

Selected Loss of Tolerance Evidenced by Crohn's Disease–Associated Immune Responses to Auto- and Microbial Antigens

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Background & Aims: Previous studies in Crohn's disease suggest global loss of tolerance with sonicated bacteria preparations containing hundreds of antigens. Monoassociation studies show that a solitary bacterium can induce colitis in one animal model, whereas another is responsible in other models. Among patients with Crohn's disease, serum responses have been documented to microbial and autoantigens (antibodies to the *Escherichia coli* outer-membrane porin C and the *Pseudomonas fluorescens*-associated sequence I2, antisaccharomyces cerevisiae antibody (ASCA), and perinuclear antineutrophil cytoplasmic antibodies). Our aim was to determine whether there are heterogeneous responses to these specific antigens. **Methods:** Sera from 330 Crohn's patients were analyzed. Immunoglobulin A enzyme-linked immunosorbent assays to ASCA, outer-membrane porin C, or I2 and immunoglobulin G enzyme-linked immunosorbent assay to ASCA and ANCA determined the presence and level of antibodies. Perinuclear antineutrophil cytoplasmic antibodies were determined by immunofluorescence. **Results:** ASCA was detected in 56% of patients; 55% were seroreactive to outer-membrane porin C, 50% were seroreactive to I2, and 23% were perinuclear antineutrophil cytoplasmic antibody positive. Eighty-five percent responded to at least 1 antigen; only 4% responded to all 4. Among microbial antigens, 78% responded to at least 1, and 57% were double positive, but only 26% responded to all 3. The level of response was stable over time and with change in disease activity. Among patients with the same qualitative antigen-response profiles, quantitative response differed. Cluster analysis of these antibody responses yielded 4 groups: ASCA, outer-membrane porin C/I2, perinuclear antineutrophil cytoplasmic antibodies, or no/low response. **Conclusions:** Rather than global loss of tolerance, there seem to be patient subsets with differing responses to selected microbial and autoantigens.

Numerous genetically engineered and T-cell manipulated animal models of intestinal inflammation have been generated over the last 10 years.^{1–3} Two com-

mon pathogenic mechanisms important for expression of these diseases have been defined. First, alteration of T-cell regulation renders the mucosa particularly susceptible to chronic inflammation. Second, expression of colitis in these animals is completely dependent on the presence of commensal colonic bacteria.^{1–3} In some models, a single bacterial strain can induce colitis. However, the same strain in a different animal model may have no effect on the development of colitis. For example, the addition of *Bacteroides vulgatus* to a group of 5 bacteria isolated from Crohn's disease (CD) patients induced colitis in HLA B27 transgenic rats⁴ but did nothing in interleukin (IL)-10 knockout mice⁵ or in E26 transgenic mice (B. Sartor, personal communication, July 2001). Because the HLA B27 rats and the IL-10 knockout mouse are different species and have different genetic backgrounds, direct comparisons may be difficult. Even so, IL-10 knockout mice monoassociated with *Streptococcus faecalis* develop colitis.⁶ A second study in IL-10 knockout mice from one laboratory developed colitis after *Helicobacter* reintroduction.⁷ These studies show that some animal systems develop colitis as a result of sensitivity to particular bacteria. In fact, Cong et al.⁸ have shown in the C3H/HEJ/BIR mouse model that serological and T-cell sensitivity occurs in response to a limited selection of the multitude of bacterial antigens in lysates of cecal flora.

The role of bacterial antigens in human inflammatory bowel disease has been studied with several approaches.

Abbreviations used in this paper: ASCA, antisaccharomyces cerevisiae antibody; BSA, bovine serum albumin; CSMC, Cedars–Sinai Medical Center; ELISA, enzyme-linked immunosorbent assay; GST, glutathione S-transferase; IL, interleukin; OmpC, outer-membrane porin C; pANCA, perinuclear antineutrophil cytoplasmic antibody; SDS, sodium dodecyl sulfate.

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In CD, some patients experience ameliorated symptoms from manipulation of bacteria by antibiotics.^{9,10} Rutgeerts et al.¹¹ showed that reintroduction of the diverted fecal stream into a segment of bowel can reactivate CD within a short time period. Many specific organisms have been proposed as directly contributing to the development of CD, including mycobacteria,^{12–17} *Bacteroides*,^{18,19} and *Listeria*²⁰; however, immune responses to antigens from these specific microbes have been variable. By using serological and T-cell responses as measures, it has been proposed that patients with CD or ulcerative colitis have lost tolerance to their own bacterial populations.^{21–23} All of these studies have been performed with sonicates of multiple bacteria that yield a multitude of bacterial antigens as the test antigenic stimuli for the loss of tolerance.^{21–23} However, the heterogeneity of antigenic responses in individual patients with CD has not been addressed.

We have recently shown immune responsiveness to several specific microbial antigens in patients with CD and ulcerative colitis.^{18,24–27} Using phage display technology, we have developed monoclonal antibodies that have similar reactivity to polyclonal antibodies seen in the sera of ulcerative colitis patients who are perinuclear antineutrophil cytoplasmic antibody (pANCA) positive (pANCA⁺).^{28–31} We have determined that these monoclonal antibodies cross-react with specific bacterial antigens, such as the outer-membrane porin C (OmpC) from *E. coli* and OmpW from *Bacteroides*.¹⁸ Recently, we showed that a subset of patients with CD express an immunoglobulin A (IgA) response to OmpC.³² Several groups have shown reactivity to an oligosaccharide from *Saccharomyces cerevisiae* in CD patients.^{24,26,33–37} This likely represents cross-reactivity to cell wall structures of multiple bacteria. We have also recently reported that the bacterial sequence of I2 is present in the mucosa of patients with active CD.³⁸ Reactivity to this antigen as measured by IgA is far more frequent in CD than ulcerative colitis or control inflammatory conditions.²⁴ Recently, this sequence has been shown to be associated with *Pseudomonas fluorescens*³⁹; however, an exhaustive search of the other *Enterobacteriaceae* species to determine the specificity of this association has yet to be completed.

The purpose of this study was to define the Ig immune response to *S. cerevisiae*, OmpC, I2, and pANCA in CD patients. Results in our study show heterogeneity in immune responses as well as the magnitude of that response within patients with CD. Rather than a global loss of tolerance to bacteria, as has been previously described, there seem to be subsets of CD patients with differing immune responses to selected microbial and

autoantigens. The relationship of the pattern (presence and magnitude) of these serological responses in a given patient to disease behavior is yet to be determined.

Materials and Methods

Study Populations

Cedars-Sinai Medical Center clinical cohort. The primary cohort consisted of 151 consecutively ascertained patients with an established diagnosis of CD evaluated by the Cedars-Sinai Medical Center (CSMC) Inflammatory Bowel Disease Center and who consented to have serum drawn for research purposes. This study was reviewed and approved for human subject participation by the Cedars-Sinai Institutional Review Board. Diagnosis of CD was rigorously defined by the presence of a combination of established features from at least 2 of the following categories: (1) clinical—perforating or fistulizing disease, obstructive symptoms secondary to small-bowel stenosis, or stricture; (2) endoscopic—deep linear or serpiginous ulcerations, discrete ulcers in normal-appearing mucosa, cobblestoning, or discontinuous or asymmetric inflammation; (3) radiographic—segmental disease (skip lesions), small-bowel or colon strictures, stenosis, or fistula; and (4) histopathologic—submucosal or transmural inflammation, multiple granulomas, marked focal cryptitis or focal chronic inflammatory infiltration within and between biopsies, or skip lesions, including rectal sparing in the absence of local therapy. Patients with primary sclerosing cholangitis and autoimmune hepatitis and those with chronically increased transaminase or alkaline phosphatase levels were excluded to avoid confusion with non-inflammatory bowel disease ANCA.^{40,41}

Cedars-Sinai Medical Center longitudinal cohort.

A second, similarly defined cohort of 73 patients with CD under treatment with infliximab was followed up over time and assessed for disease activity. Of the 73 patients in this cohort, there were 26 patients who experienced a Crohn's Disease Activity Index (CDAI) change of at least 70 at time points at least 4 months apart. These 26 were studied for changes in marker antibody expression over time relative to disease activity.

Additional Crohn's disease patients for cluster analysis (Cedars-Sinai Medical Center genetics cohort).

The CSMC genetics cohort of 153 patients with CD diagnosed by standard clinical, radiographic, endoscopic, and histopathologic criteria was grouped with the CSMC clinical cohort to enlarge our population for adequate cluster analysis.

Determination and Characterization of Serum Antisaccharomyces Cerevisiae Antibody (ASCA)

Sera were analyzed for ASCA expression in a blinded fashion by Prometheus Laboratories (San Diego, CA) with a fixed enzyme-linked immunosorbent assay (ELISA). High-binding polystyrene microtiter plates were coated with purified phosphopeptidomannans extracted from yeast *S. uvarum*, a

subspecies of *S. cerevisiae*. Coded patient sera were diluted and added to the wells. After incubation and washing, alkaline phosphatase-conjugated goat anti-human F(ab') fragment-specific IgG (Pierce, Rockford, IL) or alpha chain-specific IgA (Jackson ImmunoResearch Labs, Inc., West Grove, PA) was added. After incubation, wells were washed, and *p*-nitrophenol phosphate substrate was added. Color development was proportional to concentrations of antibody present in the sera. Samples were read at 405 nm with a reference wavelength of 650 nm on a Molecular Devices (Menlo Park, CA) E-Max microtiter plate reader. Levels were determined and results expressed as ELISA units relative to a Prometheus Laboratory standard, which was derived from a pool of patient sera with well-characterized CD found to have reactivity to this antigen. Sera showing ASCA reactivity (IgG, IgA, or both) exceeding the reference range were termed *ASCA positive* (ASCA⁺).

Serum ANCA Determination and ANCA Subtype Characterization

Serum ANCA expression and ANCA subtype characterization were performed in a blinded fashion by Prometheus Laboratories. ANCA presence was determined by fixed neutrophil ELISA as previously described.⁴² Polystyrene microtiter plates were coated with 2.5×10^5 normal human donor whole peripheral blood neutrophils per well, which were then fixed with 100% methanol. Cells were incubated with bovine serum albumin (BSA; 0.25%) in phosphate-buffered saline (PBS) to block nonspecific antibody binding. Next, control and coded sera were added at a 1:100 dilution. After incubation and washing, goat F(ab')₂ anti-human IgG (γ chain-specific) antibody (Jackson ImmunoResearch) conjugated to alkaline phosphatase was added at a 1:1000 dilution to label neutrophil-bound antibody. Substrate solution containing *p*-nitrophenol phosphate was then added. Color development was allowed to proceed until absorbance at 405 nm, with a reference wavelength of 650 nm on a Molecular Devices E-Max microtiter plate reader, in the positive control wells was 0.8–1.0 optical density units greater than that in blank wells. Levels were determined relative to a Prometheus Laboratory standard consisting of pooled sera obtained from well-characterized pANCA⁺ ulcerative colitis patients. Results were expressed as ELISA units. Sera with circulating antineutrophil cytoplasmic IgG antibody exceeding the reference range value were termed ANCA⁺. Numerical values that were below the reference range were termed ANCA⁻.

ANCA⁺ sera were further subtyped via indirect immunofluorescence staining to determine the ANCA neutrophil binding pattern. Glass slides containing approximately 10^5 normal human donor whole peripheral blood neutrophils per slide were prepared by cytocentrifugation (Shandon Cytospin, Cheshire, England). Cells were fixed in 100% methanol, air-dried, and stored at -20°C. The coded patient sera were diluted (1:20) and then layered over the fixed neutrophils, incubated at 37°C, and washed. Fluorescein-labeled F(ab')₂ γ chain-specific goat anti-human antibody was added and incubated and then washed, as previously described.⁴² The slides

were examined by fluorescence microscopy by using an epifluorescence-equipped Nikon microscope (Garden City, NJ). Sera showing perinuclear highlighting which then lost this characteristic staining pattern with deoxyribonuclease treatment were termed pANCA⁺.⁴³

Bacterial Antigen Purification

Outer-membrane porin C purification. Trimeric OmpC was biochemically purified from an OmpF[↑]-/⁻/OmpA^{-/-} disruptive insertion mutant *E. coli* K12 (provided by R.M.). Mutant *E. coli* glycerol stocks were inoculated into 10–20 mL of Luria-Bertani broth supplemented with 100 μg/mL of streptomycin (LB-Strep, Teknova, Half Moon Bay, CA) and cultured vigorously at 37°C for approximately 8 hours to log phase, followed by expansion to 1 L in LB-Strep over 15 hours at 25°C. Cells were harvested by centrifugation, washed twice with 100 mL of ice-cold 20 mmol/L of Tris-Cl pH 7.5, and resuspended in cold spheroplast-forming buffer (20 mmol/L Tris-Cl, pH 7.5; 20% sucrose; 0.1 mol/L EDTA, pH 8.0; 1 mg/mL lysozyme). Spheroplasts were allowed to form for 1 hour on ice with occasional mixing and then lysed by 14-fold dilution into ice-cold 10 mmol/L of Tris-Cl pH 7.5, 1 mg/mL of deoxyribonuclease I, and vigorous vortexing followed by pulse sonication (4 × 30 seconds; on time, 1 second at high power). Cell debris was obtained by low-speed centrifugation, and membrane preparation was collected by ultracentrifugation at 100,000g in a swing bucket rotor. Membrane pellet was resuspended by homogenizing into 20 mmol/L Tris-Cl pH 7.5 and extracted for 1 hour in 20 mmol/L Tris-Cl pH 7.5 and 1% sodium dodecyl sulfate (SDS) by rotating at 37°C. Pre-extracted membrane preparation was then pelleted by ultracentrifugation and resuspended by homogenizing into 20 mmol/L Tris-Cl pH 7.5, as previously, and OmpC was extracted for 1 hour rotating at 37°C with 20 mmol/L Tris-Cl pH 7.5, 3% SDS, and 0.5 mol/L NaCl. Membrane was then pelleted by ultracentrifugation, and the supernatant containing trimeric OmpC was collected. SDS was removed from OmpC preparations by detergent exchange dialysis against more than 10,000 volumes of 0.2% Triton X-100 followed by dialysis against more than 10,000 volumes of Tris-Cl pH 7.5. Purified OmpC was quantified with the Bradford reagent (Bio-Rad, Hercules, CA), and purity of >95% was validated by SDS polyacrylamide gel electrophoresis and silver staining (Bio-Rad). Purified protein was aliquoted and stored at -20°C until use.

I2 purification. Antigen purification and ELISA techniques have been previously published.²⁴ Briefly, the 100–amino acid open reading frame of I2 was subcloned into pGEX-KG⁴⁴ and expressed in *E. coli* XL-1 blue (Stratagene, La Jolla, CA). I2/glutathione *S*-transferase (GST) fusion protein was present as an inclusion body and was purified according to the manufacturer's instructions by differential solubilization in 0.1% SDS. The GST control was produced with unmodified pGEX-KG and XL-1 blue cells and was present approximately 50% in the soluble and inclusion body fractions. The latter was purified exactly as I2-GST, and the former was purified by G

Sephacryl (Amersham-Pharmacia, Piscataway, NJ) affinity chromatography. All protein preparations were >90% pure by SDS polyacrylamide gel electrophoresis and Coomassie blue protein staining. The presented data used the soluble material; however, both preparations of the GST control yielded the same level of reactivity with patient sera.

Determination and Characterization of the Outer-Membrane Porin C/12 Response

Human IgA antibodies that bind I2 or OmpC were detected by direct ELISA assays. Plates (Greiner, USA Scientific, Ocala, FL) were coated overnight at 4°C with 100 μ L per well of GST alone and I2-GST (5 μ g/mL) or OmpC (0.25 μ g/mL) in borate-buffered saline, pH 8.5. After 3 washes in 0.05% Tween 20 in PBS, the plates were blocked with 150 μ L per well of 0.5% BSA in PBS, pH 7.4, for 30 minutes at room temperature. The blocking solution was then discarded, and 100 μ L per well of sera diluted 1:100 was added and incubated for 2 hours at room temperature. The plates were washed as before, and alkaline phosphatase-conjugated goat anti-human IgA (α chain specific) or IgG (γ chain specific; Jackson ImmunoResearch) at a dilution of 1:1000 in BSA/PBS was added for 2 hours at room temperature. The plates were washed 3 times with 0.05% Tween 20 in PBS followed by another 3 washes with Tris-buffered normal saline, pH 7.5. Substrate solution (1.5 mg/mL of disodium *p*-nitrophenol phosphate [Amresco, Solon, OH], 2.5 mmol/L MgCl₂, and 0.01 mol/L Tris, pH 8.6) was added at 100 μ L per well, and color was allowed to develop for 1 hour, at which time the plates were read at 405 nm. Nonspecific bindings of sera to GST alone (typically <0.1) were subtracted from raw values of I2/GST binding to obtain I2-specific absorbances.

Statistical Methods

Test stability of antibodies. From the longitudinal cohort, patients who experienced a CDAI change of at least 70 at time points at least 4 months apart were analyzed. The statistical significance of the mean difference between patients was evaluated by analysis of variance. To evaluate the relationship between changes in antibody levels and disease activity (CDAI), nonparametric Spearman's correlation was calculated. To measure the stability of antibody levels within a patient relative to the variation among patients, the coefficient of intraclass correlation⁴⁵ was calculated for each antibody [$r = \sigma_B^2 / (\sigma_B^2 + \sigma_w^2)$]. A high value (maximum is 1) of the coefficient means that there is relatively little variability within a patient. A nonsignificant correlation between changes of CDAI and changes in antibody titers indicates the independence of antibody level and disease activity within each patient.

Test heterogeneity of antibody distribution in Crohn's disease patients. Cluster analysis⁴⁶ of quantitative antibodies in CD patients (from the combined CSMC clinical cohort and CSMC genetics cohort, $n = 304$) was performed by using the *k*-means method to characterize the patient clusters by minimizing within-cluster variance. Two to 10 clusters were evaluated. The pseudo-F statistic was used, and the

cluster number corresponding to the largest value was determined to be the optimal number of clusters. The correlation between antibodies was calculated by using nonparametric Spearman's correlations.

The antibody levels were log-transformed for all analyses and also standardized to mean 0 and variance 1 for cluster analysis. All statistical analyses were performed by SAS Version 6.12 (SAS Institute Inc., Cary, NC).

Results

Relationship of Qualitative Serum Reactivity to ASCA, OmpC, I2, and pANCA in the CSMC Clinical Cohort of CD Patients

We and others have previously published the relationship of the expression of serum antibodies to ASCA and pANCA in patients with CD. Forty to fifty-six percent were reactive to either IgG or IgA ASCA with similar ELISA-based technologies (reviewed in Vasiliauskas et al.⁴⁷). In addition, 10%–40% of CD patients were shown to have serum reactivity to pANCA with different technologies.²⁵ Furthermore, we have recently shown that 54% of CD patients are reactive to the CD-related bacterial antigen I2 and that 56% are reactive to OmpC.³²

Our next goal, therefore, was to determine the relationship of serum reactivity to these different antigens in the CSMC clinical cohort. Figure 1 shows the serum reactivity to each of these antigens in this cohort of 151 CD patients. Although ASCA and pANCA assays were performed previously on this cohort for different analyses,^{25,47} serum samples were reassayed for this study. I2 and OmpC were run for the first time on this cohort.

Fifty-six percent of patients in this group were serum reactive to ASCA, 55% reacted to OmpC, 50% reacted to I2, and 23% reacted to pANCA (Figure 2A). With the relatively high frequency of reactivity in this cohort, the question arose as to whether the same patients would react to all 3 of the microbe-related antigens. In such a case, the circles in this Venn diagram (Figure 2B) would completely overlap. However, as seen in Figure 2B–D, there is a complex relationship of serum reactivity to these antigens in any given patient. Only a relatively small percentage of patients are reactive to all antigens. By using combinations of these antigens, 79%–84% of the population reacts to 1 or more of these antigens, and only 16%–22% did not react (Figure 2B–D).

Looking at the expression of all 4 antigens at once (Figure 3), the complexity of this relationship can further be seen. The expression of the 3 microbe-associated antigens is lower among pANCA⁺ patients compared with pANCA⁻ patients. However, the groupings of these

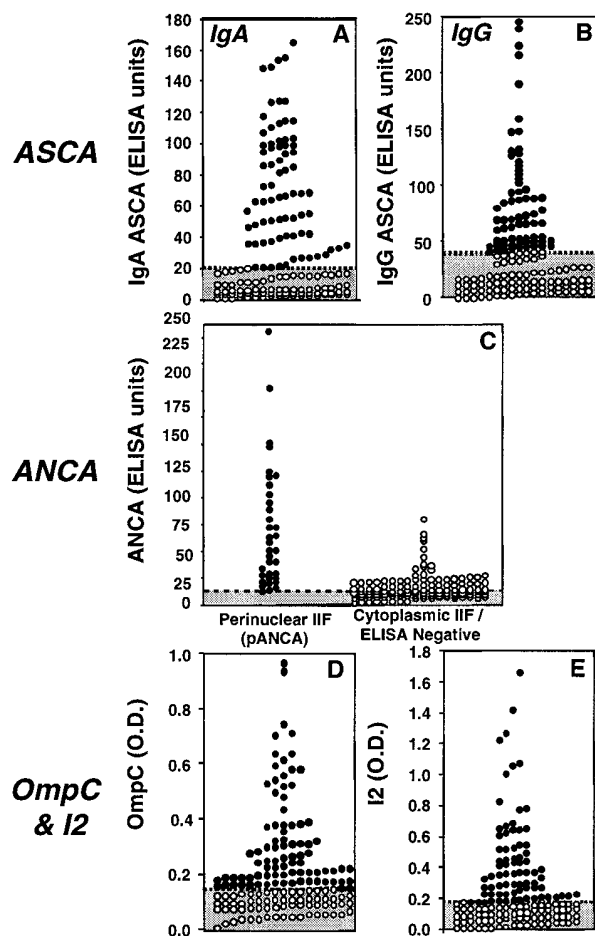


Figure 1. Level of antibody response in the CD cohort to previously described antigens: IgA ASCA (A), IgG ASCA (B), ANCA (C), OmpC (D), and I2 (E). In each panel, the dashed line indicates the division between positive and negative, as defined by 2 SD above the mean of normal controls; (●) ASCA-, pANCA-, OmpC-, and I2-positive samples. O.D., optical density.

antigens are similar in the pANCA⁺ and pANCA⁻ groups.

Antibody Stability

To address the relationship between the expression and level of these antibody responses over time and also in relationship to clinical disease activity, we took advantage of a cohort of 73 CD patients that we had followed up prospectively before and after infliximab treatment of CD (CSMC longitudinal cohort). Within this cohort, 26 patients responded by a decrease of at least 70 in their CDAI (range, 85–375; median, 179) and had an interval in between serum draws of at least 4 months (range, 4–14; median, 6.1 months). These criteria would allow a definition of a durable response after infliximab treatment and allow enough time for changes in the Ig expression after treatment response. The results

for these 26 patients showed that even though there is variation in the antibody levels before and after the therapy within a patient, the intraclass correlation was high for all 5 antibodies (0.69, 0.82, 0.96, 0.99, and 0.87 for OmpC, ANCA, IgA ASCA, IgG ASCA, and I2, respectively; all $P < 0.0001$). Figure 4 shows that the vast majority of patients did not change antibody expression. Those patients who did change expression had negative levels that changed to very low levels of positivity or were low-level positives that changed to negatives. However, the magnitude of responses among the patients with higher-level reactivity to these antigens stayed stable over time despite great changes in disease activity. These results, combined with the previous data, emphasize the importance of assessing both the magnitude and the qualitative expression of these responses.

Evidence for Selective, Not Global, Loss of Tolerance to Bacterial Antigens in Patients With CD

The results presented previously show the heterogeneity of serum reactivity in patients with CD to a series of microbial and autoantigens. Articles in the literature using serum Ig response to multiple bacterial antigen preparations have suggested that all CD patients have a global loss of tolerance to these commensal bacteria.^{19,21,22} In this study, we used 3 antigens of microbial origin and 1 that is likely to be composed of several autoantigens (pANCA), all likely to have multiple cross-reactivities with commensal bacterial antigens.^{18,27,31} Given the heterogeneous responses we have shown in patients with CD, it is likely that the degree of loss of tolerance is not equivalent in all patients with CD.

This wide variation in both the number and magnitude of serum responses within the CSMC clinical cohort ($n = 151$) is shown by 2×2 analysis (Figure 5). For example, patient 1 (Figure 5A–C) does not have reactivity to ASCA or I2 but does have high reactivity to OmpC. In contrast, there are other CD patients who have a high level of response to both I2 and ASCA (see patient 2, Figure 5A–C), but no response to OmpC.

Even among those CD patients who are responsive to all 3 microbial antigens ($n = 39$)—ASCA, I2, and OmpC—there is variability in the magnitude of the response. There is moderate correlation among these antibodies. The correlation between IgA ASCA and OmpC is 0.079 ($P = 0.643$), between I2 and OmpC is 0.208 ($P = 0.216$), and between IgA ASCA and I2 is 0.306 ($P = 0.065$). The variability in response is shown by the distribution of quartile sums in Figure 6, in which the level of reactivity to the different antigens is divided into quartiles with scores of 1, 2, 3, and 4, assigned to

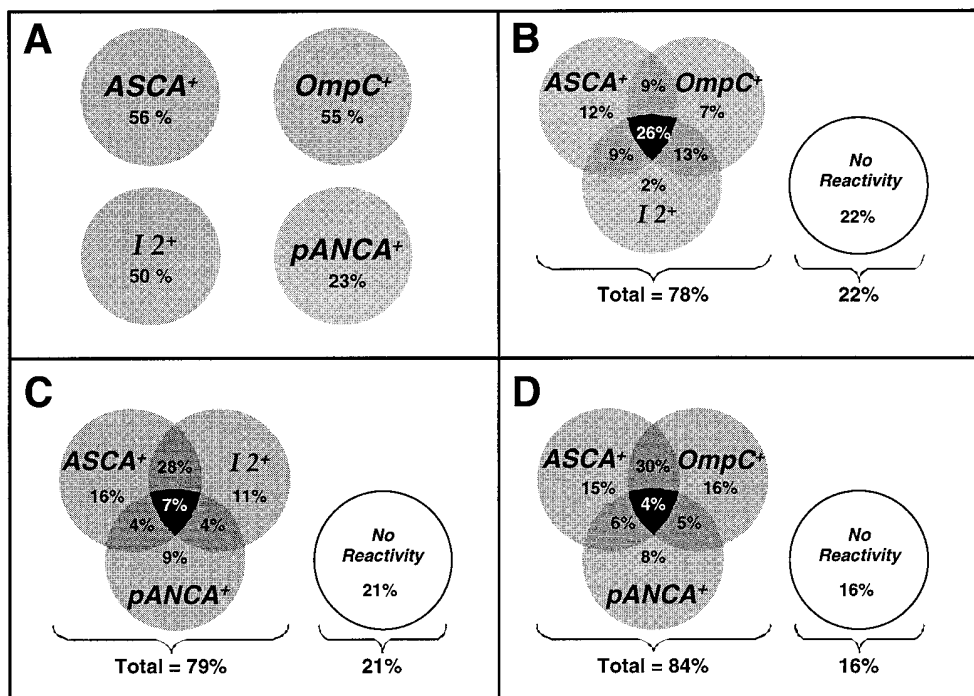


Figure 2. Relationships between marker antibodies in the CD cohort by presence vs. absence. (A) Percentage of the CD cohort that is positive for each marker. (B–D) Intersections of those groups. In each panel, the percentage of the entire cohort defined by the Venn diagram is shown.

quartiles 1, 2, 3, and 4, respectively. If a particular patient has the highest reactivity to all 3 antigens (fourth quartile), the sum of the quartiles would be 12. In contrast, if a given patient had a low reactivity to all 3 antigens (first quartile), the sum of the quartiles would be 3. Any other variability in the level of reactivity to these antigens would have a sum of the quartiles somewhere between 12 and 3. As can be seen in Figure 6, very

few patients have the highest or lowest reactivity to all 3 antigens. The vast majority (89.7%; n = 35; 95% confidence interval, 79.6%–99.8%) had levels of reactivity in between.

In addition, we used cluster analysis as a separate mathematical approach to evaluate patient responses without bias to antibody distribution. This cluster analysis evaluated the quantitative levels of the 5 antibodies among the combined group of patients in the CSMC clinical cohort and the genetics cohort (total n = 304). The pseudo-F statistic ranged from 87.6 to 152.38 among 2 to 10 clusters. On the basis of the largest pseudo-F statistic, we considered 4 clusters to be optimal in this sample. As shown in Figure 7, cluster 1 was characterized by high pANCA, cluster 2 by both high IgA ASCA and high IgG ASCA, and cluster 3 by high OmpC and I2; cluster 4 consisted of individuals with low reactivity in all 4 antibodies. Table 1 shows the mean values of the standardized levels of antibody response for each cluster displayed in Figure 7. This shows that among CD patients, the multidimensional distribution of these antibodies shows distinct clusters rather than random overlaps. Thus, these clusters represent those individuals in whom 1 or 2 reactivities predominate at very high levels. These results in conjunction with the heterogeneous positive responses reported previously show a difference in the degree and magnitude of serum

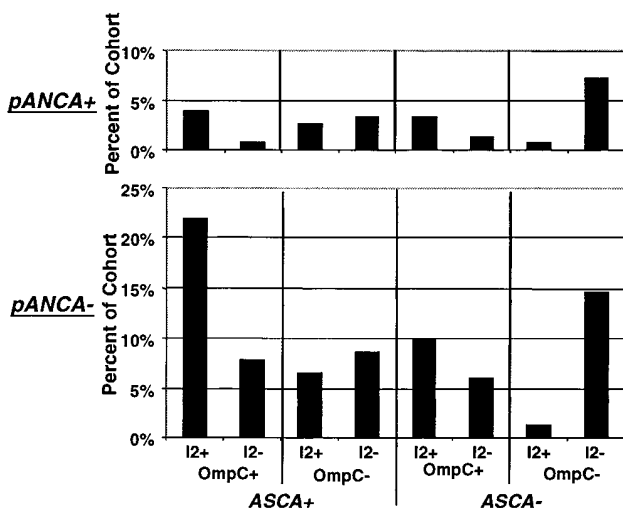


Figure 3. Population distribution of patients as defined by marker antibody presence. Bars represent the percentage of the CD cohort that fits the described category.

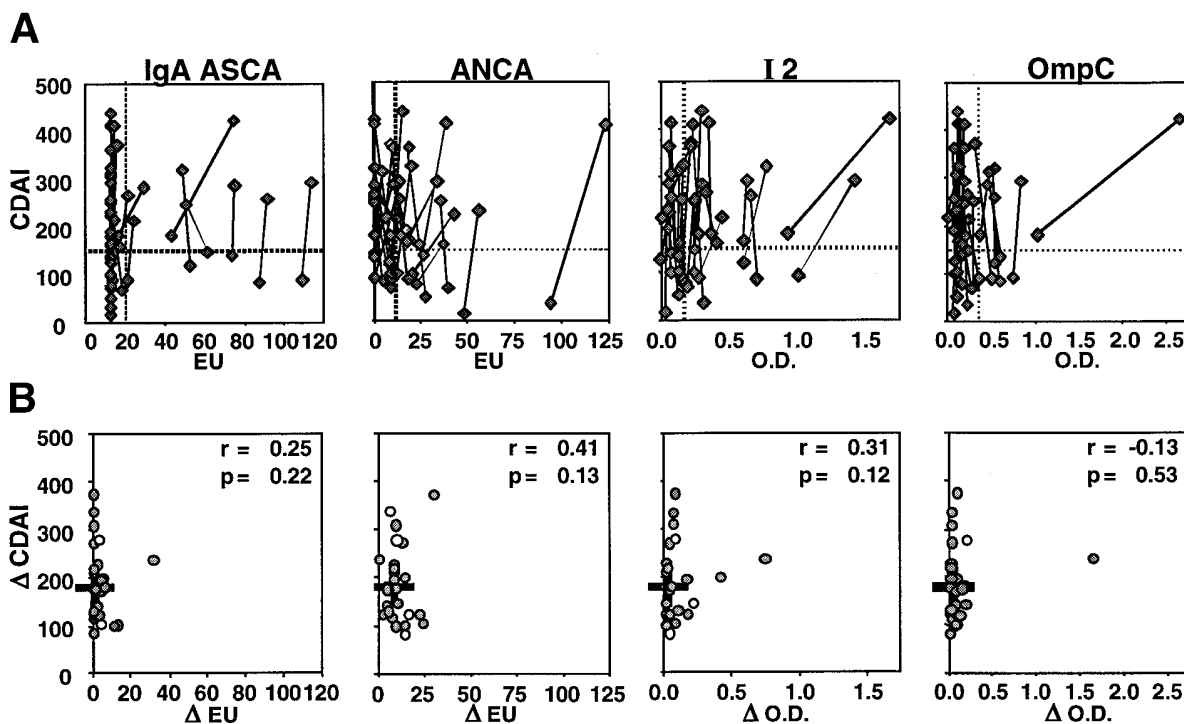


Figure 4. Relationship of antibody expression level to disease activity over time. (A) CDAI and antibody expression levels are shown for 26 patients at 2 connected time points. *Dashed lines* indicate CDAI level of 150 for reference and the division between negative and positive for the antibody tested as previously defined. (B) Change in CDAI and antibody expression level between the time points shown in (A). The median change in CDAI and antibody expression level is depicted by crosses; (○) change in antibody expression status from negative to positive or vice versa; (●) no change. The Spearman correlation between Δ CDAI and change in antibody level are shown. EU, ELISA unit; O.D., optical density.

reactivity among patients with CD to a limited panel of microbial antigens. Therefore, the loss of tolerance to microbial antigens is relatively selective, not global, and varies among CD patients.

Discussion

In this study, we show variable serologically determined responses to a panel of microbial and autoantigens. Twenty-four percent to 56% of CD patients have an immune response to a single antigen. We showed that the expression of immune responses to these antigens in a given CD patient is heterogeneous. The vast majority of patients respond to at least 1 antigen; however, some are responsive to several. The vast majority of patients also had stable expression of these markers over time, independently of disease activity. The magnitude of the immune response to specific antigens varies within a given patient. Even among those patients who have serum responses to all antigens, there is variability in the magnitude of their response. Further independent analysis of the magnitude of antibody reactivity showed 4 distinct clusters of response.

We have previously shown that not only the presence, but also the magnitude, of individual seroreac-

tivity may relate to disease behavior in both CD and ulcerative colitis.^{25,47,48} CD patients with high levels of IgG and IgA ASCA and the absence of pANCA have more aggressive, small-bowel fistulizing and fibrosing disease, and patients with high-level pANCA, in the absence of ASCA, have an ulcerative colitis-like colonic disease.^{25,47,48} In ulcerative colitis, those patients with very high levels of pANCA have a much greater chance of developing chronic bacteria-associated, antibiotic-dependent pouchitis.⁴⁸ Therefore, serum reactivity to these antigens may indicate sensitivity in a given patient to selected antigens within the mucosa that could generate different forms and severity of disease. However, this could reflect the transmural nature of the inflammation in some patients with CD. Furthermore, not answered by this study is whether these immune responses initiate or sustain inflammation patterns of disease or are a sequela, simply marking for a form of inflammation.

Reactivity to bacterial antigens was first shown by Blaser et al.⁴⁹ in 1984, who showed a modest increase in serum antibodies to 7 bacterial pathogens in a group of CD patients. Subsequently, several investigators have suggested serum responses to various bacterial and my-

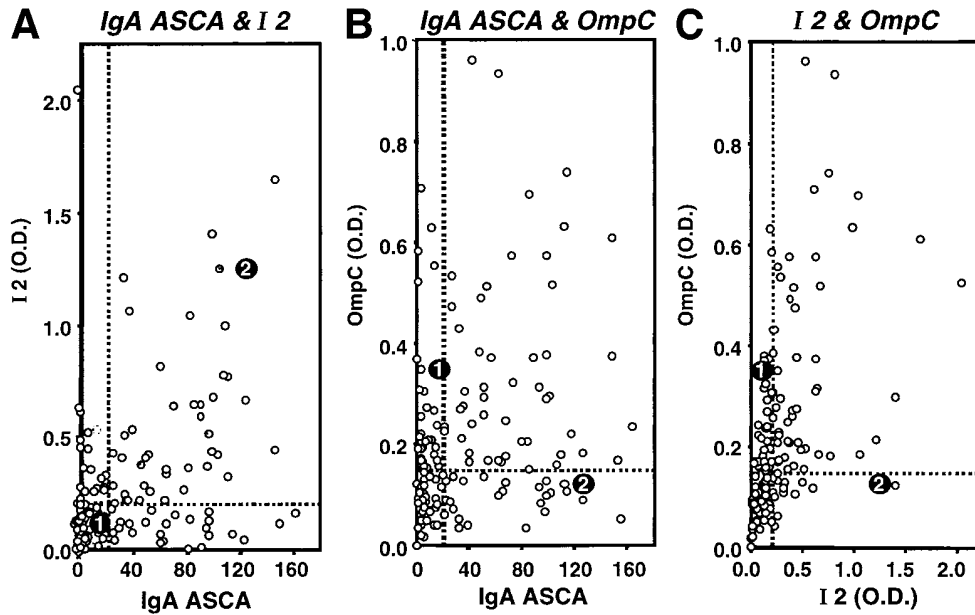


Figure 5. Relationship between marker antibodies in the CD CSMC clinical cohort (n = 151) by level of response. In each panel, the dashed line indicates the division between positive and negative. Two individual patients (1 and 2) are highlighted. O.D., optical density.

cobacterial antigens.^{12,17,50–55} All of these studies used a limited number of CD patients as well as sonicated preparations of multiple bacteria as antigens to test for immune responses. None of these compared the magnitude of the immune response. A series of studies by Duchmann et al.^{19,23,50,55,56} has shown a loss of tolerance to pathogenic as well as commensal bacteria in clones derived from peripheral and lamina propria T cells from patients with CD. Sonication of specific pathogenic bacteria or separated aerobic and anaerobic bacteria was the source of antigens for these clones. Results from all these studies proposed a global loss of tolerance to bacterial

antigens among patients with CD. However, none of these studies addressed the existence of heterogeneous responses within individuals. This study adds to these findings by showing that immune responses to specific antigens, as measured by Ig levels, are not uniform among CD patients. However, clustering of responses to these antigens does define subsets of CD patients.

Studies in animal models of immune responses to commensal bacterial antigens have shown that among

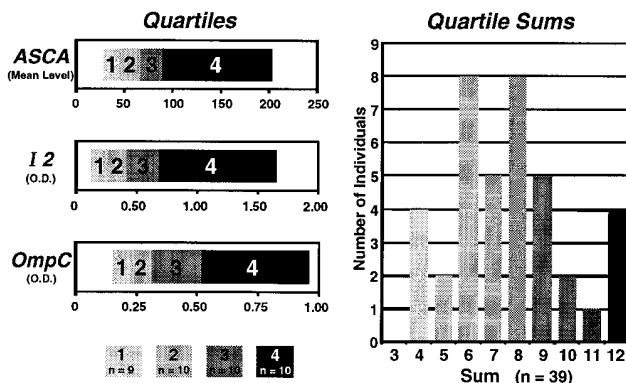


Figure 6. Quartile analysis of population in a cohort that is positive for all 3 microbial antigens (ASCA, I2, and OmpC). This population was subdivided into 4 quartiles by ASCA (top left), I2 (middle left), and OmpC (bottom left) binding levels. Quartile sums were calculated by the addition of each individual's quartile values. The distribution of the quartile sums is shown (right panel). O.D., optical density.

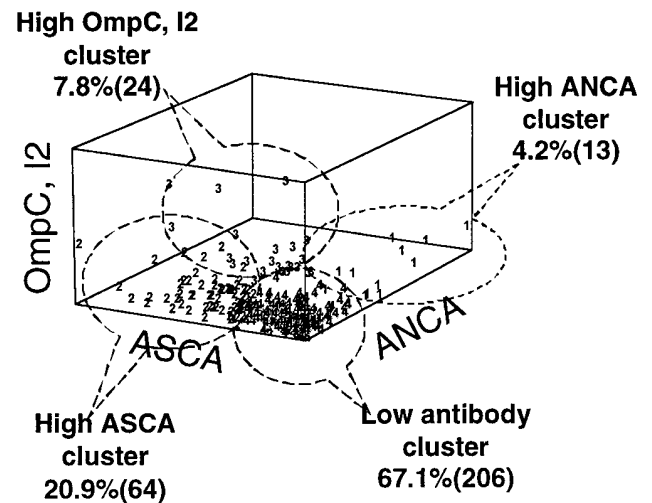


Figure 7. Three-dimensional scatterplot of the 4 clusters represented by high ANCA, high ASCA IgA and IgG, high OmpC and I2, and low values of all 5 antibodies. To represent the 5 antibodies in this figure, the average of ASCA IgA and ASCA IgG is plotted on the x axis, the average of OmpC and I2 is plotted on the y axis, and ANCA level is plotted on the z axis.

Table 1. Mean Standardized Antibody Levels for 4 Clusters

Cluster	pANCA	IgA ASCA	IgG ASCA	OmpC	I2
1	3.99	-0.60	-0.59	0.08	-0.10
2	-0.21	1.48	1.39	0.09	0.20
3	-0.15	0.43	0.04	2.41	2.43
4	-0.15	-0.48	-0.40	-0.30	-0.34

the large numbers of bacterial antigens within the commensal bacterial population, there is a relatively selective serum and T-cell immune response in a given animal model to a limited number of bacterial antigens.^{8,57} Furthermore, when mice are raised in germ-free environments, monoassociation by a single bacterium can induce colitis.^{4,5} Different bacteria are capable of inducing expression of colitis in different rodent models. These findings suggest that a limited number of bacterial antigens are capable of initiating disease, and the dominant antigens may differ in 2 separate models, both of which express intestinal inflammation yet may have different disease expression and severity.^{4,5}

The relevance of these findings in animals to the diversity of human disease seen among CD patients has yet to be defined. Which particular immune alterations and bacterial sensitivity associated with different animal models are represented among the different clinical expressions of disease seen in CD is also unknown. The findings in this study suggest, as reflected among the different animal models, that there is a diversity of patterns of immune responses to environmental and bacterial antigens that differs widely among groups of CD patients. The relationship of these different patterns of immune responses to clinical behavior is not yet clear. However, it is possible that certain antibiotics might be most effective in those patients who have the most robust, i.e., numbers and magnitude of, responses to bacterial antigens. For example, Duchmann et al.⁵⁶ showed that muting the T-helper-1 response in animal models of colitis by using antibodies to IL-12 or the infusion of IL-10 reversed the T-cell responses to commensal bacterial antigens. It is possible that alteration of the relevant bacterial flora in this model could have accomplished a similar treatment. Likewise, the most robust responses to the broadest number of these antigens may define those patients who can best be treated by manipulation of the bacterial flora. If the antigens to which an individual is sensitive are among the anaerobic or microaerophilic bacteria, then metronidazole may be more effective. If these antigens are among the aerobic bacteria, then these patients may best be treated with ciprofloxacin. Finally, patients who are immunoreactive to both aerobic and anaerobic populations may require a combination of both metronidazole and ciprofloxacin.

In conclusion, serum immune responses to microbial and autoantigens are not uniform among CD patients. Defining how these antibody reactivities relate to clinical behavior and response to therapeutic modalities will require larger numbers of phenotypically well-characterized patients.

References

- De Winter H, Cheroutre H, Kronenberg M. Mucosal immunity and inflammation. II. The yin and yang of T cells in intestinal inflammation: pathogenic and protective roles in a mouse colitis model. *Am J Physiol* 1999;276:G1317-G1321.
- Strober W, Ludviksson BR, Fuss IJ. The pathogenesis of mucosal inflammation in murine models of inflammatory bowel disease and Crohn disease. *Ann Intern Med* 1998;128:848-856.
- Bhan AK, Mizoguchi E, Smith RN, Mizoguchi A. Colitis in transgenic and knockout animals as models of human inflammatory bowel disease. *Immunol Rev* 1999;169:195-207.
- Rath HC, Herfarth HH, Ikeda JS, Grenther WB, Hamm TE Jr, Balish E, Taurog JD, Hammer RE, Wilson KH, Sartor RB. Normal luminal bacteria, especially *Bacteroides* species, mediate chronic colitis, gastritis, and arthritis in HLA-B27/human beta2 microglobulin transgenic rats. *J Clin Invest* 1996;98:945-953.
- Sellon RK, Tonkonogy S, Schultz M, Dieleman LA, Grenther W, Balish E, Rennick DM, Sartor RB. Resident enteric bacteria are necessary for development of spontaneous colitis and immune system activation in interleukin-10-deficient mice. *Infect Immun* 1998;66:5224-5231.
- Kim SC, Tonkonogy SL, Bayliss E, Warner T, Sartor RB. IL-10 deficient mice monoassociated with non-pathogenic *Enterococcus faecalis* develop chronic colitis (abstr). *Gastroenterology* 2001;120:A82.
- Fox JG, Gorelick PL, Kullberg MC, Ge Z, Dewhirst FE, Ward JM. A novel urease-negative *Helicobacter* species associated with colitis and typhlitis in IL-10-deficient mice. *Infect Immun* 1999;67:1757-1762.
- Cong Y, Brandwein SL, McCabe RP, Lazenby A, Birkenmeier EH, Sundberg JP, Elson CO. CD4⁺ T cells reactive to enteric bacterial antigens in spontaneously colitic C3H/HeJBir mice: increased T helper cell type 1 response and ability to transfer disease. *J Exp Med* 1998;187:855-864.
- Prantera C, Kohn A, Zannoni F, Spimpolo N, Bonfa M. Metronidazole plus ciprofloxacin in the treatment of active, refractory Crohn's disease: results of an open study. *J Clin Gastroenterol* 1994;19:79-80.
- Prantera C, Zannoni F, Scribano ML, Berto E, Andreoli A, Kohn A, Luzi C. An antibiotic regimen for the treatment of active Crohn's disease: a randomized, controlled clinical trial of metronidazole plus ciprofloxacin. *Am J Gastroenterol* 1996;91:328-332.
- Rutgeerts P, Goboos K, Peeters M, Hiele M, Penninckx F, Aerts R, Kerremans R, Vantrappen G. Effect of faecal stream diversion on recurrence of Crohn's disease in the neoterminal ileum. *Lancet* 1991;338:771-774.
- Wayne LG, Hollander D, Anderson B, Sramek HA, Vadheim CM, Rotter JI. Immunoglobulin A (IgA) and IgG serum antibodies to mycobacterial antigens in Crohn's disease patients and their relatives. *J Clin Microbiol* 1992;30:2013-2018.
- Sanderson JD, Moss MT, Tizard ML, Hermon-Taylor J. *Mycobacterium paratuberculosis* DNA in Crohn's disease tissue. *Gut* 1992;33:890-896.
- Moss MT, Sanderson JD, Tizard ML, Hermon-Taylor J, el-Zaatari FA, Markesich DC, Graham DY. Polymerase chain reaction detection of *Mycobacterium paratuberculosis* and *Mycobacterium avium* subsp *silvaticum* in long term cultures from Crohn's disease and control tissues. *Gut* 1992;33:1209-1213.

15. Fidler HM, Thurrell W, Johnson NM, Rook GA, McFadden JJ. Specific detection of *Mycobacterium paratuberculosis* DNA associated with granulomatous tissue in Crohn's disease. *Gut* 1994; 35:506–510.
16. El-Zaatari FA, Naser SA, Graham DY. Characterization of a specific *Mycobacterium paratuberculosis* recombinant clone expressing 35,000-molecular-weight antigen and reactivity with sera from animals with clinical and subclinical Johne's disease. *J Clin Microbiol* 1997;35:1794–1799.
17. Clarkston WK, Presti ME, Petersen PF, Zachary PE Jr, Fan WX, Leonardi CL, Vernava AM III, Longo WE, Kreeger JM. Role of *Mycobacterium paratuberculosis* in Crohn's disease: a prospective, controlled study using polymerase chain reaction. *Dis Colon Rectum* 1998;41:195–199.
18. Cohavy O, Bruckner D, Gordon LK, Misra R, Wei B, Eggena ME, Targan SR, Braun J. Colonic bacteria express an ulcerative colitis pANCA-related protein epitope. *Infect Immun* 2000;68:1542–1548.
19. Duchmann R, May E, Heike M, Knolle P, Neurath M, Meyer zum Buschenfelde KH. T cell specificity and cross reactivity towards enterobacteria, bacteroides, bifidobacterium, and antigens from resident intestinal flora in humans. *Gut* 1999;44:812–818.
20. Liu Y, van Kruiningen HJ, West AB, Cartun RW, Cortot A, Colombel JF. Immunocytochemical evidence of *Listeria*, *Escherichia coli*, and *Streptococcus* antigens in Crohn's disease. *Gastroenterology* 1995;108:1396–1404.
21. Weiner HL, Friedman A, Miller A, Khoury SJ, al-Sabbagh A, Santos L, Sayegh M, Nussenblatt RB, Trentham DE, Hafler DA. Oral tolerance: immunologic mechanisms and treatment of animal and human organ-specific autoimmune diseases by oral administration of autoantigens. *Annu Rev Immunol* 1994;12: 809–837.
22. MacDonald TT. Breakdown of tolerance to the intestinal bacterial flora in inflammatory bowel disease (IBD). *Clin Exp Immunol* 1995;102:445–447.
23. Duchmann R, Kaiser I, Hermann E, Mayet W, Ewe K, Meyer zum Buschenfelde KH. Tolerance exists towards resident intestinal flora but is broken in active inflammatory bowel disease (IBD). *Clin Exp Immunol* 1995;102:448–455.
24. Sutton CL, Kim J, Yamane A, Dalwadi H, Wei B, Landers C, Targan SR, Braun J. Identification of a novel bacterial sequence associated with Crohn's disease. *Gastroenterology* 2000;119:23–31.
25. Vasiliauskas EA, Plevy SE, Landers CJ, Binder SW, Ferguson DM, Yang H, Rotter JJ, Vidrich A, Targan SR. Perinuclear antineutrophil cytoplasmic antibodies in patients with Crohn's disease define a clinical subgroup. *Gastroenterology* 1996;110:1810–1819.
26. Main J, McKenzie H, Yeaman GR, Kerr MA, Robson D, Pennington CR, Parratt D. Antibody to *Saccharomyces cerevisiae* (bakers' yeast) in Crohn's disease. *BMJ* 1988;297:1105–1106.
27. Cohavy O, Harth G, Horwitz M, Eggena M, Landers C, Sutton C, Targan SR, Braun J. Identification of a novel mycobacterial histone H1 homologue (HupB) as an antigenic target of pANCA monoclonal antibody and serum immunoglobulin A from patients with Crohn's disease. *Infect Immun* 1999;67:6510–6517.
28. Eggena M, Targan SR, Iwanczyk L, Vidrich A, Gordon LK, Braun J. Phage display cloning and characterization of an immunogenetic marker (perinuclear anti-neutrophil cytoplasmic antibody) in ulcerative colitis. *J Immunol* 1996;156:4005–4011.
29. Gordon LK, Eggena M, Targan SR, Braun J. Definition of ocular antigens in ciliary body and retinal ganglion cells by the marker antibody pANCA. *Invest Ophthalmol Vis Sci* 1999;40: 1250–1255.
30. Gordon LK, Eggena M, Targan SR, Braun J. Mast cell and neuroendocrine cytoplasmic autoantigen(s) detected by monoclonal pANCA antibodies. *Clin Immunol* 2000;94:42–50.
31. Eggena M, Cohavy O, Parseghian MH, Hamkalo BA, Clemens D, Targan SR, Gordon LK, Braun J. Identification of histone H1 as a cognate antigen of the ulcerative colitis-associated marker antibody pANCA. *J Autoimmun* 2000;14:83–97.
32. Landers CJ, Cohavy O, Yang H, Lin S, Braun J, Targan SR. Selected, not global, loss of tolerance using Crohn's disease (CD)-associated immune responses to auto- and specific bacterial antigens (abstr). *Gastroenterology* 2001;120:A519.
33. Barnes RM, Allan S, Taylor-Robinson CH, Finn R, Johnson PM. Serum antibodies reactive with *Saccharomyces cerevisiae* in inflammatory bowel disease: is IgA antibody a marker for Crohn's disease? *Int Arch Allergy Appl Immunol* 1990;92:9–15.
34. McKenzie H, Main J, Pennington CR, Parratt D. Antibody to selected strains of *Saccharomyces cerevisiae* (baker's and brewer's yeast) and *Candida albicans* in Crohn's disease. *Gut* 1990; 31:536–538.
35. Sendid B, Colombel JF, Jacquinet PM, Faille C, Fruit J, Cortot A, Lucidarme D, Camus D, Poulain D. Specific antibody response to oligomannosidic epitopes in Crohn's disease. *Clin Diagn Lab Immunol* 1996;3:219–226.
36. Sendid B, Quinton JF, Charrier G, Goulet O, Cortot A, Grandbastien B, Poulain D, Colombel JF. Anti-*Saccharomyces cerevisiae* mannan antibodies in familial Crohn's disease. *Am J Gastroenterol* 1998;93:1306–1310.
37. Quinton JF, Sendid B, Reumaux D, Duthilleul P, Cortot A, Grandbastien B, Charrier G, Targan SR, Colombel JF, Poulain D. Anti-*Saccharomyces cerevisiae* mannan antibodies combined with antineutrophil cytoplasmic autoantibodies in inflammatory bowel disease: prevalence and diagnostic role. *Gut* 1998;42:788–791.
38. Landers CJ, Yang H, Lin Y, Vasiliauskas EA, Braun J, Targan SR. IgA serum antibody reactivity with I2, a novel Crohn's disease marker: anti-I2 level is independent from ASCA and ANCA levels even though IgA ASCA positivity is correlated with anti-I2 positivity (abstr). *Gastroenterology* 2000;118:A348.
39. Wei B, Huang T, Dalwadi H, Braun J. The Crohn's disease lesion derived gene I2 encodes the core peptide of a transcriptional regulator of *Pseudomonas fluorescens* in the functional domain of a novel T cell superantigen (abstr). *Gastroenterology* 2001; 120:A53.
40. Duerr RH, Targan SR, Landers CJ, LaRusso NF, Lindsay KL, Wiesner RH, Shanahan F. Neutrophil cytoplasmic antibodies: a link between primary sclerosing cholangitis and ulcerative colitis. *Gastroenterology* 1991;100:1385–1391.
41. Targan SR, Landers C, Vidrich A, Czaja AJ. High-titer antineutrophil cytoplasmic antibodies in type-1 autoimmune hepatitis. *Gastroenterology* 1995;108:1159–1166.
42. Saxon A, Shanahan F, Landers C, Ganz T, Targan S. A distinct subset of antineutrophil cytoplasmic antibodies is associated with inflammatory bowel disease. *J Allergy Clin Immunol* 1990; 86:202–210.
43. Vidrich A, Lee J, James E, Cobb L, Targan S. Segregation of pANCA antigenic recognition by DNase treatment of neutrophils: ulcerative colitis, type 1 autoimmune hepatitis, and primary sclerosing cholangitis. *J Clin Immunol* 1995;15:293–299.
44. Guan KL, Dixon JE. Eukaryotic proteins expressed in *Escherichia coli*: an improved thrombin cleavage and purification procedure of fusion proteins with glutathione S-transferase. *Anal Biochem* 1991;192:262–267.
45. Sokal RR, Rohlf FJ. *Biometry*. 3rd ed. New York: WH Freeman & Co, 1997.
46. Dillon W, Goldstein M. *Multivariate analysis*. New York: John Wiley and Sons, 1984.
47. Vasiliauskas EA, Kam LY, Karp LC, Gaiennie J, Yang H, Targan SR. Marker antibody expression stratifies Crohn's disease into immunologically homogeneous subgroups with distinct clinical characteristics. *Gut* 2000;47:487–496.
48. Fleshner PF, Vasiliauskas EA, Kam LY, Karp LC, Gaiennie J, Yang H, Targan SR. High level perinuclear antineutrophil cytoplasmic

- antibody (pANCA) in ulcerative colitis patients before colectomy predicts the development of chronic pouchitis after ileal pouch-anal anastomosis. *Gut* 2001;49:671–677.
49. Blaser MJ, Miller RA, Lacher J, Singleton JW. Patients with active Crohn's disease have elevated serum antibodies to antigens of seven enteric bacterial pathogens. *Gastroenterology* 1984;87:888–894.
 50. Duchmann R, Marker-Hermann E, Meyer zum Buschenfelde KH. Bacteria-specific T-cell clones are selective in their reactivity towards different enterobacteria or *H. pylori* and increased in inflammatory bowel disease. *Scand J Immunol* 1996;44:71–79.
 51. Naser SA, Hulten K, Shafran I, Graham DY, El-Zaatari FA. Specific seroreactivity of Crohn's disease patients against p35 and p36 antigens of *M. avium* subsp. *paratuberculosis*. *Vet Microbiol* 2000;77:497–504.
 52. Metcalf J. Is measles infection associated with Crohn's disease? *BMJ* 1998;316:166.
 53. Macpherson A, Khoo UY, Forgacs I, Philpott-Howard J, Bjarnason I. Mucosal antibodies in inflammatory bowel disease are directed against intestinal bacteria. *Gut* 1996;38:365–375.
 54. Lindberg E, Magnusson KE, Tysk C, Jarnerot G. Antibody (IgG, IgA, and IgM) to baker's yeast (*Saccharomyces cerevisiae*), yeast mannan, gliadin, ovalbumin and betalactoglobulin in monozygotic twins with inflammatory bowel disease. *Gut* 1992;33:909–913.
 55. Duchmann R, Neurath MF, Meyer zum Buschenfelde KH. Responses to self and non-self intestinal microflora in health and inflammatory bowel disease. *Res Immunol* 1997;148:589–594.
 56. Duchmann R, Schmitt E, Knolle P, Meyer zum Buschenfelde KH, Neurath M. Tolerance towards resident intestinal flora in mice is abrogated in experimental colitis and restored by treatment with interleukin-10 or antibodies to interleukin-12. *Eur J Immunol* 1996;26:934–938.
 57. Saparov A, Kraus LA, Cong Y, Marwill J, Xu XY, Elson CO, Weaver CT. Memory/effector T cells in TCR transgenic mice develop via recognition of enteric antigens by a second, endogenous TCR. *Int Immunol* 1999;11:1253–1264.

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Meigs of Meigs' Syndrome



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Joe Vincent Meigs (1892–1963) was born in Lowell, Massachusetts, the son of a family doctor who conducted an active surgical practice. He received his B.A. degree from Princeton and his M.D. degree from Harvard. Trained at the Massachusetts General Hospital and its affiliated institutions, where he spent his entire professional career, he gained fame for his modification of the Wertheim procedure for eradication of cancer of the uterine cervix. He also was an early and vigorous promoter of the Papanicolaou technique for detection of uterine neoplasia. Although the syndrome that bears his name (ascites and hydrothorax associated with ovarian fibroma and other pelvic tumors) is not strictly a gastroenterologic problem, the condition often enters the differential diagnosis of abdominal swelling. Affable and unaffected in his mien, Meigs was meticulous in his work. His career ended with a fatal myocardial infarction suffered aboard a plane on which he was flying home to Boston from a medical meeting in Rochester, New York. He died on his 71st birthday.

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