Deficient interleukin 2 dependent proliferation pathway in T lymphocytes from active and inactive ulcerative colitis patients

L Manzano, M Alvarez-Mon, J A Vargas, J A Girón, L Abreu, A Fernández-Corugedo, L I Román, F Albarran, A Duránte

Abstract

There is increasing evidence that ulcerative colitis is associated with an abnormality of the immune system. Although the aetiology remains unknown, it has been suggested that the immune system of these patients is implicated in the pathogenesis of their disease. T cell function was investigated in ulcerative colitis patients and defective phytohaemagglutinin induced T cell mitogenesis was found. The DNA synthesis induced by stimulation with phorbol esters plus ionophore (ionomycin), however, was normal. These changes cannot be ascribed to either decreased interleukin 2 synthesis or to a defective interleukin 2 receptor expression after cellular activation. Moreover, this defective proliferative response of the T lymphocytes was observed even in the presence of saturated concentrations of exogenous interleukin 2. These results emphasise that the interleukin 2 dependent proliferation pathway is deficient in T lymphocytes from ulcerative colitis patients.

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cascade that leads T lymphocytes to progress through the cell cycle.⁹ Protein kinase C activation can also be directly induced in vitro by phorbol esters.¹⁰ This cellular process is associated with activation of the interleukin 2 (IL-2) pathway that regulates T cell proliferation. The progression of T lymphocytes from the G0 to the G1 cell cycle phase is associated with the activation of the genes that regulate the synthesis of the IL-2 receptor (IL-2R) chains as well as the IL-2 molecule.¹¹ Secretion of this molecule and its interaction with the specific T cell surface receptor induces the progression of the T lymphocyte along the final phases of the cell cycle and regulates subsequent proliferation.^{11 12}

We have investigated the functional characteristics of peripheral blood T lymphocytes from patients with ulcerative colitis and have analysed the following functions in purified T cells from these patients and healthy controls: (1) the proliferative response to polyclonal mitogenic stimulus (plant lectins and phorbol esters); (2) the subsequent expression of IL-2R and other cellular activation antigens; and (3) the IL-2 production.

Methods

PATIENT POPULATION

Fifty two patients (31 men and 21 women with a mean (SD) age of 40 (15) years) with ulcerative colitis and 31 age and sex matched healthy controls were studied. Each gave his consent of the experimental protocol. The diagnosis of ulcerative colitis was established on the basis of clinical symptoms and on the endoscopic and pathological demonstration of abnormal, inflamed colonic mucosa. Disease activity in each patient was analysed according to Truelove's criteria.¹³ Twenty five patients (14 men and 11 women, aged 14-54 years) had active disease (14 mild, nine moderate, and two severe). There were 27 patients with inactive disease (17 men and 10 women, aged 23-76 years). When the experimental study was performed, 17 patients had been taking steroids (14 plus sulphasalazine) for at least three weeks. The remaining 35 patients had not taken steroids. Of these, 27 were taking sulphasalazine and eight were receiving no specific treatment.

Exclusion criteria for this study were: (1) the existence of clinical, serological, microbiological or pathological proof of inflammatory

Service of Internal Medicine, Department of Medicine, Hospital Universitario Príncipe de Asturias, Universidad de Alcalá, Madrid, Spain

L Manzano
M Alvarez-Mon
L I Román
F Albarran

Services of Internal Medicine I and Gastroenterology, Clínica Puerta de Hierro, Universidad Autónoma, Madrid, Spain

J A Vargas
L Abreu
A Fernández-Corugedo
A Duránte

Department of Internal Medicine, Universidad de Cadiz, Spain
J A Girón

Correspondence to: Professor M Alvarez-Mon, Departamento de Medicina, Facultad de Medicina, Universidad de Alcalá de Henares, Alcalá de Henares, Madrid, Spain.

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Ulcerative colitis is a chronic systemic inflammatory disease that mainly affects the mucosa of the rectum and colon and whose natural history is characterised by remissions and exacerbations. Changes in the immune system have been implicated in the predisposition and pathogenesis of the disease and this mechanism has been supported by clinical, pathological, therapeutic, and experimental findings.¹⁻³

T lymphocyte activation and proliferation is a complex process in which several surface molecules, intracytoplasmic enzyme systems, and cytokines (monokines and lymphokines) are involved.^{4 5} Activation begins with the specific interaction between the antigen and the clonotypic T cell receptor.⁵⁻⁷ The activation effect of this interaction can be mimicked in vitro by the interaction of different polyclonal mitogens, such as vegetal lectins, with molecules of the T lymphocyte surface.⁸ These different molecular interactions provoke the activation of several intracytoplasmic metabolic pathways which induce the activation and translocation of protein kinase C. Several proteins phosphorylated by this enzyme induce the initiation of the gene activation

intestinal disease other than ulcerative colitis and (2) the presence of intercurrent diseases or drugs, excepting steroids, that could change the results of the immunological studies performed.

REAGENTS

Several monoclonal antibodies (MoAb) that recognise specific molecules on the surface of certain cellular subpopulations were used in the studies of phenotypic characterisation and activation: OKT11 (anti-CD2), OKT3 (anti-CD3), OKT4 (anti-CD4), OKT8 (anti-CD8), OKT9 (anti-CD71) (Ortho-mune, Orthodiagnostic Systems, NJ, USA); B1 (anti-CD20), Mo2 (anti-CD14), TAC (anti-CD25) (Coulter Clone, Coulter Immunology, FL, USA); and Leu-11B (anti-CD16), Anti-HLA-DR (Becton Dickinson, CA, USA).

CULTURE MEDIUM

RPMI 1640 (Flow Lab, CA, USA) supplemented with 1% L-glutamine (Flow Lab), 0.5% HEPES (Flow Lab), and 1% penicillin-streptomycin was used for cultures. This will be referred to as 'complete medium'.

ISOLATION OF LYMPHOID CELLS

Peripheral blood mononuclear cells (PBMNC) were obtained from the heparinised venous blood of the subjects by Ficoll-Hypaque gradient centrifugation (Nyegaard Co, Oslo, Norway). T cells were purified by double rosetting as previously described,¹⁴ the purity being greater than 95% in every case. After counting, cells were resuspended in complete medium supplemented with 10% heat inactivated fetal calf serum (FCS; Gibco, NY, USA), and checked for viability by trypan blue exclusion. In other experiments, supplementation with autologous or pooled AB serum (10% and 20% v/v) was performed.

PROLIFERATION STUDIES

T lymphocytes (250 000 cells/ml) were cultured on 96 flat bottom culture plates. Soluble phytohaemagglutinin (PHA; 10 µg/ml; Difco Lab, Detroit, MI, USA), or phorbol-myristate-acetate (PMA; 10 ng/ml; Sigma, St Louis, MO, USA) plus ionomycin (5 µg/ml, Sigma) in the presence or absence of recombinant interleukin 2 (rIL-2; 100 IU/ml; provided by Dr J Farrar and Dr P Sorter; Hoffman-La Roche, NJ, USA) were added at the beginning of proliferation studies. Each reagent was tested in dose/response titrations before use. Cultures were incubated at 37°C in a humidified atmosphere containing 5% CO₂ for three, five, and seven days. Eighteen hours before the end of incubation, 1 µCi of tritiated thymidine (Radiochemical Centre, Amersham, UK) was added. The cells were harvested and the results expressed as mean (SD) counts per minute (cpm) of triplicate cultures.

LYMPHOKINE PRODUCTION

Lymphokine enriched supernatants were obtained by culturing T cells at 37°C at a density of 5×10⁶/ml in complete medium supplemented with 10% FCS. Cultures were incubated in the presence or absence of PHA (10 µg/ml) and supernatants were harvested after 24 and 72 hours of incubation, sterilised by filtration through a 0.22 µm filter (Millipore Company, Bedford, CA, USA), and stored at -70°C until use.

IL-2 activity was determined in the supernatants of T cell suspensions by evaluating the dose dependent proliferation induced in the cytotoxic murine line CTTL-2 by the lymphokine present in the cultures.¹⁵

IMMUNOFLUORESCENCE ANALYSIS

Immunofluorescence staining of cell surface antigens was performed using a standard method as previously described.¹⁶ The fluorescence of 10 000 viable lymphoid cells stained for each different MoAb was quantified using an EPICS C flow cytometer (Coulter).

STATISTICAL ANALYSIS

The data from the groups were compared using the Mann-Whitney U test. A p value of less than 0.05 was considered statistically significant.

Results

PROLIFERATIVE RESPONSE BY T LYMPHOCYTES FROM ULCERATIVE COLITIS PATIENTS IS DEFECTIVE TO MITOGENIC SIGNALS THAT INTERACT WITH MEMBRANE RECEPTORS BUT NORMAL TO INTRACYTOPLASMATIC MITOGENIC SIGNALS

The blastogenic response of PBMNC from ulcerative colitis patients and healthy controls to the stimulation signal given by PHA was analysed first. As can be seen in Figure 1A, PBMNC from ulcerative colitis patients showed a significantly reduced mitogenic response to PHA in respect of the response in healthy controls, after either three or five days of culture ($p < 0.05$). After seven days of culture in the presence of PHA, however, there were no significant differences between the proliferative response of PBMNC from ulcerative colitis patients and healthy controls.

The T lymphocyte population in the PBMNC from ulcerative colitis patients was purified to rule out a possible inhibitory effect of the non-T cells present in the PBMNC from these patients upon the proliferative response of the T lymphocytes. The PHA induced tritiated thymidine uptake by purified T cells from ulcerative colitis patients was also considerably impaired in respect of that in purified T lymphocytes from healthy controls after three and five days of culture ($p < 0.05$) (Fig 1B). The optimum mitogenic dose of PHA for T lymphocytes from controls as well as from patients was the same (10 µg/ml). Since the maximal mitogenic response was

Figure 1: Kinetics of the blastogenic response of (A) peripheral blood mononuclear cells (PBMNC) and (B) T lymphocytes from 18 ulcerative colitis patients and 12 healthy controls to stimulation with phytohaemagglutinin for three, five, and seven days. Results are expressed as mean (SD) counts per minute (cpm). Statistical differences between both groups ($p<0.05$) were found at three and five days of culture.

detected on day five in T lymphocyte cultures from both ulcerative colitis patients and healthy controls, the proliferative response was analysed on this day of culture in subsequent experiments. It was found that the blastogenic response to PHA of the purified T lymphocytes from the ulcerative colitis patients was significantly less than that observed in the healthy control group ($p<0.05$) (Fig 2). This hypoproliferative response cannot be ascribed to a change in the distribution of the mononuclear population or in the T cell subsets studied (Table).

Figure 2: Tritiated thymidine uptake by T cells from ulcerative colitis patients and healthy controls in response to phytohaemagglutinin (PHA) and phorbol-myristate-acetate (PMA) + ionomycin for five days. Results are expressed as mean (SD) counts per minute (cpm). Statistically significant differences were found between the mitogenic response of patients and controls in the presence of PHA ($p<0.05$). However, there were no statistically significant differences ($p>0.05$) between patients and controls when the mitogenic association PMA + ionomycin (Ion) was used.

Phenotypic study of the peripheral blood mononuclear cells (PBMNC) and purified T cells from ulcerative colitis patients and healthy controls

	Patients Mean (SD) (%)	Controls Mean (SD) (%)
PBMNC:		
T lymphocytes (CD3+)	60.6 (9.1)	59.4 (8.7)
B lymphocytes (CD20+)	14.2 (4.7)	15.5 (5.4)
Monocytes (CD14+)	21.7 (8.6)	20.8 (7.9)
Natural killer (CD16+)	14.0 (3.2)	13.2 (5.1)
T lymphocytes:		
CD2+	95.5 (2.8)	96.1 (2.6)
CD3+	85.2 (5.7)	87.2 (6.3)
CD4+	49.0 (8.1)	50.5 (7.5)
CD8+	33.4 (6.7)	34.1 (7.2)
CD4+/CD8+	1.46 (0.3)	1.48 (0.4)

PBMC were studied by flow cytometry with OKT3 (anti-CD3), OKT4 (anti-CD4), OKT8 (anti-CD8), OKT11 (anti-CD2), B1 (anti-CD20), Mo2 (anti-CD14), and Leu-11b (anti-CD16) Moab, and a second step fluoresceinated reagent, as indicated in the methods section. Table gives the percentage of cells staining above the second step reagent background.

To analyse the hypothesis that an inhibitory factor present in the ulcerative colitis patients' serum could be involved in the defective proliferation found in the T lymphocytes, T lymphocyte cultures were performed simultaneously in the presence of either fetal calf, heterologous AB, or autologous serum. Neither ulcerative colitis patients nor healthy controls showed a significant difference in the PHA induced T cell mitogenic response, regardless of the serum used ($p>0.05$). In all the different experimental conditions analysed, the T cell blastogenesis in ulcerative colitis patients (FCS 62 358 (15 312) cpm, heterologous AB serum 65 563 (14 834) cpm, autologous serum 69 422 (16 513) cpm) was found to be defective in respect of that found in healthy controls (FCS 116 671 (16 324) cpm, heterologous AB serum 120 514 (15 410) cpm, autologous serum 125 412 (17 211)) ($p<0.05$). We also tested the possibility that T cells isolated from ulcerative colitis patients will recover a normal proliferative response to PHA after a period of rest in culture before stimulation. In three cases, we did not find that the decreased proliferative response of T lymphocytes from ulcerative colitis patients was restored when they were rested after 48 and 72 hours in culture (data not shown). Finally, the T cell proliferative response was analysed after stimulus with mitogenic molecules that directly interact with protein kinase C and intracellular Ca^{++} : PMA and ionomycin. The proliferative response of T cells from ulcerative colitis patients and healthy controls was similar in these experimental conditions ($p>0.05$) (Fig 2). Possible variations in the proliferative response of T cells from ulcerative colitis patients to stimulation with PHA were also investigated with regard to clinical parameters. There were no significant differences between the PHA induced proliferative response of T lymphocytes from patients with active and inactive disease ($p>0.05$). Likewise, there were no significant differences between the PHA induced proliferative response of T lymphocytes from patients with proctitis versus those with total colon involvement, nor between those patients studied within the first

Figure 3: Proliferative response of T lymphocytes from ulcerative colitis patients receiving different treatments to stimulation with phytohaemagglutinin for five days. Results are expressed as mean (SD) counts per minute (cpm). No statistical differences ($p>0.05$) were detected in the proliferative response of T cells between the different groups. SPZ, sulphasalazine.

five years of diagnosis *versus* those with disease of more than five years' duration. Nor were differences observed between patients with the chronic continuous evolutive type of the disease and those with the intermittent form. The hypoproliferative response of T lymphocytes to PHA stimulation was similar in ulcerative colitis patients with and without colectomy ($p>0.05$), and the blastogenic response of T cells from patients taking steroids was similar to that in patients who were not taking these drugs ($p>0.05$) (Fig 3).

IL-2 PRODUCTION OF T CELLS FROM ULCERATIVE COLITIS PATIENTS IS NORMAL
Three different steps can be defined in the activation and proliferation of T cells. Firstly, IL-2 is produced; secondly, IL-2R is expressed;

Figure 4: Expression of (A) interleukin 2 receptor (CD25), (B) transferrin receptor (CD71), and (C) major histocompatibility complex class II molecules (DR) on the cytoplasmic membrane of T cells from ulcerative colitis patients and healthy controls, under basal conditions and after activation with phytohaemagglutinin for 24, 72, or 106 hours. The columns represent the mean (SD) of the percentage of cells expressing the different cell surface molecules. Similar results were detected in the different groups ($p>0.05$).

and thirdly, upon binding to its specific receptors, IL-2 promotes T cell mitogenesis.

There were no statistical differences in the low rates of spontaneous production of IL-2 by T cells from ulcerative colitis patients (mean (SD) 1210 (213) cpm) and healthy controls (1514 (312) cpm) ($p>0.05$). PHA stimulated T cells from the two populations also showed similar levels of IL-2 production (patients at 24 hours 24 619 (5985) cpm; 72 hours 21 304 (7703) cpm) (controls at 24 hours 26 316 (5324) cpm; 72 hours 24 638 (6334) cpm) ($p<0.05$).

T LYMPHOCYTES FROM ULCERATIVE COLITIS PATIENTS SHOW NORMAL IL-2R EXPRESSION, BUT DEFICIENT BLASTOGENIC RESPONSE TO THIS LYMPHOKINE

The simultaneous findings of a hypoproliferative response and normal IL-2 production after PHA stimulation of T cells from ulcerative colitis patients could be attributed to defective expression of the IL-2R or some change in the mitogenic response induced by the interaction of IL-2 with its receptor, or both. To investigate the first suggestion, quantitative flow cytometric studies were performed after T cell staining with monoclonal antibodies specific for the IL-2R (anti-CD25). Antibodies to CD71 structure and DR molecules were also employed for a broader analysis of the T cell activation process. In basal conditions and after PHA stimulation for one, three, or five days, the proportion of stained cells and the intensity of the expression of CD25 antigen were similar in the T cell cultures from ulcerative colitis patients and healthy controls ($p>0.05$) (Fig 4). Similar patterns were also found in the T cell membrane expression of CD71 and DR molecules in both ulcerative colitis patients and healthy controls.

Finally, we investigated the proliferative response of T cells from ulcerative colitis patients in the presence of saturant concentrations of exogenous IL-2. In the absence of stimulation with PHA, similar low mitogenic responses to exogenous IL-2 were found in T lymphocyte cultures from ulcerative colitis patients and controls ($p>0.05$). Moreover, exogenous addition of saturant concentrations of IL-2 to the PHA stimulated T cell cultures from ulcerative colitis patients neither returned to normal nor significantly increased their blastogenic response ($p>0.05$) (Fig 5).

Discussion

We have demonstrated that T lymphocytes from ulcerative colitis patients show a defective proliferative response to the mitogenic signals given by lectins that interact with surface molecules, but a normal blastogenic response to intracytoplasmatic protein kinase C activators. This T lymphocyte deficiency is independent of the clinical activity of the ulcerative colitis and cannot be ascribed to defective IL-2 production but rather to an impaired proliferative response by T lymphocytes to this lymphokine.

Figure 5: Effect of supplementation with recombinant interleukin 2 (rIL-2) on the blastogenic response of T lymphocytes from ulcerative colitis patients and healthy controls in the presence and absence of phytohaemagglutinin (PHA). Results are expressed as mean (SD) count per minute (cpm). The exogenous addition of rIL-2 to the PHA stimulated T cell cultures neither returned to normal nor significantly increased the defective blastogenic response in ulcerative colitis patients ($p > 0.05$).

Previous reports have presented contradictory results concerning the proliferative capacity of peripheral blood T lymphocytes from ulcerative colitis patients to be stimulated by mitogenic lectins.^{17–21} Likewise, IL-2 production by T cells has been reported as either normal^{22–23} or reduced.²⁴ These contradictory reports, can be partly explained by the small number of cases analysed. Furthermore, several studies have analysed patients with ulcerative colitis together with those with Crohn's disease with no good clinical definition between the two groups.

Our data show that ulcerative colitis patients have defective T lymphocyte blastogenesis after mitogenic stimulation. The hypoproliferative response cannot be ascribed to an anomalous distribution of CD4 and CD8 T cell subsets, the presence of a soluble inhibitory factor in patients' sera, or to a suppressive effect of non-T cells from ulcerative colitis patients. Nevertheless, the possibility that there are more functionally active CD8 T suppressor cells in ulcerative colitis patients than in healthy controls cannot be excluded.

These T lymphocytes, however, proliferated normally after direct activation of protein kinase C (by the phorbol ester PMA) and calcium mobilisation (by the ionophore ionomycin). These results strongly suggest that T cells from ulcerative colitis patients have a functional defect that interferes with the generation of the second messengers of the T lymphocyte activation process.

It is relevant that this functional abnormality is associated with neither defective IL-2 production by T cells nor with decreased IL-2R expression. Moreover, the normal expression of IL-2R by activated T lymphocytes from ulcerative colitis patients was associated with a defective proliferative response to IL-2, even in the presence of saturant amounts of exogenous IL-2. It may be that a change in the metabolic process of transduction of the activation signal subsequent to the interaction of IL-2 with its specific receptor on the cytoplasmic membrane of T lymphocytes is involved in this defective mitogenesis.

The functional change shown in T lymphocytes from ulcerative colitis patients is not related to the activity of the disease, since it is also present in asymptomatic patients without clinical evidence of disease activity. Moreover, colectomised patients presented a similar defective proliferative response to the mitogenic signals.

These findings show that ulcerative colitis is not only associated with a local immune abnormality in the intestine but also with a systemic change in the immune system.^{25–27} We have recently shown that this immunological change is not restricted to the T lymphocyte population, since natural killer cells are also impaired in symptomatic ulcerative colitis patients.²⁸ However, the T cell deficiency, unlike the change in natural killer cells, remains independently of the clinical activity in these patients. This T cell immunodeficiency has been found previously in other autoimmune diseases^{29–30} and it may be implicated in the development of the impaired immune system regulation that plays a part in the pathogenesis of the intestinal and extraintestinal lesions observed in these patients.

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