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Cellular Infiltration and Cytokine Expression Correlate with Fistulizing State in Crohn's Disease[▽]

Saleh A. Naser,[1]* Claudia Romero,[1] Princess Urbina,[1] Najih Naser,[2] and John Valentine[3]

Department of Molecular Biology and Microbiology, Burnett School of Biomedical Sciences, College of Medicine, University of Central Florida, Orlando, Florida[1]; Division of Biomedical Research, BioSynse, Cary, North Carolina[2]; and Department of Medicine, University of Florida, Gainesville, Florida[3]

The purpose of this study was to determine the degree of infiltration of different cell subpopulations (tissue dendritic macrophages, T-helper cells, cytotoxic T lymphocytes, monocytes, neutrophils, and B cells) and the expression of the cytokines interleukin-12 (IL-12) and tumor necrosis factor alpha (TNF-α) in inflamed and noninflamed resected tissues from Crohn's disease (CD) and non-CD patients. Twenty-one resected full-thickness intestinal tissue specimens representing 13 subjects (8 CD and 5 non-CD patients) were included in this study. Sections of 20 μm in thickness were cut and then stained using immunohistochemistry. The sections were analyzed using confocal laser scanning microscopy (CLSM). Patterns of staining for inflamed CD and noninflamed CD tissues versus non-CD tissues demonstrated significant differences in the macrophage and T-helper subpopulations. Surprisingly, the T-helper subset was decreased significantly in the inflamed CD sections compared to the noninflamed CD and non-CD sections. The staining patterns also suggested differences in the expression of both IL-12 and TNF-α between the groups, with cytokine overexpression directly relating to the fistulizing state in CD patients. Cytokine expression is upregulated in chronic CD patients; therefore, the degree of inflammation and tissue damage in CD is dependent on the expression of specific cytokines within the tissue. Differentiation of cell subpopulations may be important for establishing a direct relationship with each state of CD (inflammatory, stricturing, and fistulizing states).

Crohn's disease (CD) is a chronic inflammatory bowel disease (IBD) of uncertain etiology affecting more than 500,000 people in the United States alone (5). This disorder affects the small intestine and/or the large intestine. Inflammation extends through all layers of the gut wall to involve adjacent lymph nodes. The inflammatory process is frequently discontinuous, with sections of normal bowel separating portions of diseased bowel. CD is a complex condition that may be the result of interactions between susceptibility genes, bacteria (if any), gut barrier defects, and immunological factors. There are a variety of cellular processes and proinflammatory mediators that influence the pathogenesis of the disease. It is believed that there is a sustained immune response in the gut in response to stimuli. CD patients suffer from marked immune system dysregulation, especially with upregulation of proinflammatory interleukin-12 (IL-12) and downregulation of IL-10, but it is not clear whether this is a cause or result of the disease.

Under normal conditions, the human gut mucosa is infiltrated by a large number of mononuclear cells. This is a reflection of the fact that the human intestine is continuously subjected to a massive stimulation by luminal antigens. It has been suggested that intracellular intestinal pathogens such as *Mycobacterium avium* subsp. *paratuberculosis* may be impli-

cated in the pathogenesis of CD, based on the pathological similarity of CD with Johne's disease in animals (8).

Evidence indicates that the type of inflammatory response occurring in the intestines of patients with CD reflects a Th1 response with high levels of IL-12 and gamma interferon (IFN-γ) production (2, 3, 11). IL-12 has also been proposed as a marker of T-cell activation in CD (12). It has been shown that phagocytic leukocytes respond to a variety of bacterial products, including Gram-negative bacterial lipopolysaccharides (LPS) and mycobacterial lipoarabinomannan (LAM). The LPS and LAM systems both require CD14 and LPS binding protein (LBP) for cellular recognition, but the LAM signaling system appears to require an additional receptor component whose expression is restricted to cells of hemopoietic origin (14). LPS derived from the outer membrane of Gram-negative bacteria interacts with CD14 on the surface membrane of macrophages, thus triggering a signal cascade which leads to the production and release of proinflammatory cytokines such as tumor necrosis factor alpha (TNF-α), IL-1, IL-6, and IL-8 (1). It has been suggested that a product from intestinal pathogens, such as the *M. avium* subsp. *paratuberculosis* cell wall, may have a similar effect on macrophages and, furthermore, may also be resistant to killing by dendritic macrophages (6, 10). These effects would result in the characteristic granulomas seen in the intestines of CD patients.

Ulcerated lesions in CD patients are accompanied by a prominent infiltrate of inflammatory cells, including T lymphocytes, macrophages, and neutrophils (17). Macrophages in CD patients have been shown to express an activated phenotype (CD14+) that is not normally expressed in a healthy intestine (17). Furthermore, mechanisms involved in recruiting and activating inflammatory cells are thought to involve a complex

* Corresponding author. Mailing address: Department of Molecular Biology and Microbiology, Burnett School of Biomedical Sciences, College of Medicine, University of Central Florida, Orlando, FL 32816. Phone: (407) 823-0955. Fax: (407) 823-0956. E-mail: nasers@mail.ucf.edu.

TABLE 1. Demographic data and information for patients and tissue specimens included in this study[a]

Patient	Sex	Age (yr)	Diagnosis	Site of disease	Length of disease (yr)	State of disease	No. of surgeries	Medicines taken	Presence of inflamed tissue[b]
1	F	25	CD	Colon	4	F	2	6MP	X
2	M	26	CD	Small bowel	9	I	1	Steroids	X
3	F	68	CD	Bowel-colon	42	F, S	3	None	X
4	F	56	CD	Bowel-colon	11	S	1	6MP	X
5	NA	NA	CD	Small bowel	20	S	1	Steroids	X
6	M	60	CD	Colon	5	I	0	Steroids	X
7	M	43	CD	Small bowel	14	I, F, S	1	Steroids	X
8	F	39	CD	NA	19	I, F, S	3	Steroids, infliximab	X
9	F	79	Non-CD	NA	NA	NA	1	None	NA
10	NA	NA	Non-CD	NA	NA	NA	1	None	NA
11	M	64	Non-CD	NA	NA	NA	1	None	NA
12	M	73	Non-CD	NA	NA	NA	2	None	NA
13	F	76	Non-CD	NA	NA	NA	1	None	NA

[a] Abbreviations: F, female; M, male; CD, Crohn's disease; non-CD, no Crohn's disease; I, inflammatory; F, fistulizing; S, stricturing; 6MP, 6-mercaptourine; X, tissue specimen available; NA, not available/applicable.
[b] Noninflamed tissue was available for all patients.

interplay of inflammatory mediators such as chemokines and cytokines (9).

Current theories predict that the nature and extent of inflammation in CD are dependent on the expression of specific cytokines and on the infiltration of specific cell subsets into the tissue. To test and confirm this hypothesis, we examined the degree of infiltration of different cell subsets (tissue dendritic macrophages, T-helper cells, cytotoxic T lymphocytes, monocytes, neutrophils, and B cells) and the expression of the cytokines IL-12 and TNF-α in inflamed and noninflamed resected tissues from CD and non-CD patients.

MATERIALS AND METHODS

Sample collection. Twenty-one resected full-thickness intestinal tissue specimens from 13 subjects (8 CD and 5 non-CD patients) were included in this study. Table 1 lists the demographic and tissue information for all patients and specimens included in this study. This study was reviewed and approved by the Institutional Review Board at the University of Florida (Gainesville, FL). Written consent was obtained from each patient prior to surgery.

Tissue sectioning. Twenty-micrometer-thick sections were cut using a cryostat and were placed onto glass microscopic slides. The sections were kept at 4°C until further use.

Immunohistochemistry. To fix the sections, 4% paraformaldehyde (PFA) was applied to the slides for 30 min at room temperature. The slides were rinsed 3 times for 5 min each with phosphate-buffered saline (PBS), and 100 mM NH₄Cl was applied to each slide for 15 min at room temperature to eliminate autofluorescence. Blocking serum (10% goat serum) was applied to the slide for 30 min and then drained, and the slide was rinsed 3 times for 5 min each with PBS. Primary antibody was applied at a final level of 1 μg and left for 1 h. Primary antibodies used in this study were monoclonal mouse anti-human IL-12 (Invitrogen), monoclonal mouse anti-human TNF-α (Invitrogen), monoclonal anti-CD1a (detects tissue dendritic macrophages; Immunotech), and monoclonal anti-CD4 (detects T-helper/inducer cells; Immunotech). The slides were rinsed 3 times for 5 min each with PBS, and fluorescently labeled secondary antibody (Oregon green-labeled goat anti-mouse IgG; Invitrogen) was applied for 1 h. The slides were rinsed 3 times for 5 min each with PBS, air dried, and mounted with antifade solution.

Image analysis. Sections were analyzed using confocal laser scanning microscopy (CLSM). The detector on the microscope was set to include light from the negative isotypic control and was kept constant for all test samples. Slides were scanned serially (20 section stacks) five times across the tissue. Using Zeiss image analysis software, a threshold was applied to eliminate pixels with no fluorescent marker (black), as well as residual noise, from the detector. The volume of tissue occupied by a fluorescent marker was then calculated. The percentage of tissue occupied by the fluorescent marker was calculated by dividing the total volume of pixels remaining after subtracting the background by the total volume of tissue

scanned. This was then expressed as a percentage to indicate the proportion of tissue occupied by the marker of interest.

Statistical analysis. The average percentage of tissue bound by each fluorescent marker was calculated for each population. The variance and mean percentage were calculated for each marker. To evaluate the significance of the differences between populations, we performed one-way analysis of variance (ANOVA) on the data. Differences with P levels of <0.05 were considered significant.

RESULTS

The mean percentages of tissue occupied by specific antigens representing individual cell subsets or cytokines were calculated for the samples listed in Table 2. Furthermore, all surface markers and cytokines were compared between each of the populations, and the significance of variation between groups was calculated and expressed as P values (Table 2). IL-12 and TNF-α expression was increased significantly ($P <$ 0.05) in both CD groups compared to the non-CD group. Similarly, the expression of tissue dendritic macrophages was increased significantly ($P < 0.05$) in inflamed CD tissue compared to non-CD tissue, but it was not significantly increased or decreased between noninflamed CD tissue and non-CD tissue samples. Surprisingly, the expression of the T-helper (CD4) subset was decreased significantly ($P < 0.05$) in inflamed CD tissue compared to noninflamed CD tissue and non-CD tissue samples.

DISCUSSION

Our results highlight an important role of cytokines such as IL-12 and TNF-α in CD, as there was an increased expression of these cytokines in both inflamed CD and noninflamed CD tissues compared to non-CD samples. Upregulated cytokine expression in CD suggests that there are links between cytokine expression and disease severity. The highest level of expression of these cytokines was observed consistently with the fistulizing type of disease, not with the expected inflammatory type. Another interesting finding was that the degree of infiltration by different cell subsets was directly correlated with the degree of gravity of the disease. The greater the infiltration, the worse pathology was observed in terms of the inflamma-

TABLE 2. Percentages of tissue occupied by different cell subtypes, surface antigens, and cytokines, with calculated statistical significance (*P* values), for specimens from CD and non-CD patients[a]

Cell subtype, antigen, or cytokine	% of tissue occupied			*P* value for inflamed CD vs noninflamed CD tissue	*P* value for inflamed CD vs non-CD tissue	*P* value for noninflamed CD vs non-CD tissue
	Inflamed CD tissue	Noninflamed CD tissue	Non-CD tissue			
B cells	0.5394	0.6631	0.4192	0.714	0.066	0.097
Macrophages	1.0164	0.6344	0.383	0.648	**0.041**	0.086
Monocytes	0.715	0.66	0.627	0.507	0.523	0.961
Neutrophils	0.8119	1.31	2.441	0.69	0.731	0.396
CD8	0.5706	0.695	0.236	0.68	0.067	0.091
CD4	0.5706	0.9933	0.7502	**0.014**	0.899	0.223
TNF-α	0.4175	0.3481	0.12	0.208	**0.049**	**0.023**
IL-12	0.3581	0.3969	0.196	0.368	**0.003**	**0.017**

[a] *P* values of <0.05 were considered significant and are shown in bold.

tion, stricturing, and fistulizing states. These results are in agreement with previous studies investigating other activation markers (4, 7, 9).

Expression of dendritic macrophages was increased only in inflamed CD lesions. Tissue dendritic cells and macrophages normally accumulate at sites of infection and inflammation (13), suggesting the activation of some mechanism in response to stimuli. One could assume that these dendritic macrophages actively process and display antigenic fragments on their surfaces in order to become professional antigen-presenting cells (APCs), ready to activate T cells upon contact. Therefore, this result is in accordance with the occurrence of CD lesions, which are thought to have an ongoing inflammatory response present. It would be expected to find an increase in the monocyte and neutrophil infiltration of inflamed tissues as well. Interestingly, the expression of these two cell subsets was not significantly different between any of the populations. The results most likely suggest that even in the presence of a significant number of cells, there is impairment in the function of these subsets.

There were no significant differences for most cell subsets studied between inflamed CD and noninflamed CD tissues, except for the CD4 T-helper phenotype. In previous studies where lymphocyte subpopulations in the intestinal mucosae of patients with CD were investigated, over 80% of cells were of the suppressor-cytotoxic phenotype (CD8), and only a small population was of the CD4 helper type (15). Maintenance of an equilibrium among the different cytokines produced by CD4$^+$ cells is important because intestinal inflammation can result if such equilibrium is disrupted. T cells are highly enriched in subpopulations of activated memory cells with helper functions. T-helper cells recognize their antigens in association with major histocompatibility complex (MHC) class II molecules, indicating primary activation by extracellular antigens such as those that occur as part of bacterial cell walls. They regulate subsequent immune responses by recognizing and activating other cells, such as cytotoxic T lymphocytes (CTLs), B cells, and macrophages. This macrophage reciprocal activation is very important in combating intracellular pathogens such as *Mycobacterium* (16). Activation of infected macrophages stimulates their ability to kill the internalized pathogen.

In conclusion, dysregulation of the intestinal immune system at the cellular level constitutes an important element in the multifactorial pathogenesis of CD. This study demonstrated significantly elevated cytokine expression in tissues from patients with CD; however, patients under immunosuppressant therapy still require surgical procedures as a form of treatment. This indicates that immunosuppressant and cytokine expression-blocking therapies are perhaps not the solution to the problem as currently thought. We observed sustained elevated levels of cytokines in the CD population as well as a significant decrease in the T-helper cell subset, which may impair immune system function as a whole. Furthermore, the infiltration of different cell subsets into the tissue may lead to persistence of inflammation. This may not be due to the number of cells present in the diseased tissue but rather to the inability of these cells to function properly. What matters is not the quantity of cells present but the efficacy with which each cell subset performs in the affected tissue. Further insights into the distributions of different cell subsets, as well as the cytokines and chemokines released during inflammation, need to be revealed. We are now pursuing a similar investigation on white blood cells isolated from CD and non-CD patients.

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