Defective release of C5a related chemo-attractant activity from complement in Crohn's disease

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SUMMARY Complement was studied in 20 untreated cases of Crohn's disease and in 20 healthy volunteers by an *in vitro* activation of the cascade reaction. Total haemolytic complement was normal in all patients. In contrast, activation of the alternative pathway lead to a decreased release of C5a related chemo-attractant activity together with a subnormal utilisation of the main complement component C3. This abnormality of complement function was not related to the activity of the disease, site of involvement or to disease duration. The results suggest that an inadequate stimulation of important neutrophil functions may result when bacterial lipopolysaccharides and other macromolecules activating the alternative pathway penetrate the gut mucosa. A delayed clearance from the tissue of such foreign material could be a further pathogenic factor in Crohn's disease leading to granulomatous inflammation by a foreign body reaction.

The migration of neutrophils into areas of acute, experimental inflammation is markedly decreased in Crohn's disease,¹⁻³ and in another granulomatous condition sarcoidosis.⁴ This abnormality of leucocyte function is specific for these two groups of patients,¹⁻³ and probably reflects an inadequate release of chemotactic mediators as the neutrophils themselves seem to be competent.¹⁻³

A normal function of complement seems to be essential for the accumulation of neutrophils in inflammation. Experimental animals, deplete of complement components, are inefficient in localising granulocytes to diverse inflammatory sites.⁵⁻⁷ A delayed migration of neutrophils into skin windows has been reported in a patient with complete absence of the third component of complement, C3.⁸ The peptide C5a, which is released from native C5 during activation of complement,⁹ is likely to be responsible for this migration of neutrophils in inflammation. C5a and its degradation product C5a_{des arg} in highly purified preparations are potent activators of important neutrophil functions as chemotaxis^{10 11} and they stimulate the accumulation of neutrophils in skin window chambers.¹¹

The present series of experiments was conducted to investigate the release of chemotactic factors during activation of complement in Crohn's disease.

Methods

PATIENTS

Twenty consecutive outpatients with Crohn's disease were selected for the study, excluding patients suffering from infection, rheumatic diseases, and other conditions known to affect the immune system. None had received any medical treatment within three months before the study. The diagnosis was based on typical clinical and radiological findings in eight patients and confirmed by histology in 12 patients. The disease involved the ileum in 13 patients, the ileum and the colon in five, and the colon only in two. Two patients had arthralgia. Disease activity was graded according to Harvey and Bradshaw 1980.¹² Seven patients were in complete remission, whereas 13 were in an active stage (score 1-12, median 4). None of the patients showed systemic reactions as fever or loss of bodyweight, and none was confined to bed. Median disease duration was eight years, range three months to 36 years. Forty healthy volunteers with an age and sex distribution similar to the patients served as controls.

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MATERIAL AND ANTIBODIES

Gey's solution was prepared according to Wilkinson 1974¹³ and included disodium EDTA (10 mM) unless otherwise stated. Nylonfibres from a Leukopak^(R) apparatus (Fenwal, Deerfield, Illinois, USA) casein 'alkalilöslich' from Merck, Darmstadt, FR Germany, purified human-albumin and standard human serum from Behringwerke, Marburg, FR Germany and Sephadex^(R) G 75 gel and proteins for gel calibration from Pharmacia Fine Chemicals, Uppsala, Sweden were used. The rabbit immunoglobulins: monospecific antibodies to human C5 and to C3c and the immunoglobulin fraction from non-immunised rabbits prepared identical to the antibodies were all from DAKO immunoglobulins, Copenhagen, Denmark, and haemolysin: rabbit antibodies to goat erythrocytes were from the Pasteur Institute, Paris, France. The assay for protein determinations was purchased from Bio-Rad Laboratories, Richmond, USA.

PREPARATION OF CHEMO-ATTRACTANTS

For activation the freshly prepared heparin plasma (2 IU/ml) was incubated one hour at 22°C with sterile nylonfibres (100 g/l), and then removed from the fibres. Nylonfibres for leukapheresis is a well established activator of alternative pathway complement.¹⁴ They were selected for the experiments to exclude the possibility of adding biological materials to the plasma which might interfere with the assay of chemo-attractant activity. As control served non-activated heparin plasma incubated without nylonfibres.

To prevent spontaneous activation of complement, disodium EDTA (10 mM) was added to the plasma samples immediately after incubation. The samples were analysed in a fresh state or they were frozen in liquid nitrogen and kept at -80°C until examined. Casein (5 g/l) was solubilised in modified Gey's solution at pH 12 according to Wilkinson 1974.¹³

ASSESSMENT OF CHEMO-ATTRACTANT ACTIVITY

Neutrophil migration was quantified by the micropore filter assay¹³ and the leading front method.¹⁵ Neutrophils were isolated from peripheral blood drawn in disodium EDTA (10 mM). After a methyl-cellulose sedimentation leucocytes from the buffy coat were washed thrice and resuspended in Gey's solution without EDTA and including human-albumin (20 g/l). One million cells from this suspension containing on the average 80% neutrophils was added to each 3 µm Millipore^(R) filter and was incubated at 37°C for one hour. Results represent the median of five

determinations on each of two filters.

The chemo-attractant activity of 10% dilutions of the plasma samples in Gey's solution was assessed in two series of experiments: with autologous neutrophils and with heterologous neutrophils from normal subjects. The neutrophils from normal subject were challenged with pairs of plasma preparations from patients and healthy volunteers selected by random numbers. Neutrophils run against solubilised casein and against Gey's solution acted as controls in each experiment. Eluates from Sephadex^(R) chromatography and fractions from antibody incubation studies were tested undiluted with autologous neutrophils.

CHARACTERISATION OF THE CHEMOTACTIC FACTOR

Five millilitre plasma samples from three normals and from three Crohn patients were eluted from a G 75 Sephadex^(R) column (5 cm² × 60 cm) with Gey's solution at 10 ml per hour. Fractions of the eluate were tested for chemo-attractant activity and for protein content by the Bio-Rad^(R) assay. The column was calibrated with proteins: ribonuclease A (MW: 13 700) chymotrypsinogen A (MW: 25 000), and ovalbumin (MW: 43 000) to permit estimations of molecular weight.

Antibody to human C5 and immunoglobulins from non-immunised rabbits were incubated overnight at 4°C with pooled fractions of activated plasma from Sephadex^(R) chromatography eluted at the chemo-attractant peak. After incubation these fractions were tested for chemo-attractant activity in the Millipore^(R) filter assay.

IMMUNOCHEMICAL DETERMINATIONS

C3 and C5 concentrations in non-activated plasma were assessed by immunoelectrophoresis according to Laurell.¹⁶ The antibodies specific to human C3c and to C5 were included in 1% agarose gel, and electrophoresis was performed at 3 V/cm for 18 hours. Standard antigen for C3 determinations was human serum from Behring Werke with a known content of C3c. Concentrations of C5 were expressed in percent of values obtained with a plasma pool from healthy blood donors.

Crossed immunoelectrophoresis of corresponding samples of activated and of non-activated plasma were run simultaneously for the quantitative assessment of C3 utilisation during activation. Complete separation of native C3 and its conversion products was achieved in the first run at 20 V/cm for 1.5 hours.¹⁷ The second electrophoretic dimension was performed at 3 V/cm for 18 hours in antibody to C3c. Utilisation of C3 (in percent) represents the decrease in precipitation area of native C3.

HAEMOLYTIC ASSAY

CH 50. Total haemolytic complement was determined by the ability of serum dilutions to lyse sheep erythrocytes coated with haemolysin. In these experiments serum was allowed to clot for one hour at room temperature.

STATISTICS

The results were evaluated with non-parametric statistics: Mann-Whitney's U test, Wilcoxon's matched-pairs signed-ranks test and Spearman's rank correlation test.

Results

The chemo-attractant activity of nylonfibre incubated plasma from Crohn's disease patients was markedly depressed as assessed with both autologous neutrophils and with neutrophils from normal subjects ($p < 0.005$, Fig. 1). The responses of neutrophils to non-activated plasma from patients and from normals were not different ($p > 0.10$, Fig. 2). Neutrophils run against casein and Gey's solution reacted identically in patients and healthy volunteers (Table 1).

The chemo-attractant activity of nylonfibre

Fig. 1 Decreased chemo-attractant activity of nylonfibre incubated plasma from patients with Crohn's disease (CD) compared with reference values from normals (N) ($p < 0.005$). ● – Crohn's disease, ○ – normals.

Fig. 2 The chemo-attractant activity of plasma incubated without nylonfibres was equal in patients and in healthy volunteers. ● – Crohn's disease, ○ – normals.

incubated plasma was confined to a single, low molecular weight, derivative from C5, having the characteristics of C5a.¹⁸ A single peak of chemo-attractant activity appeared in fractions of nylonfibre incubated plasma eluted from the Sephadex^(R) column (Fig. 3). Fractions of non-activated plasma showed no chemo-attractant activity. The factor responsible for the peak of activity had an apparent molecular weight of 15 000 daltons as estimated according to Andrews.¹⁹ The biological activity eluted in the MW 15 000 region was abolished by incubation with antibody specific to human C5 (Fig. 4) but remained unaffected by incubation with immunoglobulins from non-immunised rabbits.

The utilisation (in percent) of C3 during activation was decreased in Crohn's disease patients ($p < 0.005$, Fig. 5). Concentrations of C3, of C5, and total haemolytic complement (CH 50) were normal in the patients (Table 2). The normal level of C3 in Crohn's disease plasma permitted the direct comparison of the percentual C3 utilisation obtained in patients and normal controls.

A positive correlation was seen between the C3 utilisation and the release of chemo-attractant

Table 1 Neutrophil migratory response in μm per hour to casein and Gey's solution. Median values are given with ranges in parentheses.

	Casein 5 g/l	Gey's solution
Crohn's disease patients (n=20)	75 (60–100)	20 (0–37)
Healthy volunteers (n=40)	75 (65–115)	21 (0–35)

Fig. 3 Nylonfibre incubated plasma was eluted from a Sephadex^(R) column, and the fractions were tested for chemo-attractant activity (individual values) and for protein content (curve). Both in patients and in normal controls a single peak of chemo-attractant activity appeared in MW 15 000 region. Arrows indicate the elution volume of calibration proteins: I ovalbumin, II chymotrypsinogen A, III ribonuclease A. Results represent median values from three experiments. ● – Crohn's disease, ○ – normals.

factors as assessed with autologous neutrophils ($r_s=0.484$, $p<0.005$, $n=38$, Fig. 6) and with heterologous neutrophils from normal subjects ($r_s=0.319$, $p<0.05$, $n=38$). Trace amounts of C3 conversion products was detected in four samples of non-

activated plasma and may reflect the instability of C3 at 37°C.

The C3 utilisation and the chemo-attractant activity released during nylonfibre incubation had no relation with the disease duration, disease

Fig. 4 Fractions of nylonfibre incubated plasma eluted from Sephadex^(R) column in MW 15 000 region (chemo-attractant peak) was incubated with antibody to human C5 or alone, constituting control (C). Biological activity was abolished by incubation with 1 to 5 dilution of antibody, that is titre 5. ● – Crohn's disease, ○ – normals.

Fig. 5 Decreased utilisation (percent) of C3 during nylonfibre incubation of plasma from Crohn's disease (CD) compared with reference values from normals (N) ($p<0.005$). ● – Crohn's disease, ○ – normals.

Table 2 Median values and ranges in parentheses of plasma C3 and C5 concentrations and total haemolytic complement titres

	C3 µg/ml	C5 percent	CH 50 titre
Crohn's disease patients (n=20)	920 (650-1370)	104 (74-180)	32 (16-64)
Healthy volunteers (n=20)	915 (620-1275)	91 (52-145)	32 (16-32)

activity or the involvement of the bowel ($p>0.10$). The release of chemo-attractant activity in patients grouped according to the stage of disease activity is shown in Fig. 6.

Discussion

The present study shows a dysfunction of plasma complement in Crohn's disease. A decreased release of chemo-attractant activity was found during an *in vitro* activation of the alternative pathway. This abnormality was not related to the site and severity of the disease or to disease duration. The specificity of this finding has to be evaluated in further experiments including patients with infections and inflammatory conditions as rheumatoid arthritis, sarcoidosis, and ulcerative colitis.

Fig. 6 Utilisation of C3 during incubation with nylonwool correlated positively with the chemo-attractant activity released as assessed with autologous neutrophils ($p<0.005$). Individual values for patients and for normal controls are given. ● – Crohn's disease, ○ – normals.

Fig. 7 Relation of chemo-attractant activity released during complement activation to stage of disease activity shown. Disease activity graded according to Harvey and Bradshaw.¹² ● – Crohn patients.

Steroids, sulphasalazine, and its active metabolite 5 amino salicylic acid are all potent inhibitors of leucocyte motility.²⁰ During treatment with steroids a decreased chemo-attractant activity of complement activated serum has recently been shown in Crohn's disease.²¹ We were able to extend these results by detecting a similar dysfunction of complement in a group of patients which had not received any drugs for at least three months.

The chemo-attractant activity released from complement in our experiments was confined to a low molecular weight derivative from C5, that is to C5a or to its degradation product C5a_{des arg}. Accordingly, the decreased release of chemo-attractant activity in Crohn's disease might reflect (1) a depressed release of C5a, (2) an increased degradation of C5a to the less potent C5a_{des arg} by carboxypeptidase B known to be present in plasma,¹⁸ or (3) the effect of cell directed inhibitors of neutrophil migration in Crohn's disease plasma. The associated finding in our patients of a subnormal utilisation of the main complement component C3, correlated positively to the decreased release of chemo-attractant activity, favours the hypothesis of a decreased release of C5a as explanation. Furthermore, experiments following the procedures generally outlined for the detection

of chemotactic factor inactivators and for inhibitors of neutrophil chemotaxis has been negative in Crohn's disease.² Immunochemical methods for a quantitative assessment of C5a has to be developed to settle this question definitely.

A primary cellular defect of the neutrophil produces histological changes of the gut in chronic granulomatous disease, closely resembling those seen in Crohn's disease,²² but the two conditions are obviously dissimilar. Neutrophils from Crohn's disease patients are competent in that they behave normally in the NBT reduction test^{1 3 23} and their oxygen consumption response to bacteria is normal.² Our results indicate that in Crohn's disease a neutrophil dysfunction may be caused by an inadequate activation of neutrophil chemotaxis, and of other important cellular function of neutrophils, by C5a. This may further explain a well known decreased migration of neutrophils into skin windows in Crohn's disease.¹⁻³ A defective phagocyte function delaying the clearance of gut luminal macromolecules, which are known to penetrate the mucosa,²⁴ could be a pathogenic factor in Crohn's disease.

The normal total haemolytic complement (CH 50) in our patients confirms previous findings.²⁵ Complement dysfunction in Crohn's disease may be confined to the alternative pathway as a diminished consumption of complement components C3 to C9 after activation of the alternative pathway, and a normal consumption after activation of the classical pathway has been reported in this condition.²⁵

A general lack of complement components caused by the chronic complement activation present in Crohn's disease^{26 27} could not explain our findings. Normal concentrations of C3 and C5 in the present study are in agreement with earlier reports of normal or raised levels of C3²⁵⁻²⁸ and factor B²⁵⁻²⁹ in Crohn's disease. An assessment of the functional activity of individual complement components is required to identify the factor(s) responsible for complement dysfunction in Crohn's disease.

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