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Abstract
The exact causes of inflammatory bowel disease (IBD) are not yet fully defined. From a vast body of literature, we know that the immune response has long been involved in the pathogenesis of IBD, including both ulcerative colitis and Crohn’s disease. A variety of specific alterations can lead to immune activation and inflammation directed to the colon, as revealed by some animal models. Current research has focused on the role of antibodies in downstream events and mechanisms of autoimmunity and inflammation. It is not well known whether the production of antibodies is a serologic consequence of IBD, or if it is a result of barrier dysfunction induced by inflammation. Here, we present a new hypothesis to distinguish the complex links between genetic susceptibility, barrier dysfunction, commensal and pathologic microbial factors and inflammatory response (especially autoantibodies) in the pathogenesis of IBD. In our hypothesis, we suggest that prior activation of adaptive immunity against microbial flora antigens could initiate an IBD-like chronic inflammation if something like ethanol disturbs barrier function. If this hypothesis is supported with further experiments, it would illustrate unknown aspects of IBD pathogenesis. On this basis, we have developed a new immune-based model of IBD with the presence of antibodies against enteric bacterial antigens.

INTRODUCTION

Etiology
Investigations have demonstrated that the pathophysiology of inflammatory bowel disease (IBD) is multifactorial, but briefly host (e.g., genetics, intestinal barrier and immune system function) and exogenous factors (e.g,
normal luminal flora) are two basic themes. The normal intestine contains a large number of immune cells in a chronic state of so-called physiologic inflammation to control the gut and to prepare it for any immunologic response. Lack of immune responsiveness to luminal antigens may be a result of oral tolerance. Multiple mechanisms are involved in the induction of oral tolerance. For instance, deletion or anergy of antigen-reactive T cells or activation of regulatory T cells suppresses gut inflammation through secretion of inhibitory cytokines such as interleukin (IL)-10 and transforming growth factor β (TGF-β). In addition, a selectively permeable barrier prevents unwanted solutes, microorganisms, and luminal antigens from confronting the immune system in the internal mucosa. In IBD, this tolerance is altered and leads to an uncontrolled inflammation; thus, IBD is considered as a breakdown in the regulatory constraints on mucosal immune response to the microbial flora or their products within the intestine. Most of this process is mediated through components of the autoimmune response to self-antigens.

A variety of specific alterations can lead to immune activation and inflammation directed to the colon, as revealed in animal models demonstrating murine genetic models (transgenic models). These models showed us that deleting loci of specific cytokines (e.g., IL-2, IL-10, TGF-β) or their receptors or T cell antigen recognition molecules (e.g., T cell antigen receptors) or interfering with intestinal barrier integrity (e.g., mucus glycoprotein, deleting N-cadherin or nuclear factor κB) leads to inflammation.

It has been suggested that the continuous penetration of luminal antigens and unremitting stimulation of the mucosal immune system due to an increased permeability of the intestine epithelial cells may be the primary defect in patients suffering from IBD. Therefore, if we consider increased epithelial permeability as the trigger, the tragedy of IBD initiates after a disruption occurring in the mucosal integrity. Then lots of macromolecule antigens in the lumen penetrate into internal compartments of the mucosa and submucosa, and subsequently become recognized by the gut immune system. Later, interstitial macrophages and dendritic cells are locally activated and release cytokines to recruit more macrophages and monocytes from the systemic circulation. In normal subjects, this acute response is subsided after repair of the first alteration in intestinal permeability.

**Microbial factors**

Microorganisms are a likely factor in the initiation of inflammation in IBD. However, the unanswered question in this area is whether microorganisms involved in the pathogenesis of IBD are commensal flora or invasive microbial pathogens?

Normal intestinal microflora may contribute to the development of IBD in susceptible individuals. This finding has been demonstrated repeatedly in murine models of IBD. As an example, animals which are genetically altered (e.g., deficient in IL-2 and IL-10) to be susceptible to IBD do not develop the disease when raised under germ-free conditions. Also, intestinal lesions in IBD typically predominate in areas of the highest bacterial exposure (e.g., in distal ileum and colon with 10^10 organisms/g).

On the other hand, a number of studies have evaluated the possible role of specific infectious agents in the pathogenesis of IBD. This role has been evaluated in two ways: the relation between specific microorganisms and IBD (e.g., presence of specific antibodies in serologic findings of IBD patients), and the association between some acute gastroenteritis and IBD.

Pathogens that could be directly responsible for initiating IBD are those that the mucosal immune system may fail to control in terms of the inflammatory response (e.g., *Salmonella* sp., *Shigella* sp). These bacteria are rich in peptides having chemotactic properties (e.g., formyl-methionyl-leucyl-phenylalanine). The super-antigens capable of global T-lymphocyte stimulation and subsequent inflammatory response, and those producing toxins (necrotoxins, hemolysins, and enterotoxins), cause mucosal damage. In summary, an acute infection with specific pathogens leads to a permanent uncontrollable perturbation in intestinal integrity, even though after the acute phase there is perhaps mediation of some cytokines (e.g., IFN-γ), and permeability changes across the epithelium are induced. This results in continuous exposure and stimulation of the mucosal immune system with commensal flora antigens.

**Immune regulation and inflammatory cascade defects in IBD**

As discussed later, the mucosal immune system is normally nonresponsive to luminal contents due to oral tolerance. Once inflammation is initiated, the immune inflammatory response is propagated by T cell activation in the lamina propria. CD4 T cells are of three major types: TH1, TH2, and TH17 cells. The TH1 cells secrete predominantly IFN-γ, TNF-α, IL-2, and IL-12, which
activate cell-mediated immunity by CD8 T cells (cytotoxic) residing in transmural granulomatous inflammation resembling CD. Meanwhile, the TH2 cells can induce B-cell differentiation and humoral immunity by secreting predominantly IL-4, IL-5, and IL-13 with superficial mucosal inflammation features resembling UC[25]. TH17 cells secrete predominantly IL-17, IL-6, and granulocyte colony-stimulating factor and seem responsible for neutrophilic recruitment[8,23]. After activation of these cells, they produce specific cytokines and, consequently, the epithelial barrier permeability (e.g., IFN-γ) is increased. Some of these cytokines have destructive and apoptotic effects on mucosal cells, which eventually allow more antigens to pass and produce more agitation of immune cells amplifying the inflammatory cascade[3,4]. In normal situations, an activated response is subsided with regulatory T cells, including designated TH3, Tr1, and CD4, and CD25 cells[28]. Their function is blocking or down-regulating the response of TH1 and TH2 either by producing specific cytokines (IL-10 and TGF-β) or via cell-cell contact. There is evidence which demonstrates some defects in this regulatory system in IBD-susceptible subjects[24,25].

INTESTINAL BARRIER DYSFUNCTION

The intestine is covered by a monolayer of simple columnar and non-ciliated epithelial cells that are a type of brush border cells. These are joined together by intercellular and circumferential tight junctions to form a selectively permeable membrane. This barrier prevents unwanted solutes, microorganisms, and luminal antigens from entering the internal parts. They are also part of the immune system, acting as a first-line pathogen-recognition system because they present antigens similar to classical APC. They also express toll-like receptor (TLR) 4 and, furthermore, secrete antimicrobial peptides (e.g., cryptidins and defensins)[3,4,8,26]. However, the epithelial barrier has some guards of the innate immune system to ensure permanent immune responsiveness (e.g., DC, interstitial macrophages)[27]. If anything alters the barrier function, lots of luminal antigens could pass through the submucosal layer resulting in recruitment of neutrophils and macrophages. If these cells can control the invasion, it is not necessary to call adaptive immunity components, but if the invasion takes long then adaptive immune response component will be activated. In this process, if the regulatory systems are not able to overcome the inflammatory cascade, the secreted cytokines will deteriorate and amplify the first defect in the epithelial barrier by inducing apoptosis and necrosis in the epithelial cells. In addition, a number of studies have shown that inflammatory cytokines like TNF-κ and IFN-γ may have a role in increasing intestinal barrier permeability[3,4,8]. Some animal models of IBD have shown alterations in barrier function as the first trigger contributing to pathogenesis of IBD. Furthermore, abundant evidence indicates an increased intestinal permeability in IBD patients suggesting the permanent stimulation of the mucosal immune system as the primary defect in the pathogenesis of IBD[3,4,8,26].

STEPS OF AUTOIMMUNITY IN IBD

The pathogenesis of IBD and most of its extra-intestinal manifestations is immunologically mediated and appears to be mainly due to an autoimmune-related process[9,31]. As discussed, after a permanent alteration in barrier function, various antigens pass through the interstitial space which finally activates T cells. In normal subjects, the response is directed definitively against the specific epitope of antigens, but commensal organisms in the lumens have adhesive antigens (e.g., flagellar antigens) which adhere to the surface proteins of mucosal cells. If there are some predisposing factors, then there is a chance for APCs to process epitopes of these antigens, with parts of the mucosal surface proteins, which activate T lymphocytes against mucosal cell surface protein[9,31]. Another scenario happens when the response to the specific epitopes of antigens is cross-reactive to auto-antigens. There is evidence demonstrating relations between precise human leukocyte antigen (HLA) molecules and cross-reactive cellular antigens[8,9,31]. However, in the TH2-mediated immune response in UC, it is thought that perhaps development of self-reactive B cells, which are triggered to produce mucosal IgG autoantibodies, results in an inflammatory response. Meanwhile, TH1 cell-mediated immunity and auto-reactive T cells (CD4 or CD8) may be primed by microbial antigens that are cross-reactive to autoantigens[10].

A long series of studies demonstrated that IBD patients possess autoantibodies, some of which became serologic biomarkers to diagnose or distinguish subtypes of this disease, such as anti-lymphocyte, anti-goblet cell, pancreatic autoantibodies, the autoantibody against tropomyosin isoform 5 (a cytoskeletal protein found in colon epithelial cells), and antibodies against red blood cell membrane antigens that cross-react with entero-pathogens such as Campylobacter sp[1,8,34,35].

We will now discuss some of the known autoantibodies in IBD pathogenesis. There is a form of perinuclear antineutrophil cytoplasmic antibody (pANCA) which is non-reactive to myeloperoxidase. It is well defined that 60%-70% of UC patients and 5%-15% of their first-degree relatives are pANCA-positive, whereas this applies to only 2%-3% of the general population. There is a relation between positive pANCA antibody status and severity of UC disease and other complications. Interestingly, pANCA in CD is associated with colonic disease that resembles UC[9,34,35]. The definite antigens to which these antibodies are directed have not been identified, but they have cross-reactions with enteric bacterial antigens.

Other studies demonstrated the presence of another
autoantibody, which is specific to patients with UC; it is an IgG autoantibody bound to a subtype of tropomyosin of colonic epithelial cell antigen. The capability of this antibody to initiate extracellular signal-regulated kinase (ERK) 1/2 signaling and up-regulating of the TLR and production of cytokines, and also the correlation between the titers of this antibody and the severity of colitis, suggest the possibility that such a protein could represent autoantigen- or complement-mediated responses.[13,31]

Although the presence of antibodies directed against microbial antigens has been illustrated in the serum of CD patients, a shared epitope among the host antigens is not clearly defined. For example, 55% of CD patients have antibodies against outer membrane porin C of Escherichia coli, and 50% have immunoglobulins that are reactive to a homologue of the bacterial transcription-factor families from a Pseudomonas fluorescens-associated sequence (I2). Around 50% of CD patients have serum reactivity to Cbir1, an immunodominant antigen of the enteric microbial flora. This antigen can strongly induce B cells and CD4+ T cell responses. Transferring of Cbir1-specific CD4+ TH1 T cells to C3H/SCID mice generates a severe colitis dependent on exogenous expression of Cbir1 flagellin in the colon. In 60%-70% of CD patients, anti-Saccharomyces cerevisiae antibodies have been found. A mannose sequence in the cell wall of this commensal flora has been defined[35,36].

HYPOTHESIS

Although the above-mentioned studies support the concept of the presence of antibodies against enteric bacterial antigens in IBD, we propose a model to investigate whether the production of antibodies is a result of barrier dysfunction induced by inflammation or a serologic finding secondary to IBD. The hypothesis would result in a reliable model of IBD studies in animals. Our hypothesis suggests the possibility of subcutaneous vaccination of animals with administration of all or specific enteric bacterial antigens. In this way, production of immunoglobulin against these antigens would prevent intestinal inflammation. Anything that alters the function of this barrier and increases barrier permeability would result in inflammatory responses. To test this hypothesis, we have designed a pilot study and examined the model in male Wistar rats, which were immunized with anaerobic and aerobic enteric bacteria with and without an adjuvant. After assessing the IgG titers in the rats’ plasma, well-immunized rats were anesthetized and then chitosan and ethanol were instilled intrarectally as a tight junction opener and a barrier breaker, respectively. This protocol induced a chronic inflammatory response with inflammatory features in the ethanol group with persistent le-
sions. We propose that this model of chronic intestinal inflammation would be a reliable model of human IBD. Of course, further studies would need to prove immunization with specific bacteria (Figures 1 and 2).

CONCLUSION

In this article, we addressed some known immune derangements involved in the initiation and pathogenesis of IBD. The following general principles are highlighted for better understanding of the possible mechanisms involved in the IBD pathogenesis.

Increased barrier permeability secondary to a genetic susceptibility, a specific infectious pathogen or their toxins and activation of T cells create a positive feedback to amplify the first barrier dysfunction and initiate an inflammatory cascade.

Two common features of autoimmunity processes may differ in activation of autoreactive T or B cells, involving a variety of imbalances in cytokine production and the development of autoantibodies. In IBD, these antibodies are directed against shared enteric flora antigens and epithelial cell-surface proteins.

In this study, we focused on autoantibodies. It is not well defined whether various autoantibodies found in the serologic assessment of IBD patients are destructive or involved in pathogenesis of the disease, or whether they are produced after tissue damage due to releasing of sequestered antigens[31]. We suggest that antibodies which are secreted in UC are catastrophic and are involved in the inflammatory response, but antibodies which are produced in CD are not involved in the pathogenesis and are secreted post-release of sequestered antigens. However, antibodies in both UC and CD patients are involved in extraintestinal complications, while there are various overlaps between these two subtypes.

In our hypothesis, we suggest that prior activation of adaptive immunity against microbial flora antigens in the way described could initiate an IBD-like chronic inflammation (especially in UC). Further experiments are essential to test various aspects of the method and unknown points of IBD pathogenesis.

Empirical data

After developing the hypothesis, we designed a pilot study. Six groups of male rats containing three rats in each group were considered. An extemporaneous vaccine was prepared with a mixture of heat-treated colonic commensal bacteria, which were obtained from cultured samples, and complete Freund's adjuvant. This vaccine was injected subcutaneously into nine rats on days 0 and day 14. On day 28, a blood sample was taken from each rat to assess immunoglobulin titers. All of the test animals showed an elevated titer. Then these rats were divided into three groups; intra-colonic ethanol 30% was instilled in two groups, and in the third group, normal saline was instilled instead of ethanol and this group was assigned as the vaccine group. The two groups which
The animals were sacrificed, and colon samples were removed for histopathological assays. Details of microscopic assessments are described in Figure 3.

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