Abnormalities of B-cell activation and immunoregulation in patients with Crohn’s disease

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SUMMARY We have studied B-lymphocyte function in 39 patients with Crohn’s disease and 35 normal individuals using a reverse haemolytic plaque assay as the effector system. Ten patients had active Crohn’s disease, the others being in an inactive state of the disease. Compared with normal individuals, the Crohn’s disease patients – especially those in the active state of the disease – had markedly raised numbers of spontaneous immunoglobulin secreting cells and severely decreased responses to the polyclonal activator pokeweed mitogen. The differences between the reactivity of patients with active disease and those with inactive disease were statistically significant. These findings indicate an in vivo polyclonal B-cell activation in Crohn’s disease patients, possibly due to antigen(s) or infectious agent(s). In vitro experiments were performed with separated lymphocytes in order to characterise the mechanism responsible for the altered immune reactivity in Crohn’s disease. These revealed an intrinsic B-cell defect as well as an impaired T-helper cell capacity in patients with Crohn’s disease. Findings supporting the hypothesis of an increased suppressor activity in Crohn’s disease patients could not be observed, and marker analyses revealed normal proportions with the exception of raised Leu 7 positive cells that mediate ‘natural killer’ and ‘killer’ cytolysis. We conclude that immune dysfunction in peripheral blood lymphocytes of Crohn’s disease patients involves B-cells as well as T-helper cells.

The finding of various abnormalities of the immune response in patients with Crohn’s disease has led to the concept that immune mechanisms are involved in the pathogenesis of this disease. Both humoral and cell-mediated reactivity to several gut- and bacteria-derived antigens have been shown in patients with Crohn’s disease. Moreover, complex immunoregulatory influences are involved in immune reactions, and disturbances of immunoregulation have been found to be associated with many diseases in which immune mechanisms may be important. Controversial results have been obtained from experiments with peripheral blood mononuclear cells from patients with Crohn’s disease: Hodgson reported a defective suppressor cell function, whereas Elson et al. found a covert suppressor cell activity and Holdstock et al. a normal suppressor cell activity. On the other hand, intestinal lymphocytes have been reported to show enhanced or reduced suppressor-cell activity. Similarly conflicting results were obtained when the proportions of circulating T- and B-lymphocytes were studied.

In view of these divergent findings, the present study was performed with the aim of investigating peripheral blood lymphocyte function in a series of Crohn’s disease patients by means of a reverse haemolytic plaque assay. We measured spontaneous immunoglobulin secreting cells, reflecting in vivo conditions, as well as pokeweed-mitogen-induced immunoglobulin secreting cells, reflecting the transforming capacity of resting B-cells. To define immunoregulatory influences of T-helper and T-suppressor cells, coculture experiments were done with isolated T- and B-cell populations obtained from normal individuals and patients with Crohn’s disease.

Methods

Subjects Thirty nine patients (21 women, 18 men, mean age 1255
37 years) with Crohn's disease were studied. The diagnosis of Crohn's disease was based on characteristic clinical, endoscopic, radiological, and histological features. The extent of the disease was determined by radiological and/or colonoscopic findings. Fifteen patients had colonic involvement; 12 had small bowel disease and 12 had small bowel and colonic involvement. At the time of investigation, 10 patients had active disease with Crohn's disease activity index (CDAI)20 higher than 150 (186–380, median 320); six were untreated, and four were treated with prednisolone and sulphasalazine for a short period of one to four days. Twenty-nine patients had inactive disease (CDAI <150), and all of them were untreated for at least five months except for sulphasalazine in those patients with colonic involvement. Thirty-five healthy control subjects were also studied. Their average age was 32 years (19 women, 16 men). All blood samples were obtained between 8.00 and 10.00 am and studied on the same day.

CELL SUSPENSIONS
Heparinised blood was taken, and peripheral blood mononuclear cells were obtained by Ficoll-Hypaque gradient centrifugation. In some experiments, T- and B-cell-enriched lymphocyte suspensions were purified by E-rosette separation as described.21

MONOCLONAL ANTIBODY ANALYSIS OF PERIPHERAL LYMPHOCYTES
Seven monoclonal antibodies previously described by others were used. Briefly, OKT 3 binds to all mature T-cells and BA 1 to the whole B-cell subset. Regulatory T-helper/inducer and T- cytotoxic/suppressor cells are restricted among OKT 4 positive and OKT 8 positive cells, respectively. OKM 1 identifies an antigen expressed on myelomonocytic lineage, whereas OK1a 1 recognises Ia-like antigens, and Leu 7 identifies cells that mediate 'natural killer' and 'killer' cytolysis.

To determine the frequency of positive cells, peripheral blood lymphocytes (2×10⁶ cells) were incubated with the appropriate concentrations of each monoclonal antibody for 30 minutes at 4°C in phosphate-buffered saline, washed and counterlabelled with an FITC-conjugated F(ab') fragment of goat anti-mouse IgG, washed again and then examined with a Zeiss ultraviolet microscope equipped with a vertical illuminator as described.22

CULTURE CONDITIONS
Cells were suspended in RPMI 1640 medium (Seromed, Germany) supplemented with 10% heat inactivated fetal calf serum (Gibco Lab, USA), L-glutamine and antibiotics. The cells were cultured in 16×125 mm Falcon plastic tubes at a concentration of 1×10⁶ cells/ml in a total volume of 3 ml. Pokeweed mitogen (Gibco Lab, USA) was added at a concentration of 5 µl/ml when cultures were first set up. The cells were harvested after six days of incubation (5% CO₂ in air, 37°C). In coculture experiments, 1-5×10⁶ cells from a healthy volunteer were incubated together with 1-5×10⁶ cells from the patient. In experiments with separated B and T cells, cocultures were set up at a ratio of 20:80. In some experiments performed for defining suppressor activity of the patient's T-cells (after B/T separation), cocultures between normal B-cells, normal T-cells and patient's T-cells were set up at a ratio of 20:60:20. All cultures or cocultures were done in duplicate. Cell viability evaluated by trypan blue exclusion ranged between 84 and 98%.

REVERSE HAEMOLYTIC PLAQUE ASSAY
The details of the reverse haemolytic plaque assay have been described previously.19 Briefly, protein-A-coupled sheep red blood cells were used as indicator cells and incubated in agar with cultured cells. After addition of a developer antiserum with specificity for α, γ and μ (Medac, Germany) and complement (Behring, Germany), the number of plaques was determined and expressed as the total number of plaques per 10⁷ lymphocytes (mean of duplicate determination). Controls were done in each experiment by omitting cells, complement, developer antiserum, or all three entities.

STATISTICAL ANALYSIS
Results were compared using the Wilcoxon's test.

Results
SPONTANEOUS IMMUNOGLOBULIN SECRETING CELLS IN PATIENTS WITH CROHN'S DISEASE
Spontaneous immunoglobulin secreting cells were estimated immediately after cell separation using the reverse haemolytic plaque assay. As shown in Figure 1, patients with active Crohn's disease (CDAI >150) showed a significant increase in spontaneous immunoglobulin secreting cells compared with normal individuals as well as Crohn's disease patients with inactive disease (2934 vs 313 vs 554). In patients with inactive Crohn's disease, we also found an increase in spontaneous immunoglobulin secreting cells, which was not statistically significant. In active Crohn's disease, there was no difference between patients with short term treatment and untreated patients.
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POKEWEED-MITOGEN-INDUCED IMMUNOGLOBULIN SECRETING CELLS IN PATIENTS WITH CROHN'S DISEASE
When peripheral blood mononuclear cells were stimulated with pokeweed-mitogen over a period of six days, the number of immunoglobulin secreting cells was found to show a statistically significant reduction in patients with either active or inactive disease as compared with normal individuals (903 vs 3241 \( vs \) 18 934). Moreover, there was also a statistically significant difference in the reactivity between active and inactive Crohn's disease. The overall results are depicted in Figure 2. In active Crohn's disease, there was no difference between patients with short-term treatment and untreated patients.

COCULTURES BETWEEN PERIPHERAL BLOOD MONONUCLEAR CELLS FROM CROHN'S DISEASE PATIENTS AND NORMAL INDIVIDUALS
Crohn's disease patients' lymphocytes were cultured together with lymphocytes from normal donors at a ratio of 1:1. Such coculture experiments are useful tools to elucidate increased suppressor or helper activity, as the responses of peripheral blood mononuclear cells from two HLA-non-identical individuals in coculture are additive – that is, not influenced by HLA diversity. Thus, cocultures between peripheral blood mononuclear cells from normal individuals and patients with active (n=5) or inactive (n=9) Crohn's disease always lead to responses within the predicted (calculable) range.

EXPERIMENTS WITH SEPARATED LYMPHOCYTE SUBPOPULATIONS
In order to investigate the cellular mechanism responsible for impaired immunoglobulin secreting cells generation in patients with Crohn's disease, experiments were done in which E-rosette-separated lymphocyte subpopulations from normal individuals were cocultured with those from patients with Crohn's disease at a B:T ratio of 20:80. The results shown in Table 1 show a clear intrinsic B-cell
defect in almost all patients with Crohn’s disease in the pokeweed-mitogen-driven system. Moreover, there was a defective T-helper cell capacity in most experiments, as shown in coculture experiments between normal B-cells and patients’ T-cells. As in the coculture experiments with unseparated peripheral blood mononuclear cells, an increased suppressor cell activity could never be observed when isolated normal B-cells, normal T-cells and patients' T-cells were cocultured at a ratio of 20:60:20 (see Table 1). In two experiments, the proportion of patients’ T-cells was even increased up to 50% (ratio 20:30:50), which did not influence B-cell reactivity. Likewise, no suppressor activity could be shown when patients’ T-cells were cocultured with unseparated lymphocytes from normal individuals at a ratio of 50:50.

**MARKER ANALYSES**

Results of monoclonal antibody studies on peripheral blood lymphocytes from 12 patients with Crohn’s disease compared with 50 healthy age- and sex-matched subjects are listed in Table 2. Four of the patients were in an active and eight in an inactive disease state. Because both groups showed similar values, they are depicted together. Except for data obtained from Leu 7 studies, no statistically significant differences were seen in lymphocyte subpopulations when they were compared with those from a group of normal individuals. Relative numbers of Leu 7 positive cells mediating ‘natural killer’ and ‘killer’ cytolyis, however, were increased in the Crohn’s disease group (17.5±4.1 vs 9.9±6.3 in normal subjects, p<0.01).

**Discussion**

Immunological investigations in patients with Crohn’s disease have revealed a number of phenotypic and functional abnormalities that might be involved in the initiation or perpetuation of this disease.

Previous functional studies using peripheral blood lymphocytes from Crohn’s disease patients have suggested such contradictory findings as reduced spontaneous and/or concanavalin-A-induced

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**Table 1**  Pokeweed mitogen (PWM)-induced generation of immunoglobulin-secreting cells (ISC) in co-culture of lymphocyte subpopulations from normal individuals and patients with Crohn’s disease (CD)

<table>
<thead>
<tr>
<th>Experiment number</th>
<th>ISC/10⁶ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CDAI</td>
</tr>
<tr>
<td>Unseparated lymphocytes</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td></td>
</tr>
<tr>
<td>Patient</td>
<td></td>
</tr>
<tr>
<td>Separated lymphocytes</td>
<td></td>
</tr>
<tr>
<td>Bₙ×Tₙ (20:80)</td>
<td></td>
</tr>
<tr>
<td>Bₙ×Tₙ (20:80)</td>
<td></td>
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<tr>
<td>Bₙ×Tₙ (20:80)</td>
<td></td>
</tr>
<tr>
<td>Bₙ×Tₙ ×Tₙ (20:60:20)</td>
<td></td>
</tr>
</tbody>
</table>

T and B lymphocyte subpopulations were purified by E-rosette separation and cocultured at different ratios. The isolated T and B fractions alone did not respond to PWM. CDAI = Crohn’s disease activity index; B = separated B-cells; T = separated T-cells; N = normal individual; CD = patient with CD; nd = not done.

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**Table 2**  Percentage of peripheral blood lymphocytes determined by E-rosette formation or by monoclonal antibody analysis

<table>
<thead>
<tr>
<th></th>
<th>E-Ros</th>
<th>OKT 3</th>
<th>OKT 4</th>
<th>OKT 8</th>
<th>OKM 1</th>
<th>OKIa 1</th>
<th>Leu 7</th>
<th>BA 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal individuals</td>
<td>66-4*</td>
<td>66-7</td>
<td>40-1</td>
<td>19-2</td>
<td>16-3</td>
<td>14-4</td>
<td>9-9</td>
<td>9-7</td>
</tr>
<tr>
<td>(n=50)</td>
<td>±7-0</td>
<td>±4-0</td>
<td>±3-1</td>
<td>±2-9</td>
<td>±4-9</td>
<td>±4-6</td>
<td>±6-3</td>
<td>±6-4</td>
</tr>
<tr>
<td>Crohn’s disease patients</td>
<td>62-6</td>
<td>64-2</td>
<td>38-5</td>
<td>20-2</td>
<td>14-2</td>
<td>15-0</td>
<td>17-5</td>
<td>12-0</td>
</tr>
<tr>
<td>(n=12)</td>
<td>±2-0</td>
<td>±3-4</td>
<td>±2-4</td>
<td>±4-5</td>
<td>±2-6</td>
<td>±2-0</td>
<td>±4-1</td>
<td>±3-6</td>
</tr>
<tr>
<td>p</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>&lt;0-01</td>
<td>ns</td>
</tr>
</tbody>
</table>

* Mean values ±1 SEM (standard error of the mean); ns = not significant. E-Ros = sheep erythrocyte rosette-forming cells. The reactivity pattern of the monoclonal antibodies is described in Methods.
suppressor activity, normal suppressor activity or even increased suppressor activity. Similarly discrepant results have been revealed at the mucosal level: increased suppressor activity, normal suppressor activity or reduced suppressor activity. No information is available on T-helper cell function in peripheral blood lymphocytes of Crohn’s disease patients, as, in the experiments of Elson et al., a covert suppressor activity manifested after the T/B-cell separation procedure could have masked a possibly normal T-helper cell function.

Characterisation of peripheral blood lymphocytes and T-lymphocyte subpopulations in Crohn’s disease patients with conventional or monoclonal markers revealed the following briefly summarised results: normal absolute T-cell number, decreased absolute T-cell numbers, normal proportion of T-cell subsets, reduced T(γ) and/or T(μ) positive subsets, peripheral blood B-lymphocytes were reported to be reduced, normal or increased. At the mucosal level, ReMine et al. described decreased T(γ) subpopulations, and Brandtzæg and Baklien reported increased numbers of IgG positive cells and decreased numbers of IgA positive cells.

After this confusing enumeration of contradictory data, it is necessary to state that lymphocyte functions evaluated in different assay systems can yield different results. Therefore it is not acceptable to generalise from the results obtained in a specific assay system.

For better characterisation of B cell as well as T-helper and T-suppressor cell abnormalities in one defined assay system corresponding as closely as possible to the in vivo situation, we used a reverse haemolytic plaque assay which enabled us to measure the following parameters: (1) Spontaneous immunoglobulin secreting cell numbers reflecting the in vivo situation, in which B-cells can transform into immunoglobulin secreting cells under the influence of T-helper and T-suppressor cells. (2) Pokeweed-mitogen-induced immunoglobulin secretion indicating the capacity of B-cells to differentiate into immunoglobulin secreting cells under the influence of T-helper and T-suppressor cells in vitro after stimulation with the polyclonal B-cell activator pokeweed-mitogen. (3) Function of B-cells as well as T-helper and T-suppressor cells in vitro after B/T-cell separation and reconstitution with autologous or allogeneic cells followed by stimulation with pokeweed-mitogen.

The most exciting finding of the present study was a marked significant increase of spontaneous immunoglobulin secreting cells in patients with active Crohn’s disease (approximately 10 × normal values) and, to a lesser extent, in patients with inactive Crohn’s disease (approximately 2 × normal values) (Fig. 1). Correspondingly, there was a significant inability of peripheral blood lymphocytes from both active and inactive Crohn’s disease patients to differentiate into immunoglobulin secreting cells after stimulation with pokeweed-mitogen in vitro (Fig. 2). These findings are consistent with a marked degree of polyclonal B-cell activation in vivo and agree on the whole with the results of Holdstock et al. and Macdermott et al., who estimated IgA, IgM and IgG secreted during a cultivation period of seven or 12 days in cell culture supernatants using an ELISA or a radioimmunoassay respectively. Such polyclonal B-cell activation in vivo may—among other possibilities—for example, antigen stimulation—be suggestive of viral stimulation or transformation and has been reported during infection with some herpes viruses, most notably the Epstein-Barr virus. Other disease states in which polyclonal B-cell activation has been demonstrated is the acquired immunodeficiency syndrome and certain connective tissue diseases.

To explain the phenomenon of polyclonal B-cell activation on a cellular basis, three abnormalities have been proposed: (1) excessive T-cell help, (2) decreased T-cell suppression, or (3) direct B-cell activation. To discriminate between these possibilities, coculture experiments were performed: unseparated, pokeweed-mitogen-stimulated lymphocytes from a normal individual and a patient with active or inactive Crohn’s disease were cocultured. These pilot experiments revealed no evidence for an altered function of T-suppressor cells in Crohn’s disease patients. Furthermore, in experiments with separated T- and B-cells from normal individuals and Crohn’s disease patients, evidence of an intrinsic B-cell defect as well as a defective T-helper cell capacity was manifest in almost all patients. Defective helper cell capacity in Crohn’s disease patients was easily demonstrable in coculture experiments between normal B-cells and patients’ T-cells (Table 1). On the other hand, normal T-cells could not help patients’ B-cells to differentiate into immunoglobulin secreting cells (Table 1), and patients’ T-cells did not exert abnormal suppressor activity, even in various coculture experiments between normal B-cells, normal T-cells and patients’ T-cells (Table 1).

Therefore, we would postulate an intrinsic B-cell defect defined by the pokeweed-mitogen-driven system in almost all Crohn’s disease patients, whereas Elson et al. interpreted the inability of patients’ B-cells to differentiate as a consequence of suppressor activity. Because the suppressor activity in the experiments of Elson et al. was not dependent on the technique used for T-cell
separation, this discrepancy could only be explained by the different effector systems or culture conditions. Elson et al.,\textsuperscript{10} using a radioimmunoassay, measured supernatant IgM produced during the whole culture period of seven days; whereas, in the present study, immunoglobulin secreting cells were estimated after a culture of six days. Finally, these experimental differences might also explain the finding of Holdstock et al.,\textsuperscript{26} who reported increased activity of a prostaglandin-dependent suppressor system in peripheral blood mononuclear cells of patients with inflammatory bowel disease.

In general, our findings make the assumption of excessive T-cell help or defective T-cell suppression in Crohn's disease patients seem unlikely. This conclusion is also supported by numerous marker analyses\textsuperscript{14-17, 30} including the present one (Table 2). Therefore we would favour the hypothesis of a direct polyclonal B-cell activation in patients with Crohn's disease, which is substantiated by the observation that the extent of polyclonal B-cell activation was not affected by short-term treatment (one to four days) in four patients with active disease. Thus, one might speculate that polyclonal activation, possibly due to an infectious agent(s), may initiate or perpetuate an autoimmune process leading to the presence of circulating antibodies to bowel epithelial cells.\textsuperscript{39}

An unresolved problem is whether the abnormal immune reactivity in the peripheral blood is relevant to the pathogenesis of the intestinal inflammatory process or only a concomitant phenomenon. As it is difficult to obtain well defined intestinal lymphocyte populations, there are very few and contradictory observations in humans, as previously mentioned. Nevertheless, direct access to the intestinal lymphoid system is a prerequisite for better interpretation of immunologic abnormalities in Crohn's disease. Further elucidation of both local and systemic alterations of immune functions in Crohn's disease may provide new insights into the pathogenesis of this disease, thus leading to new approaches in therapy.

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Abnormalities of B-cell activation and immunoregulation in patients with Crohn’s disease

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