

Functional impairment of natural killer cells in active ulcerative colitis: reversion of the defective natural killer activity by interleukin 2

L Manzano, M Alvarez-Mon, L Abreu, J Antonio Vargas, E de la Morena, F Corugedo, A Duràntez

Abstract

We have studied the functional characteristics and clinical importance of the natural killer (NK) cytotoxicity of peripheral blood mononuclear cells (PBMNC) from patients with ulcerative colitis. Normal NK activity was observed in PBMNC from patients with inactive disease, but a pronounced decrease was found in those with active disease. Clinical change from active to inactive disease was associated with enhancement of the depressed NK activity. The impairment of NK cytotoxicity found in patients with active disease could not be ascribed to a deficient number of NK cells as the amounts of HNK-1⁺, CD16⁺ (Leu 11), and CD11b (OKM1) cells in PBMNC were within normal ranges. This defective cytotoxic PBMNC activity was normalised by short term (18 hour) incubation with recombinant interleukin 2 (rIL-2). Moreover, long term (5 day) incubation of these effector cells with rIL-2 induced strong cytotoxic activity against NK resistant and NK sensitive target cells in patients with active and inactive disease. We also found that both precursors and effectors of cytotoxic activity promoted by short term and long term incubation with rIL-2 of PBMNC from the patients showed the phenotype of NK cells (CD16⁺, CD3⁻). Taken together, these results show that active ulcerative colitis is associated with a defective function of NK cells that is found to be normal in the inactive stage of the disease. The possible pathogenic and therapeutic implications of these findings are discussed.

Natural killer (NK) cells are subpopulations of in vivo activated lymphocytes that exert spontaneous cytotoxic activity against tumoural and virus infected cells in a major histocompatibility complex (MHC) unrestricted fashion.¹⁻⁴ The NK effectors are large granular lymphocytes that characteristically express the CD16 surface antigen.¹ These NK cells have been implicated in immunosurveillance against growth and haematogenous spread of tumours and in resistance to viral and other microbial diseases.¹⁻⁵ The cytotoxic activity of NK cells can be induced or enhanced by certain lymphokines, especially interleukin 2 (IL-2).⁶⁻⁸ IL-2 can also generate strong non-MHC restricted cytotoxic activity, referred to as lymphokine activated killer (LAK) cytotoxicity,⁹⁻¹¹ in peripheral blood mononuclear cells (PBMNC) after longterm (5

day) incubation. There is increasing evidence that the precursors of this LAK cytotoxicity are mainly CD3⁻, CD16⁺ NK cells.^{12,13}

Ulcerative colitis is a chronic systemic inflammatory disease affecting principally the mucosa of the rectum and colon, with a clinical evolution characterised by remissions and exacerbations. The aetiology of this disease remains unknown, but it has been suggested that infectious agents might be involved in the induction of the colonic lesion.¹⁴⁻¹⁹ A possible pathogenic role of the immune system has also been proposed.^{14,20,21}

We have investigated the functional characteristics and the clinical importance of the NK activity of PBMNC from patients with ulcerative colitis.

Methods

PATIENT POPULATION

Forty eight patients, 28 men and 20 women, mean (SD) age 40 (15) years, with ulcerative colitis diagnosed clinically, radiologically, endoscopically, and pathologically, were studied. Thirty one healthy, age and sex matched subjects were used as control subjects. Disease activity in each patient was analysed according to the criteria of Truelove and Witts.²² Twenty four patients, 13 men and 11 women, aged 14-54 years, had active disease (13 mild, 8 moderate, 3 severe). There were 24 patients with inactive disease, 15 men and nine women, aged 23-76 years. When the experimental study was performed 14 patients had been taking steroids plus sulphasalazine for at least three weeks. At the time of the study the remaining 34 patients had not taken steroids for at least three months. Of these, 28 were taking sulphasalazine and six were receiving no specific treatment.

TABLE I Decreased NK cytotoxicity is found in peripheral blood mononuclear cells from patients with ulcerative colitis

	Effector to target ratio		
	50:1	25:1	12:1
Patients	28 (17)	22 (15)	15 (13)
Controls	48 (12)	36 (12)	27 (11)
p value	<0.01	<0.01	<0.01

Peripheral blood mononuclear cells from 48 patients and 31 control subjects were used as effector cells in cytotoxic assays against ⁵¹Cr-labelled K-562 target cells at the indicated effector to target ratio. The results are expressed as the mean (SD) specific lysis in experiments performed in triplicate in the two groups of subjects.

Service of Internal Medicine I and Service of Gastroenterology, Clinica Puerta de Hierro, Universidad Autónoma de Madrid; Service of Internal Medicine, Department of Medicine, Hospital Universitario Príncipe de Asturias, Universidad de Alcalá de Henares, Madrid, Spain
L Manzano
M Alvarez-Mon
L Abreu
J Antonio Vargas
E de la Morena
F Corugedo
A Duràntez

Correspondence to: Professor Melchor Alvarez-Mon, Departamento de Medicina, Carretera Madrid-Barcelona, km 33,600, Universidad de Alcalá de Henares, 28871 Alcalá de Henares, Madrid, Spain.

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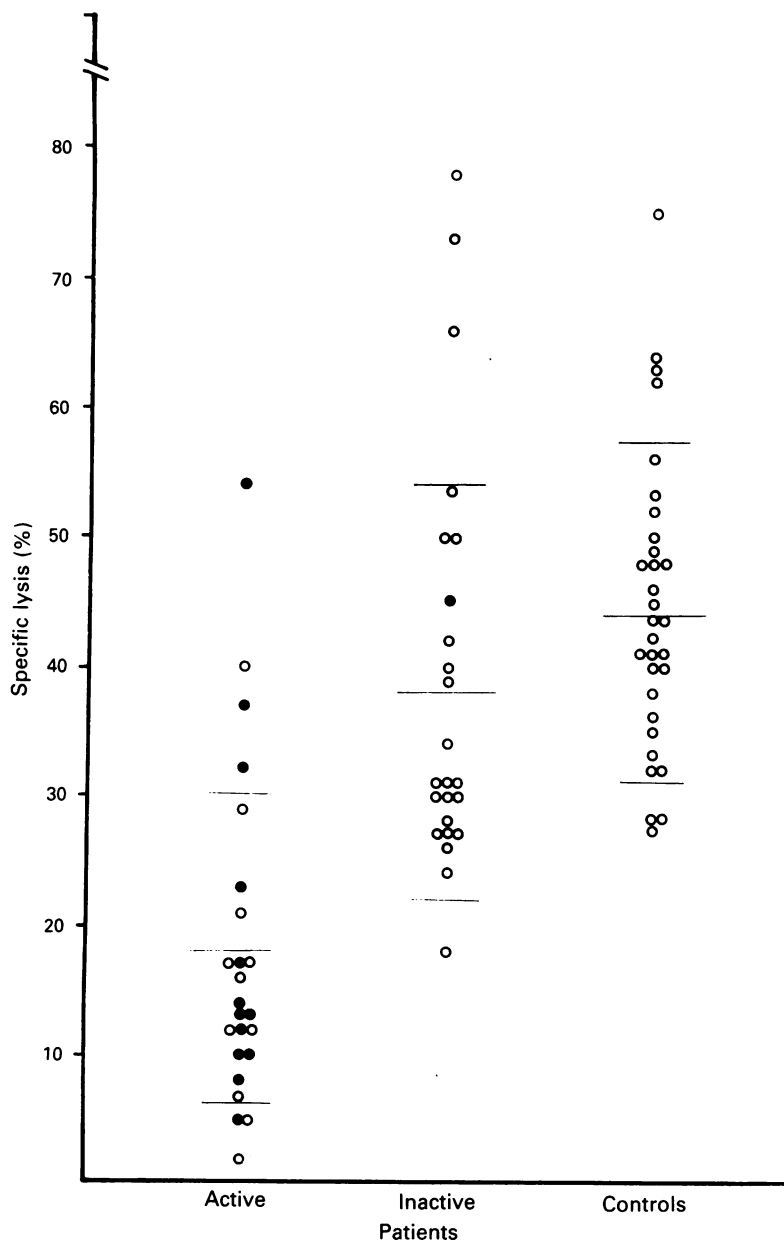


Figure 1: Ulcerative colitis patients with active, but not inactive, disease show impaired NK cytotoxicity in PBMNC. PBMNC from patients with active (24) and inactive (24) disease and from control subjects (31) were used as effector cells against ^{51}Cr -labelled K-562 target cells. Closed circles represent patients taking steroids. Each symbol indicates the mean specific lysis of triplicate cytotoxic assays performed at a 50:1 effector to target ratio for an individual in each group.

REAGENTS

Human recombinant interleukin 2 (rIL-2) was provided by Dr J Farrar and Dr P Sorter (Hoffman-La Roche, Nutley, NJ). Anti-CD16 (Leu 11) and Leu 7 monoclonal antibodies were obtained from Becton-Dickinson (Mountain View, CA). Anti-CD11b (OKM₁) was purchased from Ortho (Raritan, NJ) and anti-CD3 monoclonal antibody STV-T3b was kindly provided by Dr De Vries (University Hospital, Leiden, The Netherlands).

CULTURE MEDIUM

RPMI 1640 (Gibco, Paisley, UK) supplemented with 1% L-glutamine (Flow Lab, Irvine, UK), 0.5% HEPES (Flow Lab), and 1% penicillin-streptomycin (Difco Lab, Detroit, MI) was used for cultures. This will be referred to as complete medium.

ISOLATION OF LYMPHOID CELLS

Peripheral blood mononuclear cells were obtained from the heparinised venous blood of the subjects by Ficoll-Hypaque gradient centrifugation. They were washed twice in phosphate buffered saline (0.15 mol/l, pH 7.2), counted, and resuspended in complete medium. Where indicated, PBMNC were treated with anti-CD3 or anti-CD16 monoclonal antibodies plus rabbit complement (Behringwerke AG, Marburg, Germany) as previously described.²³ These PBMNC lacked surface expression of CD3 or CD16 antigens (<1%), as quantitated by indirect fluorescence and flow cytometry.

CELL CULTURES

PBMNC (2×10^6 cells/ml) were set up in 24 macrowell plates (Costar, Cambridge, MA) in complete medium supplemented with 10% fetal calf serum (Gibco, Glasgow, UK), in the presence or absence of different concentrations of IL-2. The cultures were carried out for 18 hours or 5 days at 37°C in 5% CO₂ humid atmosphere. The incubated cells were washed twice in complete medium and used as effector cells in the cytotoxicity assays.

CYTOTOXICITY ASSAYS

Cytotoxicity was quantified by a ^{51}Cr -specific release assay using the K-562, JY, Molt-4, P815, and LGL lines as target cells, as previously described.²⁴ The different effector cells used were resuspended in complete medium supplemented with 10% heat inactivated fetal calf serum. Target cells were labelled by incubating 2 to 3×10^6 cells with 150 μCi of ^{51}Cr for 90 minutes at 37°C, washed twice, and resuspended in complete medium, supplemented with 10% fetal calf serum, at a concentration of 5×10^6 cells/ml. Then 5×10^3 target cells (0.1 ml) were mixed with effector cells (0.1 ml) at different effector/target ratios (50/1, 25/1, 12/1, and 6/1) in triplicate round-bottom microwell plates (Linbro, Hamden, CT). Controls included targets incubated either with complete medium supplemented with 10% fetal calf serum (spontaneous release) or with detergent (total release). Plates were incubated for four hours at 37°C in a 5% CO₂ atmosphere; 0.1 ml from each well was collected and counted in a gammacounter. The percentages of specific cytotoxicity were calculated as follows:

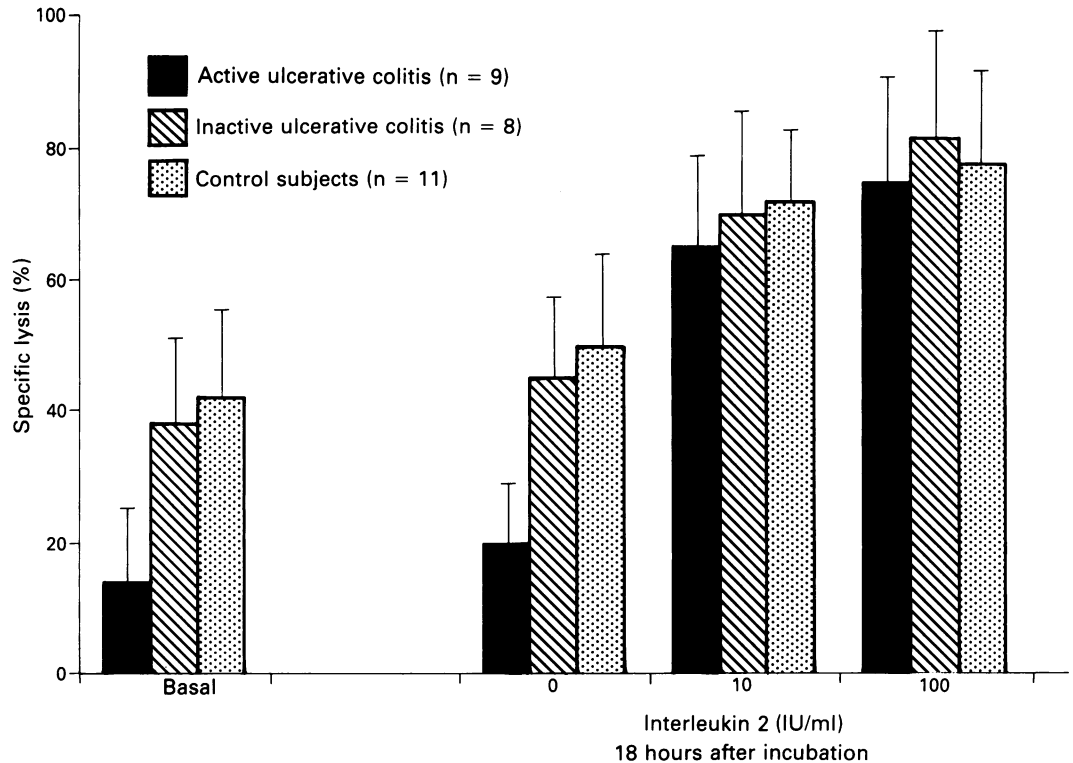
$$\frac{A - S}{T - S} \times 100$$

where A=mean cpm of test samples, S=mean cpm of spontaneous release, and T=mean cpm of total release. In all the experiments included, the mean cpm of spontaneous release was always less than 12% of the mean cpm of total release.

QUANTITATIVE FLOW CYTOMETRIC STUDY

Cells were incubated with the specific antibodies, followed by subsequent incubation with a second fluorescein isothiocyanate coupled reagent (Kallestad, Austin, TX), as described elsewhere.²⁵ The procedure was performed at 4°C in

Figure 2: rIL-2 restores the NK cytotoxicity in PBMNC from patients with active ulcerative colitis. 2×10^6 PBMNC/ml from 17 patients – nine active and eight inactive – and 11 control subjects were incubated in the presence of the indicated amounts of rIL-2 for 18 hours. These cultured cells were used as effectors against ^{51}Cr -labelled K-562 target cells. Results represent the mean (SD) of specific lysis in triplicate cytotoxic assays performed at a 50:1 effector to target ratio in the different groups studied.



the presence of 0.01% sodium azide. Surface immunofluorescence was quantified using an EPICS-C flow cytometer (Coulter) equipped with a 2 W Argon-laser tuned at the 488-nm line.

STATISTICAL METHODS

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

Results

PATIENTS WITH ACTIVE ULCERATIVE COLITIS HAVE QUANTITATIVELY NORMAL BUT FUNCTIONALLY DEFECTIVE NK CELLS IN PBMNC

First, the NK cytotoxic activity of PBMNC from patients with ulcerative colitis was analysed. As Table I illustrates, PBMNC from these patients show a clearly impaired NK cytotoxicity when compared with those of healthy control subjects ($p < 0.01$). Next, we investigated separately the NK activity of PBMNC from ulcerative colitis patients with active and inactive disease. Impairment of cytotoxic activity was evident in patients with active disease, being significantly depressed compared with both patients with inactive disease and healthy control subjects ($p < 0.01$) (Fig 1). However, there were no significant differences in the NK activity of PBMNC from patients with inactive disease when compared with healthy controls ($p > 0.05$). Interestingly, the NK cytotoxicity of PBMNC from patients with active ulcerative colitis who were taking steroids overlapped with that of patients who were not ($p > 0.05$).

We also investigated the possible variations in NK activity occurring in PBMNC from ulcer-

ative colitis patients who progress from active to inactive disease. The depressed NK activity found in PBMNC from patients with active disease (mean (SD) 11 (4)% of specific lysis at a 50:1 effector to target cell ratio in six patients) was significantly enhanced ($p < 0.01$) when the disease became inactive (30 (6)% of specific lysis at a 50:1 effector to target cell ratio). This increase in NK cytotoxicity was also found in one inactive patient who remained on steroid treatment (data not shown).

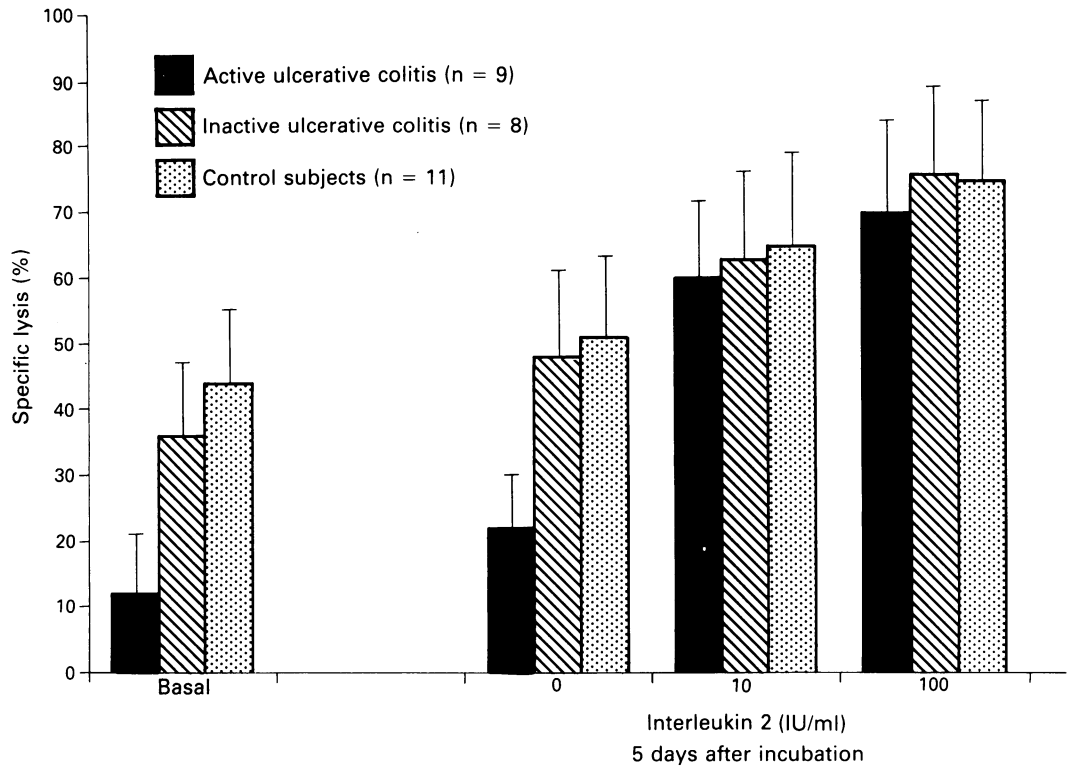
Quantitative flow cytometry studies were performed simultaneously to ascertain the number of phenotypically defined NK cells (Table II). The percentages of CD16⁺ and HNK-1⁺ cells were similar in PBMNC samples from patients with either low or normal NK cytotoxic levels and from healthy control subjects ($p > 0.05$). The numbers of cells bearing CD11b, a surface heterodimer shared by NK cells and monocytic lineages, were also similar in the three groups of subjects ($p > 0.05$). Consequently, patients with active ulcerative colitis had quantitatively normal but functionally defective NK cells in PBMNC.

IL-2 NORMALISES THE IMPAIRED FUNCTIONAL ACTIVITY OF NK CELLS

It has been shown that short term incubation with IL-2 can enhance the cytotoxic activity of normal NK cells. Along these lines, we tested the effect of titrated amounts of rIL-2 on the NK cytotoxicity of PBMNC from patients after 18 hours of incubation. In a dose dependent manner, IL-2 enhanced the NK activity of patients with inactive disease and also increased and normalised the defective NK activity of patients with active disease (Fig 2).

It could be argued that the cytotoxicity promoted by rIL-2 against K-562 found in

Figure 3: Longterm incubation with rIL-2 induces strong lytic activity in PBMNC from ulcerative colitis patients. 2×10^6 PBMNC/ml from 17 patients – nine active and eight inactive – and 11 control subjects were incubated in the presence of the indicated amounts of rIL-2 for 5 days and used as effectors against ^{51}Cr -labelled K-562. The columns represent the mean (SD) of specific lysis at a 50:1 effector to target ratio in the different groups studied.



PBMNC from ulcerative colitis patients is mediated by lymphocytes other than NK cells. To investigate whether precursor and effector cells in the IL-2-promoted cytotoxicity belong to NK lineage, PBMNC were selected by means of CD16 or CD3 monoclonal antibodies and

complement, and tested by cytotoxic assay. Depletion of CD16⁺ NK cells, but no CD3⁺ T lymphocytes, abrogated the lytic activity at both precursor and effector levels in PBMNC from patients with active and inactive disease (Table III).

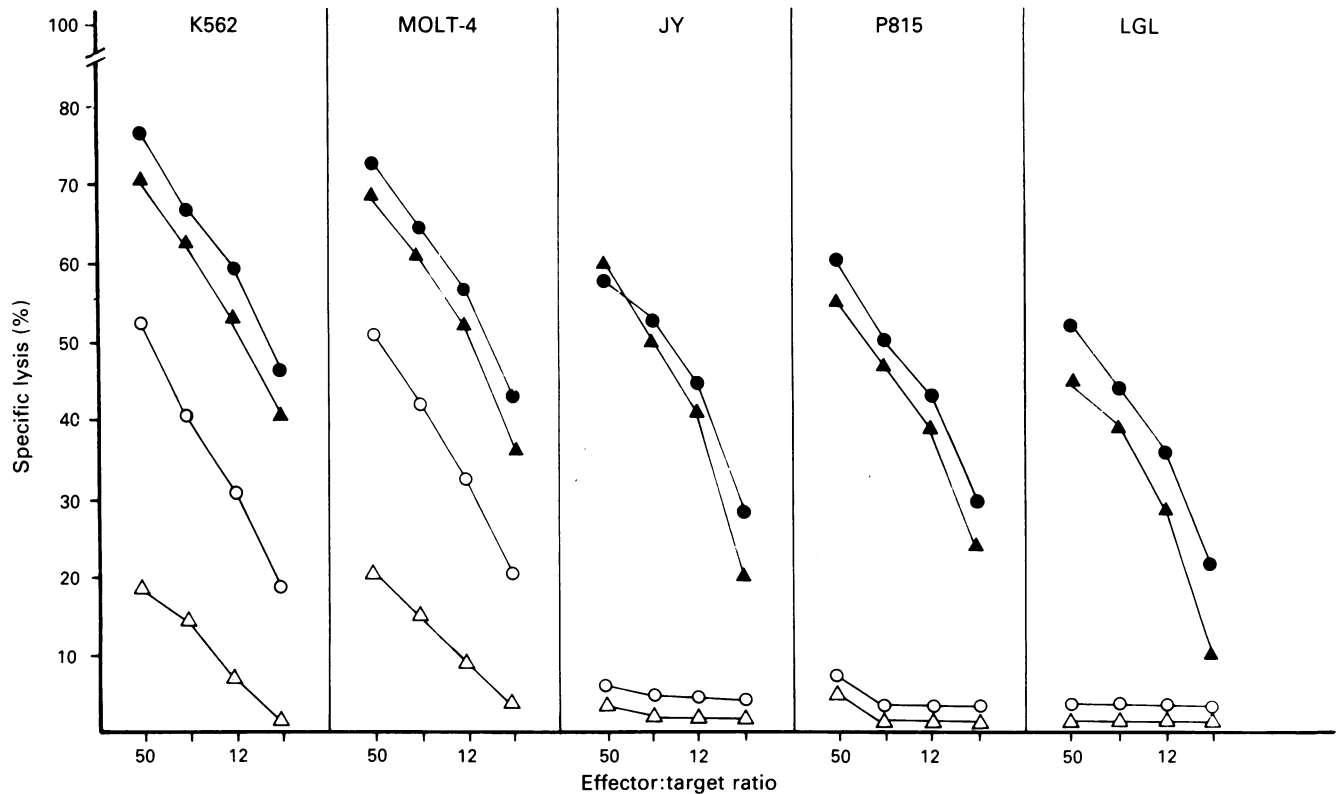


Figure 4: Non-MHC restricted cytotoxicity against both NK sensitive and NK resistant target cells (LAK activity) is promoted in PBMNC from ulcerative colitis patients by longterm incubation with rIL-2. In basal conditions (open symbols) or after incubation with 100 IU/ml rIL-2 (closed symbols) for five days, PBMNC from ulcerative colitis patients (Δ) or control subjects (○) were used as effector cells against different ^{51}Cr -labelled NK sensitive and NK resistant target cells. Results represent the mean specific lysis in triplicate cytotoxicity assays performed at a 50:1 effector to target ratio with the different targets studied. A representative experiment of the three performed in patients with active disease is shown.

TABLE II Impaired NK activity in patients with ulcerative colitis does not correlate with reduced numbers of phenotypically defined NK cells

	HNK-1	CD16	CD11b
Patients	21 (5)	14 (3)	34 (6)
Active	19 (5)	12 (3)	31 (7)
Inactive	23 (4)	15 (4)	37 (6)
Controls	18 (4)	13 (5)	36 (4)

Peripheral blood mononuclear cells from 37 ulcerative colitis patients with active (19) or inactive (18) disease and 25 control subjects were studied by indirect immunofluorescence with Leu 7 (HNK1), Leu 11 (CD16), and OKM1 (CD11b) monoclonal antibodies, and a second step fluoresceinated reagent, as indicated in Methods. The table gives the percentage of cells staining above the second step reagent background as quantitated by an EPICS-C flow cytometer. The data are the mean (SD) of each group studied.

TABLE III The precursor and effector lymphocytes of the NK promoted cytotoxicity in peripheral blood mononuclear cells (PBMNC) from patients with ulcerative colitis are CD3⁺, CD16⁺ NK cells

	Patients		Control subjects
	Active	Inactive	
PBMNC (a)	60	68	65
Precursor cells (b)			
CD3 ⁺ , CD16 ⁺	68	72	70
CD3 ⁺ , CD16 ⁻	12	14	10
Effector cells (c)			
CD3 ⁺ , CD16 ⁺	72	75	72
CD3 ⁺ , CD16 ⁻	8	10	12

PBMNC from three patients with active disease and three patients with inactive disease and three control subjects were left untreated (a) or were treated with anti-CD3 (CD3⁺, CD16⁺) or anti-CD16 (CD3⁺, CD16⁻) monoclonal antibodies plus complement (b). 2 × 10⁶ cells/ml were incubated in the presence of 100 IU/ml rIL-2 for 18 hours. At the end of the culture period, aliquots of untreated PBMNC were treated with anti-CD3 (CD3⁺, CD16⁺) or anti-CD16 (CD3⁺, CD16⁻) monoclonal antibodies plus complement (c). Cultured cells were used as effectors against ⁵¹Cr-labelled K-562 target cells at a 25:1 effector to target ratio. The results are expressed as the mean specific lysis of triplicate experiments performed with the different cellular subpopulations studied in each group of subjects.

TABLE IV CD3⁺, CD16⁺ NK cells are the main precursor and effector lymphocytes of LAK activity

	Patients			Controls
	Active	Inactive		
Peripheral blood mononuclear cells (a)	45	43		47
Precursor cells (b)				
CD3 ⁺ , CD16 ⁺	52	55		56
CD3 ⁺ , CD16 ⁻	4	6		9
Effector cells (c)				
CD3 ⁺ , CD16 ⁺	50	48		52
CD3 ⁺ , CD16 ⁻	5	4		8

PBMNC from three patients with active disease and three patients with inactive disease and three control subjects were left untreated (a) or were treated with anti-CD3 (CD3⁺, CD16⁺) or anti-CD16 (CD3⁺, CD16⁻) monoclonal antibodies plus complement (b). 2 × 10⁶ cells/ml were incubated in the presence of 100 IU/ml rIL-2 for 5 days. At the end of the culture period, aliquots of untreated PBMNC were treated with anti-CD3 (CD3⁺, CD16⁺) or anti-CD16 (CD3⁺, CD16⁻) monoclonal antibodies plus complement (c). Cultured cells were used as effectors against ⁵¹Cr-labelled NK resistant JY target cells at a 25:1 effector to target ratio. The results are expressed as the mean specific lysis of triplicate experiments performed with the different cellular subpopulations studied in each group of subjects.

LAK ACTIVITY IS INDUCED IN PBMNC FROM PATIENTS WITH ACTIVE AND INACTIVE DISEASE

Longterm incubation with IL-2 can induce non-MHC restricted cytotoxic activity directed against either NK sensitive or NK resistant target cells in PBMNC.⁹ Thus we investigated the effect of titrated quantities of rIL-2 on the non-MHC restricted lytic cytotoxicity of PBMNC from ulcerative colitis patients after five days of incubation. As shown in Figure 3,

IL-2 induced strong lytic activity against the NK sensitive K-562 target cells in PBMNC from patients with either active or inactive disease. Furthermore, this cytotoxic activity is also directed against different NK sensitive and NK resistant target cells in a non-MHC-restricted fashion (Fig 4).

It has been shown that the lymphocytic precursors and effectors of LAK activity in normal control subjects are mainly CD16⁺, CD3⁻ NK cells.¹² We also investigated whether precursor and effector cells of the LAK activity induced in PBMNC from ulcerative colitis patients belong to this lymphocytic subset. We performed a negative selection of PBMNC using CD16 or CD3 monoclonal antibodies plus complement and tested them in the cytotoxicity assay. Both pre- and post-IL-2 incubated PBMNC from ulcerative colitis patients treated with CD16 monoclonal antibody and complement failed to mediate cytotoxic activity. However, the lytic activity of PBMNC was not modified by the initial or final treatment with CD3 monoclonal antibody and complement (Table IV).

Discussion

In this study we have shown that the levels of NK cytotoxicity in PBMNC from ulcerative colitis patients are related to the clinical activity of the disease. Active disease is associated with the presence in PBMNC of NK cells in normal numbers but functionally defective. However, in patients with inactive disease, these NK cells display normal cytotoxic activity. We also found that the NK cell functional defect found in patients with active disease can be reversed upon *in vitro* incubation with IL-2.

Previous reports have presented contradictory results concerning the NK activity of PBMNC from ulcerative colitis patients.²⁶⁻³¹ Our findings of normal NK cytotoxicity in patients with inactive disease but deficient lytic levels in patients with active disease clarify the heterogeneous results in previous series dealing with small numbers of cases which, clinically, were only partially defined.

Our results show that the percentages of NK cells in PBMNC from patients with either active or inactive disease are normal as defined by monoclonal antibodies HNK-1, CD16, and CD11b, and by quantitative flow cytometry. The simultaneous finding of low levels of NK cytotoxic activity in PBMNC from active patients indicates a diminished lytic efficiency in NK cells from these ulcerative colitis patients. However, we have found *in vivo* normalisation of the cytotoxicity in patients whose disease progresses from active to inactive. We also show here how short term *in vitro* incubation of PBMNC with IL-2 from patients with active disease reverses the deficient NK activity. Furthermore, long-term incubation of PBMNC with this lymphokine generates a strong LAK cytotoxicity that is mainly induced and displayed by CD16⁺, CD3⁻ NK cells in patients with active and inactive disease. Taken together, these results could suggest that in patients with active disease there is a functional defect in the NK cell maturation

from non-cytotoxic precursors to cells that are already cytotoxic and/or in the activation of these lytic effector cells.

NK cells have been involved in the immunosurveillance against neoplasias and infections.^{1,2} In tumour patients there is increasing evidence that diminution of NK activity in PBMNC is associated with local progression and dissemination of the disease.^{32,33} Defective NK activity in PBMNC has also been associated with a high incidence of severe viral infections.³⁴ Low levels of NK activity have been found in PBMNC from patients with chronic viral infections.³⁵ However, the pathogenic importance of the NK cell deficiency observed in different inflammatory diseases of unknown aetiology with pronounced disorders of the immune system remains unclear.^{36,37} Thus our demonstration of the existence of altered NK cells in patients with active ulcerative colitis could be implicated in a predisposition toward the suggested pathogenic action of microbial agents at the intestinal wall. Nevertheless, the functional defect in the NK cells could also be secondary to the pathogenic action of these microbiological agents. Further studies are needed to define the molecular bases and the cause of the functional impairment of NK cells found in patients with active disease. It is feasible to suggest that the defect in NK activity of PBMNC from such patients could be corrected in vivo by treatment with biological immunomodulators such as IL-2, with possible clinical improvement of the disease.

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