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Local Immune Responses to the *Campylobacter* Flagellin in Acute *Campylobacter* Gastrointestinal Infection

IRVING NACHAMKIN* AND XIAO-HE YANG

Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, 3400 Spruce Street, Philadelphia, Pennsylvania 19104-4283

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Intestinal immunoglobulin A (IgA) anti-campylobacter flagellin antibodies were measured by an enzyme-linked immunosorbent assay in patients with and without campylobacter infections. The level of fecal IgA antiflagellin antibodies was significantly higher in patients than in controls, but only 12 of 29 patients (41.4%) with a campylobacter infection had detectable IgA antibodies against flagellin (optical density, ≥0.100). Further testing of fecal IgA antibodies from 10 patients with homologous and heterologous purified flagellins showed strain specificity in 1 patient. Cross-reactivity of fecal IgA antibodies with heterologous flagellins did not correlate with the Lior serotype of the isolate.

Studies on the pathogenesis of campylobacter infection have implicated an important role for the flagellum in the colonization of the host and protective immunity. Flagellar variants of *Campylobacter jejuni* and *C. coli* have a reduced ability to cause infection, as studied in a number of animal models (2, 23), and protection against infection may, in part, involve flagellar epitopes (1, 23). Immune responses to campylobacter infection have been studied in humans with respect to serum immunoglobulin responses (3, 4, 14, 20), and protection against infection has been associated with antiflagellin antibodies (13). Preliminary data also suggest that breast milk antiflagellin antibodies may have some protective effect (18). Few studies have examined the intestinal immunoglobulin A (IgA) response during human campylobacter infection (9, 24). The purpose of our study was to further characterize the intestinal IgA response to campylobacter flagellin during human infection and to determine whether the response was type specific or broadly reactive with heterologous flagellins.

Stool samples from 29 patients with acute *C. jejuni* infections (range, 2 to 6 days after the onset of symptoms) were used for the study. Stool samples from 11 patients and from which *Salmonella* spp. ($n = 6$), *Shigella* spp. ($n = 2$), *Giardia* spp. ($n = 1$), and *Aeromonas* spp. ($n = 2$) were isolated were used as controls. Only one sample per patient was used for the study. All stool samples were frozen at −70°C in aliquots before use in the study. Fecal IgA concentrations were measured by immunoturbidimetric analysis with a Roche Cobas biocentrifugal analyzer. The SPQ IgA test system (Atlantic Antibodies, Scarborough, Maine) was used as described by the manufacturer. Specific IgA antibodies against *C. jejuni* flagellin were measured by an enzyme-linked immunosorbent assay (ELISA) as previously described (20), with slight modifications. For the initial ELISA, stock antigen was obtained from an isolate of *C. jejuni* (Lior type 7) that has been characterized (7, 20). All dilutions of stool filtrates for ELISA testing were made with phosphate-buffered saline and phenylmethylsulfonyl fluoride at 100 μg/ml (final concentration) as a protease inhibitor. The ELISA was standardized by measuring the activity of filtrates at an IgA concentration of 6.7 μg/ml (150 μl per assay). This concentration was chosen after preliminary dilution studies of filtrates from patients with infections were done and was within the linear portion of the titration curve. Antibodies to each antigen were detected with alkaline phosphatase-conjugated goat anti-human IgA (alpha chain specific) (Cappel Laboratories). All assays were run in duplicate for each sample with test wells (containing antigen) and control wells (containing no antigen). After the wells were blanked, the optical density (OD) was measured (Dynatech model MR500 ELISA reader) and the final OD was calculated as the average OD of test wells minus the average OD of control wells. The upper 95% confidence interval (CI) for control samples (campylobacter-negative patients) was an OD of 0.098. Thus, we chose an OD of ≥0.100 to indicate positivity in the assay. Relative specific antibody activity against flagellin was expressed as the OD obtained in the ELISA. Serotyping of isolates was performed with the heat-labile system of Lior et al. (11). The Mann-Whitney U test was used to assess differences between groups.

Patients with campylobacter infections had a higher mean fecal IgA level (61.6 mg/dl; 95% CI, 50.4 to 72.8) than did controls (48.2 mg/dl; 95% CI, 22.6 to 73.8), but the difference was not statistically significant. When specific IgA anti-campylobacter flagellin antibodies were measured, the mean IgA activity was significantly higher in patients (OD, 0.338; 95% CI, 0.109 to 0.567) than in controls (OD, 0.051; 95% CI, 0.004 to 0.098) ($P = 0.02$). However, only 12 of 29 patients (41.4%) had antibody levels above the control cutoff (OD, ≥0.100), with a mean ELISA OD of 0.755 (95% CI, 0.265 to 1.25). Two of 11 control patients (18.2%) had IgA activity at an OD of ≥0.100 (ODs, 0.21 and 0.17). These values, however, were below the 95% CI for positive results seen with patients with campylobacter infections.

Since the antigen preparation used for the ELISA was derived from a single strain of *C. jejuni* (Lior type 7), we wanted to know whether the responses observed or the lack of a response was due to type specificity of the IgA response. To answer this question, we further analyzed fecal IgA from 10 patients with campylobacter infections tested in the initial ELISA against flagellin purified from the patient's own isolate (homologous) and heterologous isolates from the other 9 patients (Table 1). For testing, we selected from the original 29 patients samples that showed a range of antibody

* Corresponding author.

TABLE 1. Cross-reactivity of fecal IgA antiflagellin antibodies with homologous and heterologous flagellin antigens

Antigen (Lior type)[a]	ELISA OD[b] for stool sample from patient:										No. of patients positive with antigen
	1	2	3	4	5	6	7	8	9	10	
1 (36)	**0.10**	0.03	0.24	0.00	0.16	2.00	1.69	0.37	0.46	2.00	8
2 (8)	0.06	**0.03**	0.19	0.01	0.23	2.00	1.66	0.72	0.37	2.00	7
3 (36)	0.08	0.04	**0.19**	0.06	0.16	2.00	1.49	0.23	0.66	2.00	7
4 (36)	0.11	0.04	0.29	**0.00**	0.16	2.00	2.00	0.63	0.65	2.00	8
3 (36)	0.09	0.02	0.12	0.02	**0.10**	0.69	0.29	0.09	0.21	0.46	6
6 (36)	0.01	0.00	0.06	0.00	0.00	**0.30**	0.12	0.04	0.03	0.26	3
7 (NT)	0.11	0.01	0.11	0.02	0.08	0.95	**0.60**	0.16	0.14	0.79	7
8 (36, 4)	0.24	0.07	0.18	0.04	0.27	1.64	0.31	**0.30**	0.31	1.33	8
9 (8)	0.09	0.06	0.36	0.01	0.38	2.00	1.67	0.92	**1.00**	2.00	7
10 (NT)	0.07	0.02	0.13	0.00	0.09	1.12	0.64	0.16	0.22	**1.20**	6
No. of patients with a positive sample	4	0	9	0	7	10	10	8	9	10	

[a] NT, nontypeable.
[b] Boldfacing indicates the OD for homologous flagellin.

activity in the ELISA, from no detectable binding to very high binding.

Two patients (no. 2 and 4) who showed no antibody activity in the initial ELISA did not exhibit detectable IgA against flagellin when the homologous or heterologous flagellin preparations were used. Patients 1, 3, 5, and 8 showed intermediate levels of IgA ranging from reactivity just above the cutoff OD to an OD of <1.00. Patients 6, 7, 9, and 10 exhibited ODs of ≥1.00 to one or more flagellins tested. Flagellins from patients with detectable IgA antibodies generally showed cross-reactivity with other flagellins, but this cross-reactivity was not related to the Lior serotype of the isolate. For example, patients 6, 7, and 10 showed high IgA activity against two isolates of Lior type 8 but not against all isolates of Lior type 36.

One patient, however, exhibited a strain-specific fecal IgA response (no. 9). Fecal IgA from patient 9 had an ELISA OD of 1.00 against the homologous flagellin (Lior type 8) but showed lower reactivity with heterologous flagellins, including that from another patient isolate of the same Lior serotype (from patient 2). Although strain- or type-specific responses were not observed with the other patients tested, fecal IgA reactivities with different flagellins varied significantly. Cross-reactivity of fecal IgA antibodies with homologous and heterologous flagellins did not appear to correlate with the Lior serotype of the particular isolate.

We have examined the intestinal IgA response during acute campylobacter infection to flagellin, the major subunit protein of the flagellar filament. Flagellin has a molecular mass of ca. 62 kDa and varies in molecular mass and amino acid composition among different *Campylobacter* strains (17). The genes involved in the expression of flagellin, *flaA* and *flaB*, in *C. jejuni* and *C. coli* have been recently described (8, 12, 21), and flagellin predominantly appears to be the product of *flaA* in *C. jejuni* (8, 21). The N- and C-terminal regions of the protein are highly conserved, whereas the internal region is highly variable (18). Common and type-specific epitopes of flagellin have also been defined with monoclonal antibodies (19, 22).

We showed that for acute campylobacter infection, IgA antiflagellin antibodies were detected in approximately 40% of patients whose fecal specimens were submitted for bacterial culturing. Lane et al. (9) found that IgA anticampylobacter antibodies were detected in 5 of 10 patients whose samples were also submitted for culturing; however, the antigens used for their assay were not specified. They did find that fecal IgA ELISA titers were highest in samples collected 6 to 10 days after the onset of enteritis and quickly declined thereafter. Similar results were obtained by Burr et al. (6) with a rabbit model in which IgA antiflagellin antibodies were maximal at approximately 1 week postchallenge and then declined rapidly. Windsor et al. (24) examined the fecal IgA antibody response to campylobacter proteins in stool samples obtained during the acute stage of disease by Western blot (immunoblot) analysis and found that five of eight patients had IgA antibodies directed against flagellin as well as other outer membrane proteins but that five control patients did not show a reaction with any campylobacter proteins. Our samples were collected 2 to 6 days after the onset of disease. It is likely, therefore, that many of the samples did not have detectable antiflagellin antibodies because the samples were collected too early in the disease course, before antibody levels rise.

The local IgA antibody response to flagellin, as studied in a limited number of patients, did not appear to be type specific, on the basis of a lack of correlation with the Lior serotype. One patient (no. 9) clearly had a strain-specific antiflagellin response when the acute-phase stool sample was tested, suggesting that conserved flagellin domains were not highly antigenic in early infection. Furthermore, fecal IgA from patient 9 did not show equivalent reactivity with the heterologous Lior type 8 strain. This result suggests that other undefined type-specific epitopes that are expressed on flagellin accounted for this observation. The pattern of reactivity of fecal IgA antiflagellin antibodies with different flagellins also suggested that even within a single Lior serotype, such as type 36, there was significant heterogeneity of epitope expression. In patients whose samples contained flagellins that exhibited cross-reactivity with heterologous flagellins, we cannot rule out the possibility that cross-reactions were due to previous *Campylobacter* infections.

Identifying specific epitopes on flagellin that elicit either type-specific or cross-reactive antibodies will be important for future studies. Several studies have found that protective immunity against campylobacter infection is associated with the Lior antigen complex (1, 23), which may involve flagellin. Burr et al. (5) found a strong association between the development of intestinal IgA and serum antiflagellin antibodies and protection against homologous infection by using

the adult rabbit diarrhea model. Cross-protection studies done by Pavlovskis et al. (23) with the rabbit model of infection implicated the flagellum as the component that elicited protective immunity.

Although we could not assess the local immune response in patients during the course of infection or determine whether patients developed immunity to infection, the observations of strain-specific antiflagellin antibody production in one patient and restricted cross-reactivity in other patients are in concert with experimental and human evidence for type-specific immunity in campylobacter infection. Since motility is an important prerequisite for the ability of campylobacters to colonize the intestinal tract (10, 16) and antibodies present in mucus may have a protective effect against colonization (15), the identification of protective flagellar epitopes will be useful for designing future vaccine strategies.

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REFERENCES

1. **Abimiku, A. C., and J. M. Dolby.** 1988. Cross-protection by *Campylobacter jejuni*: importance of heat-labile serotyping (Lior) antigens. J. Med. Microbiol. **26**:265–268.
2. **Aguero-Rosenfeld, M. E., X.-H. Yang, and I. Nachamkin.** 1990. Infection of adult Syrian hamsters with flagellar variants of *Campylobacter jejuni*. Infect. Immun. **58**:2214–2219.
3. **Blaser, M. J., R. E. Black, D. J. Duncan, and J. Amer.** 1985. *Campylobacter jejuni*-specific serum antibodies are elevated in healthy Bangladeshi children. J. Clin. Microbiol. **21**:164–167.
4. **Blaser, M. J., and D. J. Duncan.** 1984. Human serum antibody response to *Campylobacter jejuni* infection as measured in an enzyme-linked immunosorbent assay. Infect. Immun. **44**:292–298.
5. **Burr, D. H., M. B. Caldwell, A. L. Bourgeois, H. R. Morgan, R. Wistar, and R. I. Walker.** 1988. Mucosal and systemic immunity to *Campylobacter jejuni* in rabbits after gastric inoculation. Infect. Immun. **56**:99–105.
6. **Burr, D. H., D. T. Kerner, C. S. Blanco, A. L. Bourgeois, and R. Wistar.** 1987. Gastric lavage: a simple method to obtain IgA rich intestinal secretions from the rabbit. J. Immunol. Methods **99**:277–281.
7. **Fischer, S. H., and I. Nachamkin.** 1991. Common and variable domains of the flagellin gene, flaA, in *Campylobacter jejuni*. Mol. Microbiol. **5**:1151–1158.
8. **Guerry, P., S. M. Logan, S. Thornton, and T. J. Trust.** 1990. Genomic organization and expression of *Campylobacter* flagellin genes. J. Bacteriol. **172**:1853–1860.
9. **Lane, E. M., R. A. Batchelor, A. L. Bourgeois, D. H. Burr, and J. G. Olson.** 1987. Urine and fecal IgA response during naturally acquired infection with *Campylobacter jejuni*. Lancet **i**:1141.
10. **Lee, A., J. L. O'Rourke, P. J. Barrington, and T. J. Trust.** 1986. Mucus colonization as a determinant of pathogenicity in intestinal infection by *Campylobacter jejuni*: a mouse cecal model. Infect. Immun. **51**:536–546.
11. **Lior, H., D. L. Woodward, J. A. Edgar, L. J. Laroche, and P. Gill.** 1982. Serotyping of *Campylobacter jejuni* by slide agglutination based on heat-labile antigenic factors. J. Clin. Microbiol. **15**:761–768.
12. **Logan, S. M., T. J. Trust, and P. Guerry.** 1989. Evidence for posttranslational modification and gene duplication of *Campylobacter* flagellin. J. Bacteriol. **171**:3031–3038.
13. **Martin, P. M. V., J. Mathiot, J. Ipero, M. Kirimat, A. J. Georges, and M. C. Georges-Courbot.** 1989. Immune response to *Campylobacter jejuni* and *Campylobacter coli* in a cohort of children from birth to 2 years of age. Infect. Immun. **57**:2542–2546.
14. **Mascart-Lemone, F. O., J. R. Duchateau, J. Oosterom, J.-P. Butzler, and D. L. Delacroix.** 1987. Kinetics of anti-*Campylobacter jejuni* monomeric and polymeric immunoglobulin A1 and A2 responses in serum during acute enteritis. J. Clin. Microbiol. **25**:1253–1257.
15. **McSweegan, E., D. H. Burr, and R. I. Walker.** 1987. Intestinal mucus gel and secretory antibody are barriers to *Campylobacter jejuni* adherence to INT407 cells. Infect. Immun. **55**:1431–1435.
16. **Mooroka, T., A. Umeda, and K. Amako.** 1985. Motility as an intestinal colonization factor for *Campylobacter jejuni*. J. Gen. Microbiol. **131**:1973–1980.
17. **Nachamkin, I., R.-H. Angeletti, and M. B. Prystowsky.** 1988. Biochemical similarity among serologically distinct flagellins of *Campylobacter jejuni*. FEMS Microbiol. Lett. **51**:149–152.
18. **Nachamkin, I., S. H. Fischer, X.-H. Yang, O. Benitez, and A. Cravioto.** 1990. Protective effect of breast milk directed against *Campylobacter jejuni* during the first year of life, abstr. B-50, p. 35. Abstr. 90th Annu. Meet. Am. Soc. Microbiol. 1990.
19. **Nachamkin, I., and A. M. Hart.** 1986. Common and specific epitopes of *Campylobacter* flagellin recognized by monoclonal antibodies. Infect. Immun. **53**:438–440.
20. **Nachamkin, I., and X.-H. Yang.** 1989. Human antibody response to *Campylobacter jejuni* flagellin protein and a synthetic N-terminal flagellin peptide. J. Clin. Microbiol. **27**:2195–2198.
21. **Nuijten, P. J., F. J. Van-Asten, W. Gaastra, and B. A. van der Zeist.** 1990. Structural and functional analysis of two *Campylobacter jejuni* flagellin genes. J. Biol. Chem. **265**:17798–17804.
22. **Nuijten, P. J. M., B. A. M. van der Zeijst, and D. G. Newell.** 1991. Localization of immunogenic regions on the flagellin proteins of *Campylobacter jejuni* 81116. Infect. Immun. **59**:1100–1105.
23. **Pavlovskis, O. R., D. M. Rollins, R. L. Haberberger, A. E. Green, L. Habash, S. Strocko, and R. I. Walker.** 1991. Significance of flagella in colonization resistance of rabbits immunized with *Campylobacter* spp. Infect. Immun. **59**:2259–2264.
24. **Windsor, D. K., J. J. Mathewson, and H. L. DuPont.** 1986. Western blot analysis of intestinal secretory immunoglobulin A response to *Campylobacter jejuni* antigens in patients with naturally acquired *Campylobacter* enteritis. Gastroenterology **90**:1217–1222.