Deposits of terminal complement complex (TCC) in muscularis mucosae and submucosal vessels in ulcerative colitis and Crohn's disease of the colon

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SUMMARY Extensively washed, ethanol fixed and paraffin embedded colonic specimens from 15 patients with ulcerative colitis (UC) and nine patients with Crohn's disease (CD) of the colon, ileal specimens from six patients with CD of the ileum, and histologically normal control specimens obtained from 10 patients operated for colonic carcinoma, were examined by immunohistochemistry with a monoclonal antibody specific for a neopeptide in the C9 part of the terminal complement complex (TCC). The submucosal blood vessels in inflammatory bowel disease (IBD) showed significantly more TCC positivity than the controls, and vascular TCC deposition was statistically related ($p < 0.001$) to degree of inflammation. Five of the six ileal CD specimens contained likewise vascular TCC deposits. In addition, five UC specimens and one colonic CD specimen contained TCC-positive fibrils in the muscularis mucosae or submucosa. There was no significant difference in vascular TCC deposits between UC and CD. The results suggested that terminal complement activation takes place in the intestinal lesions of IBD.

The aetiology of inflammatory bowel disease (IBD) is unknown. Several possibilities have been suggested, including infectious agents, autoimmunity and local immune complex deposition.^{1,2} Despite the lack of definite evidence, it is generally believed that immunological mechanisms contribute to the pathogenesis of ulcerative colitis (UC) and Crohn's disease (CD). Immunohistochemical studies of diseased mucosa have revealed a marked local over production of IgG in IBD³ with a significantly higher IgG1-to-IgG2 proportion in UC than in CD.⁴ To some extent the locally produced IgG shows specificity for intestinal microorganisms.^{5,7} Moreover, local IgG has been found to react with an intestinal epithelial antigen in UC but not in CD.⁸

As IgG can activate complement, the striking local IgG response seen in IBD is of considerable pathogenetic interest. In 1974 Ballard and Shiner⁹ proposed that immune complexes formed at the epithelial basement membrane might activate complement and attract polymorphonuclear cells;

the release of proteolytic enzymes from these cells could then lead to epithelial destruction.

Immune complexes induce C3 activation either by the classical or alternative pathway. C3b participates in the C5 convertase which initiates the terminal pathway when C5 is cleaved. The terminal complement complex (TCC) is formed when C5b reacts with C6, C7, C8, and C9.

On biological membranes TCC appears in the form of the cytolytic, pore forming C5b-9(m).¹⁰ In a fluid phase, however, S-protein (vitronectin) binds to C5b-7 and the non-cytolytic soluble form of TCC (SC5b-9) is formed.¹¹ Both C5b-9(m) and SC5b-9 express TCC neopeptides which are absent from the native components. The purpose of this study was to examine lesions of IBD for the expression of TCC neopeptide suggestive of local complement activation.

Methods

TISSUE SPECIMENS

Colonic tissue from inflamed lesions were obtained immediately after surgical resection in 15 patients

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Table 1 Clinical information about patients with IBD and TCC deposits in their colonic specimens

Patient no	Age (yrs)	Sex	Diagnosis	Medication		Topographic affection	Disease duration	Specimen site	Local inflam.	Vascular TCC*	Fibrillar TCC
				Pred	Sala.						
1	33	F	UC	No	Yes	Total	9 years	Sigmoidium	+	++/++	0
2	34	F	UC	UN	UN	Total	5 years	Sigmoidium	+++	+++ / +++	0
3	39	F	UC	Yes	Yes	Total	9 years	Sigmoidium	+++	+++ / +++	0
4	27	M	UC	No	Yes	Total	10 years	Transversum	+++	+++ / +++	+++
5	50	M	UC	Yes	Yes	Total	5 years	Transversum	+++	+++ / +++	0
6	33	M	UC	Yes	Yes	UN	UN	Rectum	+++	+++ / +++	++
7	29	F	UC	Yes	No	Total	24 years	Ascendens	+++	+++ / +++	+++
8	17	F	UC	No	Yes	Total	3 years	Ascendens	++	++ / +++	+
9	47	M	UC	Yes	Yes	Total	1 year	Rectum	++	++ / ++	++
10	26	F	UC	No	Yes	Total	9 years	Sigmoidium	+++	+++ / +++	0
11	20	F	UC	No	Yes	Total	6 years	Ascendens	++	+/+	0
12	43	F	UC	Yes	Yes	UN	17 years	Descendens	++	0/0	0
13	28	F	UC	No	No	Total	1 year	Ascendens	+++	+++ / ++	0
14	30	F	UC	No	No	Total	3 weeks	Rectum	+++	+/+	0
15	44	F	UC	Yes	Yes	Total	UN	Sigmoidium	++	++ / +	0
16	23	F	CD	Yes	No	Seg.	UN	Transversum	+	++ / ++	0
17	20	M	CD	Yes	Yes	Seg.	UN	Transversum	+++	+++ / ++	0
18	64	F	CD	UN	UN	Total	UN	Transversum	+++	+++ / +++	++
19	36	M	CD	UN	No	Seg.	UN	Sigmoidium	+++	++ / ++	0
20	30	F	CD	Yes	No	Total	UN	Rectum	++	0/0	0
21	27	F	CD	Yes	No	Total	2 years	Transversum	++	++ / ++	0
22	35	F	CD	Yes	No	UN	UN	Sigmoidium	++	+++ / +++	0
23	15	M	CD	No	No	Seg.	3 years	Rectum	+	++ / ++	0
24	18	M	CD	No	No	Seg.	1 year	Ascendens	++	+/+	0

Pat.=patient; Pred.=prednisolone; Sala.=salazopyrin; UC=ulcerative colitis; UN=unknown; CD=Crohn's disease of the colon; Seg.=segmental. *The vascular TCC score from two blind examinations.

with UC (average age, 33 years; range, 17–50) and nine with CD of the colon (average age, 30 years; range, 15–64, Table 1). In addition, we collected ileal samples from six patients with CD of the ileum (average age, 31 years; range, 18–43). The diagnoses were based on clinical and pathological criteria. Control material was obtained from histologically normal mucosa of 10 patients (average age, 71 years; range, 51–80) undergoing colonic resection because of carcinoma. These specimens were taken well away from the tumour.

All specimens were collected in ice cold isotonic saline and brought to the laboratory within one hour. Thin tissue slices were washed for 48 h at 4°C in 0.01 M phosphate buffer (pH 7.5) containing 0.15 M NaCl (PBS) to remove diffusible proteins. Thereafter the tissue was fixed for 18 h in cold 96% ethanol, dehydrated in absolute ethanol, cleared in xylene and embedded in paraffin for three to four hours at 56°C.¹² The paraffin blocks were stored at 4°C until used.

IMMUNOHISTOCHEMISTRY

Dewaxed tissue sections (6 µm) were incubated for 30 minutes at room temperature with ascitic fluid of a murine monoclonal antibody (MoAb) to a C9

neoeptope of TCC.¹³ The reagent (aE11) was applied at an IgG concentration of 2.5 mg/ml (1:4000 dilution in PBS containing bovine serum albumin at 125 g/l). Rabbit antimouse IgG conjugated with fluorescein isothiocyanate (molar F/P ratio=1:2) was used at 0.46 g/l for 30 min in an indirect immunofluorescence technique. In addition, the alkaline phosphatase antialkaline phosphatase (APAAP) method was used as detailed elsewhere¹⁴ to examine selected specimens.

MICROSCOPY AND PHOTOGRAPHY

Immunofluorescence features were observed in a Leitz Orthoplan microscope equipped with an XBO 150 W lamp. The results were recorded on Agfa 1000 ASA daylight film.

IMMUNOHISTOCHEMICAL EVALUATION

The immunofluorescence examination was performed blindly twice by the same investigator. Each specimen was given a semi-quantitative score ranging from quite faint fluorescence in a few submucosal vessels (0) to intense staining in many vessels through the submucosa (+++). One tissue section from each immunohistochemical series was stained with haematoxylin and eosin and was examined blindly.

Fig. 1 Staining for the TCC neopitope in washed ethanol-fixed paraffin embedded colonic specimens from a patient with ulcerative colitis. Intense fluorescence staining was observed in the submucosal vessel walls (arrows). (Magnification $\times 44$).

Each specimen was given a semiquantitative inflammatory score from negative (0) to intense (+++) on the basis of the cellular infiltrate density.

CONTROLS

Incubation with murine control ascites at a dilution comparable to that used for the MoAb (1:4000), did not produce detectable staining. Occasional weak autofluorescence was seen, however, in the internal elastic membrane of some vessels.

STATISTICAL ANALYSIS

Comparisons between specimen categories were based on the Mann-Whitney non-parametric two-tailed test. Correlation analyses were performed by the Kendall's t test.

Results

Intense staining for TCC in submucosal blood vessels was found by indirect immunofluorescence in 14 of 15 UC specimens and in eight of nine colonic CD

specimens (Fig. 1 and Table 1). Five of six ileal CD specimens were likewise positive for TCC (Table 2). By contrast, only one of 10 histologically normal colonic control specimens showed positive vascular staining for TCC ($p < 0.01$) (Table 3). Re-examination with the sensitive APAAP method, however, revealed some deposits in occasional submucosal vessels in every control specimens.

The staining intensity of individual vessel walls and the number of positive vessels varied considerably in the inflamed specimens. Nevertheless the TCC positivity scores obtained from the two blind examinations were well correlated ($t = 7.7$; $p < 0.001$). Furthermore, there was a positive correlation between inflammatory score and vascular TCC deposits ($t = 8.05$; $p < 0.001$, Table 1).

In specimens from two UC patients we observed thrombosed submucosal blood vessels with TCC positivity both in the vessel wall and in the thrombus (Fig. 2). These arterioles were located beneath densely inflamed and damaged mucosa of patients who were severely ill when colectomy was performed.

Terminal complement complex deposits were detected on fibrillary elements (Fig. 3) in the muscularis mucosae or submucosa of five UC specimens (nos 4, 6, 7, 8, 9) and in one specimen from a

Table 3 TCC deposits in control specimens without inflammation

Patient no	Age (yrs)	Sex	Specimen site	Vascular TCC	Fibrillar TCC
29	87	F	Sigmoidium	0/0	0
30	51	M	Rectum	0/0	0
31	57	M	Sigmoidium	0/0	0
32	64	M	Sigmoidium	0/0	0
33	56	F	Rectum	0/0	0
34	86	M	Rectum	0/0	0
35	78	F	Sigmoidium	++/+++	0
36	78	F	Rectum	0/0	0
37	70	F	Rectum	0/0	0
38	80	F	Sigmoidium	0/0	0

Table 2 Clinical information about patients with Crohn's disease in the ileum and TCC deposits in their ileal lesion

Patient no	Age (yrs)	Sex	Medication		Topographic affection	Disease duration	Local inflam.	Vascular TCC*
			Pred.	Sala				
19	36	M	UN	UN	C+I	UN	+++	+/+
25	26	M	UN	UN	I	UN	++	+/+
24	18	M	No	No	C+I	1 year	++	++/+++
26	43	M	No	No	C+I	3 years	+++	+++ / ++++
27	25	F	Yes	No	C+I	10 years	++	+++ / +++
28	40	M	UN	UN	I	UN	+	0/0

Pred. = prednisolone; Sala. = salazopyrin; UN = unknown; C = colon; I = ileum. *The vascular TCC score from two blind examinations.

Fig. 2 Positive staining for TCC in a thrombosed submucosal vessel. Note TCC positivity both in the thrombus and in the vessel wall (arrows). APAAP staining on ethanol-fixed paraffin-embedded colonic specimens counterstained with haematoxylin from UC patients No. 7. (Magnification $\times 100$).

patient (no 18) with CD of the colon. Additional sections from these tissue blocks were re-examined and stained for elastin. The similarity of the staining patterns suggested that the TCC positivity was associated with the elastic fibrils. The majority of such fibrils, however, were TCC-negative. TCC-positive fibrils were not observed in histologically normal mucosa.

Germinal centres of secondary follicles from ileum (Peyer's patches) and colon was positively stained for TCC in a dendritic pattern. This staining feature was shown to be associated with the follicular dendritic cells and could be observed in histologically normal mucosa and other lymphoid tissues as well.¹⁵

Fig. 3 TCC positivity on fibrillary elements in muscularis mucosae (arrows). Identical TCC positive fibrils were also observed in the submucosa. APAAP staining of washed, ethanol-fixed and paraffin-embedded colonic specimens counterstained with haematoxylin from a patient with ulcerative colitis. (Magnification $\times 162$).

Discussion

The demonstration of substantially more TCC in colonic submucosal blood vessel walls in IBD than in histologically normal controls, is apparently a new observation of pathogenetic interest. There was no significant difference in vascular TCC deposits between UC and CD of the colon. The inflammatory lesions might in fact have contained more TCC than that observed, because we usually observed less vascular TCC positivity in paraffin blocks stored for more than 10 years. Eight of the UC, six of the colonic CD, and all the ileal CD blocks were older than 10 years, whereas none of the control blocks were older than six years.

The vascular TCC deposition should not be considered specific for IBD because trace amounts were seen in the controls, particularly with the sensitive APAAP method. Other workers have likewise observed immunohistochemical staining for TCC in arterioles and arteries in cryosections of normal human renal tissue¹⁶ and myocardium.¹⁷

Vascular complement deposits are often regarded as signs of a type III hypersensitivity reactions. Circulating immune complexes may become deposited in the vessel walls of inflamed areas. When rabbits with formalin induced rectal mucosal damage are injected intravenously with human serum albumin-anti-albumin immune complexes, the result is a severe colitis and crypt abscesses.¹⁸ Human endothelial cells have been shown to express receptors for C3b and Fc γ *in vitro* when infected with herpes,¹⁹ cytomegalo- or influenza virus.²⁰ Bovine pulmonary endothelial cells express such receptors *in vitro* when incubated with white cell lysate.²¹ It is unknown whether inflammation *per se* will induce such receptors, but their expression may explain why circulating immune complexes preferentially become deposited in blood vessel walls of inflamed areas. It is controversial, however, whether patients with IBD have raised levels of circulating immune complexes.²²

Other mechanisms could well be involved in the observed submucosal vascular TCC deposition. Locally produced IgG antibodies might complex with luminal antigens *in situ* and induce a local immune complex disease or Arthus type reaction as suggested by the presence of TCC-positive thrombosed submucosal blood vessels in two UC patients. Although vascular TCC deposits have been demonstrated in leucocytoclastic vasculitis of the skin,²³ no cellular infiltrates or fibrinoid necrosis of the TCC-positive submucosal vascular walls were observed. The majority of our patients, however, received immunosuppressive medication which could have modified such reactions.

Previous immunohistochemical studies of complement factors in IBD have been based on antisera which do not distinguish between native and activated components. Also, tissue specimens with unpredictable amounts of retained extracellular proteins have been used. It is therefore uncertain whether previously observed C1q²⁴ and C3²⁴ deposits in the intestinal epithelial basement membrane zone represented immune complexes or retained native components.²⁵ We used extensively washed tissue specimens from which extracellular diffusible proteins had been extracted; and our MoAb to a neoeptope of TCC reacted exclusively with terminal activated complement. We observed virtually no TCC positivity in the basement membrane zone.

Mucosal terminal complement activation may, however, take place in the fluid phase. Although soluble immune complexes may induce complement activation and extensive C3 consumption, relatively little soluble TCC (SC5b-9) is produced.²⁶ Furthermore, soluble SC5b-9 is most likely removed by our tissue washing procedure. We therefore believe that the vascular TCC was present in a tissue bound form and not as free soluble SC5b-9.

In addition to its well known biological effects, complement activation products such as C3a and C5a may trigger other biological systems that participate in the inflammatory reaction. C3b, iC3b, and C3c have been shown to stimulate human macrophages to release prostaglandin E₂ (PGE₂),^{21,27} and C3a and C3b induce thromboxane B₂ (TXB₂) release.^{27,28} C3a, C5a, and C5a_{des Arg} have been shown to stimulate the production of leucotriene B₄ in guinea pig ileum and in lung tissue cultures.^{29,30} Furthermore, generation of TCC on cell membranes have been shown to stimulate human macrophages to release PGE₂ and TXA₂.³¹ Such TCC also induces the production of free oxygen radicals by cultured rat mesangial cells.³² The possibility therefore exist that the increased amounts of TXB₂ and PGE₂ produced *in vitro* by rectal mucosa from UC³³ and the raised concentrations of the PGE₂ and leukotriene B₄ shown by *in vivo* equilibrium dialysis of rectum in relapsing UC,³⁴ may have been induced by complement activation products. The TCC positive fibrillary elements observed in the muscularis mucosae and submucosa in five UC specimens and in one colonic CD specimen may have been associated with S-protein positive elastic fibrils as elastic fibres of human skin have been shown to stain for S-protein.³⁵

In conclusion, our results suggested that *in situ* terminal complement activation takes place in the intestinal lesions of UC and CD. Regardless of the mode of complement activation and the nature of the target attacked, generation of biologically active

products (C3a, C5a, and TCC) will cause inflammation. Local terminal complement activation may therefore be of pathogenetic importance in IBD.

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