Mucosal complement deposition in inflammatory bowel disease

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TS HALSTENSEN, P BRANDTZAEG. Mucosal complement deposition in inflammatory bowel disease. *Can J Gastroenterol* 1993;7(2):91-101. Mucosal deposition of activated complement and immunoglobulin (Ig) in inflammatory bowel disease (IBD) was examined by indirect two- and three-colour immunofluorescence staining applied on sections of ethanol-fixed or frozen tissue specimens from patients with ulcerative colitis or Crohn's disease. Monoclonal antibodies (mAbs) to IgG subclasses and neopeptides of activated C3b or terminal complement complex (TCC) were used in combination with rabbit antiserum to various complement components (C1q, C3c, C3dg, C4c). Activated C3b was found on the luminal face of the surface epithelium in the most affected ulcerative colitis specimens from 91% of 23 patients, together with cytolytic TCC in 8.1%. Similar deposition was observed in 50% of 18 patients with Crohn's disease. However, co-deposition of the IgG1 subclass and complement components involved in the classical activation pathway (C1q and C4c) was seen only in ulcerative colitis and in complement components involved in the classical activation pathway (C1q and C4c). Moreover, in ulcerative colitis these epithelial immune complexes often co-localized with a previously identified Mr 40 kDa putative autoantigen (mAb 7E12H12). Additional type III immune reaction might take place in both diseases because evidence of continuous vascular complement activation has been seen in submucosal blood vessels. The results demonstrated that local complement activation takes place in IBD lesions. While epithelial deposition of IgG1 and activated complement suggested an autoimmune attack in ulcerative colitis, the absence of IgG1, C1q and C4c in Crohn's disease was rather consistent with the alternative activation pathway.

Key Words: Activated complement, Complement deposition, IBD, Pathogenesis

Dépôt du complément au niveau de la muqueuse dans la maladie intestinale inflammatoire

RÉSUMÉ: Les dépôts de complément et d'immunoglobuline (Ig) activés au niveau muqueux dans la maladie intestinale inflammatoire ont été examinés à l'aide d'une coloration indirecte par immunofluorescence à deux et à trois couleurs, effectuée sur des sections de spécimens de tissu congelés ou fixés à l'éthanol obtenu de patients porteurs de maladie de Crohn ou de colite ulcéreuse. Les anticorps monoclonaux dirigés contre des sous-classes d'IgG et de nouveaux déterminants génétiques de C3b activés ou le complexe de complément terminal ont été utilisés en combinaison avec des antiséras de lapin sur différentes com-

THE LESIONS OF INFLAMMATORY bowel disease (IBD) are infiltrated with lymphocytes, macrophages, immunoglobulin (Ig)-producing plasma cells (mainly of the complement activating IgG1 subclass) and a striking accumulation of neutrophils (1-3). These features suggest that local complement activation takes part in the pathogenesis.

Complement system: Complement is a complex multifactorial defence system composed of at least 26 soluble and membrane-bound proteins with the potential of forming an activated cascade. Such activation may be induced by complexing IgG or IgM antibodies with luminal or epithelial antigens (classical pathway) (Figure 1), or by direct activation of C3 by components of the local microbiota (alternative pathway) (4). Both pathways induce C3 cleavage and merge in the terminal activation step with the assembly of the terminal complement complex (TCC), ie, C5b-9 (5-7).

Classical activation pathway: The classical pathway components consist of the C1 complex (C1qrs), C2 and C4. This pathway is activated by binding of C1qrs with the Fc portion of IgG or IgM bound to antigen. IgM has a superior activation potential and of the IgG subclasses, IgG3 seems to be more potent than IgG1. The complement-activating potential of IgG2 is somewhat controversial, but may depend on antigen density (8). IgG4 (9), IgA (10) and IgE (11) do not activate human complement by the classical pathway. Ac-

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posantes du complément (C1q, C3c, C3dg, C4c). Le C3b activé a été observé sur la face luminale de l'épithélium de surface des spécimens de colite ulcéreuse les plus touchés de 91 % des 23 patients, le complexe de complément terminal cytolytique étant présent chez 8,1 %. Un dépôt similaire a été observé chez 50 % des 18 patients atteints de maladie de Crohn. Cependant, le dépôt conjoint d'une sous-classe d'IgG1 et de composantes du complément associées à la voie d'activation classiques (C1q et C4c). De plus, dans la colite ulcéreuse, ces complexes immuns épithéliaux se trouvaient localisés avec un autoantigène Mt 40kDa possiblement déjà identifié (mAb 7E12H12). Une autre réaction immunitaire de type III peut avoir lieu dans les deux maladies parce que l'on observe une activation continue du complément vasculaire dans les vaisseaux sanguins sous-muqueux. Nos résultats ont démontré que l'activation du complément local a lieu dans des lésions de la maladie intestinale inflammatoire. Bien que les dépôts épithéliaux d'IgG1 et l'activation du complément suggèrent une attaque auto-immune dans la colite ulcéreuse, l'absence d'IgG1, de C1q et de C4c dans la maladie de Crohn concorde plutôt avec la voie d'activation alternative.

tivated C1 catalyzes the assembly of the classical C3 convertase, C4b2a (12). Complement activation may be controlled at this stage by the C4b-binding protein (C4bp) or factor I in plasma and by complement receptor 1, decay-ac-

celerating factor and membrane cofactor protein on cell membranes (7,13).

Alternative activation pathway: The alternative pathway is activated by certain polysaccharides, viruses, fungi, bacteria, cell organelles and aggregated

Ig. It consists of four serum proteins: C3, factor B, factor D and properdin (14,15).

The alternative pathway is, in fact, always active. Spontaneous hydrolysis of the internal thioester bond in C3 generates a modified C3b-like molecule, C3(H₂O), which may associate with factor B. The C3(H₂O)B complex is activated by factor D and factor B is cleaved into Ba and Bb. The product C3(H₂O)Bb is the alternative pathway by which C3 convertase cleaves C3; the anaphylatoxin C3a is released and C3b binds covalently to the target with its very reactive thioester bond. Regardless whether C3b is generated by the classical or alternative pathway, it may bind another factor B, thereby activating the alternative amplification loop, or bind to factor H and be cleaved by factor I, which terminates further activation (Figure 2) (16,17). It is the nature of the surface that determines whether C3b preferentially binds to factor B or to the inhibitory factor H. Factor P is not essential for the alternative pathway but stabilizes the alternative C3 convertase, resulting in more efficient activation.

The two activation pathways are not strictly separated because antibodies and immune complexes may also activate the alternative pathway (18), and agents (such as cardiolipin, C-reactive protein and bacterial surface antigens) may activate the classical pathway under certain circumstances (18-22).

Terminal activation pathway: When target-bound C3 convertase (C4bC2a or C3bBb) binds its own product (C3b), it becomes the C5 convertase, which initiates terminal pathway activation by generating C5a and C5b. Whether surface-bound or in the fluid phase, C5b reacts with C6, C7, C8 and C9 to form TCC. When C7 binds to C5b-6 on biological membranes, it exposes hydrophobic regions and becomes inserted into the lipid bilayer. Thereafter C8 and C9 bind to C5b-C7. The lytic activity of the C5b-8 complex is limited to erythrocytes, whereas killing of nucleated cells requires at least one C9 within the membrane-bound TCC

Figure 1) Schematic depiction of various complement activation steps. Classical pathway is antibody-dependent; alternative pathway is induced by various components of the target surface (for details see text). Both pathways produce the C3 convertases C4bC2a and C3bBb, respectively, which cleave C3 to yield C3a and C3b. The latter split product binds to the C3 convertases and participates in C5 cleavage, which initiates terminal pathway activation by generation of C5a and C5b. The C3b reacts with C6, C7, C8 and C9 to form terminal complement complex (TCC). On biological membranes, TCC is generated as the cytolytic (pore-forming) C5b-9(m). Conversely, in the fluid phase (ie, in plasma or interstitial tissue fluid) S protein or Sp 40,40 (clusterin) binds to TCC at the C5b-7 level and cytolytically inactive soluble SC5b-9 results. S-protein may also be present in membrane-incorporated TCC as SC5b-9(m) (30)

(C5b-9[m]) (23). Two membrane molecules have been shown to inhibit TCC formation on cell membranes: the homologous restriction factor (24), also called C8-binding protein (C8bp) (25), and the M_r 20 kDa homologous restriction factor (CD59) (26,27).

When the terminal pathway is activated in the fluid phase (ie, in plasma or intestinal tissue fluid), the S protein (vitronectin) and/or Sp 40,40 (clusterin) binds to TCC at the C5b-7 level; cytolytically inactive soluble SC5b-9 is generated (28, 29). S protein was previously named 'serum spreading factor' because it promotes adhesion of cells to extracellular substrates. Small amounts of S protein may also be present in membrane-incorporated TCC (SC5b-9[m]) (30).

C3b inactivation: C3b – bound to either complement receptor 1 or 2 (CR1 or CR2) or serum factor H – becomes partly degraded by serum factor I, and a small M_r 2 kDa fragment C3f is released from the α chain (Figure 2) (31-33). The inactivated product (iC3b) is degraded to a small M_r 40 kDa fragment, C3dg, and a major M_r 150 kDa fragment, C3c. The latter is released to the fluid phase whereas C3dg (which contains the C3b thioester bond) remains covalently bound to the target surface (34). C3dg may be further cleaved by trypsin to C3d (M_r 30 kDa) and C3g (M_r 10 kDa) in vitro (35).

Neopeptides in the complement system: The structural alteration of complement components induced during activation exposes new antigenic determinants (eg, monoclonal antibodies [mAbs]) to neopeptides; these react selectively with the activated components and can therefore be used for immunohistochemistry on directly fixed or frozen tissue to reveal in situ complement activation.

The mAb bH6 reacts with a neopeptide in the C3c part of C3b or with its inactivated form iC3b (36). Terminal activation may be disclosed by mAb aEll reacting with a C9 neopeptide exposed during C9 polymerization in membrane-incorporated TCC (C5b-9[m]) or when C9 binds to C8 in the soluble S protein-

Figure 2 Schematic depiction of C3 activation and regulation. When C3 is activated either by the immunoglobulin (Ig)-dependent classical or the Ig-independent alternative pathway, the anaphylatoxin C3a is released and C3b becomes covalently bound to the target. The physicochemical properties of surface determines the fate of target-bound C3b. It may either bind factor B (B), which induces the amplification loop, or it may be inactivated if bound to factor H (H) or membrane cofactor protein (MCP) and cleaved by factor I (I). The complex C3b*B is activated by factor D (D) and Ba is released to the fluid phase. The product Bb*C3b, also named the alternative pathway C3 convertase, can activate C3 alone, but factor P (P) stabilizes the complex, leading to prolonged activation. Conversely, cells that express decay accelerating factor (DAF) and MCP (like vascular endothelium) will rapidly inactivate the complex. While DAF releases factor Bb from the complex, MCP binds to C3b and acts as a cofactor for factor I. The small fragment C3f is first released and the inactivated form of C3b (iC3b) is cleaved further to form C3c (released to the fluid phase) and the small fragment C3dg (remains attached to the target)

bound form of TCC (SC5b-9) (37). These two forms of TCC, therefore, cannot be distinguished by mAb aEll.

Previous immunohistochemical studies of complement deposition in IBD were based on antisera to native complement components applied on tissue specimens that contained unpredictable amounts of extracellular serum proteins (38,39). In fact, there may be quite high concentrations of interstitial native C3 in inflamed tissue with increased vascular permeability (40).

The purpose of the authors' previous studies (41-45) has been to trace the site and nature of mucosal complement activation in IBD. In addition, we re-examined the tissue distribution of the previously identified M_r 40 kDa putative autoantigen in ulcerative colitis (46,47) by using the mAb 7E12H12 for

immunofluorescence two- and three-colour staining.

MATERIALS AND METHODS

Tissue and immunological reagents: The clinical material consisted of colonic biopsy or resection specimens from 39 patients with ulcerative colitis, 18 with Crohn's colitis, and 26 controls (mainly histologically normal mucosa from patients with colonic carcinoma). Ileal samples from 14 patients with Crohn's ileitis and six controls were also included.

Immunohistochemical staining for IgG and complement on directly fixed tissue specimens may represent retained extravascular serum proteins rather than immune complexes (40). To overcome this problem, the authors used extensively prewashed specimens

Figure 3) Intense staining for the terminal complement complex (TCC) was observed in vessel walls (arrows). Prewashed, ethanol-fixed and paraffin-embedded colonic specimen from a patient with active ulcerative colitis is shown. (original magnification $\times 44$)

from which most diffusible extracellular proteins, such as IgG and complement, had been extracted (48). In addition, mAbs specific for neopeptides of activated complement were used on both prewashed and directly ethanol-fixed and paraffin-embedded specimens to resolve whether the observed deposits represented tissue-bound or soluble immune complexes. Additional tissue specimens were immediately placed in ice-chilled tissue culture medium (RPMI 1640, Gibco, Paisley, Scotland) or in a 0.5% to 1% paraformaldehyde-lysine-periodate fixative and brought to the laboratory within 2 h. The biopsy specimens were properly oriented on a thin slice of carrot, embedded in optimum cutting temperature (Tissue-Tek, Miles Laboratories, Indiana), snap-frozen in isopentane cooled in liquid nitrogen and stored at -70°C until cryosectioning ($4\ \mu\text{m}$) (45).

Dewaxed paraffine sections ($6\ \mu\text{m}$) were incubated with mAb to a C3c neopeptide exposed in C3b, iC3b and C3c (C3b-neo, mAb bH6), (36), a C9 neopeptide in TCC (mAb aE11) (37) or S protein (Cytotech, California) in

Figure 4) Scatter plot of vascular terminal complement complex% (TCC%) versus C3c%. The two variables were well correlated ($P < 0.005$, Kendall's $\tau = 0.57$). Controls O; Ulcerative colitis ●; Crohn's disease of the colon ■

combination with rabbit antiserum to C1q (1:500, Dakopatts, Glostrup, Denmark), C3c (1:500, Behringwerke AG, Marburg, Germany), C3d, C4c and C5 (all 1:500, Dakopatts), C9 (1:500, Behringwerke AG), cytokeratin (1:100) (49), von Willebrandt factor (1:350, Dakopatts) or S protein (1:5000) (50). A three-step two-colour biotin/avidin-enhanced immunofluorescence staining procedure was principally performed as described previously (51). Sections from prewashed tissue were, in addition, examined with mAbs to the four IgG subclasses: (clones: 267, GO M2, CBI-AH7, RJ4, respectively) (52) in combination with rabbit antiserum to C3c and C4c.

Because inflamed tissue contains more vessels than normal tissue, vascular TCC and C3c positivity was scored on a semiquantitative scale and related to the number of blood vessels in the actual section. Each specimen was given a vascular TCC and C3c percentage score as detailed elsewhere (42). With regard to epithelial deposits, a semiquantitative score was applied for each immunological marker ranging from no epithelial staining (–) to in-

tense staining on many epithelial cells (3+). Histopathological examination was performed to grade each specimen with regard to inflammation from negative (–) to intense (3+).

Three-colour immunofluorescence staining: Selected colonic and ileal specimens from five patients with Crohn's disease and five with ulcerative colitis having apical complement deposition (see below) were subjected to three colour staining in which mAb to human IgG1 (murine IgG1) was combined with mAb bH6 to C3b (IgG2a) or mAb aE11 to TCC (IgG2a) and mixed with rabbit antiserum to C3c or C4c. Secondary reagents were biotinylated and FITC-conjugated subclass-specific goat antimouse IgG2a and IgG1 (Southern Biotechnology, Alabama) followed by 7-amino-4-methylcoumarin-3-acetic acid (AMCA)-conjugated goat antirabbit IgG (1:20, Vector Laboratories, location) in combination with Streptavidin-Texas Red (0.0025 g/L; Bethesda Research Laboratories, Gaithersburg, Maryland).

Additional sections of selected ethanol-fixed and paraffin-embedded or paraformaldehyde-lysine-periodate-fixed cryosections from ulcerative colitis patients were likewise subjected to three-colour staining; the murine IgM mAb 7E12H12 to the M_r 40 kDa colonic protein was mixed with rabbit antiserum to cytokeratin and combined with either mAb bH6 to C3b (murine IgG2a), mAb aE11 to TCC (IgG2a) or mAb to human IgG1. Preselected saline-extracted and ethanol-fixed specimens from five ulcerative colitis patients were also subjected to three-colour staining in which the murine IgM mAb 7E12H12 was combined with mAb to IgG1 and rabbit antiserum to C3c. Secondary reagents were biotinylated goat subclass-specific antimouse IgG1 or IgG2a (0.01 g/L) (Southern Biotechnology) combined with FITC-conjugated goat class-specific antimouse IgM (0.05 g/L) (Southern Biotechnology). Both combinations were applied for 1 h and were followed by incubation (30 mins) with AMCA-conjugated goat antirabbit IgG (1:10) (Vector Laboratories, Bur-

Figure 5 Three-colour staining for (a) immunoglobulin (Ig) G1 (b) C3b (c) terminal complement complex (TCC) in submucosal vessel from patient with Crohn's disease of the colon showing segmental staining for immunoglobulin G1 associated with the complement deposition (arrow). The same vessel also contained C1q and C4c (not shown), suggesting classical pathway activation. The section is from a prewashed ethanol-fixed and paraffin-embedded colonic specimen from a patient with Crohn's disease of the colon (original magnification $\times 106$)

lingame, California) in combination with Streptavidin-Texas Red (0.0025 gL).

The intestinal distribution of the M_r 40 kDa putative autoantigen in ulcerative colitis was examined on formalin-fixed and paraffin-embedded routine tissues blocks from various levels of normal gastrointestinal fractions. Two-colour immunofluorescence staining combining the mAb 7E12H12 to the M_r 40 kDa-antigen with rabbit serum to cytokeratin was performed as described above.

RESULTS

Vascular terminal complement complex deposits: There was significantly more ($P < 0.01$) complement deposition (C3d and TCC) in the colonic submucosal blood vessels from patients with IBD than in controls (Figure 3). The median TCC percentage increased

from 11% in controls to 31% in ulcerative colitis and to 46% in Crohn's colitis. Some of the TCC-positive vessels showed segmental co-staining for C3b (C3b-neo and C3c). The median C3c percentage (1% in controls) increased to 23% in ulcerative colitis and to 39% in Crohn's colitis. There was good correlation between the C3c and TCC percentages (Kendall's $\tau = 0.57$, $P < 0.0005$) (Figure 4).

The vascular complement deposits did not generally contain detectable Ig components. However, submucosal vessels in two patients with Crohn's colitis contained C1q, C3b, C4c, TCC and weak segmental IgG1 positivity (Figure 5).

Diverse complement positivity: Sub-epithelial deposition of TCC, S protein and C3d was often observed in affected specimens from both diseases. Some specimens of all categories contained

C3d and TCC, associated with S protein-positive elastic fibrils in the lamina muscularis mucosae, submucosa and perivascular sheets, although such staining was observed more often and with more intensity in IBD patients than in controls. The contents of fissure ulcers and crypt abscesses stained for C3d and TCC, but were mostly negative for C3b. The follicular dendritic cells in the germinal centres stained for C3d, TCC and S protein, but not for C3b. This feature was observed in normal and diseased colon and was similar to that seen in normal lymphoid tissue (53). Numerous cells in the lamina propria contained cytoplasmic granules that stained for C3c, C4c, C1q and occasional IgG1 (Figure 6).

Distribution of the M_r 40 kDa antigen in the normal gastrointestinal tract: The mAb 7E12H12 did not react with epithelium of esophagus, stomach,

Figure 6 Two-colour staining for (a) C3c and (b) immunoglobulin (Ig) G1 in the submucosa from a patient with ulcerative colitis. Note many IgG1-producing plasma cells (arrows) and some cells with cytoplasmic granules containing both IgG1 and C3c (open arrow) (possibly immune-complex-containing macrophages). Section from prewashed, ethanol-fixed and paraffin-embedded colonic specimen from a patient with active ulcerative colitis (original magnification $\times 106$)

Figure 7 Immunofluorescence staining for the M_r 40 kDa (mAb 7E₁₂H₁₂) in (a) ascending colon (x44) and (b) rectum (x106) on section of formalin-fixed routine specimens from control colon (x106). Whereas goblet cells are selectively stained in the ascending colon, they are virtually negative in the rectum (closed arrow). Enterocytes, however, are intensely stained in the rectum but not in the ascending colon (open arrows). Dashed line indicates epithelial basement membrane zone

duodenum or proximal jejunum. The enterocytes of jejunum and ileum (including the terminal ileum) also did not react. Occasional goblet cells with strong staining were observed in ileal crypts of specimens taken about 110 cm proximal to the cecum. The frequency of such positive goblet cells appeared to increase in distal direction; many crypt goblet cells in the terminal ileum were stained. In the cecum and ascending colon, most goblet cells were positive as was the apical face of surface epithelium (Figure 7a). The enterocytes of the colon increased their apical cytoplasmic staining in distal direction, and the crypt and surface enterocytes of the rectum were usually intensely positive throughout the cytoplasm (Figure 7b). The goblet cell staining was unaltered in the transverse and descending colon but decreased in the sigmoid and virtually vanished in the normal rectum (Figure 7b).

Epithelial immune deposits – ulcerative colitis: C3L was observed on the colonic surface epithelium in 91% of the 23 ulcerative colitis patients studied; 81% of these patients had cytolytic TCC deposited within the C3b. Both the frequency (Figure 8) and the intensity (Kendall's $\tau=0.56$, $P<0.001$)

of immune deposition were well correlated with the topical degree of inflammation.

Because colon-specific autoantibodies have been reported in IBD (54–60), the authors examined prewashed tissue specimens from 11 ulcerative colitis and 19 Crohn's colitis patients for co-deposition of complement components and IgG subclasses. Patients with ulcerative colitis (63%) showed selective binding of IgG1 apically on colonic epithelial cells; C3b and TCC often were present within these IgG1 deposits (Figure 9). Moreover, the M_r 40 kDa putative autoantigen co-localized with the immune complexes apically on the surface epithelium in ulcerative colitis.

Epithelial immune deposits – Crohn's disease: Somewhat more granular epithelial deposits of C3b and TCC were observed on the luminal face of the surface epithelium in 10 of 18 patients with Crohn's colitis. Specimens from eight of 14 patients with ileal involvement showed intense staining for C3b within the surface mucus layer. No epithelial IgG, C1q or C4c deposition was detected in Crohn's disease (Figure 10), contrasting the co-deposition of IgG1, C3b, C4c, TCC

and, occasionally, C1q observed in ulcerative colitis. Additional globular elements with membrane positivity for C3b were seen attached to the epithelium in one-half of the Crohn's patients; aggregates of C3b-positive globular elements were seen in the lamina propria in directly fixed specimens from three patients.

The ileal surface mucin stained for C3b in eight of 14 patients with Crohn's disease, four of whom also stained for TCC on the apical face of the epithelium. Epithelial complement deposits were not seen in 96% of 26 colonic controls, nor in six ileal controls. Epithelial deposition of IgG was not observed in any control.

DISCUSSION

Vascular complement activation: Vascular complement deposits are usually regarded as a sign of immune complex formation, often associated with vasculitis (61). However, the authors observed no cellular infiltration or fibrinoid necrosis along with TCC or C3b, although thrombosed submucosal vessels positive for TCC and C3b were seen in two patients (42). The vascular TCC was apparently in a membrane-bound form because it was retained after

extensive prewashing of the tissue specimens, suggesting that it represented the lytic, membrane-incorporated C5b-9 (m). However, there was a striking co-localization of TCC and S protein, suggesting that it represented the soluble S protein-associated form of TCC (42). Nevertheless, the possibility for soluble SC5b-9 (passively retained from the circulation) was apparently excluded by the extensive washing procedure applied, which removed most diffusible molecules of comparable size (eg, IgM, M_r 1000 kDa) from the tissue. Another possibility was an S protein-vitronectin-mediated binding of SC5b-9 to S protein receptors on fibroblasts or/and smooth muscle cells in the vascular walls (62), but this apparently was contradicted by the parallel staining for TCC and C3d, suggesting that both early and late phase complement activation had occurred *in situ*. Moreover, the vascular immune deposits in ulcerative colitis, and especially in Crohn's disease, contained significantly more C3b than control colon.

Because breakdown of C3b is a rapid process, the presence of both C3b and TCC suggested that vascular complement activation is a continuous process in IBD. Although the vascular immune deposits did not generally stain for Ig (63), segmental co-deposition of IgG1, C1q and C4c in C3b- and TCC-positive vessels observed in two samples from Crohn's colitis suggested antibody-mediated classical activation (12).

Hemorrhagic necrosis induced by a local Shwartzman's reaction depends on both C5a and tumour necrosis factor (64). Complement activation might initiate this reaction as C5a stimulates macrophages to tumour necrosis factor transcription (65). Such activity could induce thrombosis of submucosal vessels in IBD, and larger vessels are probably involved in Crohn's disease because of the transmural inflammation; perhaps this explains why vascular thrombosis and intestinal infarction seem to be a feature of Crohn's disease (66). The diffuse C3b positivity observed in the mucin layer of patients with Crohn's ileitis might reflect increased local synthesis and external

Figure 8 Epithelial complement deposits in ulcerative colitis. Percentage of specimens with epithelial C3b and terminal complement complex (TCC) deposition in relation to actual severity of inflammation in ulcerative colitis (range 0 to +++). All specimens with intense (3+) inflammation showed epithelial C3b deposition. Neg Negative

transfer of C3, as was shown recently to take place even in unaffected mucosa in this disease (67).

Epithelial IgG1 and complement deposition: The epithelial deposition of IgG1 along with activated complement (C4c, C3b and TCC) in active ulcerative colitis could reflect binding of autoantibodies to brush border-associated antigen(s) and complement-mediated epithelial attack (43,44). This was further supported by the frequent co-localization of the epithelial immune complexes and the M_r 40 kDa colonic protein, which has previously been proposed to be an autoantigen in ulcerative colitis (45-47).

The expression of this antigen, also observed in ileal mucin (45), might explain 'backwash ileitis' (68) and ileal pouchitis (69), commonly seen in ulcerative colitis patients. The colonic distribution of the M_r 40 kDa antigen, from mainly goblet cell positivity in the proximal colon to increasing enterocyte expression in the distal direction, further supports its putative role as an autoantigen in ulcerative colitis. Moreover, the mAb to the M_r 40 kDa antigen and the classical serum anticolon antibodies (60) produce a similar staining pattern in human and rat colon, and ulcerative colitis patients have serum antibodies to both the M_r 40 kDa

antigen (70) and rat colon extracts (54); this suggests that the M_r 40 kDa protein may be associated with the antigen for the classical anticolon antibodies.

Although epithelial cells are partly protected against autologous complement attack by CD59 (71,72), this survival mechanism may not be sufficient in IBD mucosa. The enterocytes will probably also try to escape IgG-mediated complement attack by rapid shedding of damaged plasma membranes (73). This event may involve loss of the epithelial brush border in ulcerative colitis; the putative consequence is reduced surface area and hampered absorptive function. Epithelial damage, in addition, will increase leakage of autoantibodies and complement components into the colonic lumen where transport takes place distally. Because the putative autoantigen is located at the apical surface, epithelium located downstream may be subjected to an IgG1-mediated complement attack continuously. Perhaps this explains why ulcerative colitis is typically a continuum in distal direction. In addition, autoantibodies may complex with mucin-associated antigen and cause luminal complement activation. The anaphylatoxins (C4a, C3a, C5a) produced may contribute to

Figure 9 Immunofluorescence staining for (a) immunoglobulin (Ig) G1, (b) IgG3 and (c) activated C3b marker C3c in section of extensively prewashed colonic mucosa from a patient with ulcerative colitis. Small arrows indicate plasma cells producing IgG3. Only IgG1 was deposited on the apical surface of the epithelium, often in conjugation with C3b (large arrows). Dashed lines indicate epithelial basement membrane zone (original magnification $\times 100$)

Figure 10 Two-colour immunofluorescence staining for a immunoglobulin G1 and b activated complement C3c in section of extensively prewashed colonic mucosa from patient with Crohn's disease of the colon. No immunoglobulin (Ig) G1 (or any other IgG subclass) was observed within the C3b-positive epithelial deposits in Crohn's disease (arrows). Dashed lines indicate epithelial basement membrane zone (original magnification $\times 100$)

transepithelial migration, activation and degranulation of granulocytes in active ulcerative colitis.

Epithelial complement deposition is not specific for ulcerative colitis; it was also observed in approximately one-half of patients with Crohn's colitis (44). However, lack of co-deposition of IgG and classical complement activation pathway components (C1q and C4c) suggests that complement was activated by the alternative pathway in Crohn's disease.

Subepithelial deposition of TCC, S protein and C3d, was mainly seen directly beneath or adjacent to epithelial destruction in ulcerative colitis (43), but also under intact

epithelium in Crohn's disease (44). Although this might be a secondary phenomenon, soluble immune complexes generated in the basement membrane zone could have induced subepithelial complement activation. The possibility that the C3b-positive globular elements observed in mucosa with Crohn's colitis represented bacteria, such as those previously identified by electron microscopy in this lesion (74,75) or the isolated wall-deficient spheroblasts of *Mycobacterium paratuberculosis* (76), needs further elucidation.

Consequences of mucosal complement activation: Regardless of the mode of complement activation and the nature

of the target attacked, generation of the anaphylatoxins (C3a, C4a and C5a) causes inflammation and release of other inflammatory mediators such as leukotrienes and prostaglandins (77-83). The elevated levels of these substances found in the ulcerative colitis lesion (84-86) could therefore be secondary to complement activation. Both leukotriene B₄ and anaphylatoxin C5a are highly chemotactic for granulocytes and are probably involved in the massive mucosal mobilization of such cells seen in ulcerative colitis; lysosomal enzymes and toxic oxygen radicals liberated from neutrophils may also attack the epithelium. The intraluminal and crypt staining for TCC and C3d in ulcerative colitis might have been induced by autoantibodies reacting with the Mr 40 kDa autoantigen in mucin, shedding of attacked cell-membranes, as well as being induced by the alternative activation pathway, perhaps secondary to epithelial damage (the latter may dominate in Crohn's disease).

Expression of the Mr 40 kDa autoantigen by the colonic surface epithelium was often decreased in the surface epithelium in active ulcerative colitis and in patients with inactive disease of long duration. This suggests that IgG1-mediated complement attack might exert a selective pressure on the epithelium, thereby allowing cells with low expression of this antigen to survive. One of the consequences could be positive selection of dysplastic epithelium in ulcerative colitis. Such a putative mechanism would explain the increasing risk for developing colonic carcinoma after long disease duration. This possibility is testable and should be explored in the future.

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