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# *In vitro* testing of immunoresponsiveness in patients with inflammatory bowel disease: prevalence and relationship to disease activity immunoresponsiveness in IBD

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SUMMARY

Abnormalities in the numbers and function of thymus-derived and bone marrow-derived lymphocytes (T and B cells) and K cells were determined in sixty-nine consecutive patients with Crohn's disease or ulcerative colitis. Rosetting techniques to identify subpopulations of lymphocytes showed a significant decrease in E-rosettes (T cells) and significant increase in EA- and EAC-rosettes (B cells) in patients with inflammatory bowel disease when compared to normals. *In vitro* lymphocyte transformation responses to mitogens and antigens were depressed to a variable degree. Mean levels of K cell activity were not significantly different from normal controls. A considerable degree of individual variation was noted in all groups. When the results of each group were considered, none of the laboratory variables correlated with the site, duration or activity of disease, therapy, presence of iron deficiency anemia, weight loss or hypoalbuminaemia.

Thus, *in vitro* evidence of abnormal immune responses in patients with inflammatory bowel disease cannot be directly related to clinical or laboratory variables and probably reflects a multifactorial aetiology.

## INTRODUCTION

The aetiology of inflammatory bowel disease (IBD) is unknown and the role of cell-mediated immunity remains controversial. There have been reports of alterations in the numbers and functions of T and B cells in patients with Crohn's disease (CD) and ulcerative colitis (UC), yet others have been unable to confirm these results. The subject has been reviewed by Whorwell & Wright (1976). This discrepancy may be partly explained by differences in methods and in the number of tests of cell-mediated immunity (CMI) performed on each patient. An additional explanation rests with potential differences in the groups of patients studied. In particular, only a few studies have attempted to correlate alterations in CMI with the patient's clinical condition (Meuwissen *et al.*, 1975; Meyers *et al.*, 1976; Sachar *et al.*, 1973). Accordingly, seven *in vitro* laboratory tests of CMI were performed in sixty-eight consecutive patients with inflammatory bowel disease. The resultant data was correlated with thirty selected clinical, haematological, biochemical and pharmacological variables.

## MATERIALS AND METHODS

*Patients.* Sixty-eight consecutive patients referred to the Division of Gastroenterology with inflammatory bowel disease were studied; half were hospitalized and half were out-patients. Standard clinical, radiological and histological criteria were used to diagnose Crohn's disease in forty-seven patients and ulcerative colitis in twenty-one. There were thirty-two

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males and thirty-six fameles studied. The mean age of the patients was 32 years, with a range of 14–76 years. Age, sex and frequency distribution of Crohn's disease and ulcerative colitis were similar to that reported by others (MacPherson, Alberlini & Beeken, 1976; Bolton *et al.*, 1974; Asquith, Kraft & Rothberg, 1973). The patients were on standard regimes of treatment: 50% were on salazopyrine and prednisone; 20% were on either salazopyrine or prednisone alone. No patients were receiving azathioprine or other immunosuppressive drugs. None of the patients had recently undergone surgery and none had clinical or laboratory evidence of liver or renal disease.

The activity of the inflammatory bowel disease was assigned a score according to the method of Best *et al.* (1976). From a subset of a number of variables fulfilling a combination of constraints, these workers used a multiple regression computer programme to derive an equation for the prediction of the physician's overall ratings of how well the patient with Crohn's disease was doing. This equation, numerically simplified and utilizing either selected predictor variables, is the Crohn's Disease Activity Index. Most of the predictor variables, such as number of liquid or soft stools, abdominal pain, general well being, and extra-intestinal complications are experienced with at least equal frequency in chronic ulcerative colitis, whereas other predictor variables, such as abdominal mass or perianal disease, are distinctly less common. For convenience the activity index was calculated by the same method for both Crohn's disease and for chronic ulcertaive colitis, but these indices are not directly comparable. Furthermore, in ulcerative colitis it is not yet possible to designate a numerical value for the activity index representing mild, moderate or severe disease.

*Laboratory techniques.* Standard laboratory techniques were used to determine the haemoglobin concentration, haematocrit, red blood cell count, cellular indices, white blood count, marrow iron stores, serum folate activity, serum B12 concentration, serum iron, iron binding capacity, sedmentation rate and albumin concentration.

*Cellular immune studies.* Lymphocytes were isolated on Ficoll-Hypaque (Böyum, 1968) and washed three times in M199 with 10% FCS. Cells were cultured in pooled AB serum.

*Rosetting techniques. Preparations of sheep erythrocytes.* Sheep erythrocytes were obtained from the Provincial Laboratories, University of Alberta, Edmonton and stored at 4°C in Modified Alsever's Solution.

*E- and EAC-rosettes (T and B cells).* A standard E-rosetting technique was used to prepare E- and EAC-rosettes according to the method of Weiner, Bianco & Nussenzweig (1973). A lymphocyte was considered rosetted if at least three sheep erythrocytes adhered to it.

*EA-rosettes.* The number of EA-rosettes were determined using the technique previously described by Zeylemaker *et al.* (1974).

*Lymphocyte transformation.* Lymphocyte concentrations were adjusted to $10^6$ cells/ml for PHA and Con A responses and to $2 \times 10^6$ cells/ml for Varidase, these being optimum for our laboratory. Cells were suspended in RPMI 1640 (Grand Island Biological Co., Burlington, Ontario, Canada); 100λ of cell suspensions were distributed into flat-bottomed microtitre plates. Mitogens or antigens were added to cell suspensions in triplicate (Hagen & Frøland, 1973; Foad *et al.*, 1974). The cells were cultured for 144 hr for both mitogens and antigens. Six hr before harvesting, 10 μl of $^3$H-thymidine were added to the cultures (concentration 20 μCi/ml, sp. act. 40 mCi/mmol). Viability of cultures was routinely determined by the uptake of Trypan blue stain in the remaining cells and by checking the yield of lymphocytes in unstimulated cells in parallel with stimulated cells. Harvesting was performed using a multi-sample, semi-automated harvester (Skatron, Lierbyen, Norway).

Cultures of unstimulated cells from the same patient were made at all times in order to determine the rate of spontaneous transformation. The optimal concentrations of mitogens and antigens for lymphocyte transformation in our laboratory have been previously determined in a random selection of six normals and six IBD subjects. In these groups there was no discernible difference in optimum doses which were PHA 14 μglml (Difco Laboratories, Detroit, Michigan, USA), Con A 45 μg/ml (Calbiochem, Los Angeles, California, USA), Varidase 24–45 u/ml (Lederle Products, Montreal, Quebec, Canada).

After harvesting, the fibre discs were removed, placed on 10 ml of scintillation fluid and the emissions counted in a Beckman L.S. 120 scintillation counter. Results were expressed as the log of the ratio between stimulated and unstimulated cells from the same subject (Ziegler *et al.*, 1974).

*K cell activity. Targets.* A monolayer of human amnion embryo cells were infected with HSV1 and harvested after 48 hr. Normal uninfected cells and infected cells were removed from culture flask with 0·25% tryspin, washed and labelled with $^{51}$Cr for 2 hr at 37°C. Targets were washed four times in $Ca^{++}$ and $Mg^{++}$ free Hanks' solution with 10% FCS (Russell Percy & Kovithavongs, 1975; Perlmann & Perlmann 1970). $25 \times 10^6$ lymphocytes were incubated with $25 \times 10^4$ targets in the presence of 0·1 ml of serum from a herpes simplex positive control for 3 hr at 37°C. After incubation, 1·0 ml of cold media was added to neutralize the mixture. The tubes were centrifuged and the supernatant decanted. The supernatant and pellets were counted and specific $^{51}$Cr release was calculated after correction for the mean background release (about 5%).

*Statistical methods.* The statistical significance of the difference between the means of groups was determined by the method of paired and unpaired samples. Analysis of variance was performed by multiple range student Newman–Keuls procedure. The correlation coefficient and discriminant function were also computed (Winer, 1971; Nie, Hull & Jenkins, 1970).

The normal range of values of the many *in vitro* tests of cell-mediated immunity (CMI) was determined in a large group of healthy control personnel. The results of the tests of CMI in patients with idiopathic ulcerative colitis (UC) or Crohn's disease (CD) were compared with these normal values. For any given test, the magnitude of abnormalities was assessed by comparing the mean value of normals with the mean value of the group with UC or CD. Because of the non-Gaussian distribution of the results of the test of lymphocyte transformation, these results were expressed logarithmically (Ziegler *et al.*, 1974).

## RESULTS

*Rosetting techniques and K cell activity*

Absolute numbers of peripheral blood lymphocytes (PBL) were normal in all patients at the time of study (Table 1). The mean level of E-rosetting cells was significantly reduced in both UC and CD when compared to normals. In contrast, the mean level of both EA- and EAC-rosettes was significantly increased in both disease (Table 1). As the absolute number of PBL were normal, percentage changes in E-, EA- and EAC-rosettes represented significant changes in absolute numbers of lymphocyte subclasses.

TABLE 1. Mean levels of lymphocyte subpopulations in normals, Crohn's disease and ulcerative colitis

| | Normals (Mean ± 1 s.d.) | Crohn's disease (Mean ± 1 s.d.) | Ulcerative colitis (Mean ± 1 s.d.) |
|---|---|---|---|
| E-rosettes (T cells) | 50±5 | 32±13* | 29±12* |
| EA-rosettes | 26±5 | 35±6* | 33±7* |
| EAC-rosettes (B cells) | 22±4 | 29±11* | 26±7* |

* $P < 0{\cdot}05$, CD or UC group compared with normals.

Mean±1 s.d. of E-, EA- and EAC-rosettes determined by counting 200 cells in a haemocytometer. Levels in CD and UC are compared to those in normal controls.

TABLE 2. Mean levels of K cell activity in normals, Crohn's disease and ulcerative colitis

| Normals (Mean ± 1 s.d.) | Crohn's disease (Mean + 1 s.d.) | Ulcerative colitis (Mean ± 1 s.d.) |
|---|---|---|
| 45% | 43%±20* | 44%±24* |

*No statistically significant difference ($P < 0{\cdot}05$) between CD or UC and normals.

K cell activity expressed as mean ± 1 s.d. of percentage $^{51}Cr$ released from labelled target cells in CD and UC compared to normals.

It was not possible to demonstrate a statistically significant difference between the mean value of $^{51}Cr$ release in controls and K cell activity as assessed by ADCC in UC or CD (Table 2).

In all four tests there was no statistically significant difference between the two groups with CD or UC. A considerable degree of individual variability in results was noted with all tests, and was particularly noticeable with ADCC. However, there was no correlation between increased EA-rosettes (K cells) and K cell activity as assessed by antibody-dependent cell-mediated cytotoxicity (ADCC).

*Lymphocyte transformation to mitogens and antigens*

Spontaneous lymphocyte transformation responses were not significantly different in IBD patients when compared to normal controls, although a wide range of individual variation was noted in both groups. In UC, the mean levels of lymphocyte transformation responses were not significantly reduced in response to PHA, Con A and Varidase. In CD, the mean levels of lymphocyte transformation were significantly reduced in response to PHA and Varidase, but not to Con A. The mean value of each of these tests of lymphocyte transformation was similar in UC and CD (Table 3). As with other tests, a considerable degree of individual variability was seen with some patients showing evidence of suppressed responses compared to others.

TABLE 3. Mean levels of lymphocytes responses to PHA, Con A and Varidase in normals, Crohn's disease and ulcerative colitis

| | Normals (Mean) | Crohn's disease (Mean) | Ulcerative colitis (Mean) |
|---|---|---|---|
| PHA | 1·32±0·53 | 1·01±0·75* | 1·16±0·71 |
| Con A | 1·07±0·51 | 0·74±0·60 | 0·67±0·69 |
| Varidase | 0·62±0·56 | 0·19±0·39* | 0·34±0·36 |

* Statistically significant difference ($P < 0·05$) between CD or UC and normals.

Mean levels of lymphocyte responses expressed logarithimically, to PHA, Con A and Varidase, compared to normal controls.

*Correlation with clinical and laboratory variables*

A correlation was sought between the results of each test and a wide range of individual laboratory and clinical variables. In both UC and CD there was no statistically significant correlation between disease activity index and the individual values in any of the groups of tests of CMI. Even when only those patients with abnormal values were considered, there was no correlation between disease activity and the values of the *in vitro* immune function tests. It was not possible to demonstrate any correlation between the tests of CMI and weight loss or hypoalbuminaemia, anaemia or iron deficiency, duration of illness, site of disease or different medications (Table 4).

## DISCUSSION

We have demonstrated evidence of depressed cell-mediated immunity in some patients with inflammatory bowel disease based on results of *in vitro* immunological testing, and have attempted to determine the significance of these changes by relating them to the patient's clinical status. This study has shown that the proportions of subpopulations of circulating lymphocytes are different from those in normals and that their *in vitro* function may be abnormal. E-rosettes were significantly reduced in both Crohn's disease and ulcerative colitis. EAC-rosettes, on the other hand, were statistically increased in Crohn's disease compared to normals and were slightly higher than normal in ulcerative colitis. EA-rosettes were found to be significantly increased in both Crohn's disease and ulcerative colitis. Similar

TABLE 4. Correlation coefficients of index of disease activity with E-, EA- and EAC-rosettes, K cell activity and lymphocytes transformation responses to PHA, Con A and Varidase

| | Diagnosis | Coefficient |
|---|---|---|
| E-rosettes | CD | −0·25 |
| | UC | −0·35 |
| EA-rosettes | CD | 0·33 |
| | UC | 0·22 |
| EAC-rosettes | CD | −0·14 |
| | UC | 0·22 |
| K cell activity | CD | −0·01 |
| | UC | −0·18 |
| PHA response | CD | −0·07 |
| | UC | 0·24 |
| Con A response | CD | −0·22 |
| | UC | −0·35 |
| Varidase response | CD | −0·5 |
| | UC | −0·49* |

* $P < 0·05$.

results with E-rosettes have been reported by Strickland *et al.*, (1974), although they were only demonstrated in patients with Crohn's disease.

Suppressed T cell function was demonstrated by lymphocyte responses *in vitro* to mitogens and antigens. With the specific antigen Varidase there was a marked decrease when patients with Crohn's disease and ulcerative colitis were compared to the normal group. This was only significantly different in the Crohn's disease patients. However, when the non-specific mitogens were used, only the lymphocyte response to PHA demonstrated a statistically significant difference between Crohn's disease and the normal group, although again there was a marked decrease in lymphocyte responsiveness to the mitogens in both disease when compared to the normal group. We have also shown that K cell function is not significantly different from normals in inflammatory bowel disease, as assessed by their effector role in ADCC.

A number of possible factors have been suggested to explain these abnormalities. Skinner & Whitehead (1974) found no evidence of diminished cell-mediated immunity, suggesting that the depressed PHA response in Crohn's disease does not necessarily reflect a depressed immune response. Bird & Britton (1974) found no evidence of decreased lymphocyte reactivity in Crohn's disease and they concluded that lymphocyte anergy is not a necessary feature of Crohn's disease. They speculated that malnutrition may be the cause of the previously observed lymphocyte anergy. The effect of malnutrition has been previously reported (Chandra, 1972; Law, Dudrick & Abdou, 1973), but we could not demonstrate any correlation between loss of weight or low serum albumin with any of the immune tests performed. We conclude from our observations that although malnutrition may occur in some patients with inflammatory bowel disease, an even larger group who did not fit into the generally accepted criteria of malnutrition have abnormal immune function tests. Joynson *et al.* (1972) have reported defects of cell-mediated immunity in patients with iron deficiency anaemia. It was also suggested that serum iron levels may affect immunological competence and the presence of iron deficiency may potentiate an existing primary or secondary immunodeficiency. In our study, a number of haematological variables were measured and we were unable to demonstrate any significant difference in immunological tests between patients with normal haematology and those with abnormal levels. In particular, reduced and absent bone marrow iron stores failed to correlate with depressed CMI.

Sachar *et al.* (1973) found that azulfadine had no effect on PHA responsiveness, whereas there was a high prevalence hyporesponsiveness among patients on steroid therapy. MacPherson *et al.* (1976) noted that immunosuppressive agents exerted little effect on PHA and PWM lymphocyte transformation tests in patients with Crohn's disease. Approximately 70% of our patients were taking steroids alone or in combination with either salazopyrine or azathioprine, and about 70% were taking salazopyrine alone or in combination with other medications. It was not possible in this study to determine clearly whether changes in CMI could be directly related to medications.

Most investigators in this field would agree that it is possible to demonstrate abnormalities in immunological tests in some patients with inflammatory bowel disease. In spite of the extensive studies which have been performed, the role of these abnormalities in the aetiology and pathogenesis of IBD remains unclear. It is not known for certain whether these changes are primary or secondary. Our studies have confirmed some of these observations, but our attempts to correlate these abnormalities with state of nutrition, body iron stores therapy or a number of other biochemical or haematological variables have been unsuccessful. In addition there was also no absolute correlation in the group as a whole between laboratory variables and clinical disease activity as assessed by the Crohn's Disease Activity Index.

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