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Characterization of Immunologic Defects in Patients with Common Variable Immunodeficiency (CVID) with Intestinal Disease

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Abstract

Background—Common variable immunodeficiency (CVID) is a heterogeneous disorder commonly presenting with recurrent sino-pulmonary infections. In all, 6%–10% of CVID patients develop an inflammatory bowel disease (IBD)-like disorder, making these patients a unique population to investigate immune-mediated gastrointestinal disease. This study examined whether defects in peripheral and/or intestinal lymphocytes are involved in disruption of the intestinal mucosa in CVID patients with inflammatory intestinal diseases.

Methods—Peripheral blood (PB) T cells from healthy controls; CD or UC; CVID; and CVID with IBD were stimulated for 48 hours with anti-CD3+CD28 or phytohemagglutinin (PHA) + phorbol 12-myristate 13-acetate (PMA); cytokine production was measured by enzyme-linked immunosorbent assay (ELISA). Cytokine expression from unstimulated lamina propria lymphocytes (LPLs) was compared by real-time polymerase chain reaction (PCR). Immunohistochemistry of mucosal biopsies was performed. Cell populations were quantified by morphometry.

Results—CVID/IBD PB T cells stimulated by anti-CD3+CD28 had trends for reduced IL-2, IL-10, IFN- γ , and TNF- α compared to controls. These differences were not apparent following stimulation by PHA/PMA. Constitutive production of inflammatory cytokines by LPLs was not detected. Histologically, CVID patients had reduced/absent plasma cells with reductions in intestinal IgM and IgA. CVID patients with and without gastrointestinal (GI) disease exhibited increased CD3+ T cells, specifically CD8+, in the colon compared to normal and IBD controls, suggesting immune dysregulation.

Conclusions—Intestinal inflammation in CVID patients with IBD-like disease may be mediated by abnormal cytokine production through a T-cell receptor-mediated pathway. However, the variability observed suggests multiple, rather than singular, mechanisms are involved. Histologic features such as reduced intestinal plasma cells and lack of intestinal immunoglobulins may be useful markers in diagnosing CVID in a patient with GI disease refractory to conventional therapies.

Keywords

antibody deficiency; common variable immunodeficiency; gastrointestinal disease; inflammatory bowel disease

Common variable immunodeficiency (CVID) is the most common symptomatic primary immunodeficiency. The diagnosis of CVID is established based on reduced levels of two serum immunoglobulins, IgG and IgA and/or IgM, at least two standard deviations below the age-specific mean values, and impaired specific antibody production in response to either vaccination in vivo or recent infection.¹ The hypogammaglobulinemia results from the failure of B cells to differentiate into plasma cells; however, T-cell abnormalities and defective cytokine production have also been described.²⁻⁵

Most commonly, CVID patients present with recurrent sinopulmonary infections such as bronchitis, sinusitis, and pneumonia, although autoimmunity and gastrointestinal (GI) disease are also quite prevalent and may be the initial presentation of disease.^{6,7} Several studies have reported the incidence of GI diseases in patients with CVID ranging from 20%–60%.⁶⁻¹⁰ This is not unexpected, given that the GI tract with its large surface area contains the most immunoglobulin-producing cells in the body; IgA being the major immunoglobulin in the gut. GI diseases observed in CVID patients include chronic diarrhea,¹⁰⁻¹² inflammation of the small or large intestine resembling Crohn's disease (CD) or ulcerative colitis (UC),¹³⁻¹⁵ villous flattening resembling celiac sprue,^{6,8,16,17} pernicious anemia,¹⁸ nodular lymphoid hyperplasia,¹¹ lymphoma, and gastric adenocarcinoma.⁸

Immune abnormalities in CVID include impaired antibody production, disruption in T-cell function,¹⁹⁻²³ and defects in innate immunity.²⁴⁻²⁷ However, few studies have examined specifically what immune parameters and mechanisms predispose these patients to GI tract disease. Recent studies have suggested that GI infections are more frequent in patients with undetectable serum IgA (47 [36%] of 131 patients) compared to patients with residual IgA production.²⁸ However, unlike X-linked agammaglobulinemia (XLA) and IgA deficiency, GI disease is far more common in CVID, suggesting that T-cell dysfunction contributes to the pathogenesis of intestinal disease. Furthermore, immunoglobulin replacement, which replaces antibody (predominately IgG) but not T-cell defects, improves the infectious complications of CVID; however, GI symptoms often persist and progress further, supporting the complexity of GI disease pathogenesis in the setting of CVID.

As a human model of primary immunodeficiency with disruption of mucosal immunity, CVID patients represent a unique population to investigate immune-mediated GI disease. In this study we examined whether defects in peripheral and intestinal lymphocytes exist that may be involved in the disruption of the mucosal immune response in CVID patients with inflammatory diseases of the gut. In addition, we examined the cellular composition in the gut of these patients with CVID and GI disease to better understand the nature of the intestinal inflammation. We show that CVID patients with inflammatory GI disease have trends toward greater reduction in serum IgG compared to non-GI affected CVID patients, as well as reduced/absent plasma cells in both the small intestine and colon with consequent reductions in intestinal IgM and IgA. CVID patients with and without GI disease exhibited increased CD3+ cells, specifically CD8+ T cells, in the colon compared to normal and inflammatory bowel disease (IBD) controls, supporting the potential for immune dysregulation. The results presented here represent a limited patient population and the variability in data further support that the defects in the immune system that predispose these patients to mucosal inflammation are complex, likely involving both T- and B-cell immunity, rather than a single mechanism.

MATERIALS AND METHODS

Patients

Patients with a diagnosis of CVID, according to established criteria,²⁹ were obtained from the Immunodeficiency Clinic at the Mount Sinai Medical Center, a large referral center for adult and pediatric patients with immunodeficiency. All protocols and procedures were approved by the Mount Sinai Institutional Review Board. Patient charts were reviewed for documentation of clinical history of GI symptoms including diarrhea, malabsorption, weight loss, nausea/vomiting, or abdominal pain. Demographic and pertinent clinical data including quantitative immunoglobulins at the time of diagnosis prior to administration of intravenous immunoglobulin (IVIg), lymphocyte subsets, in vitro antigen and mitogen stimulation, and GI pathology reports were collected.

Because CVID is a heterogeneous disorder with variability in the degree of antibody and T-cell defects we chose to distinguish our patient groups according to underlying GI disease. Two patient groups were studied: CVID patients with inflammatory GI disease and CVID patients without GI disease. Non-CVID patient control groups included: healthy disease-free individuals and patients with IBD such as CD or UC.

Peripheral T-cell Isolation and Cytokine Production

Cytokine production was measured in isolated T cells from four groups of patients: healthy controls, IBD patients (CD or UC), CVID patients with inflammatory GI disease, and CVID patients without GI disease. All CVID patients were receiving IVIg; however, peripheral blood samples were drawn prior to each IVIg infusion for CVID patients or anti-TNF therapy (infliximab) for IBD patients. Peripheral venous blood was suspended in an equal volume of RPMI-1640 and peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation over a Ficoll-Hypaque density gradient. T cells were isolated following rosetting with sheep red blood cell as described.³⁰

Various modes of T-cell activation were used to determine whether defects in specific activation pathways existed. One $\times 10^6$ T cells were stimulated in a 24-well flat-bottom culture plate under the following conditions: 1) RPMI 1640 with 10% FCS and penicillin/streptomycin/glutamine; 2) 1% monoclonal antibody 446 (IgG1 anti-CD3); 3) costimulation with 1% monoclonal antibody mAb446 with 1 μ g/mL commercially prepared purified mouse IgG1 anti-CD28 obtained from BD Biosciences Pharmingen (San Jose, CA); and 4) costimulation with 10 ng/mL phorbol 12-myristate 13-acetate (PMA) and 1 μ g/mL phytohemagglutinin (PHA). Cultures were maintained for 48 hours in a 37°C, 5% CO₂ incubator in a 95% humidified atmosphere. After 48 hours cells were collected and centrifuged at 500g for 2 minutes at 4°C. Cell free supernatants were collected and stored at -20°C. Cytokines, IL-2, IL-10, TNF- α , and IFN- γ from 48-hour cultures of T cells were measured by enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Lamina Propria Lymphocytes (LPLs)

Mucosal samples were obtained from either surgically resected or biopsied colons from UC or CD, CVID/IBD patients, and normal control patients who had GI symptoms warranting a biopsy but normal histology. Colonic tissue was subjected to enzymatic digestion with dispase and collagenase to collect LPLs. Freshly isolated LPLs (unstimulated condition) were stored at -80°C in Trizol until RNA was extracted. Quantitative real-time polymerase chain reaction (PCR) was performed using SYBR Green at 40 cycles for the following cytokines: IFN- γ , TNF- α , IL-10, and EF-1 as a housekeeping gene. The differences in target

gene expression were determined by calculating the fold change expressed as $2^{\Delta\Delta CT}$, where $\Delta\Delta CT = \text{average of } \Delta CT_{\text{normals}} - \Delta CT_{\text{CD,UC,CVID/IBD}}$.

Pathologic Analysis

Pathology archived specimens were reviewed for paraffin-embedded blocks of CVID with and without inflammatory GI disease, IBD, and normal control patients. Available tissue blocks were collected and unstained slides were prepared for immunohistochemistry from small intestine and/or colon biopsy specimens fixed in 10% formalin. Hematoxylin and eosin-stained (H&E) sections were reviewed by the GI pathologist who was blinded to the patient's disease/diagnosis.

Immunohistochemistry/Immunofluorescence

For immunohistochemistry, tissue sections were dewaxed and rehydrated in xylene and ethanol, respectively, and washed in phosphate-buffered saline (PBS). Antigen retrieval was performed by heating for 20 minutes at 97°C using commercial antigen retrieval solution (Target Retrieval Solution, Dako, Carpinteria, CA). Slides were washed in PBS after antigen retrieval and prepared for blocking. Slides were blocked with 10% BSA/TRIS/0.1% Triton X) for 40 minutes. Specimens were covered with commercial primary monoclonal antibody (CD3, CD8, IgA, and IgM, Dako) diluted 1:100 in blocking serum overnight in a humid chamber. Slides were washed three times in PBS and secondary rabbit antimouse IgG antibody (Alexa Fluor by Invitrogen, La Jolla, CA) diluted 1:400 in PBS was prepared and placed on each section and incubated at room temperature in a humid chamber for 30 minutes. Slides were then washed in PBS/DAPI and a final wash with PBS. The slides were mounted with a coverslip and examined with a fluorescence microscope (Zeiss Axio-phot2) at 10×, 20×, and 40× magnification. Digital images were taken at 10× of a representative area of the specimen for morphometric analysis.

Morphometric Analysis

Cell populations were quantified using morphometric analysis software (NIS Elements BR). This software allows quantification of cells by size, circularity, and fluorescence intensity. Images were quantified without knowledge of the patient's clinical diagnosis.

Statistics

Statistical analysis was done using GraphPad Prism Software (San Diego, CA). One-way analysis of variance (ANOVA) was implemented to compare the differences between mean values for each group. If statistical significance was detected, post-hoc comparisons were done using the Tukey Multiple Comparison test to determine which group was statistically significant. A *P* value less than or equal to 0.05 was regarded as significant at the 95% confidence limits. Nonparametric tests, Kruskal–Wallis, and Dunn's Multiple Comparison were implemented to compare the differences between each group.

RESULTS

Immunologic Parameters

Among CVID patients followed at Mount Sinai, 12 patients were identified with a concomitant inflammatory GI disease, as diagnosed by endoscopy, such as villous flattening resembling sprue or IBD-like disease. As controls, we selected 16 CVID patients without GI tract disease.

Mean quantitative serum immunoglobulins (mg/dL) $\pm$ standard error (SE) of the mean at the time of diagnosis of CVID (pre-IVIg treatment) was compared among the groups as shown

in Figure 1. CVID patients without GI disease ($n = 16$), had a mean IgG of 287 ± 30 , IgA 19 ± 3 , IgM 53 ± 15 ; patients with inflammatory GI disease ($n = 12$), such as IBD or villous flattening, had a mean IgG of 221 ± 48 , IgA 24 ± 13 , IgM 35 ± 15 . While there was a trend towards lower IgG levels in CVID patients with inflammatory GI disease, this did not achieve statistical significance.

B- and T-lymphocyte populations were examined for differences in numbers and function among the CVID groups. CVID patients with and without GI disease had similar percentages of T and B cells (Table 1). However, lymphocytes from CVID patients with inflammatory GI disease demonstrated a nonstatistical significant reduction in proliferation in response to T-cell specific mitogens PHA and Con A suggesting the presence of a T-cell defect that could account for the mucosal dysregulation seen in this group of patients.

Cytokine Production

Given the absence of any statistical difference in absolute lymphocyte numbers or response to mitogen stimulation between the groups, we measured cytokine secretion from stimulated T cells by ELISA to determine whether peripheral T-lymphocyte cytokine secretion defects correlate with the disruption of mucosal homeostasis in CVID patients with specifically IBD-like disease. Defective cytokine production by CVID patient lymphocytes has been previously described.¹⁵ Cytokine secretion (IL-2, IL-10, TNF- α , IFN- γ) was measured from T cells isolated from peripheral blood and stimulated under various conditions from CVID patients without GI disease, CVID patients with IBD-like disease, normal healthy subjects, and IBD (UC and CD) controls.

T-cell stimulation by anti-CD28 and anti-CD3 provided a strong stimulus leading to absolute increases in all cytokines measured (Fig. 2). Although not statistically significant, overall the CVID/IBD group demonstrated a trend towards reduced production of all cytokines. In order to determine whether these trends toward reduced cytokine production were secondary to a defect in signaling through the T-cell receptor (TCR), we stimulated T cells with PHA/PMA, stimuli that bypass the TCR (Fig. 3). Bypassing the TCR with PHA/PMA activation resulted in an even stronger stimulus for each cytokine measured across all patient groups. In the CVID groups there was near-normal production of all cytokines measured. Thus, there appears to be a partial defect in signaling through the TCR in CVID patients and this is present to a greater extent in CVID/IBD patients.

LPLs

Findings seen in peripheral blood studies do not always mirror defects seen in affected organs. To determine if differences in immune responses exist at the level of the mucosa under physiological conditions we quantified cytokine mRNA obtained from freshly isolated unstimulated LPLs (Fig. 4). LPLs from UC specimens exhibited a 2-fold increase in expression of IL-10 and TNF- α mRNA compared to LPLs from normal, CD, or CVID/IBD patients. CD derived LPLs demonstrated a 2-fold increase in IFN- γ mRNA expression. CVID/IBD LPLs demonstrated lower overall mRNA levels for IFN- γ compared to UC and CD LPLs nearing statistical significance.

GI Pathology

We next examined the pathology of mucosal biopsies taken from the upper and/or lower GI tract from the 16 CVID patients, eight normal, and four IBD controls. All patients had GI symptoms warranting endoscopy/colonoscopy. Upon histologic review of 27 CVID specimens, a lack of plasma cells in the lamina propria was the most consistent finding in both the upper (13 specimens) and lower tract (14 specimens). This finding correlated with the reductions in serum immunoglobulins in CVID patients. In contrast, normal controls and

IBD patients had a significant number of plasma cells in the lamina propria (Fig. 5). Other notable findings in intestinal specimens from CVID/IBD patients included active chronic colitis, distortion of crypt architecture, villus blunting, nodular lymphoid hyperplasia, IEL lymphocytosis, and increased LPLs.

Immunofluorescence

We examined these specimens for the presence of intestinal immunoglobulins by immunofluorescence. There was an abundant number of IgA and fewer IgM-producing cells in the normal and IBD intestinal lamina propria. In contrast, biopsies taken from CVID patients did not demonstrate any IgA or IgM in the lamina propria, further supporting the lack of plasma cells.

We further examined the lamina propria to determine whether differences in T-cell populations existed. Grossly, all groups of patients demonstrated similar numbers of CD3+CD8+ cells in the LP populations with a 2:1 CD4/CD8 ratio (Fig. 6).

However, when we performed morphometric analysis to quantify differences in cell populations, there was a trend towards increased numbers of CD3+ cells/mm² in CVID compared to normal controls (Fig. 7). These changes reflected increases in CD8+ cell populations but not in CD4+ T cells. Within the CVID group there was no correlation with the presence or absence of inflammatory disease and the increase in CD3/CD8+ T cells.

DISCUSSION

Several prior studies have reported an increased incidence of IBD and inflammatory GI diseases in patients with CVID, ranging from 2%–13%. In fact, the presence of such concomitant disease far exceeds the incidence of these inflammatory diseases seen in the general population. The reason(s) for this observation are poorly understood but growing evidence suggests that the presence of immune dysregulation may preferentially express itself as inflammation in the intestine. This has been most visible and reinforced in the growing numbers of murine models of IBD where genetically engineered alterations in cytokines or T-cell populations result in intestinal inflammation. Given the fact that several groups have reported defects in cytokine production by T cells in CVID patients our hypothesis driving this study was that such defects are responsible for the increased incidence of mucosal inflammation seen in these patients. This study describes characteristics of a unique subgroup of patients with CVID and IBD-like disease. Like classical IBD or celiac disease patients, these patients have symptoms of chronic diarrhea, weight loss, and malabsorption. For many patients, GI disease may be the first and only clinical presentation of their underlying immunodeficiency and others may develop GI complications during the course of their disease, making recognition of such a disorder important. Why some individuals with CVID develop GI disease and others do not is still not well understood. The diverse clinical manifestations of CVID suggest that the pathogenesis of this disorder is complex, reflecting multiple dysregulated pathways. In our study we aimed to determine whether defects in peripheral and intestinal lymphocytes exist that may be involved in the disruption of mucosal homeostasis. Identification of immune parameters associated with this subgroup of patients may allow early diagnosis and help to establish more specific and targeted therapies.

By definition all patients with CVID have reduced serum immunoglobulins and impaired antibody responses; however, there is variability in the degree to which these immunological parameters are affected. One previous study had documented that the presence of villous atrophy in CVID patients is associated with anemia, malnutrition, and low blood CD4+ lymphocyte levels.¹⁶ In this cohort of patients the clinical profile inclusive of

immunoglobulin levels and lymphocyte numbers of the CVID patients with IBD-like disease was comparable to those CVID patients without GI disease, making these parameters of limited use in characterizing this subset of patients. Whether or not alterations in other cell types that are involved in the inflammatory response such as NK cells or macrophages exist will require further study. We did not measure other markers of inflammation including erythrocyte sedimentation rate and C-reactive protein; however, prior studies have reported the absence of an increase in these peripheral blood markers in CVID patients with GI symptoms.¹⁵

B- and T-cell responses to proliferative stimuli (mitogen response and cytokine production) are also variable in this heterogeneous population of patients. Studies that have attempted to define a distinct cytokine profile responsible for the inflammatory changes seen in CVID patients with IBD-like disease have been inconsistent, likely because of the variability within CVID patients itself and the fact that multiple pathways might be involved in the dysregulated state. We observed modest reductions in peripheral lymphocyte cytokine production of IL-2, IL-10, IFN- γ , and TNF- α in CVID/IBD patients following TCR-dependent stimulation that were less apparent or partially corrected following stimulation by the TCR-independent polyclonal activators PHA/PMA. However, the trends toward reduced cytokine production in this group persisted, suggesting that abnormal cytokine production may be involved in the development of intestinal inflammation. A role for T cell dysfunction in patients with immunodeficiency and GI disease is consistent with prior observations that GI inflammatory diseases are much more common in CVID patients as compared to the existence of such disorders in immunodeficiencies resulting from more pure B-cell disorders such as X-linked agammaglobulinemia.

At the mucosal level, we were unable to document constitutive production of inflammatory cytokines by LPLs in the different patient groups. The absence of such cytokines, even in patients with documented IBD, however, may reflect loss of mRNA and protein following prolonged isolation procedures used to purify these cells. Alternatively, gut inflammation in CVID patients may be mediated by cytokines that were not studied here (e.g. IL-13). Additionally, IBD patients in this study may have been receiving immunomodulatory therapies including prednisone, azathioprine, or 6-mercaptopurine in addition to infliximab; the effects of these treatments on disease remission may have played a role as to why no differences were seen. However, given the histologic changes observed in these patients, a more detailed examination of LPL phenotypes may yield more consistent findings.

The GI tract in CVID patients has been reported to display many histologic patterns whose features can mimic lymphocytic colitis, collagenous colitis, celiac disease, lymphocytic gastritis, granulomatous disease, and IBD, which is consistent with what we observed in our CVID population. Most consistently, our CVID samples demonstrated a lack of intestinal plasma cells by H&E and IgA and IgM-producing cells in the lamina propria by IF, making this a useful feature for diagnosis of CVID in someone presenting with treatment-refractory GI disease in the absence of infectious or autoimmune manifestations of CVID.

Phenotypically there were trends toward higher CD3⁺ cells/mm² in the lamina propria of CVID patients compared to normal controls, again suggesting that T cells may play a more direct role in the predisposition to GI disease seen in patients with CVID. These trends reflected changes in CD8⁺ cell populations but not in CD4⁺ T cells. However, within the CVID group there was no correlation with the presence or absence of inflammatory disease and these increases in T cells. A more comprehensive investigation of cell populations may yield phenotypic differences within patients with CVID and IBD-like disease.

In summary, the immune parameters measured in this study failed to document statistically significant differences between CVID patients with and without IBD. However, the trends observed supporting T-cell alterations imply that severe defects in immunocompetence/immunoregulation may predispose to mucosal inflammation in CVID patients and help identify these patients. To characterize more distinct differences a broader range of immune parameters and a larger population of patients will need to be studied. CVID is a disease that is, by definition, variable. Diverse clinical manifestations and underlying immune defects make the study of this disorder quite challenging.

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FIGURE 1.
Mean quantitative immunoglobulins (mg/dL) at diagnosis of CVID.

FIGURE 2.

Peripheral T cell cytokine production after anti-CD3/CD28 stimulation. CVID/IBD patients demonstrate defective cytokine secretion by anti-CD3/CD28 stimulated peripheral T cells.

FIGURE 3. Peripheral T-cell cytokine secretion after stimulation with *PHA/PMA. CVID/IBD patients demonstrate near normal cytokine secretion after stimuli that bypassing TCR.

FIGURE 4.
Cytokine mRNA real-time PCR from freshly isolated LPLs.

FIGURE 5.

Intestinal histology (H&E 40×). CVID intestinal biopsies lack intestinal plasma cells. This is in contrast to specimens collected from IBD patients as shown by the arrows.

FIGURE 6.
Immunofluorescence CD3 (green), CD8 (red), DAPI (blue) in the three patient groups.

FIGURE 7. Morphometric analysis of T lymphocyte populations. CVID patients (inclusive of patients with and without inflammatory disease) had an increase in CD3+ and CD8+ T cells in the lamina propria compared to normal or IBD controls.

TABLE 1

Lymphocyte Numbers and Response to Mitogens in CVID Patient Subgroups

Group (Normal Range)	% T cells $\pm$ SE (55–88)	% B cells $\pm$ SE (5–15)	PHA $\pm$ SE (20,000–90,000)	Con A $\pm$ SE (7500–45,000)	PWM $\pm$ SE (2500–30,000)
CVID without GI disease ($n = 16$)	74 $\pm$ 3	12 $\pm$ 3	27,004 $\pm$ 5849	11,703 $\pm$ 2961	5795 $\pm$ 1216
CVID+ inflammatory GI disease ($n = 12$)	73 $\pm$ 4	10 $\pm$ 3	20,682 $\pm$ 8331	9263 $\pm$ 3531	6988 $\pm$ 2466

Common variable immunodeficiency (CVID), gastrointestinal (GI), phorbol 12-myristate 13-acetate (PMA), phytohemagglutinin (PHA), concavalin A (Con A), pokeweed (PWM).