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Higher Proliferative Capacity of T Lymphocytes from Patients with Crohn Disease than from Ulcerative Colitis is Disclosed by Use of *Herpesvirus saimiri*-Transformed T-Cell Lines

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Background: T lymphocytes play a crucial role in the pathogenesis of inflammatory bowel disease. Achieving stable T-cell lines, rather than continuous bleeding of patients, is desirable in order to dissect their implication in the disease. **Methods:** Long-lasting T-cell lines from patients with Crohn disease and ulcerative colitis and from healthy volunteers have been obtained by transformation of T lymphocytes using the lymphotropic *Herpesvirus saimiri*. Lines were subjected to phenotypic and functional analyses, and the results compared with freshly isolated peripheral blood mononuclear cells. **Results:** Fresh cells revealed only minor differences between patients and controls, with regard to phenotype and proliferative capacity. In contrast, the use of T-cell lines showed that cells from Crohn disease patients, but not ulcerative colitis patients, over-responded to several membrane or cytoplasmic stimuli when compared to control T-cell lines. Thus, higher responses were found when stimulated with αCD3 and IL2, αCD3 and αCD28, IL2 alone, phorbol esters (PMA) and αCD3 and, finally, PMA and αCD2 ($P < 0.05$ in all instances). Further, lines from patients with Crohn disease responded more vigorously to αCD3 and αCD28 or αCD3 and PMA when compared to ulcerative colitis ($P < 0.05$ in both instances). **Conclusions:** The data obtained with these lines suggest that T cells from patients with Crohn disease differ in vivo in their proliferative capacity, as compared with those from ulcerative colitis patients, a finding that may reflect the clear Th-1 phenotype found in the former and absent in the latter.

Key words: Crohn disease; *Herpesvirus saimiri*; proliferation; T lymphocytes; ulcerative colitis

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Inflammatory bowel disease (IBD) consists of two major illnesses, ulcerative colitis (UC) and Crohn disease (CD), which are chronic inflammatory disorders of the intestine of unknown origin. By the use of clinical manifestations and histologic differences, clear distinctions between both diseases can be made (1). In fact, UC in its classical form differs so much from CD in its histopathologic and clinical presentations, that it is likely to be a different disease.

Although the precise etiology of both diseases remains unknown, there is increasing evidence for an alteration of the immune regulation mechanisms in patients with IBD, with T lymphocytes playing a crucial role in the pathogenesis, especially at the intestinal mucosal surface. Here, once again, discrepancies appear between both diseases. Thus, CD seems to be a Th-1-driven disease, whereas a Th-2 pattern is more akin to UC (2).

Active CD is characterized by an increased number of activated intestinal mucosal T lymphocytes secreting interferon γ (IFNγ) and by an increased mucosal production of IL-12 and IL-18, cytokines that activate Th-1 lymphocytes (3). Moreover, treatment with depleting anti-CD4 antibodies may induce remissions in CD (4), and this disease, but not UC, is associated with non-caseating granulomata, a main feature of cell-mediated immunity.

Initial descriptions of immunological alterations in CD have focused on peripheral blood lymphocyte function. Phenotypic analysis did not disclose any differences in the T-lymphocyte subsets between CD patients and control subjects (5), but differences were found when functional state and degree of activation were measured.

Thus, increased serum levels of soluble IL-2 receptor were found in patients with CD (6, 7), indicating an increased state of activation of peripheral blood T lymphocytes. On the other hand, proliferative assays using several mitogens (lectins, monoclonal antibodies) revealed an impaired in vitro re-

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sponse of T lymphocytes from CD patients. In more recent publications, defective responses were found when mitogens interacting with surface molecules were used, whereas T-cell blastogenic response to intracytoplasmatic protein kinase C activation was normal (8). Moreover, this responsiveness was associated with an in vivo expansion of HLA-DR+ and antigen-primed memory CD45RO+CD4+ T cells, indicating a state of basal activation of T lymphocytes.

It therefore seems relevant to study the signal transduction pathways from the cell membrane to the nucleus in patients and to compare it with control individuals. To achieve this goal, continuous bleeding from patients is required—a tedious and cumbersome task; alternatively, long-lasting T-cell lines with a durable growth in vitro are desirable.

We report the results obtained by means of a transformation procedure with a T-lymphotropic virus, *Herpesvirus saimiri* (HVS), a common lymphotropic virus of squirrel monkeys. It is known that the virus is able to transform both CD4+ and CD8+ human T lymphocytes into stable growth by, as yet, undefined mechanisms (9). HVS-transformed T lymphocytes remain IL-2 dependent but become antigen- and mitogen-independent for their continuous growth (10). These cells display normal downstream functional responses (proliferation, cytokine synthesis, induction of activation markers, cytotoxicity) to membrane (anti-TcR-CD3, anti-CD95 ligand and IL-2) and transmembrane (phorbol myristate acetate (PMA) or calcium ionophores) stimuli (11). Moreover, this methodology has been proven to be successful in attaining durable cell lines either from peripheral blood or tissue samples (12). Following this methodology, long-lasting T-cell lines from blood samples of patients with CD, UC and healthy volunteers were established. The phenotype and proliferative responses of these lines were compared with those attained with freshly isolated peripheral blood mononuclear cells (PBMC). Results obtained are reported in the present work.

Methods

Patients

Ten patients (5 M, 5 F, mean age 51 years, range 33–80 years) with UC (mean duration of the disease 9 years, range 1–20 years) and 17 patients (4 M, 13 F, mean age 38 years, range 21–50 years) with CD (mean duration of the disease 9 years, range 1–23 years) followed at the Gastroenterology Service of the Hospital '12 de Octubre' were included in the present study. IBD was inactive or mild to moderately active in all patients, and all of them were receiving anti-inflammatory or immunosuppressive drugs (5-ASA, corticoids, azathioprine). The control population consisted of adult healthy volunteers ($n = 97$).

Preparation of blood samples

PBMC were isolated by centrifugation with Lymphoprep (Axis Shield PoC AS, Oslo, Norway) and two aliquots were made. One of them was used to carry out a basic phenotypic study by cytometry and a functional study assessed by cell proliferation. Cells of the remaining aliquot were subjected to transformation with HVS.

Transformation of peripheral blood lymphocytes using Herpesvirus saimiri

The isolated lymphocytes were resuspended ($1–3 \times 10^6$ cells/mL) in a mixture (1:1 proportions) of two culture media: RPMI 1640 (Gibco BRL, Life Technologies, Paisley, UK) and Panserin 401 medium (Pan, Hamburg, Germany) with 10% FCS (Bio Whittaker, Verviers, Belgium) and 1% L-glutamine (Gibco BRL, Life Technologies), supplemented with 100 U/mL recombinant human IL-2 (rhIL-2, TECIN, kindly provided by Hoffmann-La Roche, Nutley, N.J., USA) and seeded into 24-well microplates (Sarstedt, Newton, N.C., USA).

Test cultures were exposed once (day 0) to infectious doses of HVS subgroup C-488 strain. Control cultures were treated identically but HVS was omitted. The source of the infectious virus was supernatant from cultures of a lytically infected owl monkey kidney cell line (OMK). After infection, cells were regularly fed with medium containing 50 U/mL rh IL-2. An immortalized phenotype was suggested by death of control cultures, as described (9). Two months after inoculation of HVS, the cell lines were established with a stable morphology and surface phenotype. To provide homogeneity to the results, and to ensure that differences detected could not be attributed to the age of the cell line, experiments shown herein were carried out with lines grown in culture for a period of time of between 4 and 7 months.

Flow cytometry analysis

Cytofluorometric analysis of PBMC and HVS-derived T-cell lines was carried out using standard procedures; 2×10^5 cells (whether PBMC or T-cell lines) were incubated for 30 min at 4 °C using the following monoclonal antibodies: α-CD2 (T11, Coulter, Miami, Fla., USA), α-CD3 (UCTH1, Immunotech, Marseille, France), α-CD4 (13B8.2, Immunotech), α-CD8 (B9.11, Immunotech), α-CD14 (M5E2, BD Biosciences Pharmingen, San Diego, Calif., USA), α-CD16 (Leu-11a-NKP15, Becton Dickinson, San Jose, Calif., USA), α-CD19 (SJ25-C1, Caltag Laboratories, Burlingame, Calif., USA), α-CD25 (CD25-3G10, Caltag Laboratories), α-CD28 (IOT28, Immunotech), α-CD45 (J33, Immunotech), α-CD45RA (ALB11, Immunotech), α-CD45RO (UCHL-1, Becton Dickinson), α-CD56 (N901-NKH-1, Immunotech), α-CD80 (L307.4, Becton Dickinson), α-CD86 (BU63, Caltag Laboratories), α-CD103 (LF61, Caltag Laboratories), α-HLA-DR (Immu-357, Immunotech) and α-TcRαβ (WT31, Becton Dickinson). All antibodies were directly conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE) or cyanine 5-R-phycoerythrin (Cy5-PE). Negative control used was Opticlone (Immunotech), containing a mixture of mouse IgG1-FITC, IgG1-PE and IgG1-Cy5-PE-conjugated mono-

Table I. Mitogens and concentrations used throughout the experiments

Mitogen	Concentration
PHA (PHA-L Sigma Aldrich, St. Louis, Mo., USA)	0.5 μg/mL
α-CD3 (Orthoclon OKT3, Ortho Biotech Products, Raritan, N.J., USA)	12.5 ng/mL (soluble)
α-CD3 (Orthoclon OKT3, Ortho Biotech Products, Raritan, N.J., USA)	1 μg/mL (plastic-bound)
α-CD2 (T11 1/1, clone 6G4, and 2/1, clone 4B2, CLB, Amsterdam, The Netherlands)	2.5 μg/mL
α-CD28 (KOLT-2, CLB, Amsterdam, The Netherlands)	50 ng/mL
PMA (Sigma Aldrich)	10 ng /mL when used alone, 1.2 ng/mL when combined with ionomycin
Ionomycin (Calbiochem, La Jolla, Calif., USA)	1 μM
IL-2 (rhIL-2, TECIN, Hoffman-La Roche Inc., Nutley, N.J., USA)	50 U/mL

PMA = phorbol myristate acetate. PHA = phytohemagglutinin.

clonal antibodies. Cells were washed twice with PBS (Gibco BRL, Life Technologies) and analyzed in a Coulter EPICS Elite ESP flow cytometer (Coulter). A minimum of 5000 cells was analyzed per sample, gated to exclude non-viable cells. Fluorescence intensities above the upper limit of the negative control distribution were considered positive.

Proliferative assays

PBMC or HVS-derived cell lines were stimulated with monoclonal antibodies to CD3, CD2 or CD28, alone or in combination with other mitogenic substances such as IL-2, phorbol esters (PMA) or ionomycin. PBMCs, additionally, were stimulated with phytohemagglutinin (PHA).

9×10^4 cells/well were seeded in triplicate in a 96-well plate (Sarstedt) and stimulated for 72 h (PBMC) or 48 h (T-cell lines) and then pulsed with 1 μCi ^{3}H-thymidine (Moravek Biochemicals, Brea, Calif., USA), left for a further 18 h and harvested to evaluate incorporation in cellular DNA in a beta counter (Matrix 96 TM, Packard, Canberra Company, Canberra, Australia). Results are expressed as mean counts per minute (cpm). The concentration of the mitogens used is shown in Table I and the stimuli applied to PBMCs or cell lines, in Table II

Different conditions with regard to mitogen concentrations, number of cells per well (45,000 or 90,000) and length of stimulation (48 h or 72 h), were tested when setting up the culture experiments. The conditions finally chosen and used in the present work were those that rendered the most consistent and repeatable results.

Statistical analysis

Results obtained (in the phenotype or proliferative assays) are shown as mean value $\pm s_{\bar{x}}$ (standard error of the mean). A two-tailed Mann-Whitney two-sample test was used to compare results between CD, UC and the volunteers group. Significance was reached when a P value of less than 0.05 was obtained.

Ethical considerations

All the experiments were implemented with the approval of the Ethics Committee of the institution. Blood samples were drawn upon routine follow-up of patients.

Results

Peripheral blood mononuclear cells

Phenotypic analysis. No drastic differences were found between either of the groups of patients and the control individuals in most of the leukocyte markers tested (Table III). The UC groups, however, rendered some significant deviations in CD28 and CD45RA markers. Overall, the presence of CD28+ cells diminished in UC patients when compared to CD or control individuals. Significance was reached when CD28+CD4–cells were considered, since they are found in 3% of cells in UC, as compared to 14% in CD or 17% in healthy individuals ($P = 0.006$ in both instances). Hildebrandt and colleagues (13) have also reported a lower frequency, though no statistically significant, of CD28 + CD3 + cells in these patients, as in our results.

The frequency of CD45RA+ (Table III) cells was also diminished in patients with IBD when compared to control individuals (60%), though significance was reached only in UC patients (42%, $P = 0.026$), but not in CD patients (49%). This decrease in CD45RA figures was accompanied by an

Table II. Stimuli applied to PBMC or T-cell lines

Stimulus	PBMC	T-cell lines
IL-2	+	+
PMA	+	+
α-CD3 (soluble)	+	–
α-CD3 (plastic-bound)	–	+
α-CD3 + IL-2	+	+
α-CD3 + PMA	+	+
α-CD3 + α-CD28	+	+
α-CD3 + α-CD2	–	+
α-CD2	+	+
α-CD2 + IL-2	+	+
α-CD2 + PMA	+	+
α-CD2 + α-CD28	+	+
α-CD28	+	+
α-CD28 + PMA	+	+
Ionomycin	+	–
Ionomycin + PMA	+	+
PMA + IL-2	+	+
PHA	+	–
PHA + IL-2	+	–
PHA + PMA	+	–
PHA + α-CD28	+	–

PBMC = peripheral blood mononuclear cells; PMA = phorbol myristate acetate; PHA = phytohemagglutinin.

Table III. Expression (%) of CD markers on the surface of freshly isolated PBMC from IBD patients and healthy volunteer controls. *n* is number of patients or volunteers tested. Only relevant results are shown. See text for a complete list of markers studied

	Ulcerative colitis (mean ± s_x)	Crohn disease (mean ± s_x)	Control (mean ± s_x)
CD3	79 ± 3 (n = 10)	73 ± 4 (n = 17)	77 ± 1 (n = 19)
CD4	55 ± 5 (n = 10)	47 ± 4 (n = 17)	50 ± 2 (n = 18)
CD8	24 ± 2 (n = 10)	30 ± 2 (n = 15)	29 ± 2 (n = 18)
CD45RA	42 ± 7 (n = 6)*	49 ± 5 (n = 9)	60 ± 5 (n = 11)
CD45RO	34 ± 5 (n = 6)	20 ± 6 (n = 11)	20 ± 4 (n = 11)
CD45RA+ CD45RO−	33 ± 7 (n = 6)**	45 ± 5 (n = 9)	58 ± 5 (n = 10)
CD45RA− CD45RO+	27 ± 5 (n = 6)	19 ± 4 (n = 9)	19 ± 4 (n = 10)
CD28	36 ± 10 (n = 8)	48 ± 8 (n = 13)	55 ± 6 (n = 12)
CD28+ CD3+	22 ± 11 (n = 4)	43 ± 9 (n = 9)	47 ± 8 (n = 9)
CD28+ CD4+	29 ± 16 (n = 4)	31 ± 7 (n = 9)	39 ± 7 (n = 9)
CD28+ CD4−	3 ± 1 (n = 4)***	14 ± 3 (n = 9)	17 ± 4 (n = 9)

PBMC = peripheral blood mononuclear cells; IBD = inflammatory bowel disease; CD = Crohn disease; s_x = standard error of the mean.
* $P = 0.02$ ulcerative colitis versus control.
** $P = 0.01$ ulcerative colitis versus control.
*** $P = 0.006$ ulcerative colitis versus control and versus Crohn disease.

increase of CD45RO+ cells, though significance was not reached in this instance.

These results show that although both diseases (CD and UC) seem phenotypically fairly homogeneous, slight differences can be detected.

Proliferative assay. Upon stimulation with several mitogenic substances, PBMC of patients and control individuals responded to a similar extent in all instances but one (Fig. 1). When PHA plus PMA were used, healthy subjects and UC patients responded in a similar fashion (92,649 ± 4,745 cpm and 119,441 ± 40,197 cpm, respectively), whereas CD patients showed a clear, defective response (39,066 ± 8,827 cpm $P = 0.0002$). It is presently unclear whether this represents a characteristic defect of CD.

HVS-derived T-cell lines

Seven T-cell lines from CD patients and 4 from UC patients were achieved. All cell lines from a given disease (i.e. CD or UC) behaved, phenotypically and functionally, in a similar manner, regardless of the duration or the activity of the disease and the therapy patients were receiving.

Phenotypic analysis. All cell lines obtained, whether from patients or controls, were CD8 positive. A feature of HVS transformation is that either CD8 or CD4 cell lines are obtained, CD8 being the phenotype most frequently obtained.

No significant differences were detected in any of the surface markers analyzed (data not shown). The fact that CD45RA+ cells were so scarce in the lines reflects the activation state required to achieve an effective transfection of the HVS. Thus, all lines showed a phenotype characteristic of activated T cells (increased expression of CD45RO and HLA-DR).

The overall impression is that transformation tends to homogenize T-cell populations, at least in terms of phenotype.

Proliferative assays. Contrary to the results obtained with PBMC, sharp differences were found upon stimulation of HVS-derived lines between UC patients and control subjects on the one hand, and CD patients on the other (see Fig. 1).

When compared to healthy individuals, antibodies to CD3, whether alone (190,092 ± 24,366 cpm versus 95,437 ± 7,069 cpm, $P = 0.0007$) or in combination with IL-2 (162,135 ± 24,190 cpm versus 94,410 ± 7,126 cpm, $P = 0.0078$) or antibodies to CD28 (165,119 ± 19,065 cpm versus 89,468 ± 9,242 cpm, $P = 0.0006$), always yielded higher responses in CD patients. Another membrane-based stimulus, IL-2, also produced more counts in the group of CD patients than in volunteers (68,678 ± 11,279 cpm versus 46,859 ± 3,054 cpm in controls ($P = 0.014$).

Downstream (cytoplasmic) activation also disclosed more vigorous responses in CD patients than in control lines, as revealed when PMA, whether alone (53,888 ± 9,961 cpm versus 24,207 ± 2,832 cpm, $P = 0.0017$) or in combination with antibodies to CD3 (145,973 ± 19,636 cpm versus 48,820 ± 6,261, $P = 0.0000$) or CD2 (87,825 ± 12,133 cpm versus 43,235 ± 6,905, $P = 0.0025$), were considered.

In the case of UC-derived lines, no significant differences were found in any of the stimuli used when compared to the control population. However, if compared to CD patients, differences were found in CD3-mediated stimuli. Thus, CD3 in combination with antibodies to CD28 or PMA rendered more counts in CD than in UC patients (165,118 ± 19,065 cpm versus 94,963 ± 12,268 cpm, $P = 0.0033$ and 145,973 ± 19,636 cpm versus 51,448 ± 9,541 cpm, $P = 0.0196$, respectively).

Discussion

Peripheral blood mononuclear cells

Phenotypic analysis. No major differences were found in most of the cell surface markers tested between patients and controls, or when CD patients were compared with UC.

Only in two instances was significance reached. CD45RA+ cells were present in 60% of control subjects, but only in 42%

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of UC patients ($P = 0.026$). A concordant increase in CD45RO+ cells was observed, although significance was not reached, probably owing to the low number of patients tested.

This decrease, and the CD45RO increase, may reflect an activation state of cells in IBD patients, and matches previously published data (14, 15). However, the fact that the percentage of CD28-bearing cells was diminished (whether CD28+CD3+, CD28+CD4+, CD28+4−) in patients, a finding also reported in the literature, apparently contradicts the activation status of cells (13).

CD28 is a co-stimulatory molecule required for activation of T cells. If scarcely present on cells from diseased individuals, they would be less readily activated in vivo than cells from healthy individuals. This is at odds with the low CD45RA expression on patients T cells, which suggests an activated status. This puzzling discrepancy has been also reported in multiple sclerosis (MS), another disease in which imbalances of immune cells may underlie the etiopathogenic mechanism. It was found that MS patients have a significantly decreased level of suppressor precursor (CD28−) CD8+ T cells, along with a significant increase in naïve (CD45RA+) T cells in the peripheral blood (16). Additionally, a decrease in CD28 expression (17) and expansion of CD45RO+ CD8+ T cells (18) has been described during the aging process.

Proliferative assays. Upon stimulation, cells from patients and healthy donors proliferated to a similar extent, although some differences were noted. UC patients always yielded higher, and CD patients lower responses than control cells, though significance was only reached in one instance. When the PHA+PMA combination was considered, cells from UC patients and control individuals yielded similar counts ($119{,}441 \pm 40{,}197$ cpm and $92{,}649 \pm 4{,}745$ cpm, respectively) and clearly lower counts in CD patients $39{,}066 \pm 8{,}827$ cpm ($P = 0.0002$ versus control), a finding that may reflect a distinctive feature of CD and UC cells. However, the number of patients tested will have to be increased before firm conclusions can be reached.

The fact that cells from patients respond in a similar fashion to that of control individuals may be at odds with the previous

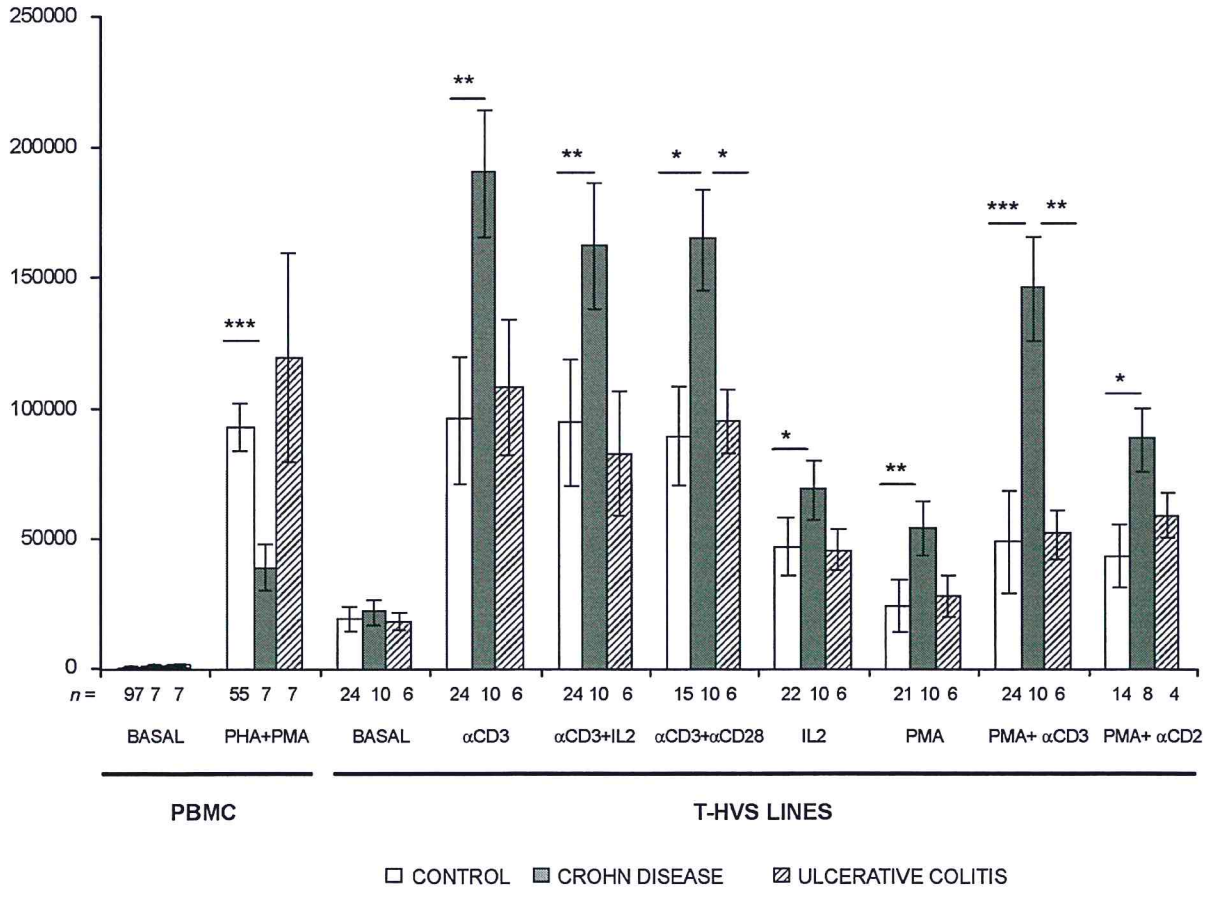

Fig. 1. Proliferative response in counts per minute (cpm) to mitogens of peripheral blood mononuclear cells (PBMC) (left) or *Herpesvirus saimiri* (HVS)-transformed T-cell lines (right). Results are given as the mean ± s_x (standard error of the mean). See text for a complete list of stimuli used. n = number of inflammatory bowel disease (IBD) patients and controls used in each stimulus.* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

phenotypic data, which show an activated state of cells. However, it can be argued that precisely activated cells are more difficult to stimulate than resting ones, or that these cells are more prone to enter apoptosis upon stimulation. Previously published reports disclosed that a defective proliferative response in UC patients was not due to apoptosis (19). Alternatively, medical treatment can alter the capacity of cells to proliferate adequately.

HVS-derived T-cell lines

Noticeably, no differences were found in terms of phenotype or proliferative capacity between the cell lines of a given disease when aspects such as duration or activity of the disease, or accomplishing treatment were considered. Certainly the number of cell lines is scarce, but some other researchers have reiterated that T-cell marker expression in patients with IBD is independent of the activity or anatomical localization of the disease, presence of inflammation and therapy used (20, 21).

Phenotypic analysis. No difference was found in any of the markers tested when comparing CD patients and control subjects. This may not be very striking since freshly isolated cells did not reveal major differences either. Moreover, it cannot be ruled out that transformation may phenotypically homogenize both populations. For instance, HVS infection selects CD4 or CD8 populations and, in our case, all cell lines, whether from patients or not, are CD8. Nevertheless, this does not disqualify the HVS model beforehand. If important phenotypic differences had existed in fresh cells, they would have appeared in the cell lines, as has been found in gastric cancer (22).

Proliferative assays. HVS T-cell lines always yielded more counts per minute than their PBMC counterparts (see basal counts, Fig. 1). Furthermore, and contrary to the findings of PBMC, lines from CD patients responded more vigorously to mitogenic stimuli, whether membrane or cytoplasmic. Surprisingly though, lines from UC patients resembled control lines more closely than CD lines.

CD3-mediated stimulation, whether the monoclonal antibody alone or in combination with IL-2, PHA or antibodies to CD28, disclosed significant differences when the CD patients were compared with the control group (Fig. 1). This increased response was also observed when cell lines were stimulated with IL-2 alone, also a membrane stimulus.

Engagement of the T-cell antigen receptor (TcR) triggers signal transduction pathways that regulate the activation of T lymphocytes. This process depends upon the activity of protein tyrosine kinases (PTKs), which induce the phosphorylation of a number of proteins. PTKs mediating these functions include the Src family members, lck and fyn, and the Syk family members, ZAP70 and syk (23). It is known that transformation with HVS activates the lck kinase by binding of the Tip protein of the virus (24). This fact, however, explains why transformed T cells, irrespective of the clinical status of donor individuals, proliferate more readily than PBMC, but cannot explain the finding of a significantly more active response in CD lines than in control and UC lines.

Interestingly, in their activation pathway, CD3- or IL-2-mediated stimuli share the lck kinase. The possibility could be envisaged that upon transformation, lck reaches an activation state which would render the cells over-responsive. Yet, CD HVS cells respond more vigorously than control or UC HVS cells, indicating that the higher proliferative response in these patients is not due to the HVS transformation process.

When transmembrane stimuli were considered, a higher response in CD patients was also found. PMA, or PMA plus antibody to CD3 or CD2, yielded more cpm in patients than in healthy individuals ($53{,}888 \pm 9{,}961$ cpm versus $24{,}207 \pm 2{,}832$ cpm, $P = 0.0017$; $145{,}973 \pm 19{,}636$ cpm versus $48{,}820 \pm 6{,}261$, $P = 0.0000$ and $87{,}825 \pm 12{,}133$ cpm versus $43{,}235 \pm 6{,}905$, $P = 0.0025$, respectively). PMA is an analogue of diacylglycerol, which binds to, and activates, protein kinase C (PKC), resulting in up-regulation in cells from CD patients.

Altogether, data obtained with HVS lines indicate that CD patients' cells are overreactive, as compared to control and UC cells. This may also be the situation in vivo. In fact, comparable results have been previously obtained using IL-2-derived T-cell lines from mucosa tissue. CD patients showed an intrinsic hyperreactivity to IL-2 (20), probably due to defective apoptosis mechanisms (21). Furthermore, the clinical data reveal clear differences between CD and UC. CD is a Th-1-based disease and UC is closer, though not neatly, to a Th-2-mediated disease. In the former, the use of monoclonal antibodies to TNFα (infliximab) confers therapeutic benefits, whereas this is not true in the latter. Consistent with our results, Agnholt and Kaltoft reported that cell lines obtained from CD patients by continuous stimulation with IL-2 produced higher amounts of IFN-γ and TNF-α than control cell lines, a production that was down-regulated with infliximab (25).

Why, then, are these differences not reflected in the proliferative assays carried out with fresh cells? It may well be due to the medical treatment of patients, some with clear immunosuppressive effects, which may hamper proliferation of cells upon stimulation. Since our cell lines had been grown for several months, any such compound would run out in the culture media and cells would be able to proliferate freely. In fact, a report from Jacquot et al. (26) disclosed that IL-2-dependent T-cell lines obtained from tissue explants of CD patients free of immunosuppressor treatment responded more vigorously to CD3-mediated activation than lines of control individuals. Their model, however, may not be totally comparable to ours, since in the same work, no differences were found when lines from PBMC, instead of tissue origin, were considered. Alternative explanations, such as increased apoptosis rates of patients' cells when stimulated, cannot be ruled out, and experiments clarifying these explanations are required.

In summary, we show here the establishment of long-

lasting T-cell lines of blood origin from patients with CD or UC, taking advantage of the transformation process with a lymphotropic virus, the HVS. Such lines revealed that cells from CD, but not UC, patients tend to overreact when stimulated with mitogens, contrary to what is achieved when fresh PBMC are analyzed. This is in keeping with the previously published data on mucosal cell lines achieved with a different methodology from ours (20, 21) and lends credence to our (HVS) model. Moreover, we have subjected our cell lines to an array of mitogens, not just IL-2, as in works elsewhere (20, 21), to dissect several signaling pathways, and our results suggest that an excessive response of immune cells may underlie the pathogenesis of Crohn disease, but not of ulcerative colitis. Finally, the model shown herein has already been used to obtain cell lines derived from intestinal origin (12) and several of them are currently being grown in our laboratory. These lines may help clarify their role in tissue damage in UC or CD.

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