1.1 M, more than that for IgG (0.92 M) or IgM (0.70 M). Our ID_{50} for IgA is comparatively higher than that reported by Roberton and coworkers (23) for *E. coli* and diphtheria toxins. This discrepancy may be due to differences in the maturation of immune response or the nature of antigens and antibodies used (5, 12). In this study, there was no direct correlation between avidity and antibody titers (Table 1), which agrees with the findings of Lopatin et al. (16). It has been reported that highly avid antibodies may be continuously skimmed off the circulation by successive exposure to the inducing agent in an area of endemicity or the presence of cross-reactive antigens (15, 16).

Recent or primary infections usually result in antibodies with low avidity (11, 18). Since our serum samples were collected within a month of disease complaint, IgG antibodies may be expected to have a relatively low avidity, compared to IgA and IgM, which appear within 1 week of infection. Hedman et al. (12) reported that low-affinity specific immunoglobulin predominates early in infection, with a subsequent increase in affinity over time. Our results indicate that avidity of IgM and IgG from symptomatic patients was greater than that for asymptomatic carriers (Table 1). This finding may be explained by the observation that acute-phase IgG was eluted off the antigen more easily than was IgG of prior immunity (12). This is also consistent with our finding that IgG had a lower avidity than IgA and IgM. Yet, a relatively high ID_{100} of the chaotropic agent (5 M) was required for IgG versus IgA and IgM (2 and 3 M, respectively). This observation may be due to differences in the specificity of antigen-binding sites, immunoglobulin polymerism, or the presence of several IgG subclasses (7, 22).

Evaluation of the immunoabsorbent effect of other cross-reactive antigens and the specificity of *C. jejuni*-reactive antibodies showed a considerable shared antigenicity between the antigens of *C. jejuni*, *C. coli*, and *C. rectus* (a periodontal pathogen that is also associated with inflammatory bowel disease). *E. coli*, *S. flexneri*, and *S. sonnei* were less cross-reactive with *C. jejuni* antibodies. These results may reflect the interaction between the immune response (in terms of antibody specificity) and pathogens of related antigenicity. *Campylobacter*-reactive antibodies have been reported to be common in areas of hyperendemicity (4, 6). However, flagellar proteins of *C. jejuni* have been reported to cross-react with antibodies produced against *C. coli*, *Helicobacter pylori* (formerly *Campylobacter pylori*) (21), and *C. rectus*. Because of this cross-reactivity, it has been suggested that antibodies against these infectious agents may not be regarded as specific without absorption studies (21,

29). While a flagellar protein in *C. jejuni* was reported to share a significant sequence with class I pilin of *N. meningitidis* and the heat-labile enterotoxin of *E. coli* (22), our results show a high cross-reactivity with *Shigella* spp.

This study demonstrates that antibodies developed against *C. jejuni* are of low avidity, probably due to considerable cross-reactivity between this organism and other microbial flora or enteric pathogens, particularly in countries where these types of infections are endemic. The relative avidity of immunoglobulin isotypes from symptomatic patients was higher than that for asymptomatic carriers.

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