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Pokeweed mitogen stimulated immunoglobulin production by peripheral blood lymphocytes *in vitro*: evidence for disordered immunoregulation in patients with ulcerative colitis and Crohn's disease

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SUMMARY

Production of immunoglobulins (G, A, M) by pokeweed mitogen stimulated peripheral blood lymphocytes was studied in 81 patients with inflammatory bowel disease and compared with 40 patients with mild gastrointestinal disorders (controls). Immunoglobulin production was dependent on the concentration of mononuclear cells in culture, being maximal at the lowest concentration used ($2{\cdot}5 \times 10^5$/ml). Adherent monocytes exerted suppression when cultures were reconstituted with more than 20% of these cells. T lymphocyte depleted cells (B cells) demonstrated T cell helper/suppressor dependence for immunoglobulin production in an isotype specific manner, the optimal T cell concentration for 'helper' activity being lowest for IgG and highest for IgM. In patients with active ulcerative colitis (UC) there was a reduction in the T cell concentration for optimal helper activity that was not isotype specific suggesting an increase in non-specific T cell helper activity. T cell helper activity reverted toward control levels in patients with UC in disease remission, except in the case of IgA production where there was a significant diminution of IgA production and of T helper activity for IgA synthesis. Patients with Crohn's disease were distinguished from both UC and control patients by: (1) reduced immunoglobulin production at low lymphocyte concentrations; (2) reduced monocyte-mediated suppression of immunoglobulin production and (3) no shift in T cell concentration for optimal helper activity for IgG and IgA with active disease.

Keywords Crohn's disease ulcerative colitis inflammatory bowel disease immunity immunoregulation mitogen stimulation

INTRODUCTION

A characteristic feature of the pathology of ulcerative colitis (UC) and Crohn's disease (CD) is the dense infiltration of lymphocytes in the bowel wall (Meuwissen *et al.*, 1976; Meijer, Bosman & Lindeman, 1979). The numbers of immunoglobulin (Ig) containing cells are also markedly increased in diseased bowel, with a disproportionate increase in IgG and IgM containing cells (Baklien & Brandtzaeg, 1975). Thus, profound alterations in the distribution of lymphocytes in general and of Ig producing cells in particular appear to take place at the mucosal level in patients with idiopathic inflammatory bowel disease (IBD). It is possible that these changes may to some degree be reflected in the recirculating population of lymphocytes.

Spontaneous and pokeweed mitogen (PWM) driven Ig production by peripheral blood

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lymphocytes in *in vitro* systems has so far been investigated in limited groups of patients with IBD, and the results have been either negative or conflicting (Elson *et al.*, 1981; MacDermott *et al.*, 1981; Holdstock, Ershler & Krawitt, 1982). The reasons for this may be that the culture conditions were varied and that the culture conditions were generally not appropriate to reveal any subtle changes in the cells regulating the production of Ig.

The aim of the present study was to define culture conditions that would reveal differences in Ig production by lymphocytes from patients with UC, CD and a group of control patients with other gastrointestinal diseases. Parameters considered included PWM stimulation, lymphocyte concentrations, T lymphocyte function and monocyte function. The influences of disease activity, nutritional status and treatment regimen of the patients were taken into account in assessing the results.

MATERIALS AND METHODS

Patients. Blood samples were taken from patients with CD and UC attending an IBD out-patient clinic, the diagnosis being based upon conventional clinical, radiological and pathological criteria. The simple clinical index (Harvey & Bradshaw, 1980) was used to assess disease activity and a score of five or more was considered to indicate active disease.

Control samples were obtained from patients attending a gastrointestinal clinic who were booked for endoscopic examination and who had active symptoms and a clinical and pathological diagnosis of oesophagitis, gastritis or benign peptic ulcer disease.

The patients were divided into three groups. Group 1: 40 patients with oesophagitis, gastritis or benign peptic ulcer disease were selected for the control group. Mean age ($\pm$s.d.) was 51 years $\pm$13, and 17 were female. No treatment other than antacids was being given. Group 2: 35 patients with UC or proctitis. Mean age ($\pm$s.d.) was 46 years $\pm$14, 18 were female, 11 were on no treatment, 18 were taking sulphasalazine and six were on sulphasalazine plus either topical steroids or prednisone. Group 3: 46 patients with CD. Mean age ($\pm$s.d.) was 37 years $\pm$16, and 23 were female. Twenty were on no treatment, four were on sulphasalazine and 22 were on prednisone with or without sulphasalazine. Thirteen patients had had small bowel resections.

When IBD patients were subdivided according to disease activity or quiescence the distribution of patients was similar in terms of the number of patients, their age, sex and treatment regimens. The nutritional status of patients with CD was assessed by standard anthropometric and biochemical criteria (Harries *et al.*, 1983).

Cell isolation and culture. Mononuclear cells were separated from diluted, heparinized blood by centrifugation over Ficoll-Hypaque (specific gravity 1·077). Absolute mononuclear cell counts were performed on whole blood using Turk's diluent (crystal violet in 1% acetic acid). T cells were identified in mononuclear cell preparations by rosetting with AET treated sheep red blood cells (Saxon, Feldhans & Robins, 1976) and excluding latex ingesting cells. Monocytes were counted separately as the proportion of latex ingesting cells. Polymorph contamination was less than 2%. Adherent cells were isolated by reversible binding to calf serum coated plastic Petri dishes (Kumagai *et al.*, 1979). The purity of monocytes was 92% (range 86–98%). T cells were isolated by separating AET-E rosettes over Ficoll-Hypaque without pre-incubation at 4°C (Madsen, Johnson & Hansen, 1980). Red cells were lysed with Tris-HCl-buffered ammonium chloride at 37°C. The co-efficient of variation for T cell estimates was 7·9%, $n=12$, and for monocyte estimates 17·3%, $n=12$. After E rosette depletion, the non-T cell fraction contained less than 5% E rosetting cells and the purity of both T and non-T cell fractions was monitored as control cultures in T–B co-culture experiments. Experiments where either the T or the non-T cell fractions produced significant amounts of Ig *in vitro* were excluded.

Cells were cultured in round bottom microtitre plates (Sterilin Ltd.) in RPMI 1640 medium (GIBCO-Biocult Ltd.) containing 10% heat-inactivated FCS (Tissue Culture Services, Slough, England) and 1% penicillin and streptomycin. Two hundred microlitres of cell suspension was added to 25 μl of medium alone or medium containing PWM (GIBCO-Biocult Ltd.) to a final dilution of 1/250. Mononuclear cells were cultured in three concentrations (2·5, 5 and 10×10^5/ml)

and monocyte: lymphocyte co-cultures at a maximum total concentration of 10^6/ml. Non-T cells were cultured at 10^5/ml and reconstituted with T cells from $1{\cdot}0$–$12{\cdot}5 \times 10^5$/ml. All samples were depleted of adherent cells. Cells were cultured in a humidified chamber at 37°C in 5% CO_2 in air for 8 days. Supernatants were stored at −70°C until assayed. The coefficient of variation in Ig production by replicate lymphocyte cultures was 22·4%, $n=8$.

Radioimmunoassays. Purified polyclonal human IgG, IgA and IgM were iodinated by the chloramine-T method (McConahey & Dixon, 1966). Specific activity was 20,000 ct/min/ng IgG, 12,000 ct/min/ng IgA and 6,000 ct/min/ng IgM. Flexible PVC microtitre plates (Dynatech Laboratories Ltd.) were coated with 25 μl rabbit anti-human heavy chain specific IgG (Dako, Mercia Brocades Ltd.) at 1/800 dilution in PBS for 1 h at 37°C. Plates were washed and blocked with 50 μl of 1% BSA/Tween 20 in PBS for 30 min at 37°C.

Supernatants were assayed for Ig by competitive binding with labelled Ig (10 ng/25 μl). IgA and IgM were assayed in the presence of 0·5 mM dithiothreitol in order to increase the sensitivity of the assay. After 1 h incubation with supernatant and then 1 h incubation with labelled Ig the wells of the plates were cut out and counted on an LKB gamma counter. Results were compared with standard Ig preparations diluted in medium and stored for comparable periods at −70°C. In this way IgG, IgA and IgM could be quantitated in duplicate from 150 μl of culture supernatant. Measurements were taken from the curve with maximum slope, routinely 10–2,000 ng/ml. The coefficients of variation in measuring the same sample on 10 different occasions were 19·4%, 13·3% and 16·0% respectively for IgG, IgA and IgM. Statistical comparisons were performed using the Student's *t*-test.

RESULTS

Peripheral blood cell populations

Table 1 summarises the leucocyte populations in the circulation of these patients and the proportions of T cells and monocytes in the final cell culture preparations. There were no significant differences in total lymphocyte, T cell and monocyte counts between the control group and patients with IBD although patients with inactive disease had higher T cell counts. The proportions of T cells and monocytes in culture were comparable in all the groups.

Ig production by monocyte depleted lymphocyte cultures

Culture of PWM stimulated peripheral blood lymphocytes from control patients showed that B cells produced less Ig per cell as the concentration increased so that Ig production at 10^6/ml was

Table 1. Cell populations in the circulation and in culture (mean ± s.d. × 10^5/ml)

	Control (43)	UC-A (35)	UC-I (36)	CD-A (60)	CD-I (54)
Lymphocytes	17·4±5·6	17·7±7·2	23·5±9·5	20·2±9·9	24·7±9·7
T cells	9·1±3·6	10·2±4·4	14·7±6·6	11·0±6·1	15·3±7·9
Monocytes	5·0±2·4	5·4±3·6	3·5±6·6	4·1±2·2	4·3±2·6
T cells in culture (%)	44±14	46±16	52±12	48±17	51±7
Monocytes in culture (%)	16±14	10±5	8±10	7±8	6±7
Monocytes removed by adherence (%)	37±18	40±28	65±23	35±22	36±22

A = active disease; I = inactive disease.

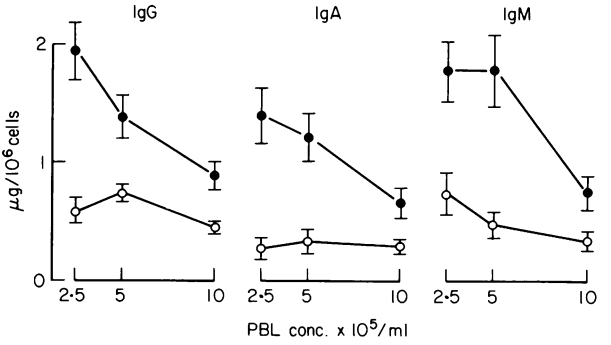

Fig. 1. *In vitro* production of immunoglobulins by peripheral blood lymphocytes from control patients: concentration-dependent effect ($n=42$). ●——● = PWM stimulated; ○——○ = unstimulated.

significantly less ($P<0{\cdot}01$) than that at $0{\cdot}25\times10^5$/ml (Fig. 1). At the latter cell concentration significant differences in Ig production were observed in cultures of lymphocytes from patients with IBD (see Fig. 2). Cells from CD patients in disease remission produced highly significantly reduced amounts of IgG ($P<0{\cdot}003$) and IgA ($P<0{\cdot}002$) compared with control patients. IgM production was not significantly reduced ($P<0{\cdot}07$). Cells from patients with active CD also produced highly significantly reduced amounts of IgG ($P<0{\cdot}0001$) as well as significantly reduced amounts of IgA and IgM ($P<0{\cdot}04$). Corresponding changes were not observed in patients with UC. When lymphocytes were cultured at 5×10^5/ml concentration the amount of IgM produced was significantly reduced in patients with active CD ($P<0{\cdot}01$) and also with active UC ($P<0{\cdot}03$). These changes in Ig production in patients with IBD were independent of corticosteroid therapy and were not directly associated with undernutrition in these patients.

Ig production by lymphocyte cultures reconstituted with monocytes

Peripheral blood mononuclear cells which contained less than 15% monocytes after adherent cell depletion (Table 1) were cultured at a concentration of 5×10^5/ml. Autologous adherent cells were added in increasing numbers up to 5×10^5/ml. With control and UC patients, adherent cells

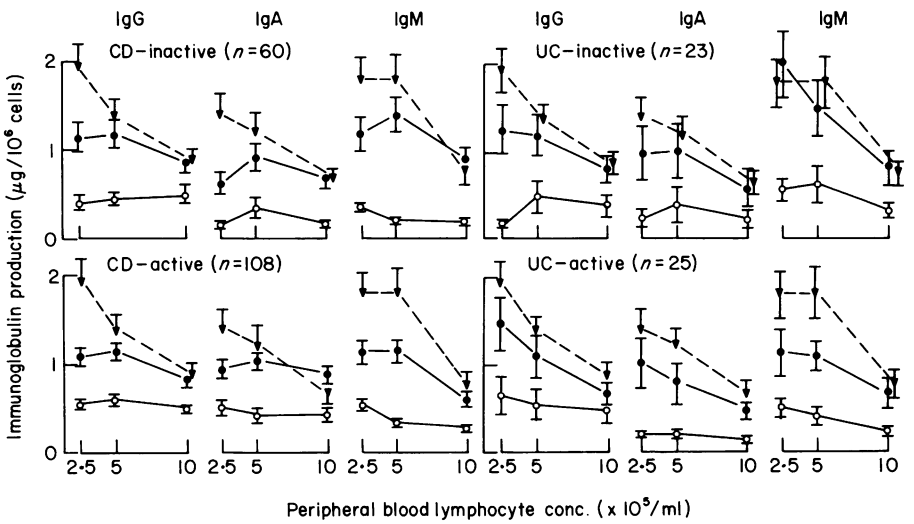

Fig. 2. *In vitro* production of immunoglobulins by peripheral blood lymphocytes from patients with IBD compared with PWM stimulated lymphocytes from control patients. ●——● = PWM stimulated; ○——○ = unstimulated; ▼——▼ = control.

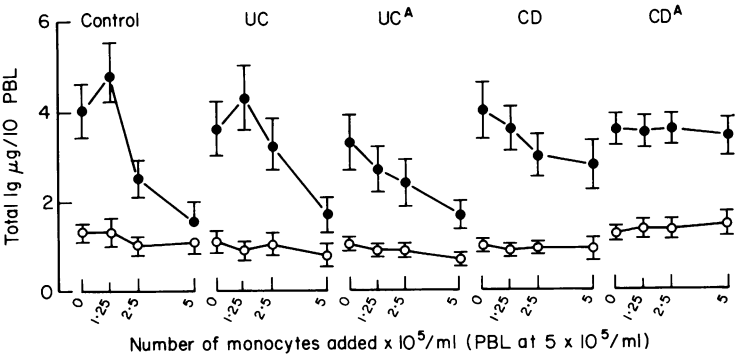

Fig. 3. Effect of addition of autologous monocytes on peripheral blood lymphocyte Ig production in patients with UC ($n=42$), CD ($n=124$) and controls ($n=36$). ●——● = PWM stimulated; ○——○ = unstimulated; A = active disease.

provided some helper activity before rapidly suppressing Ig production at higher concentrations (Fig. 3). Addition of autologous adherent cells failed to produce this suppression in CD patients. Table 2 shows the effect of co-culturing monocytes and lymphocytes from patients with IBD and those from control patients. Monocytes from CD patients failed to suppress Ig production by lymphocytes from control patients but monocytes from control patients could still suppress lymphocytes from CD patients. Monocytes from patients with UC suppressed Ig production by lymphocytes from control patients to the same degree as their own lymphocytes were suppressed. Co-culture of cells from control patients showed that monocyte-mediated suppression could take place across allogeneic barriers (see Table 2).

Ig production by B lymphocyte cultures reconstituted with autologous T lymphocytes

The addition of increasing numbers of T cells to a fixed number of autologous non-T cells (adherent cell depleted) resulted in a rise in Ig production up to an optimal level after which production was decreased (Fig. 4). The optimal T cell concentration was 5×10^5/ml for IgG and between 6 and 8×10^5/ml for IgA and IgM. This occurred when non-T cells were cultured at 10^5/ml. In patients with UC, active disease was associated with a shift to a lower optimal T cell concentration compared

Table 2. Suppression of Ig production in monocyte–lymphocyte co-culture (ratio 1:2)

Lymphocyte source	Monocyte source	*n*	Total Ig production (μg/10^6 cells; mean ± s.e.)	Percentage suppression by monocytes (mean)
Control	—	27	4·2±0·5	—
Control	Control	27	2·3±0·6	45
Control	Control-allogeneic	8	1·8±0·4	57
Control	CD	12	3·8±0·5	10
Control	UC	5	3·0±0·6	29
CD	—	77	4·0±0·6	—
CD	CD	77	3·7±0·4	8
CD	Control	14	2·3±0·5	42
UC	—	42	3·8±0·4	—
UC	UC	42	2·8±0·4	26
UC	Control	5	2·8±0·4	26

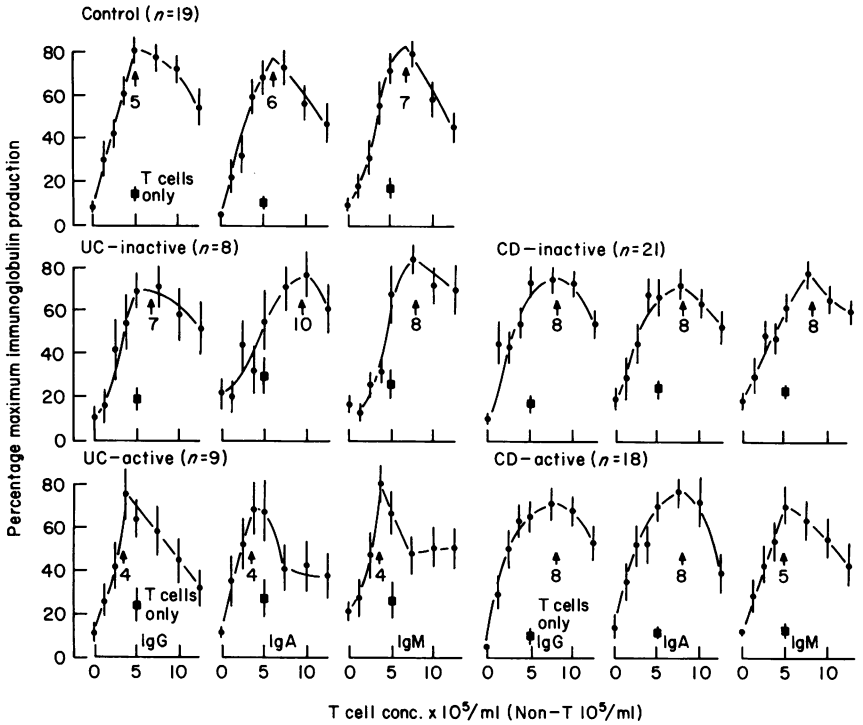

Fig. 4. *In vitro* immunoglobulin production by a fixed number of peripheral blood B lymphocytes in response to the addition of increasing numbers of autologous T lymphocytes and PWM stimulation in control patients and patients with inflammatory bowel disease.

with patients in remission for all Ig isotypes (see Fig. 4). A similar shift to a lower T cell concentration for optimal Ig production was observed in patients with active CD but only for IgM production. IgA and IgG production showed identical patterns in CD whether active or in remission.

In quantitative terms, a difference in the optimal amount of Ig produced, was observed in patients with UC who had significantly reduced IgA production especially in disease remission (Table 3). This was associated with a higher T cell concentration (10^6/ml) for optimal T helper activity for IgA. Patients with active CD produced significantly less IgM than controls even though there was a shift to a lower T cell concentration for optimal T helper activity for IgM (Fig. 4).

Table 3. Optimal Ig production in T/non-T co-cultures expressed as $\mu g/10^6$ non-T cells (±s.e.)

	Control (19)	UC-A (11)	UC-I (9)	CD-A (20)	CD-I (22)
IgG	10·8±1·5	11·4±2·5	7·3±1·5	9·9±1·6	8·2±1·0
IgA	9·3±1·3	5·1*±1·8	3·4†±1·0	8·1±1·3	7·6±1·5
IgM	14·5±1·5	12·8±2·3	9·6±2·1	8·7*±1·3	12·2±1·3

*$P < 0.02$, †$P < 0.001$ compared with control.

DISCUSSION

The way in which disease states can result in alterations in the populations of cells that regulate B cell functions can be studied *in vitro* by culturing cells under certain critical conditions (see Knight, 1982). Alterations in Ig production by peripheral blood B cells from patients with IBD were evident when cells were cultured at low cell concentrations, when the proportion of monocytes in culture was increased and when the proportion of T cells in culture was varied.

When peripheral blood lymphocytes were cultured, those from patients with CD but not UC displayed reduced Ig production relative to the control group. This was not isotype restricted and was not dependent on disease activity. This suggests that the proportion of PWM reactive B cells is reduced in these patients. Since PWM reactive B cells represent the small proportion of B cells that have already been primed *in vivo* (Stevens, Macy & Thiele, 1981) this may mean that memory B cells primed for the expression of all Ig isotypes are constantly being sequestered to mucosal sites (Meijer *et al.*, 1979) even in the absence of clinical symptoms.

Monocytes have been shown to have helper and suppressor function regarding lymphocyte Ig production (Gmelig-Meyling & Waldmann, 1981). The present studies confirm this and also reveal a lack of suppressor function that is peculiar to monocytes from patients with CD. Again the phenomenon was relatively independent of disease activity and suggests that CD may also be associated with alterations of normal suppressor mechanisms. Indeed reduced suppressor cell activity in patients with CD has already been suggested by work on mitogen-induced blastogenesis of peripheral blood and intestinal lymphocytes (Victorino & Hodgson, 1981; Goodacre & Bienenstock, 1982).

Polyclonal B cell activation by PWM is dependent on both the T/B ratio and the T cell concentration in culture (Waldmann & Broder, 1982). T cells may also exert isotype specific helper/suppressor functions (Rosenberg, 1982). In the present study, B cell function was shown to be dependent on the T cell concentration and some changes in T cell regulation were observed in patients with IBD. Some of these changes were shown to be isotype specific. Active disease resulted in increased T helper function for the production of all Ig isotypes in patients with UC but only a moderate increase in T helper function for IgM production in patients with CD. This may be related to a shift in the T helper/T suppressor ratio observed in patients with active UC (Aiso *et al.*, 1982) which has not been observed in patients with CD (Selby & Jewell, 1983).

Although there was an increase in T helper activity for IgM production in patients with active CD the optimal amount of IgM produced was decreased. This suggests that there are reduced numbers of PWM reactive IgM producing B cells in these patients (Elson *et al.*, 1981) probably due to sequestration to mucosal sites where an IgM response is particularly enhanced (Baklien & Brandtzaeg, 1975). A decrease in T helper function for IgA production but not IgG or IgM was observed in patients with UC in remission. This was also associated with reduced B cell IgA production. This suggests that UC is associated with defective IgA responses since there are less recirculating T helper cells for IgA production and less recirculating IgA memory cells which are primed at mucosal sites (Kutteh, Prince & Mestecky, 1982). The phenomenon was not apparent in patients with active symptoms probably due to the increase in non-isotype specific helper activity induced during the active disease process. We have previously shown that rectal mucosal secretion of IgA was reduced in patients with inactive disease, and that inflamed colonic mucosa secreted reduced amounts of IgM.

Therefore, either defective secretory antibody defences (in the case of patients with UC) or defective suppressor mechanisms (in the case of patients with CD) may allow increased activation of systemic immune mechanisms. These can then lead to inappropriate mucosal immune responses such as those mediated by IgG or IgM, which in turn may perpetuate the local inflammatory reaction.

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