Candidacidal activity of Crohn's disease neutrophils

F T Curran, D J Youngs, R N Allan, M R B Keighley

Abstract

The ability of normal and Crohn's disease neutrophils to kill *Candida albicans* has been studied using neutrophils isolated from peripheral blood and suspended in phosphate buffered saline at 5×10^6 cells per ml. *C. albicans* was grown to a stationary phase in broth culture and suspended in phosphate buffered saline at 10^7 organisms/ml. Neutrophils and *Candida* were then incubated together at 37°C in a shaking water bath in the presence of fresh serum. At 30 and 60 minutes samples were withdrawn, neutrophils lysed, and *Candida* survival assessed by colony counting. Results were compared with control suspensions of *Candida* incubated with serum alone. After 30 and 60 minutes in the presence of autologous serum normal neutrophils had killed significantly more *Candida* than Crohn's disease neutrophils (mean (SD) 61.0 (16.7)% v 40.5 (16.2)% at 30 minutes, $p < 0.0001$; 83.2 (7)% v 70.8 (16)% at 60 minutes, $p < 0.005$). The results did not alter significantly when normal neutrophils were incubated with *Candida* in the presence of Crohn's disease serum instead of normal serum. When Crohn's disease neutrophils were incubated with *Candida* in the presence of normal serum instead of autologous serum there was some improvement in candidacidal ability at 30 minutes (48.9 (20.6)% v 40.5 (16.2)%, $p < 0.03$) but not at 60 minutes. Phagocytosis, measured using a radiometric assay, was normal. Neutrophils from patients with Crohn's disease have an impaired ability to kill this granuloma provoking organism. It is not due to serum inhibitors or defective phagocytosis.

The inactivation of invading micro-organisms is the most important function of the neutrophil. Although various abnormalities of neutrophil function have been reported in Crohn's disease,¹ their capacity to kill granuloma provoking organisms has not been evaluated.

This study was designed to investigate the ability of Crohn's disease neutrophils to kill *Candida albicans*. The organism was chosen for study because it is present in the gastrointestinal tract of 14-47% of the healthy population^{2,3} and causes chronic inflammatory diseases, such as chronic mucocutaneous candidiasis, and leads to granuloma formation.³ It may also cause enteritis⁴ and allergic colitis.^{5,6} It is a convenient organism to culture compared to other organisms responsible for causing chronic inflammatory conditions. Whereas *C. albicans* can be cultured in two to three days, mycobacteria, for example, require four to 12 weeks. It is a relatively safe organism to handle and is the species most resistant to killing by neutrophils.⁷

Phagocytosis was studied using a sensitive

radiometric assay to determine whether impairment of candidacidal activity could be a result of this.

Methods

PREPARATION OF ORGANISMS

C. albicans from the same source were used in all experiments. The organisms were isolated from the faeces of a healthy woman who was taking no drugs. The organism was maintained in stock culture on a slope of malt extract agar (Oxoid) at room temperature. When required, a sample of the organisms was inoculated into approximately 50 ml Sabouraud liquid medium (Oxoid) and grown to a stationary phase for three to five days at 30°C. The organisms were washed twice in Dulbecco's B phosphate buffered saline (PBS), centrifuged at 1500 g for 10 minutes after each wash, counted in an improved Neubauer haemocytometer, and suspended in PBS at a concentration of 10^7 organisms per ml. Freshly cultured organisms were used in all tests.

PREPARATION OF NEUTROPHILS

Neutrophils were isolated from fresh venous blood using discontinuous density gradients of Percoll (Pharmacia) as follows. An iso-osmotic stock solution of Percoll was made by adding 1 part 1.5 M saline to 9 parts Percoll (resulting density 1.123 g/ml). Stock Percoll was further diluted with 0.15 M saline to densities of 1.070 g/ml⁸ and 1.097 g/ml⁹ for the separation of white cells. In a sterile universal container was placed 6 ml Percoll of density 1.097 g/ml, then 4 ml Percoll of density 1.070 g/ml was carefully layered on top avoiding mixing at the interphase.

Five ml heparinised blood was diluted with an equal volume of 0.15 M saline and carefully layered on top of the Percoll in the same way. The tube was centrifuged at 200 g for 25 minutes, to ensure isopycnic banding of cells at the relevant interphases; granulocytes were suspended between the two layers of Percoll. They were removed with a pipette and washed twice in PBS and centrifuged at 1500 g for 10 minutes after each wash. Any contaminating erythrocytes were lysed by hypotonic shock treatment by resuspending the cells in tris-ammonium chloride for 10 minutes before the second wash. Neutrophils were counted in an improved Neubauer chamber and resuspended in PBS at concentrations of 5×10^6 cells per ml. All neutrophil suspensions were at least 95% pure and viability, assessed by trypan blue exclusion tests,⁷ was 98%.

CANDIDACIDAL ASSAY

The study was performed in two parts using

Departments of Surgery and Gastroenterology, The General Hospital, Birmingham

F T Curran
D J Youngs
R N Allan
M R B Keighley

Correspondence to:
Mr F T Curran, Queen Elizabeth Hospital, Edgbaston, Birmingham B15.

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TABLE I Subject data – candidacidal assay

	Healthy subjects	Crohn's disease
<i>Autologous serum:</i>		
No	20	30
Mean (SD) age (years)	47 (18)	45 (16)
Sex	13 M, 7 F	10 M, 20 F
Disease activity	—	Quiescent 19 Active 11
No taking steroid treatment	—	4
<i>'Crossed' serum:</i>		
No	15	25
Mean (SD) age (years)	45 (18)	49 (14)
Sex	8 M, 7 F	8 M, 17 F
Disease activity	—	Quiescent 15 Active 10
No taking steroid treatment	—	4
<i>Phagocytosis assay:</i>		
No	20	20
Mean (SD) age (years)	40 (15)	38 (13)
Sex	9 M, 11 F	14 M, 6 F
Disease activity	—	Quiescent 12 Active 8
No taking steroid treatment	—	1

fresh serum, either autologous or 'crossed' – that is, normal neutrophils with Crohn's disease serum and vice versa. With autologous serum, 20 healthy subjects and 30 patients with Crohn's disease were studied; with crossed serum there were 15 and 25 respectively. Details of subjects are summarised in Table I. Four patients were taking steroids; none was taking sulphasalazine. Crohn's disease activity was assessed using the method of Harvey and Bradshaw.¹⁰

A 0.5 ml neutrophil suspension, 0.25 ml *C albicans*, and 0.25 ml fresh serum were brought to 37°C and mixed in a stoppered polystyrene test tube. Tubes were laid on their sides in a shaking water bath at 37°C so that the contents flowed continually from one end of the tube to the other during each cycle. A control tube containing *C albicans*, fresh serum, and 0.5 ml PBS instead of neutrophils was included in each test.

At time 0 (immediately after mixing) a 10 µl aliquot was withdrawn from the control tube for an initial colony count, so that the exact number of viable organisms used in each test was known.

Two time points were included for each assay. At 30 and 60 minutes 10 µl aliquots were

withdrawn from each tube (control, normal, and Crohn's disease) and allowed to stand for at least 10 minutes in 10 ml sterile distilled water to lyse neutrophils and release ingested organisms. This time was adequate to lyse all neutrophils, which was confirmed microscopically on several occasions – after centrifuging the water no neutrophils could be identified in the deposit. Each aliquot was serially diluted to 1×10⁴ and 1×10⁵ of the initial concentration using sterile distilled water and sterile glass containers. One ml of both the 10⁴ and 10⁵ dilutions was spread on malt extract agar and incubated at 30°C for two to three days. The number of colonies of *C albicans* on the plates (Fig 1) was counted using an illuminated colony counter. The 10⁴ dilution plate was normally counted but if the number of colonies was excessive the 10⁵ dilution plate was used instead. Any 10⁵ plate used contained at least 15 colonies.

The percentage killing was calculated using the formula:

% of yeast killed=

$$100 - \left(\frac{(\text{No of colonies at 30 or 60 minutes}) \times 100}{(\text{No of colonies at time 0})} \right)$$

The percentage of *Candida* remaining on control plates at 30 and 60 minutes was also calculated to ensure that neither normal nor Crohn's disease serum alone had candidacidal activity, using the formula:

% candida remaining on control plate=

$$\frac{(\text{No of 'control' colonies at 30 or 60 minutes})}{(\text{No of colonies at time 0})} \times 100$$

INTERASSAY VARIABILITY

Three assays were performed in triplicate on different days. The numbers of *Candida* colonies on each plate are given in Table II. The variance, calculated using the formula:

$$\text{variance} = \frac{\text{standard deviation}}{\text{mean}} \times 100,$$

was 9.5% at 30 minutes and 11.4% at 60 minutes.

PHAGOCYTOSIS ASSAY

Twenty healthy subjects and 20 patients with Crohn's disease were studied. Details are summarised in Table I. Neutrophils were suspended at 8×10⁵ cells/ml PBS and *C albicans* at 4×10⁶ organisms/ml PBS, a *Candida*:neutrophil ratio of 5:1. A 0.5 ml neutrophil suspension, 0.5 ml *Candida* suspension, and 50 µl autologous

Figure 1: *Candida albicans* on agar plates after 3 days' incubation: 1. At time 0; 2. After 30 minutes with normal neutrophils; 3. After 60 minutes with normal neutrophils; 4. After 30 minutes with Crohn's neutrophils; 5. After 60 minutes with Crohn's neutrophils.

TABLE II Interassay variability

	No of <i>Candida</i> colonies	
	30 minutes	60 minutes
Assay 1	71	51
	80	51
	73	47
Assay 2	44	30
	40	27
	52	32
Assay 3	71	27
	69	19
	60	19

serum were incubated at 37°C in a shaking water bath. The tubes were laid on their sides so that the contents continually flowed from one end of the tube to the other during each cycle. All experiments were performed in duplicate and a control tube containing 0.5 ml PBS instead of neutrophils was included in each assay. During this incubation *Candida* were phagocytosed by neutrophils.

After incubation 0.2 ml aliquots were withdrawn from each tube, tests, and controls, and transferred into microtitre wells containing 3.6 µl [5,6-³H] uridine (specific activity 46 Ci/mmol, Amersham International) made up to 20 µl with PBS. The microtitre plate was incubated at 37°C for one hour. During this time *Candida* which had not been phagocytosed incorporated ³H-uridine.

After the second incubation 0.1 ml aliquots were withdrawn from each microtitre well after mixing and transferred to a Whatman GF-B filter (pore size 1 µm) prewetted with PBS. Each filter was washed with approximately 8 ml sterile distilled water under suction from a stream of water to remove free radioactivity, so as to leave only *Candida*-associated radioactivity on the filter. To confirm that the wash was sufficient to remove all free radioactivity, ³H-uridine was placed on four filters and washed precisely as described; only background radioactivity could be detected on them.

The filters were dried overnight, placed in scintillation fluid, and the radioactivity counted. To check that no *Candida* passed through the filter the filtrate was centrifuged and the pellet examined under a microscope; it contained a few fragments of glass fibre from the filter but no organisms.

The mean number of *Candida* phagocytosed per neutrophil (the phagocytic index), was calculated using the following formula:

$$\text{Phagocytic index} = \left(1 - \frac{(\text{dpm for } \textit{Candida} + \text{neutrophils})}{(\text{dpm for } \textit{Candida} \text{ alone})} \right) \times \frac{\text{No of } \textit{Candida}}{\text{No of neutrophils}}$$

where dpm = disintegrations per minute.

STATISTICAL ANALYSIS

Results were analysed using the Wilcoxon rank sum test and are expressed as mean (SD).

Results

CANDIDACIDAL ACTIVITY

The percentages of *C. albicans* killed at 30 and 60 minutes by normal and Crohn's disease neutrophils in autologous serum and crossed serum are shown in Figures 2 and 3, and Tables III and IV. After 30 minutes normal neutrophils had killed significantly more *Candida* (mean (SD) 61 (16.7)%) than Crohn's disease neutrophils (40.5 (16.2)%) ($p < 0.0001$). After 60 minutes the proportion of *Candida* killed had increased but was

Figure 2: Neutrophil candidacidal activity after 30 minutes.

still significantly greater using normal neutrophils (83.2 (7)%) than Crohn's disease neutrophils (70.8 (16)%) ($p < 0.005$).

When normal neutrophils were incubated with Crohn's disease serum instead of normal serum the mean killing ability was not significantly altered after either 30 minutes 60.8 (18.4)% in Crohn's disease serum *v* 61 (16.7)% in normal serum or 60 minutes (78.7 (8.5)% *v* 83.2 (7)%) (Table IV).

After 30 minutes in normal serum the mean killing ability of Crohn's disease neutrophils improved from 40.5 (16.2)% to 48.9 (20.6)% ($p < 0.03$), but was still lower than that of normal neutrophils in normal serum (61 (16.7)%) ($p < 0.05$). At 60 minutes this improvement in killing ability could no longer be shown (73.7

Figure 3: Neutrophil candidacidal activity after 60 minutes.

TABLE III Percentages of *Candida albicans* killed

Normal neutrophils in normal serum		Crohn's neutrophils in Crohn's serum	
30 min	60 min	30 min	60 min
75.5	96.1	38.5	51.4*
78.4	82.0	44.7	79.3
64.4	80.0	44.0	68.0
69.1	73.2	40.8	64.4*
79.2	87.3	21.2	33.6
53.7	81.1	5.6	81.6*
48.0	86.1	36.8	44.5
32.5	87.9	63.2	54.5
49.2	82.9	44.2	63.1*
86.6	92.4	55.0	71.9*
66.1	82.7	33.2	72.6
80.4	91.5	30.0	71.6
61.0	85.0	39.7	80.4*
75.9	83.4	48.8	80.2
55.2	73.9	35.8	81.7
46.7	71.3	44.6	92.0
48.7	78.9	72.3	81.7
26.8	80.4	59.6	81.5
47.7	73.8	45.4	72.3
74.4	93.1	67.7	94.9
		28.9	75.3
		21.5	63.2*
		50.0	53.9*
		57.7	80.5
		3.0	35.2
		44.3	77.0
		25.3	56.0
		24.7	90.0*
		44.9	88.7*
		42.7	84.1*
Mean (SD)	61.0 (16.7) 83.2 (7.0)	40.5 (16.2)	70.8 (16.0)

*Denotes patient with active disease.

(14.6)% in normal serum and 70.8 (16)% in Crohn's disease serum, not significant). These findings were independent of disease activity (Figs 4 and 5, Tables III and IV). Neither normal nor Crohn's disease serum seemed to have candidacidal activity. When compared to colony counts at time 0, the proportions of *Candida* colonies remaining after 30 minutes in normal serum was 91.2 (14.3)% and in Crohn's disease serum 97.7 (8.5)%. After 60 minutes the results were 92.0 (17.2)% in normal serum and 92.1 (9.4)% in Crohn's disease serum

Figure 4: Crohn's disease neutrophil candidacidal activity at 30 minutes related to disease activity. 's' denotes patient on steroids.

TABLE IV Percentages of *Candida albicans* killed

Normal neutrophils in Crohn's serum		Crohn's neutrophils in normal serum	
30 min	60 min	30 min	60 min
30.8	82.8	53.6	52.0
69.2	81.1	58.8	62.8*
52.1	78.9	77.3	87.3*
30.0	61.7	30.8	31.5*
91.1	90.2	61.0	71.6
68.1	89.2	58.9	65.8
80.0	84.3	43.3	72.5
54.0	69.3	49.6	87.9
47.8	75.1	88.4	91.1
78.0	79.2	34.4	88.8
74.1	91.7	70.0	81.5
71.0	80.3	64.6	68.8
69.2	76.6	58.7	83.0
59.5	68.2	51.5	65.1
37.7	72.2	8.0	67.2*
		62.7	65.1*
		54.8	73.9
		13.0	70.0
		70.4	90.0
		44.0	82.0
		49.0	90.3*
		29.4	87.3*
		5.0	62.6*
		49.0	55.6*
		37.1	86.7*
Mean (SD)	60.8 (18.4) 78.7 (8.5)	48.9 (20.6)	73.7 (14.6)

*Denotes patient with active disease.

(Table V, Fig 6). These results were obtained from 17 assays with autologous serum and 14 with crossed serum at the 30 minute time point. At 60 minutes two plates from each group were unsuitable for counting so that 15 and 12 results respectively were available for analysis.

PHAGOCYTOSIS
Normal neutrophils incorporated a mean (SD) of 2.0 (0.98) *C. albicans* and Crohn's disease neutrophils a mean (SD) of 1.9 (0.88) *C. albicans* (Table VI, Fig 7). These results are not significantly different.

Figure 5: Crohn's disease neutrophil candidacidal activity at 60 minutes related to disease activity. 's' denotes patient on steroids.

TABLE V Candidacidal activity of serum

Normal serum		Crohn's serum	
30 min	60 min	30 min	60 min
84.1	76.4	108.0	88.4
70.0	78.0	96.9	90.0
76.8	56.4	94.7	105.3
95.5	67.7	96.7	100.8
96.9	94.2	107.1	107.1
83.2	87.4	96.2	97.7
100.0	97.2	87.7	85.9
97.5	111.6	95.1	91.3
119.6	107.7	87.1	91.0
98.5	110.0	91.3	90.4
95.3	83.0	100.0	84.1
101.2	101.7	89.9	73.5
78.8	91.3	99.1	NA
100.0	100.4	117.8	NA
104.2	116.5		
88.3	NA		
60.1	NA		
Mean (SD)	91.2 (14.3)	97.7 (8.5)	92.0 (17.2) 92.1 (9.4)

NA=Not available.

Discussion

Two previous studies have investigated microbicidal activity in Crohn's disease with conflicting results. Lamster *et al*¹¹ reported one patient whose neutrophils showed an enhanced ability to phagocytose and lyse *Escherichia coli* compared to a single control subject. Worsaae *et al*¹² investigated nine patients and found an 'increased intracellular survival' of *Staphylococcus aureus* in Crohn's disease neutrophils. The importance of this finding is uncertain since staphylococci do not form granulomas and patients with Crohn's disease are not unusually susceptible to bacterial infections.

Lehrer and Cline¹³ first showed in vitro the phagocytosis and killing of *C albicans* by human neutrophils and monocytes. The process is rapid; more than 90% of *C albicans* injected into the bloodstream of rabbits is removed within 10 minutes¹⁴ and more than 70% is removed within five minutes from the blood stream of rats.¹⁵ The yeast are killed intracellularly by the phagocyte peroxide/myeloperoxidase system¹⁶⁻¹⁸ and a non-oxidative fungicidal mechanism.¹⁶⁻¹⁸ There are no reports of candidacidal activity in patients with inflammatory bowel disease, but neutrophil myeloperoxidase is probably normal in Crohn's disease.¹⁹

A *Candida*/neutrophil ratio of 1:1 was used in

Figure 6: Candidacidal activity of normal and Crohn's disease serum.

TABLE VI Phagocytic index

Normal	Crohn's disease
2.5	1.1
1.5	1.0
1.8	1.3
1.7	1.4
1.4	1.2
2.0	2.4
2.7	1.9
2.5	2.3
1.8	2.2
3.5	1.3
0.8	3.3
0.7	2.6
1.6	3.2
0.4	3.7
0.7	0.3
2.3	1.5
3.9	2.8
2.3	1.5
2.7	1.9
3.5	1.3
Mean (SD)	2.0 (0.98) 1.9 (0.88)

the assay, as recommended by Babior and Cohen,⁷ since the number of *Candida* presented to neutrophils and the number killed is linear up to a ratio of 5:1.²⁰

These results suggest that neutrophils from patients with Crohn's disease have a significantly impaired candidacidal activity in vitro, even in quiescent disease, implying that patients with Crohn's disease are less able to inactivate organisms which can cause chronic inflammatory diseases. The results are not due to a serum inhibitor since adding Crohn's disease serum to washed normal neutrophils did not impair their candidacidal activity. The temporary improvement in killing by Crohn's disease neutrophils in the presence of normal serum at 30 minutes but not at 60 minutes may suggest some replenishment of a deficient plasma factor important for killing. If it was due to poor opsonisation the improvement should have been sustained and Crohn's disease serum should have impaired the candidacidal activity of normal neutrophils.

Phagocytosis was studied because any impairment in opsonisation or the ability of Crohn's

Figure 7: Neutrophil phagocytosis of *Candida albicans*.

disease neutrophils to phagocytose would result in impaired killing ability. A radiometric assay was used,^{20, 21} based on the observations that the uptake of ³H-uridine by *Candida* is an index of phagocytic function, that there is linear correlation between uridine incorporation and yeast numbers, and that neutrophils do not take up uridine in short term cultures so that *Candida* within neutrophils have no access to uridine.²¹ The assay is one of the best available for measuring phagocytosis and opsonisation,^{22, 23} and no abnormality was found.

There was no evidence that normal or Crohn's disease serum alone had candidacidal activity, a mean of 92% of *C. albicans* remaining after one hour. This confirms the findings of Wilkinson,²⁴ who reported that less than 10% of organisms should be dead after one hour. Some counts were greater than 100%, presumably because *Candida* continued to multiply during the assay – serum at 37°C is a good stimulant of *Candida* germ tube formation.^{25, 26} A few counts in normal serum were quite low at 30 and 60 minutes, due either to a sampling error or to the presence in serum of antibodies to *Candida* which result from the host's immune response to infection. There is no good published evidence for true fungistasis of *C. albicans* by serum.³

Many factors can affect neutrophil function and in patients with Crohn's disease malnutrition is an important consideration. We, however, found similar results in patients with quiescent and active disease and know of no data suggesting that patients in a quiescent phase of their disease are malnourished. Hill *et al* found that malnutrition was unlikely to be present when patients with inflammatory bowel disease were in remission or during the early stages of an acute attack.²⁷ Deficiency of micronutrients, such as iron, necessary for normal neutrophil function is unlikely to have caused the impaired killing since iron is an essential growth factor for *C. albicans*. Iron deficiency offers a competitive advantage to the host rather than the organism because iron binding to transferrin and lactoferrin renders the level of free ionic iron too low for microbial growth.^{28, 29} The growth of *C. albicans* in human serum is directly related to the percentage of iron saturation³⁰ and iron deficiency increases resistance to *C. albicans* in vivo.³¹ If our patients were iron deficient the killing of *Candida* would have been greater than that in normal subjects.

An unexplained finding is that some patients had normal neutrophil candidacidal activity, as shown by the overlapping results in Figures 2 and 3. The reason is not clear but the results imply that the defect is either intermittent or occurs only in certain patients, but these were not necessarily patients with quiescent disease or those on a particular treatment.

Neutrophils from many patients with Crohn's disease have an impaired ability to kill *C. albicans*, an organism known to cause chronic inflammatory diseases. There was no evidence of a serum inhibitor, defective opsonisation, or impaired

phagocytosis, and a cellular defect is the most likely explanation. Further work is needed to determine the reason for these findings and whether they are disease specific.

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