

The Epidermolysis Bullosa Acquisita Antigen (Type VII Collagen) is Present in Human Colon and Patients with Crohn's Disease have Autoantibodies to Type VII Collagen

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Epidermolysis bullosa acquisita is an autoimmune blistering disease of the skin characterized by IgG autoantibodies against type VII collagen. Systemic diseases are often associated with epidermolysis bullosa acquisita, Crohn's disease being the most frequent. This study sought to determine if type VII collagen, the epidermolysis bullosa acquisita autoantigen, was present in normal human colon by western blotting and immunofluorescence. The 290 kDa type VII collagen α chain was demonstrated by western blotting in four normal intraoperative colon specimens. Antibodies to type VII collagen labeled the junction between the intestinal epithelium and the lamina propria. We also used an enzyme-linked immunosorbent assay to test sera

from patients with Crohn's disease (n = 19), ulcerative colitis (n = 31), celiac disease (n = 17), rheumatoid arthritis (n = 15), and normal controls (n = 16). It was found that 13 of 19 patients with Crohn's disease and four of 31 patients with ulcerative colitis demonstrated reactivity to type VII collagen. Sera from control subjects, patients with celiac disease or rheumatoid arthritis were negative. The sera from Crohn's disease patients also reacted with type VII collagen by immunoblot analysis. It was concluded that patients with inflammatory bowel disease may have IgG autoantibodies to type VII collagen, which exists in both the skin and the gut. **Key words:** autoimmunity/inflammatory bowel disease/skin bullous diseases. *J Invest Dermatol* 118:1059–1064, 2002

Inflammatory bowel disease (IBD) has numerous cutaneous associations, including pyoderma gangrenosum, erythema nodosum, oral ulcers, annular erythemas, and vascular thrombosis (Raab *et al*, 1983). Epidermolysis bullosa acquisita (EBA), an acquired severe subepidermal blistering skin disease, is also recognized as one of the extraintestinal manifestations of IBD. IBD has been reported to be present in approximately 30% of cases of EBA, but many of these observations were made before modern diagnostic criteria for EBA were established (Sherry and Dothridge, 1962; Labeille *et al*, 1988). Crohn's disease (CD) is the most frequently associated condition with EBA, noted in at least 25 cases in the literature (Livden *et al*, 1978; Chan *et al*, 1996). Only four cases of EBA associated with ulcerative colitis (UC) have been reported (Hughes and Horne, 1988). EBA has been reported in association with a number of other systemic diseases, such as rheumatoid arthritis, Hashimoto's thyroiditis, diabetes, amyloidosis, and systemic lupus erythematosus (Chan *et al*, 1996).

The pathogenesis of IBD has not been established. Environmental factors, genetic factors, microorganisms, chemical

mediators, and defects in the gut immune system may all play a part. There is circumstantial evidence that autoimmune mechanisms may play an important part in IBD; however, a putative autoantigen has not been identified (Braegger and MacDonald, 1994). Immunomodulatory drugs are used in the treatment of patients with IBD (Hanauer, 1996). These drugs include azathioprine, mercaptopurine, corticosteroids, cyclosporine, and infliximab. These agents have effects on cellular and humoral immune function. They also inhibit the production and action of cytokines and inflammatory mediators (Hanauer, 1996).

EBA is characterized by autoimmunity to type VII collagen. Several investigators reported that type VII collagen was confined to basement membranes beneath stratified squamous epithelium such as skin, oral, anal, and vaginal mucosae (Paller *et al*, 1986; Visser *et al*, 1993). It was reported that type VII collagen was not present in normal colonic mucosa (Paller *et al*, 1986; Visser *et al*, 1993). More recently, punctuate type VII collagen staining was noted in normal colonic epithelium by immunofluorescence (Lohi *et al*, 1996). The purpose of this study was to investigate whether normal colonic epithelium contains full-length type VII collagen and to investigate whether patients with IBD have autoantibodies to type VII collagen.

MATERIALS AND METHODS

Diagnosis of EBA Fifty-one patients whom we have seen in the Dermatology Clinics at the University of North Carolina, Stanford Medical Center and North-Western University Medical Center were evaluated. The diagnosis of EBA was made based upon the following

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Abbreviations: CD, Crohn's disease; EBA, epidermolysis bullosa acquisita; IBD, inflammatory bowel disease; NC1, 145 kDa noncollagenous domain of type VII collagen; UC, ulcerative colitis.

criteria: (i) an active, chronic, mechanobullous disorder; (ii) subepidermal blisters on routine light microscopy of skin; (iii) IgG deposits detected at the dermal-epidermal junction by routine direct immunofluorescence; (iv) IgG deposits localized to the dermal floor of the patient's skin when the dermal-epidermal junction was fractured through the lamina lucida by treatment with 1 M NaCl (Woodley *et al*, 1983; Gammon *et al*, 1992); (v) IgG deposits detected within the sublamina densa zone of the dermal-epidermal junction using direct immunoelectron microscopy (Yaoita *et al*, 1981). Patients meeting the above criteria were identified and questioned for a previous history of symptoms related to bowel function. Previous medical records and endoscopy procedures were reviewed. Approximately half of these patients and their incidence of IBD were published previously (Chan *et al*, 1996).

Diagnosis of IBD Patients with IBD with no notable skin disease were seen at St James Hospital and Trinity College in Dublin, Ireland by three of the authors (N.M., D.K., and D.W.) who are faculty in the Division of Gastroenterology. The diagnoses of CD and UC were based on clinical evaluations, radiographic findings, endoscopic studies, and biopsies of intestinal mucosa sent for pathology.

A number of EBA patients who were identified in our files also carried the diagnosis of IBD, predominantly CD. The diagnosis of IBD in these EBA patients was accepted if the diagnosis of IBD was made by a specialist in gastroenterology, if endoscopic and biopsy studies were performed and if the patient had been followed and treated by the gastroenterologist for IBD for more than 1 y.

Colon extract preparation/immunoblotting Full thickness colon samples from patients were obtained immediately after resection for carcinoma. The normal epithelium was obtained at least 7 cm away from areas of tumor. The samples were placed in a cold cocktail of protease inhibitors containing phosphate-buffered saline (PBS), 10 mM ethylenediamine tetraacetic acid, and 2 mM cysteine at pH 7.5 and at 4°C. The samples were frozen at -80°C until western blotting or immunostaining was performed. The samples were then washed with a sterile solution of 2 mM ethylenediamine tetraacetic acid in cold water three times. Colonic extracts were prepared by pulverizing the tissue with liquid nitrogen. The pulverized tissue was then placed in sample buffer (0.125 M Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS) containing 10 mM dithiothreitol and 1 mM phenylmethylsulfonyl fluoride. The samples were then vortexed, sonicated, and boiled for 5 min. The supernatant was removed and subjected to western analysis (*vide infra*).

Ten micrograms of colonic extract (per lane) were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using a 6% slab gel. After the protein separation by SDS-PAGE, the proteins were electrophoretically transferred to a nitrocellulose membrane (Towbin *et al*, 1977). The membrane was blocked overnight at 4°C with 5% nonfat dry milk in 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% Tween-20 (TTBS) and then incubated for 2 h at room temperature with a polyclonal antibody to the NC1 domain of type VII collagen diluted in TTBS with 1% bovine serum albumin (BSA; 1:2000) (Chen *et al*, 1997a). The blots were washed, and then incubated for 1 h with a peroxidase-conjugated antibody to rabbit IgG at a dilution of 1:1000 in TTBS with 1% BSA (Sigma, St Louis, MO). The blots were then developed using enhanced chemiluminescence (Amersham, Buckinghamshire, U.K.). Human neonatal foreskin extract was used as a positive control. Normal rabbit serum was used as a negative antibody control at a dilution of 1:2000.

Immunofluorescence Full thickness colon samples from two patients were obtained immediately after resection and embedded in OCT compound. The tissue was cut on a Cryostat set at 5 µm and air-dried on glass slides. Sections were incubated with either LH 7.2 (Sigma), a monoclonal antibody to type VII collagen, diluted 1:25, or a polyclonal antibody to the NC1 domain of type VII collagen, diluted 1:200, at room temperature for 2 h. After the incubation period, the sections were washed three times with PBS and then overlaid with fluorescein-conjugated goat anti-mouse or anti-rabbit IgG (Dako, Carpinteria, CA), diluted 1:25, and incubated at room temperature for 1 h. The sections were then washed three times with PBS, mounted in 50% glycerol-PBS and examined with a Zeiss fluorescence microscope equipped with epilumination. Human neonatal foreskin samples sectioned and labeled with the same antibody served as positive controls. Normal colonic mucosa incubated with normal mouse IgG or normal rabbit serum, at the same dilution as the primary incubation step, served as negative controls.

In addition to immunostaining intestinal mucosa for type VII collagen, serum samples from the patients without EBA but who had IBD were

tested for their ability to label salt-split human skin and monkey esophagus by indirect immunofluorescence as previously described (Woodley *et al*, 1984). Sera were diluted in PBS containing 10% BSA at 1:5 and 1:10 dilutions and tested in a "blinded" fashion along with positive (EBA sera) and negative controls.

ELISA using recombinant NC1 A recently developed enzyme-linked immunosorbent assay (ELISA) using the recombinant NC1 domain of type VII collagen as the substrate was used to detect type VII collagen autoantibodies in the sera of patients with IBD (Chen *et al*, 1997b, >c). The ELISA was performed as described (Chen *et al*, 1997c). Celiac disease sera were used as a relevant bowel disease control. Rheumatoid arthritis sera were used as an autoimmune disease control and normal human sera were used as a negative control. All IBD and celiac disease patients had biopsy-proven disease. Information available on each patient included the clinical activity of the disease at the time of blood sampling and the level of inflammatory markers (erythrocyte sedimentation rate and C-reactive protein). All of the serum samples were obtained from a cohort of patients and normal subjects from Dublin, Ireland.

Immunoblotting of recombinant NC1 protein with CD sera Purified recombinant NC1 protein (100 ng per well) was run on a 6% SDS-PAGE, then electrophoretically transferred on to a nitrocellulose membrane. Cut strips of the nitrocellulose membrane were blocked overnight at 4°C with 5% BSA in TTBS. After washing with TTBS, the strips were incubated for 1 h at room temperature with sera from CD patients or normal control subjects. All sera were diluted 1:50 in 1% BSA/TTBS. The strips were then washed three times with TTBS. Immunoreactivity was detected using a horseradish peroxidase-conjugated goat anti-human IgG (Organon-Teknika-Cappel) diluted in TTBS with 1% BSA (1:5000) for 30 min and enhanced chemiluminescence (Amersham).

RESULTS

Twenty-five percent of EBA patients have IBD IBD has been recognized as one of the more common systemic illnesses associated with EBA (Roenigk *et al*, 1971; Chan *et al*, 1996). We have seen, identified, diagnosed and treated 51 patients with EBA since 1986. All 51 patients met the criteria outlined above for the diagnosis of EBA. Based upon complete evaluations by gastroenterologists, including endoscopy and intestinal biopsies, 12 of the 51 patients had CD and one had UC associated with EBA. These patients had their IBD diagnosed and managed by an independent gastroenterologist. Therefore, in our series the incidence of IBD in these patients with EBA is 25% (Chan *et al*, 1996). In addition, we have four EBA patients who do not have the diagnosis of IBD and have not sought medical care for gastrointestinal problems, but who were unable to tolerate even one 0.4 mg tablet of colchicine without experiencing the onset of diarrhea and cramping. Interestingly, all but two of 13 patients with EBA and IBD had the onset of their IBD at least 2 y before the onset of their EBA.

Full-length type VII collagen is detectable in colonic mucosa using western blotting Using immunoblot analysis, we demonstrated that colonic extracts contain type VII collagen. In **Fig 1**, the anti-type VII collagen antibody labeled a 290 kDa band and a 145 kDa NC1 band in all four intraoperative colon samples (lanes 2-5), whereas no bands were detected with normal rabbit serum (lane 1). The 290 kDa band was demonstrated to be type VII collagen because a comigratory band was detected in a parallel lane of authentic type VII collagen extracted from human neonatal foreskin (lane 6).

Type VII collagen is detectable in human colonic basement membrane In **Fig 2**, we used the mouse monoclonal antibody LH 7.2 and the polyclonal antibody to the NC1 domain of type VII collagen to demonstrate type VII collagen immunoreactivity in the basement membrane of the colon samples. This immunoreactivity displayed a strong linear pattern using the NC1 antibody (**Fig 2c**), and a granular pattern using the less sensitive LH 7.2 (**Fig 2d**). There is no basement membrane staining in the negative control panels using either normal rabbit (**Fig 2a**) or mouse sera (**Fig 2b**). As expected, control normal human skin displayed a strong, linear

pattern of distribution of type VII collagen at the dermal-epidermal junction using the NC1 antibody (**Fig 2e**) or LH 7.2 (**Fig 2f**).

Sera from CD patients reacted with NC1 by ELISA We previously developed a specific and sensitive ELISA to detect autoantibodies circulating in the sera of patients with EBA and bullous systemic lupus erythematosus using recombinant NC1 (Chen *et al*, 1997b). Using this assay, we tested the sera from

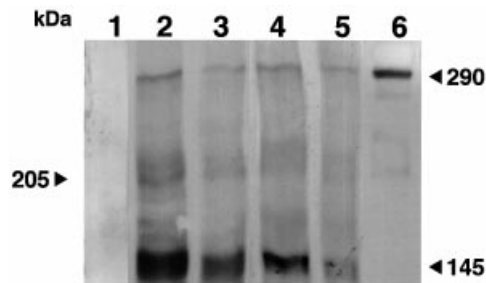


Figure 1. Colonic extracts contain type VII collagen. Colonic extracts (10 μ g per well) from postoperative colon (lanes 2–5) or foreskin extract (lane 6) were subjected to 6% SDS–PAGE and electrophoretically transferred on to a nitrocellulose membrane, before incubation with a polyclonal antibody to NC1 (1:2000) and horseradish peroxidase-conjugated antibody to rabbit IgG followed by enhanced chemiluminescence detection. Lane 1 is the postoperative colon immunoblotted with normal rabbit serum (1:2000). The positions of the 290 kDa type VII collagen, 145 kDa NC1, and the molecular weight marker are indicated.

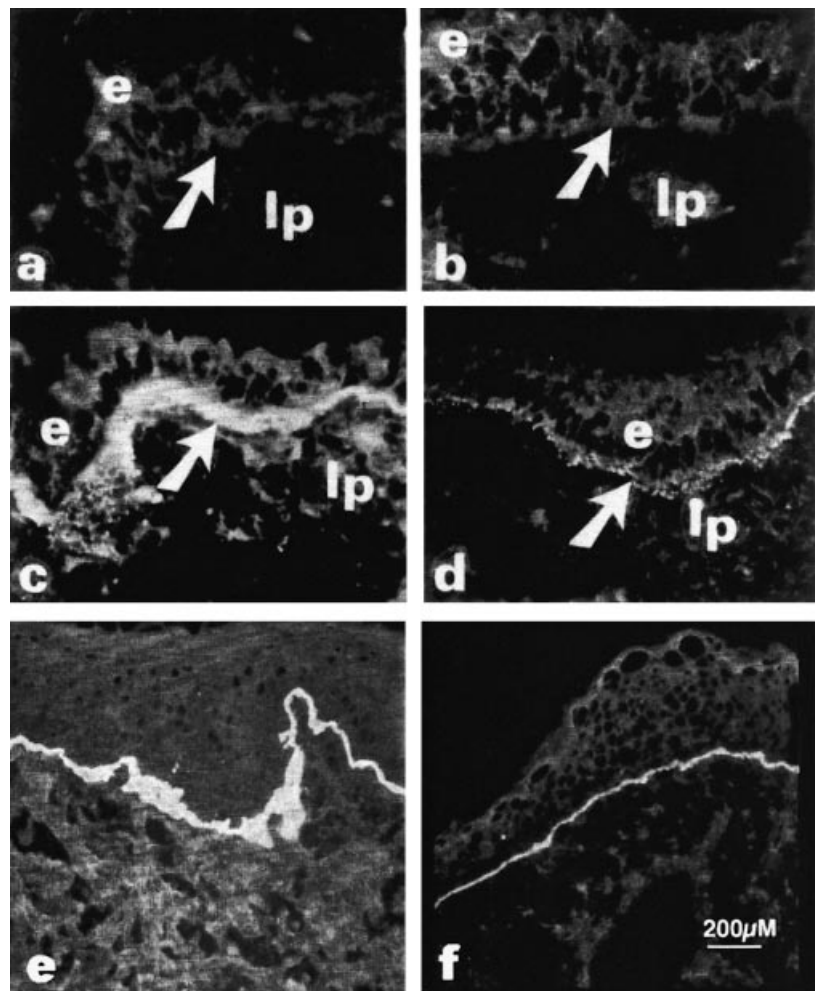
patients with CD (19), UC (31), rheumatoid arthritis (15), celiac disease (17), or normal human subjects (16 NHS). We also included 15 EBA sera as our positive control. All sera were run in the ELISA at a 1:200 dilution. The results of this analysis are shown in **Fig 3** as a scatter plot. The results from negative control sera (16 NHS) were analyzed to set an appropriate threshold for this assay. All the control sera showed very low reactivity in the ELISA with values less than 0.25. We set 0.3 OD arbitrarily as the threshold for the (ELISA) (horizontal line in **Fig 3**). All the autoimmune disease control sera (15 rheumatoid arthritis) and gastrointestinal disease control sera (17 celiac disease) showed low reactivity with OD values less than the threshold. In contrast to the controls, 13 sera from 19 CD patients and four sera from 31 UC patients exhibited reactivity with the recombinant NC1 in the ELISA. OD values ranged from 0.38 to 1.24. As expected, all 15 EBA sera showed the strong reactivity for NC1 with OD ranged from 0.9 to 1.78.

In order to determine whether the five sera from CD patients that had weak reactivity in the ELISA (OD = 0.35–0.5) at dilutions of 1:200 actually contained autoantibodies for NC1, we performed a serum dilution titer study (**Fig 4**). Five sera showed increased reactivity as the sera dilution decreased from 1:1250 to 1:10. In contrast to this dilution-dependent reactivity for NC1, sera from four NHS did not show altered reactivity at different dilutions in the ELISA.

Sera from CD patients reacted with NC1 by immunoblot analysis The CD sera were further analyzed by immunoblotting against recombinant NC1. As shown in **Fig 5**, the 145 kDa recombinant NC1 protein was recognized by seven CD sera at 1:50 dilutions (lanes 2–7) but not by normal sera (lane 1). Six sera from CD patients that had low titer in ELISA did not react with the

Figure 2. Type VII collagen is present in the basement membrane of colon.

Colonic tissue was cut on a Cryostat set at 5 μ m and air-dried on glass slides. Sections were incubated with either LH 7.2, a monoclonal antibody to type VII collagen, diluted 1:25 (panel d), or a polyclonal antibody to the NC1 domain of type VII collagen, diluted 1:200 (panel c), at room temperature for 2 h, then with a fluorescein-conjugated goat anti-mouse or anti-rabbit IgG, diluted 1:25, and incubated at room temperature for 1 h. Panels a and b were negative control sections that stained with normal rabbit serum (1:200) and normal mouse serum (1:25), respectively. Panels e and f were positive control human skin sections staining with NC1 polyclonal antibody (1:200) and LH 7.2 monoclonal antibody (1:25), respectively. The slides were mounted in 50% glycerol–PBS and examined with a Zeiss fluorescence microscope equipped with epillumination. Scale bar: 200 μ m.



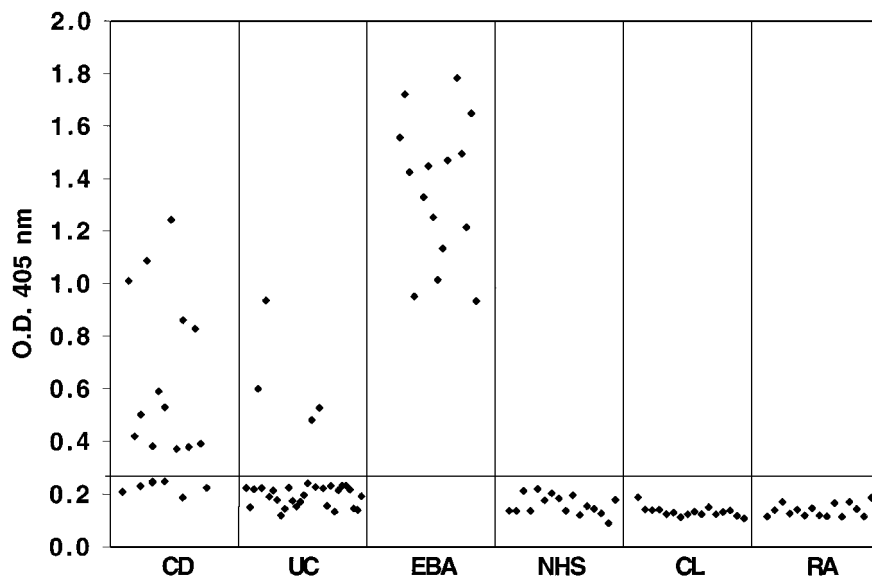


Figure 3. Scatter-plot representation of ELISA results using recombinant NC1. Patient and control sera (as indicated along the horizontal axis) (1:200 dilution) were incubated with immobilized purified recombinant NC1 domain of type VII collagen and the bound antibodies were detected with an alkaline-phosphatase-conjugated antibody against human IgG whole molecule. Each sample was run in triplicate and the points plotted on this graph represent the average of the OD₄₀₅ obtained from study serum. Similar results were obtained in three other independent studies.

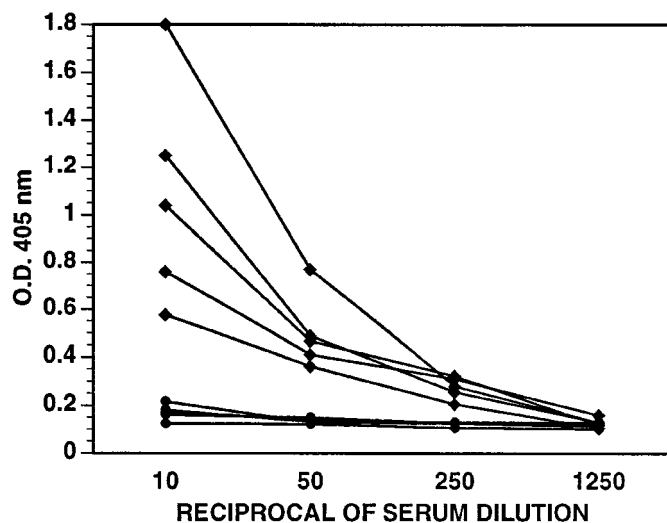


Figure 4. Titer-dependent specific reactivity of EBA autoantibodies to NC1. ELISA plate immobilized with equal amount of recombinant NC1 was incubated with increasing concentrations of five CD patient sera in dot or four NHS sera in triangle. Each data point is an average of two independent observations.

NC1 in the immunoblot analysis at this dilution (data not shown). Taken together, these data show a correlation between the results of the ELISA and the immunoblot analysis.

Indirect immunofluorescence on human skin and monkey esophagus with IBD patient serum Seventeen sera from patients with IBD that reacted with NC1 in ELISA (and no evidence of EBA) were tested for their ability to react with frozen sections of salt-spilt human skin or monkey esophagus. All sera were tested at dilutions of 1:5 and 1:10. Of the 17 IBD sera tested, none reacted with either human skin or monkey esophagus and labeled the epidermal-dermal basement membrane zone.

Lack of correlation between IBD duration and NC1 reactivity in ELISA An attempt was made to examine if there was a correlation with the duration of the patient's IBD and the level of the OD reading in the ELISA. The disease duration of each of the patients with IBD varied considerably. The shortest duration was 8 y and the longest duration was 30 y. There was no

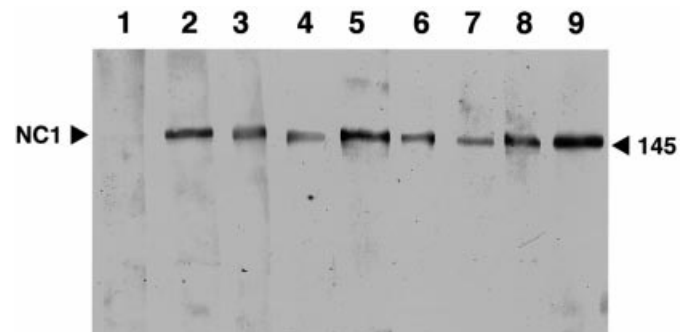


Figure 5. Immunoblot of the recombinant NC1 proteins by CD and control sera. Purified recombinant NC1 (100 ng per well) protein was separated on 6% SDS-PAGE and transferred on to nitrocellulose membranes before incubation with sera at a dilution of 1:50 and horseradish peroxidase conjugated anti-human IgG (1:5000) followed by enhanced chemiluminescence detection. Lane 1, NHS; lanes 2-8, CD serum; lane 9, EBA serum. The location of 145 kDa of recombinant NC1 is indicated.

correlation between the ELISA OD reading and the duration of the patient's IBD. For example, the longest duration patient who had IBD for 30 y had an OD of 0.248, whereas the patient with the shortest duration of IBD of 8 y had an OD of 0.502.

DISCUSSION

Patients with EBA have IgG autoantibodies to type VII collagen (Woodley *et al*, 1984). By western blotting analysis, these autoantibodies recognize two proteins in extracts of skin basement membrane: a major protein of 290 kDa and a minor protein of 145 kDa, the NC1 domain (Woodley *et al*, 1988). Using a polyclonal antibody to recombinant NC1, we demonstrated the presence of type VII (anchoring fibril) collagen in human colon by western blotting. The antibody labeled both 290 kDa and 145 kDa bands in four different samples of colon (**Fig 1**).

Visser *et al* (1993) used colon samples from four patients with normal mucosa by colonoscopy, under observation for rectal bleeding. These samples were subjected to direct immunofluorescence using two monoclonal antibodies against type VII collagen: NP-76 and LH 7.2. Type VII collagen immunoreactivity, however, was seen in mucosa adjacent to adenomas and carcinomas and

in adenomatous epithelium, close to the luminal surface, but not in normal epithelium. More recently, Lohi *et al* (1996) found that immunoreactivity for type VII collagen was confined to basement membrane of colonic epithelium in a punctate manner. Previously, other authors were unable to identify type VII collagen in colon using EBA sera and monoclonal antibodies (Paller *et al*, 1986).

In our immunofluorescence study, we were able to identify strong linear staining of the colonic basement membrane with a polyclonal antibody to recombinant NC1. Immunofluorescent staining of human colon for type VII collagen was probably missed previously because low titer EBA sera were used. Alternatively, the presence of type VII collagen in human colon was missed because the antibodies used were directed against a protease-sensitive domain of type VII collagen. The type VII collagen targets for these antibodies may have been degraded by the highly efficient proteolysis in gut samples after the tissues were obtained.

Both EBA and IBD are considered to be disorders of immunity. The etiologic events leading to either disease, however, are unknown. One theory of autoimmunity is that the rise of autoantibodies may result from molecular mimicry between microbial and host protein. The environment created by nonspecific events that cause cellular and tissue destruction may render normal tissue proteins immunogenic, as such these altered proteins become autoantigens. In this study, we found that none of the 17 IBD sera that reacted with NC1 in ELISA labeled salt-split human skin or monkey esophagus by indirect immunofluorescence. There are several possible explanations. The first is that the ELISA is more sensitive than indirect immunofluorescence in detecting low titer autoantibodies as we demonstrated previously (Chen *et al*, 1997c). Another possibility is that the IBD sera bind to certain epitopes within NC1 that are not accessible in skin basement membrane but are available to the immune system in gut basement membrane. In the ELISA, however, the NC1 epitopes would be available to bind the IBD sera. As only tissue-bound autoantibodies can become pathogenic, this may explain why none of our 17 IBD patients developed EBA.

In our patient files, we identified 13 patients who had both EBA and IBD. Eleven of these 13 patients had the onset of their IBD at least 2 y before the onset of their EBA. It is not clear why some patients with IBD have EBA and others do not. It is conceivable that the sequela of IBD autoantibodies binding to gut type VII collagen (and/or other mechanisms that establish an inflammatory nidus) is tissue damage in the gut, which alters in the structural molecules in the tissue and subsequently reveals cryptic type VII collagen epitopes that become antigenic. When autoantibodies are then generated against these antigenic neo-epitopes in gut, in certain patients they may bind to skin basement membrane and invoke EBA. If this process does not occur, EBA would not develop and the patient would only have IBD. In theory, the process could work in the other direction such that a patient with EBA generates autoantibodies to skin type VII collagen that results in the revelation of type VII epitopes shared by gut basement membrane and the induction of IBD ensues after the onset of EBA. In this scenario, the autoantibodies to the epitope in the gut would invoke inflammation and induce IBD.

In addition to the theory of pure autoimmunity causing the pathogenesis of IBD, it has also been speculated that genes related to IBD encode products that contribute to functional or structural alterations in the gastrointestinal tract. These alterations may render the tissues more susceptible to toxins, infectious agents, or autoimmune responses (Statter *et al*, 1993). Therefore, in addition to possible immune and environmental factors, genetic tendencies to develop CD and UC may be part of their disease pathogenesis (Satsangi *et al*, 1996; Hampe *et al*, 2001).

IBD has also been associated with circulating immune complexes and IgG immune deposits in the intestinal mucosa (Hodgson *et al*, 1977; Fiasse *et al*, 1978). Although, the tissue damage in IBD may be immune mediated, the condition is not strictly an autoimmune disease because the tissue damage is not directly caused by injury to a known autoantigen (Shanahan, 1992).

There is no conclusive evidence for a direct pathogenic role for any antibody in IBD. In the last decade, many different autoantibodies have been identified in UC and CD patients. These include pancreatic, anti-neutrophil cytoplasmic, anti-erythrocyte, anti-endothelial cell, anti-*Saccharomyces cerevisiae*, and anti-tropomyosin antibodies (Aldebert *et al*, 1997; Biancone *et al*, 1998; Quinton *et al*, 1998; Fricke *et al*, 1999). It is probable that antibodies in these patients are markers of underlying mucosal immune dysregulation or perhaps a response to gastrointestinal microbial antigens (Targan *et al*, 1995). This study demonstrated that type VII collagen antibodies are present in the sera of CD patients. It is likely that the presence of type VII collagen represents an epitope spreading phenomenon (Chan *et al*, 1998), in which damage to the overlying mucosa exposes antigenic epitopes of the type VII collagen molecule. These newly exposed antigenic epitopes may then invoke the immune system to mount an antibody response.

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