Leucocyte migration studies in Crohn's disease using Crohn's colon homogenate and mitochondrial and microsomal fractions

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SUMMARY Leucocytes from 38 patients with Crohn's disease were tested for evidence of migration inhibition in the presence of preparations of colon from a patient with this disease. The occurrence of migration inhibition showed a positive correlation with clinically active disease and a negative correlation with immunosuppressive treatment. It was not seen with leucocytes from 12 healthy subjects.

Impairment of cellular immunity has been described in Crohn's disease (Phear, 1958; Parent, Barrett, and Dodd Wilson, 1971; Guillou, Brennan, and Giles, 1973). This state of relative anergy, with the further resemblance to sarcoidosis of a similar non-caseating granulomatous lesion seen in both conditions, has led to investigation of cellular hypersensitivity to sarcoid spleen preparations in Crohn's disease (Willoughby and Mitchell, 1971; Richens, Gough, and Williams, 1973). These studies, using the leucocyte migration technique, showed evidence of hypersensitivity to sarcoid spleen in a proportion of the Crohn's patients. The technique had previously shown no evidence of hypersensitivity to extracts of foetal colonic and jejunal mucosa in Crohn's disease, although leucocytes from a majority of patients with ulcerative colitis had shown inhibited migration to these extracts (Bendixen, 1969).

In this study we have investigated leucocyte migration in cases of Crohn's disease, using extracts from the colon of a patient with this disease. In addition to testing leucocyte migration against colon homogenate, it seemed useful to attempt to localize a possible subcellular site of hypersensitivity by testing against mitochondrial and microsomal fractions of colonic mucosa.

Patients and Methods

PATIENTS

Thirty-eight patients with Crohn's disease were

studied: 14 males and 23 females with a mean age of 41 years. The diagnosis was established on clinical and radiological grounds alone in 21 cases and was confirmed by histological evidence in the other 17 patients who had undergone surgery. Twenty-eight patients were judged to have active disease on clinical criteria of continuing abdominal pain, weight loss, diarrhoea, anaemia, and fever, whereas the remaining 10 had none of these features and appeared well. This arbitrary classification cannot be an accurate assessment, but it was felt that an attempt should be made to correlate clinical activity of disease with the results of immunological testing.

CONTROLS

This group comprised 12 healthy subjects, six male, and six female, with a mean age of 32 years.

PREPARATION OF ANTIGENS

Under sterile conditions mucosal and submucosal layers were stripped from the muscular wall of colon removed at operation from a patient with Crohn's disease. After these had been cut into small pieces and suspended in 10 times their volume of 0.25 M sucrose solution pH 7.2 at 4°C, they were homogenized by 10 passes in a Potter Elvehjem homogeniser. The homogenate was centrifuged at 600 *g* for 10 minutes, and part of the supernatant fraction retained to serve as the colonic homogenate in this study. The remainder was centrifuged at 5000 *g* for 20 minutes. The precipitate from this centrifugation was resuspended and used as the mitochondrial fraction, after two washings in 0.25 M

sucrose, while the supernatant was again centrifuged at 104 000 *g* for one hour. The precipitate from this fraction contained the microsomes which were again resuspended. The particulate subcellular fractions were examined by negative staining in an electron microscope. Each fraction was standardized for protein content and adjusted to a concentration of 100 $\mu\text{g/ml}$ for the final culture fluid (Lowry, Rosebrough, Farr, and Randall, 1951). All the fractions were stored in aliquots at -20°C .

LEUCOCYTE MIGRATION TEST

The method of Bendixen and Soborg (1969) was used with minor modifications. The theoretical basis of this test depends on the fact that lymphocytes from a sensitized individual, on contact with the specific antigen, produce a soluble migration inhibition factor (MIF) which modifies leucocyte migration. Testing leucocytes *in vitro* against an antigen to which the individual is hypersensitive usually results

in inhibition of leucocyte migration, whereas in the absence of antigen cell migration is unaffected. Stimulation of leucocyte migration has been noted to indicate a weak hypersensitivity to the antigen used (Søborg, 1967). The method used was as follows.

Twenty-five ml of venous blood was collected and heparinized (Evans preservative-free heparin, 20 units/ml) and the blood allowed to sediment at 37°C for 45 minutes. The leucocyte-rich plasma was removed, centrifuged at 150 *g* for 10 minutes, and the cell pellet washed a further three times with phosphate-buffered saline, pH 7.2. The washed cells were then resuspended in Eagles MEM, supplemented with 15% foetal calf serum (Flow Laboratories), penicillin 100 units/ml, and streptomycin 100 $\mu\text{g/ml}$ to a concentration of 7×10^7 cells/ml. Capillary tubes (Drummond 25 μl microcaps) were filled with the cell suspensions, sealed at one end (Cristaseal, Hawksley) and centrifuged at 150 *g* for five minutes. The tubes were cut 1 mm below the cell-fluid inter-

Fig Leucocyte migration indices obtained with Crohn's diseased gut constituents in various categories of Crohn's patients and normal subjects. (Dotted lines indicate range (mean $\pm$ 2SD) of migration indices from normal subjects.)

space and the cell pellet was positioned by means of silicone grease in a leucocyte migration chamber (Sterilin Ltd). One series of at least three chambers was filled with culture medium alone, and a second series with culture medium plus antigen. The chambers were sealed with glass coverslips and incubated horizontally at 37°C for 21 hours. The migration pattern was then projected, drawn, and measured by planimetry.

The effect of antigen on cell migration was expressed as migration index (MI) as follows:

Mean migration in presence of antigen

Mean migration in absence of antigen

Results were analysed statistically using the Mann-Whitney two-sample ranking test to see if the presence of antigen had a significant effect on cell migration. The average variation between replicate cultures was 10%.

Results

The effect of crude colonic mucosal homogenate on leucocyte migration is shown in the figure. The control population gave a mean MI of 0.99 with a range (mean $\pm$ 2 SD) of 0.91 $\rightarrow$ 1.07. Eighteen of 32 Crohn's patients tested (57%) gave abnormal migration values, ie, values outside this range; two patients (6%) showed migration stimulation (1.15 $\pm$ 0.01); 16

(50%) showed inhibition (0.79 $\pm$ 0.10), and the remaining 14 (44%) gave values within the normal range (0.98 $\pm$ 0.04). The difference between the two groups, total patients and controls, is significant ($P < 0.005$).

The effect of colon mitochondrial fraction on leucocyte migration is also shown in the figure. The mean migration index for the control group is 0.95 with a range of 0.77 $\rightarrow$ 1.13. Seventeen out of 29 Crohn's patients (58%) gave abnormal migration values: three (9%) showed stimulation (1.21 $\pm$ 0.01); 14 (49%) showed inhibition (0.70 $\pm$ 0.07); and the remaining 12 (42%) gave values within the normal range (0.93 $\pm$ 0.03). The difference between the patient and control groups is again significant ($P < 0.005$).

The third section of the figure shows the effect of colon microsomal fraction on leucocyte migration. The mean migration index for the control group is 0.97 with a range of 0.83 $\rightarrow$ 1.11. Twenty-two out of 36 in the Crohn's group (61%) gave abnormal migration values: four patients (11%) showed stimulation (1.27 $\pm$ 0.17); 18 (50%) showed inhibition (0.72 $\pm$ 0.03); and the remaining 14 (39%) gave values within the normal range (0.95 $\pm$ 0.10). The difference between the patient and control groups was again significant ($P < 0.005$).

Table I shows that with each antigen tested significant alteration of migration was shown only

Patient Group	Homogenate		Mitochondria		Microsomes	
	Mean $\pm$ SD	<i>p</i>	Mean $\pm$ SD	<i>p</i>	Mean $\pm$ SD	<i>p</i>
Active phase of disease, no immunosuppression	0.83 $\pm$ 0.11 (16)	<0.005	0.75 $\pm$ 0.14 (15)	<0.005	0.83 $\pm$ 0.19 (19)	<0.005
Not affected	0.96 $\pm$ 0.01 (5)		0.86 $\pm$ 0.00 (1)		0.95 $\pm$ 0.10 (3)	
Stimulated	1.15 $\pm$ 0.00 (1)		1.23 $\pm$ 0.00 (1)		1.19 $\pm$ 0.10 (3)	
Inhibited	0.76 $\pm$ 0.04 (10)		0.70 $\pm$ 0.06 (13)		0.72 $\pm$ 0.01 (13)	
% Abnormal	69		93		84	
Active phase of disease, immunosuppressed	0.92 $\pm$ 0.13 (8)	>0.05	0.93 $\pm$ 0.14 (8)	>0.05	0.93 $\pm$ 0.13 (7)	>0.05
Not affected	0.99 $\pm$ 0.01 (3)		0.94 $\pm$ 0.07 (6)		0.82 $\pm$ 0.14 (2)	
Stimulated	1.14 $\pm$ 0.00 (1)		1.14 $\pm$ 0.00 (1)			
Inhibited	0.80 $\pm$ 0.04 (4)		0.62 $\pm$ 0.00 (1)		0.98 $\pm$ 0.14 (2)	
% Abnormal	62		25		29	
Inactive phase of disease, no immunosuppression	0.91 $\pm$ 0.17 (6)	>0.05	1.03 $\pm$ 0.16 (4)	>0.05	0.93 $\pm$ 0.26 (8)	>0.05
Not affected	0.99 $\pm$ 0.01 (4)		0.95 $\pm$ 0.01 (3)		0.91 $\pm$ 0.01 (4)	
Stimulated			1.27 $\pm$ 0.00 (1)		1.52 $\pm$ 0.00 (1)	
Inhibited	0.74 $\pm$ 0.01 (2)				0.75 $\pm$ 0.10 (3)	
% Abnormal	33		25		50	
Inactive phase of disease immunosuppressed	0.96 $\pm$ 0.01 (2)	>0.05	0.91 $\pm$ 0.08 (2)	>0.05	0.93 $\pm$ 0.01 (2)	>0.05
Not affected	0.96 $\pm$ 0.01 (2)		0.90 $\pm$ 0.01 (2)		0.93 $\pm$ 0.01 (2)	
Stimulated						
Inhibited						
% Abnormal	0		0		0	
Totals	0.89 $\pm$ 0.13 (32)	<0.005	0.85 $\pm$ 0.18 (29)	<0.005	0.88 $\pm$ 0.19 (36)	<0.005
% Abnormal	57		58		61	
Controls	0.99 $\pm$ 0.04 (12)		0.95 $\pm$ 0.09 (12)		0.97 $\pm$ 0.07 (12)	

Table I Migration indices (mean $\pm$ SD) obtained with colon preparations in different categories of Crohn's patients with significance of difference (Mann-Whitney *U* test) from control group

Subject	Rat Liver Mitochondria	Colonic Mitochondria
Controls	0.89 ± 0.19 (18)	0.95 ± 0.09 (12)
Crohn's patients	0.89 ± 0.17 (10)	0.80 ± 0.10 (29)

Table II Migration indices (mean ± SE) obtained with colonic and rat liver mitochondria in Crohn's patients and normal subjects

by those patients in a clinically active phase of the disease and not on immunosuppressive treatment with prednisone or azathioprine.

In order to check that the mitochondrial reaction was organ specific, an additional series of tests was done using rat liver mitochondria standardized to 100 µg/ml protein content in the final culture medium. The results, shown in table II, demonstrate no significant difference in leucocyte migration against this antigen between the normal and Crohn's groups.

Discussion

The leucocyte migration test is now well established as a correlate of delayed hypersensitivity *in vitro* and has frequently been used to study cell-mediated immune mechanisms in a variety of diseases of suspected autoimmune origin, such as thyroiditis (Søborg and Halberg, 1968; Calder, McLeman, Barnes, and Irvine, 1972; Wartenberg, Doniach, Brostoff, and Roitt, 1973), idiopathic Addison's disease (Nerup, Andersen, and Bendixen, 1969), pernicious anaemia (Brostoff, 1970), and Crohn's disease (Willoughby and Mitchell, 1971; Brostoff and Walker, 1971; Richens *et al.*, 1973). The results presented here indicate that a high proportion of patients in an active stage of Crohn's disease, not on immunosuppressive treatment, have circulating lymphocytes sensitized to constituents of the diseased colonic mucosa as shown by leucocyte migration inhibition. Another manifestation of this sensitization has recently been shown by the production of lymphotoxin cytotoxic to colonic epithelial cells by lymphocytes from patients with Crohn's disease of the colon and ulcerative colitis (Shorter, Huizenga, Spencer, and Guy, 1972). Attempts to identify the components of the diseased colonic mucosa show that the crude homogenate and both subcellular fractions are involved, with the mitochondrial fraction producing the most marked effect although this preparation of mitochondria was likely to have considerable microsomal contamination. Delayed hypersensitivity to mitochondria has been shown in other autoimmune diseases. It has been shown not to be

organ specific in thyroid disease (Brostoff, 1970; Calder *et al.*, 1972). However, the antigenic activity of mitochondria in Addison's disease has been reported as organ specific (Nerup *et al.*, 1969). Our results appear to show that the effect is organ-specific as leucocytes from both Crohn's disease patients and healthy subjects showed no significant alteration of migration against rat liver mitochondria.

If in fact cell-mediated immune mechanisms play a part in the production of Crohn's lesions it is likely that the initial reaction is one involving a cell-surface component, the hypersensitivity to subcellular components possibly being a sequel to this event. However, in transplantation studies the spleen microsomal fractions have been found to be rich in transplantation antigen (Al-Askari and Lawrence, 1973) so that this may be a false assumption.

While the aetiology and pathogenesis of Crohn's disease and ulcerative colitis are unknown, further study of immune mechanisms in patients with these diseases should be of value. It has been shown that extracts of fetal, colonic, and jejunoileal mucosae inhibit migration of leucocytes from patients with ulcerative colitis, but not those with Crohn's disease (Bendixen, 1969). Our studies show migration inhibition of leucocytes from patients with Crohn's disease against Crohn's colon, and a further immunological differentiation between the two conditions would be apparent if further work showed that leucocytes from ulcerative colitis patients did not react in this way.

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