Elevated flagellin-specific immunoglobulins in Crohn’s disease

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CROHN’S DISEASE (CD) and ulcerative colitis (UC), collectively referred to as inflammatory bowel disease (IBD), are characterized by seemingly aberrant mucosal immune responses. Namely, whereas the active flares of disease activity resemble both clinically and histopathologically the acute food-borne gastroenteritis caused by enteric pathogens, efforts to associate specific pathogens with IBD have been unsuccessful. IBD patients have been shown to have an elevated level of immunoreactivity to their own microflora (3) and in multiple murine models of IBD, intestinal inflammation does not develop if the mice are maintained in germ-free conditions (15). These observations support the notion that IBD is mediated by aberrant immune responses directed at presumably normal enteric microflora. Whereas the specific microbial targets to which this elevated mucosal immune response are directed have only recently begun to be described, their characterization is considered germane to diagnosing, understanding, and perhaps treating IBD (4).

For several reasons, bacterial flagellin seems a likely candidate to be involved in the mucosal immune responses associated with IBD. Being expressed abundantly by all motile bacteria, flagellin is present in substantial amounts in the intestine. Flagellin is highly antigenic being a major immunoglobulin target in a variety of infectious events (12). Specifically, recognition of flagellin by the germ line-encoded pattern recognition receptor Toll-like receptor 5 (TLR5) (7), which is expressed on the basolateral membrane of polarized intestinal epithelial cells, can directly promote a mucosal inflammatory response (5). Flagellin’s ability to activate the innate immune system allows it to function as an adjuvant (13), and this ability may also underlie its own immunogenicity. Thus the goal of this work was to test whether flagellin might be a target of the elevated adaptive mucosal immune response associated with IBD via measuring serum levels of flagellin-specific immunoglobulins. Whereas antibodies to whole flagella bear considerable ability to distinguish between bacteria that express highly homologous flagellin genes (the basis of H serotyping), we used flagellin monomers thus minimizing serotype specificity (1). Specifically, we used flagellin monomers purified from a human commensal Escherichia coli strain (F-18) (16) to serve as a “generic” flagellin and found that human sera displayed a similar pattern of recognition of such flagellin monomers whether isolated from several flagellated E. coli or Salmonella typhimurium strains.

First, we used serum from IBD patients and normal controls to immunoblot bacterial extracts of flagellated and nonflagellated enteric bacteria as well as purified flagellin. We observed that, as shown in Fig. 1, patients with CD exhibited a substantial general increase in immunoreactivity to these bacterial extracts. Whereas this increase in immunoreactivity was clearly observed for many components of these bacteria, the band appearing to be flagellin on the basis of its molecular weight clearly appeared to be one of the specific targets. The band was verified to be flagellin by observing its absence in corresponding aflagellate bacteria and by use of highly purified polyclonal and monoclonal flagellin-specific antisera (data not shown). Consistent with flagellin being one of the targets of the CD-associated immune response, immunoblotting of flagellin chromatographically purified from E. coli (Fig. 1B) or Salmonella (not shown) with these serum samples indicated CD patients had increased immunoreactivity to flagellin consistent with the fact that flagellin is a highly conserved protein in general and among these gram-negative bacteria in particular.

In light of flagellin appearing to be a target of the enhanced immune response associated with CD and flagellin’s potential to drive immune responses to bystander antigens, we performed a more quantitative and larger-scale analysis of flagellin immunoreactivity in IBD patients and control subjects. Specifically, the levels of flagellin-specific immunoglobulin (IgG and IgA) in control and IBD patients were measured by ELISA. Total levels of IgG and IgA were also measured as were levels of IgG and IgA that recognized LPS, because some studies have observed these to be elevated in IBD (8). The specificity of these interactions was verified by observing that no immunoreactivity was observed to microtiter plates coated

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with BSA (not shown). Whereas, when diluted 1:100, nearly all of the serum samples analyzed exhibited an easily quantifiable level of Ig that recognized flagellin, the levels of flagellin-specific Ig observed were significantly elevated in patients with CD (Fig. 2). Specifically, mean relative values of anti-flagellin IgG and IgA were increased by 2.5- and 3-fold, respectively (actual corresponding mean ODs for control, CD, and UC were, respectively, 0.15, 0.39, and 0.17 for IgG and 0.05, 0.16, and 0.06 for IgA, \( P < 0.001 \)), with 52% of patients with CD exhibiting levels of flagellin-specific IgA or IgG that were higher than two standard deviations beyond the mean of the range exhibited by control persons who share a similar environment (levels of 2/40 control patients also met this criteria). In general, elevations in flagellin IgA and IgG correlated closely in individual patients (\( r^2 = 0.47 \)). Whereas we have not yet observed any statistically significant differences in clinical characteristic between CD patients who do or do not exhibit flagellin immunoreactivity, similar studies (Gewitz A and Sitaraman SV, unpublished observations) on a smaller set of patients at Emory University suggest the response may correlate with disease severity and presence of fistulas. The increase in flagellin-specific Ig was despite a total 30% decrease in mean total IgG (\( P < 0.001 \)); mean total IgA decreased by 25% but the difference had only moderate statistical significance (\( P = 0.02 \)). Whereas the reason for these decreases is not known, they serve to indicate that these patients did not display gross globally elevated immunity. A similar relative increase in flagellin-specific Ig in CD was observed for serum diluted 1:500 (not shown). Consistent with previous studies, we also observed an increase in LPS-specific Ig although the relative mean increase of 1.6- and 1.7-fold for IgA and IgG, respectively (actual corresponding mean ODs for control, CD, and UC were 0.04, 0.06, and 0.04 for IgG and 0.09, 0.14, and 0.07 for IgA, \( P < 0.01 \)), and percentage of
individuals exhibiting significantly elevated responses (26% of CD subjects had LPS-specific IgG or IgA >2 SD above mean of control subjects) was less than that observed for flagellin. Furthermore, the overall level of LPS-specific Ig was approximately substantially less (assessed by comparing optical density values) than that observed for flagellin despite using 20-fold more LPS to coat the microtiter plates (necessary to get measurable responses). Thus the CD-associated immune response, and adaptive immune response in general, appears to target flagellin more than LPS. In contrast to the case for CD, patients with UC did not exhibit levels of flagellin-specific (or LPS-specific) Ig that were significantly different from control subjects indicating flagellin is thus unlikely to be a relevant antigen in that disorder.

While this work was being considered for publication, an elegant study was reported by Lodes et al. (10) that, by an unbiased noncandidate approach, also concluded flagellin is a major antigen of CD. Specifically, this group used serological expression cloning to identify a novel “Cbir flagellin”, which has minimal similarity to previously characterized flagellins, as a major antigen of a murine model of colitis. Furthermore, via ELISA, they also found Cbir flagellin to be a target of the elevated immune response associated with CD patients. In contrast to the case for CD, patients with UC did not exhibit levels of flagellin-specific (or LPS-specific) Ig that were significantly different from control subjects indicating flagellin is thus unlikely to be a relevant antigen in that disorder.

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Consider the following:

- Lodes et al. (10) concluded that flagellin is a major antigen in CD.
- CD patients exhibited elevated levels of serum antibodies to Cbir flagellin but not to a Salmonella flagellin.
- The conclusion that CD-associated immune responses may be targeted primarily toward these rare flagellins rather than typical gram-negative bacterial flagellins.
- In contrast, the authors observed a substantial and highly statistically significant elevation of serum antibodies to a common E. coli flagellin.

We speculate that antibody cross reactivity to various flagellins in these same regions of the molecule might account for the ability of individual flagellins to broadly detect anti-flagellin immunoreactivity in a diverse group of CD patients.

Whereas innate immune activity, especially polymorphonuclear leukocyte infiltration, mediates the acute flares of IBD, chronic intestinal inflammation is characterized by increased numbers of mucosal adaptive immune cells, especially CD4 T cells (2). Whereas these T cells are known to be broadly reactive to the intestinal microflora in general, the specific antigens that drive these T cells are relatively unknown. An
indirect readout of the antigenic targets of these T cells is the assessment of serum immunoglobulins, because for most antigens, including flagellin (our unpublished results), generation of antibodies requires activating antigen-specific T cells. Thus our demonstration that CD patients exhibit elevated levels of flagellin-specific Ig strongly suggests that flagellin is an antigenic target of the elevated adaptive immune response that characterizes the chronic stage of inflammation in IBD. Flagellin may be especially important for the CD-associated immune response in that it appears to be one of the major targets of the immune response to enteric bacteria in general as evidenced by our immunoblotting of whole extracts of flagellate and aflagellate bacteria with CD serum. Furthermore, flagellin has substantial adjuvant ability (13) enabling it to drive the immune responses to other bacterial antigens that by themselves are not highly immunogenic. In light of flagellin’s potentially important role in driving immune responses in the gut, it is important to define the molecular mechanisms that regulate both the innate and adaptive immune responses to this molecule.

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