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Activation of peripheral blood and intestinal lamina propria lymphocytes in Crohn's disease. *In vivo* state of activation and *in vitro* response to stimulation as defined by the expression of early activation antigens

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SUMMARY In the present study the state of activation of either peripheral blood and intestinal lamina propria mononuclear cells in Crohn's disease was defined by investigating the expression of early activation antigens (namely the 4F2 antigen, the transferrin receptor and the interleukin-2 receptor). The expression of 4F2 and T9 antigens was greatly increased – in the peripheral blood and in the intestinal lamina propria whereas the proportion of interleukin-2 receptor bearing cells was much less pronounced. The counts of early activation antigens bearing cells in the lamina propria were quite comparable with those of the autologous peripheral cells. In the peripheral blood counts of 4F2 and T9 positive cells were very high in patients with active Crohn's disease but patients with quiescent disease also had a significantly raised proportion of 4F2 and T9 bearing cells. Only in those patients with no evidence of macroscopic disease (namely those resected without recurrence) the counts of early activation antigens bearing cells were within the normal range. The *in vitro* mitogen induced expression of early activation antigens on either peripheral and intestinal mononuclear cells of patients with Crohn's disease proved to be both quantitatively and qualitatively similar to that of the controls showing the full expression of 4F2, transferrin receptor, and interleukin-2 receptor. While demonstrating that in Crohn's disease there was no intrinsic defect of generation and expression of growth factors receptors by peripheral and intestinal lymphocytes, these results showed that there was a divergence in the expression of early activation antigens *in vivo* and *in vitro*. This would indicate that in Crohn's disease there is an *in vivo* increased population of preactivated rather than fully activated lymphocytes consisting of 4F2 and T9 bearing cells. The high proportion of these cells in the peripheral blood and in the intestine suggests that a chronic immune activation is present in these patients outside as well as within the affected bowel.

There is increasing evidence that immunological mechanisms may play a role in the pathogenesis of the tissue damage in Crohn's disease. Even though no conclusive data have emerged to indicate the basis of these mechanisms, an imbalance of immunoregulatory T cells has been postulated to occur in Crohn's disease and lymphocyte function have been investigated as well as the expression of surface markers of cell differentiation.[1-8]

The activation of mature T lymphocytes seems also to be important in defining the involvement of cell mediated immunity. Several disease states thought to be immunologically mediated have been shown to be associated with an *in vivo* T cell activation.[9-11] A class of surface antigens named 'activation antigens' define the state of activation of human mature T cells. These antigens are not expressed on resting lymphocytes and can be detected using specific monoclonal antibodies on the cell surface *in vivo* and after *in vitro* stimulation.[12,13] Activation antigens can be classified as early, intermediate and late on the basis of their appearance on cell surface in relation to the DNA synthesis.[13] The subclass of early activation antigens

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include the 4F2 antigen, the transferrin receptor (T9 antigen) and the interleukin-2 (IL-2) receptor (Tac antigen).[14-18] Early activation antigens are sequentially expressed by lymphocytes after *in vitro* activation, the 4F2 being the first to appear (within four hours) and the Tac the last.[13]

We have recently reported that patients with Crohn's disease have an increased number of peripheral blood mononuclear cells expressing these antigens.[19,20] The preliminary results of these studies showed that Crohn's disease patients have a high number of circulating peripheral blood mononuclear cells expressing antigens of the very early phase of activation while the proportion of peripheral blood mononuclear cells expressing markers more directly related to cell growth – for example, the IL-2 receptor, is much less pronounced. To explore this problem further the expression of early activation antigens by peripheral blood mononuclear cells and autologous lamina propria mononuclear cells was investigated in patients with Crohn's disease *in vivo* and after *in vitro* mitogen stimulation. The relation between the prevalence of *in vivo* early activation antigens expressing peripheral blood mononuclear cells and the clinical and anatomical manifestations of the Crohn's disease was also carefully reappraised in a larger group of patients in order to evaluate the possible clinical significance of early activation antigens bearing peripheral blood mononuclear cell counts.

Methods

PATIENTS AND CONTROLS

A total of 90 patients (53 men and 37 women, mean age 36±5 years) were included in this study. In 40 of the patients the disease involved the ileum either as a primary or recurrent disease, in 28 the ileum and colon and in seven the colon only. Diagnosis was based on the usual clinical radiological and endoscopical criteria. Diagnosis was supported by endoscopic biopsies in all cases with colonic involvement and in 15 of the 40 in which the disease was limited to the ileum. Fifteen patients had had a bowel resection for Crohn's disease and did not show evidence of recurrence at the time of the study. The clinical activity of the disease was assessed by the Bristol Simple Index[21] supplemented by laboratory measurements.[22,23] Thirty six patients were studied during an active phase of the disease (and eight of these were operated upon) whereas in 39 the disease was clinically quiescent. Thirty eight of the patients were receiving steroids (mean daily dose 17 mg), 12 were on sulphasalazine only.

A disease control group included non-inflammatory gastrointestinal disorders (colonic adenomatous polyposis three, intestinal lymphangectasia two), inflammatory gastrointestinal disorders (coeliac disease two, Whipple disease one, acute appendicitis three, acute infective colitis two) and acute viral infections (20 patients).

Intestinal specimens were obtained from eight Crohn's disease patients who underwent surgery during the study period. The disease involved the ileum in five and the colon in three. Steroid treatment was stopped in all patients before surgery. As controls intestinal specimens were obtained from a miscellaneous group of patients including colonic carcinoma four, adenomatous polyposis one. Hirschsprung disease one and diverticular disease one. In addition peripheral blood drawn from 20 healthy volunteers from laboratory staff was used.

PERIPHERAL BLOOD MONONUCLEAR CELLS

Peripheral blood mononuclear cells were obtained from venous heparinised blood layered on a Ficoll-Paque (Pharmacia Fine Chemicals, Uppsala) density gradient and centrifuged at 400 *g* for 30 minutes. Cells were washed twice in Hanks Balanced Salt Solution free of calcium and magnesium (HBSS-CMF) (Flow Lab, Irvine, UK) and resuspended in complete medium (RPMI 1640, Flow Lab, 10% fetal calf serum, Flow Lab, 25mM Hepes buffer, L-glutamine, 100 µg/ml Streptomycin, 100 units/ml penicillin, 50 µg/ml Gentamicin).

LAMINA PROPRIA MONONUCLEAR CELLS

Intestinal mucosa was dissected from surgical specimens within one hour of resection. Lamina propria mononuclear cells were isolated using the Bull and Bookman enzymatic method[24] with minor modification. Briefly, strips of the mucosa (6-8 g total weight) were washed in HBSS-CMF containing 1 mM dithiothreitol (Sigma Chem, USA) and antibiotics for 15 minutes at room temperature. The strips of mucosa were then minced in approximately 3×3 mm pieces and incubated four to six times in HBSS-CMF containing 0·75 mM EDTA, 10 mM Hepes buffer and antibiotics for 45 minutes at 37 °C to remove epithelial cells. After two wash in HBSS-CMF the pieces of mucosa were incubated for 10-13 hours at 37 °C in humid 5% CO2 atmosphere in complete medium containing 25 U/ml purified Collagenase (CLSPA, Worthington, Freehold, NJ, USA). The medium containing the mononuclear cells was collected and washed twice in HBSS-CMF. The pellet was resuspended in RPMI 1640 and then layered on a Ficoll-Paque density gradient. The resulting lamina propria mononuclear cells were counted and the viability assessed using 0·1% trypan blue. Contamination by epithelial cells of the final lamina propria mononuclear cells suspension ranged between 10 and 16%.

PERIPHERAL BLOOD MONONUCLEAR CELLS AND LAMINA PROPRIA MONONUCLEAR CELLS IN VITRO ACTIVATION

Peripheral blood mononuclear cells and lamina propria mononuclear cells were resuspended in complete medium at a concentration of 2×10^6 cells/ml and placed in 2·8 ml wells of tissue cultures plates (Falcon Plastic, USA). Duplicate samples were stimulated with 2 μg/ml purified phytohaemagglutinin (Wellcome, UK). Stimulated and unstimulated cells were cultured in 5% CO2 humid atmosphere at 37 °C. After 24, 48 and 72 hours of culture cells were harvested counted, checked for viability and tested by immunofluorescence for each of the early activation antigens listed below.

ADHERENCE

The percentage of 4F2 and T9 bearing non-adherent was evaluated in patients with Crohn's disease to rule out the possibility that the increased 4F2 and T9 expression in the peripheral blood mononuclear cells was due to positive monocytes.[25] Adherent cells depleted cell suspensions were studied in 10 patients (six with active disease and four in remission). Similar experiments were carried out with four lamina propria mononuclear cells suspensions of Crohn's disease specimens. Circulating monocyte counts in these 10 patients were $344\pm168/mm^3$.

Peripheral blood mononuclear cells were washed in HBSS-CMF resuspended in 37 °C heated complete medium and placed in tissue culture dishes (35×10 mm, Falcon Plastic) at a concentration of 2×10^6/ml. After two hours incubation at 37 °C in 5% CO2 the dishes were washed three to four times with cold (4 °C) HBSS-CMF and the non-adherent mononuclear cells were harvested, counted and tested with the appropriate antisera as listed below.

SURFACE MARKERS

The following antigens were checked as appropriate: T3 (by the OKT3 monoclonal antibodies, Orthomune, Raritan NJ, USA); M1 (by the OKM1, Moab, Ortho-mune); the 4F2 antigen (by the 4F2 Moab, kindly supplied by Dr G Eisenbarth, Joslin Clinic, Boston, USA) (14); transferrin receptor (T9) (by the OKT9 Moab, Orho-mune) (15-16); IL-2 receptor (Tac antigen) (by the anti-Tac Moab, kindly supplied by Dr TA Waldmann, Bethesda, USA) (17-18).

STATISTICAL ANALYSIS

The non-parametrical two tailed Wilcoxon's rank-sum-test and the Student's *t* test were used as appropriate for the statistical analysis of the data.

Results

IN VIVO EXPRESSION OF EARLY ACTIVATION ANTIGENS BY PERIPHERAL BLOOD MONONUCLEAR CELLS IN CROHN'S DISEASE IN RELATION TO THE CLINICAL ASSESSMENT

The proportion of 4F2 and T9 bearing peripheral blood mononuclear cells were significantly higher in

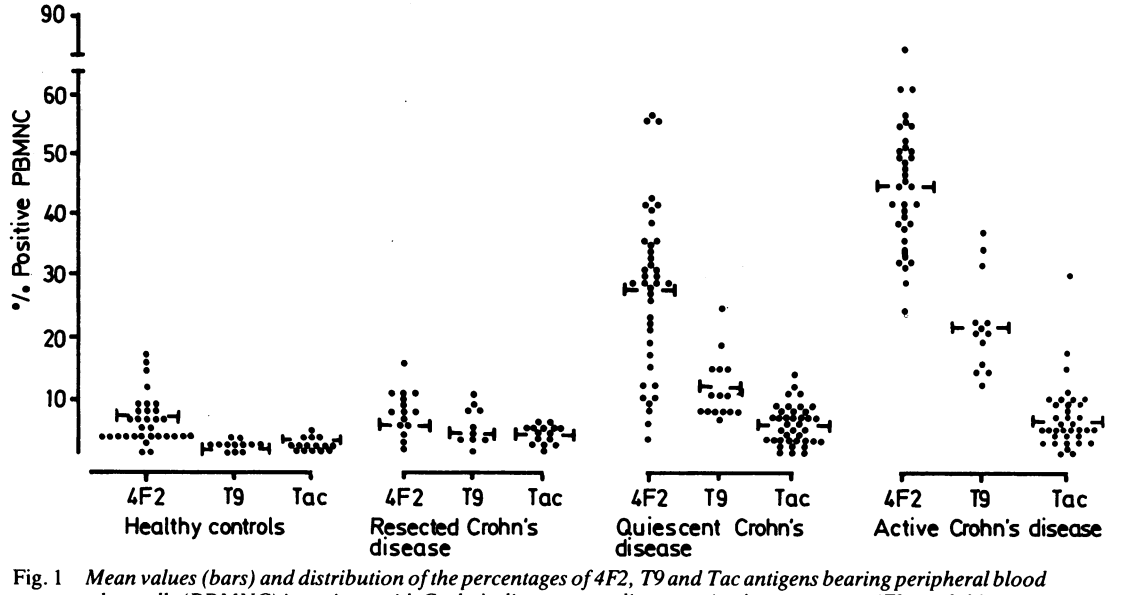

Fig. 1 *Mean values (bars) and distribution of the percentages of 4F2, T9 and Tac antigens bearing peripheral blood mononuclear cells (PBMNC) in patients with Crohn's disease according to patient's assessment. 4F2: $p<0·01$ active v quiescent v resected v controls; $p<0·001$ quiescent Crohn's v resected Crohn's v controls T9: $p<0·001$ active v quiescent v* resected Crohn's v *controls; $p<0·05$ quiescent v resected Crohn's v controls. Tac not significant active v quiescent v resected.*

patients with active Crohn's disease (45±12% and 22±7% respectively) than either in patients with quiescent disease (26±13% and 11±4%) and in patients resected not showing evidence of recurrence (7±4% and 5±3%) (Fig. 1). Even in patients with quiescent disease the percentage of peripheral blood mononuclear cells expressing the 4F2 and T9 antigens was higher than in either the healthy controls and the resected Crohn's disease patients. Longitudinal studies in five patients showed that 4F2 positive cells and T9 positive cells counts dropped from 45±10 and 22±8 respectively when the patients were active to 9±3 and 4±2 respectively after surgery. The slightly increased percentages of anti-Tac positive peripheral blood mononuclear cells did not differ in relation to the clinical assessment.

A wide range and distribution in the percentages of peripheral blood mononuclear cells expressing early activation antigens were observed in the disease control groups (Table 1). As shown in Table 2 the 4F2 positive and T9 positive adherent cells accounted for a <30% proportion of the total 4F2 and <20% of the T9 positive peripheral blood mononuclear cells.

KINETICS OF MITOGEN INDUCED EARLY ACTIVATION ANTIGENS EXPRESSION BY PERIPHERAL BLOOD MONONUCLEAR CELLS IN CROHN'S DISEASE

The peripheral blood mononuclear cells of 12 Crohn's disease patients and 11 healthy controls were cultured for three days and stimulated with purified phytohaemagglutinin. The results of this experiment, summarised in Figure 2, showed that activation by phytohaemagglutinin evoked a significant increase of 4F2, T9 and Tac positive cells in cultures of peripheral blood mononuclear cells from both Crohn's disease patients and controls. Counts at each point in the curves of stimulated cultures were significantly ($p<0.001$) higher than those at each corresponding point of the unstimulated cultures. There was no significant difference in terms of relative increase of T9 and Tac positive cells after 24, 48, and 72 hours between peripheral blood mononuclear cell cultures of Crohn's disease and controls. The proportion of 4F2 positive cells reached its peak (88±7% of positive cells) after 48 of phytohaemagglutinin stimulation in cultures of Crohn's disease peripheral blood mononuclear cells while in the control cultures the peak (81±3%) was reached after 72 hours. The relative increase from the initial to the 48 hours readings was, however, quite similar for the two culture groups. No difference between Crohn's disease and controls stimulated cultures was found when the results were expressed subtracting the corresponding unstimulated values. The main finding of this experiment was that the percentage of cultured peripheral blood mononuclear cells bearing the 4F2, the T9, and the Tac antigens after 72 hours of phytohaemagglutinin stimulation were virtually identical in Crohn's disease and controls (4F2: controls 81±3%, Crohn's disease 82±8%; T9: controls 48±8%, Crohn's disease 45±14%; Tac: controls 53±7%, Crohn's disease 59±9%).

EARLY ACTIVATION ANTIGENS BEARING LAMINA PROPRIA MONONUCLEAR CELLS IN CROHN'S DISEASE AND CONTROLS

As shown in Figure 3, in Crohn's disease the percent-

Table 1 *Percentages (M±SD) of early activation antigens bearing peripheral blood mononuclear cells in Crohn's disease and controls*

	Healthy controls	*Crohn's disease*	*Inflammatory GI disorders*	*Non-inflammatory GI disorders*	*Acute viral infections*
4F2	6±1·4 (32)	30±17 (90)*	28±9 (8)*	6±4 (5)	40±14 (20)*
T9	1±1 (12)	14±9 (38)*	7±4 (4)*	1±0·5 (3)	NT
Tac	1±1 (15)	5±4 (90)†	5±4 (8)†	1±1 (5)	9±6 (20)*

(Numbers in brackets are the patients studied); * $p<0.01$ *v* healthy controls and non-inflammatory GI disorders; † $p<0.05$ *v* healthy controls and non-inflammatory GI disorders; NT=not tested.

Table 2 *Percentages (M±SD) of non-adherent (NA) peripheral blood mononuclear cells expressing the 4F2 and T9 surface markers in Crohn's disease and controls*

	4F2 positive		*T9 positive*	
	Total PBMNC	*NA-PBMNC*	*Total PBMNC*	*NA-PBMNC*
Healthy controls (6)	4·8±3·2	0	0·8±0·6	0
Crohn's disease (10)	29·7±9·2	24±8	9·6±6	6·3±4

(Number in brackets are the patients studied).

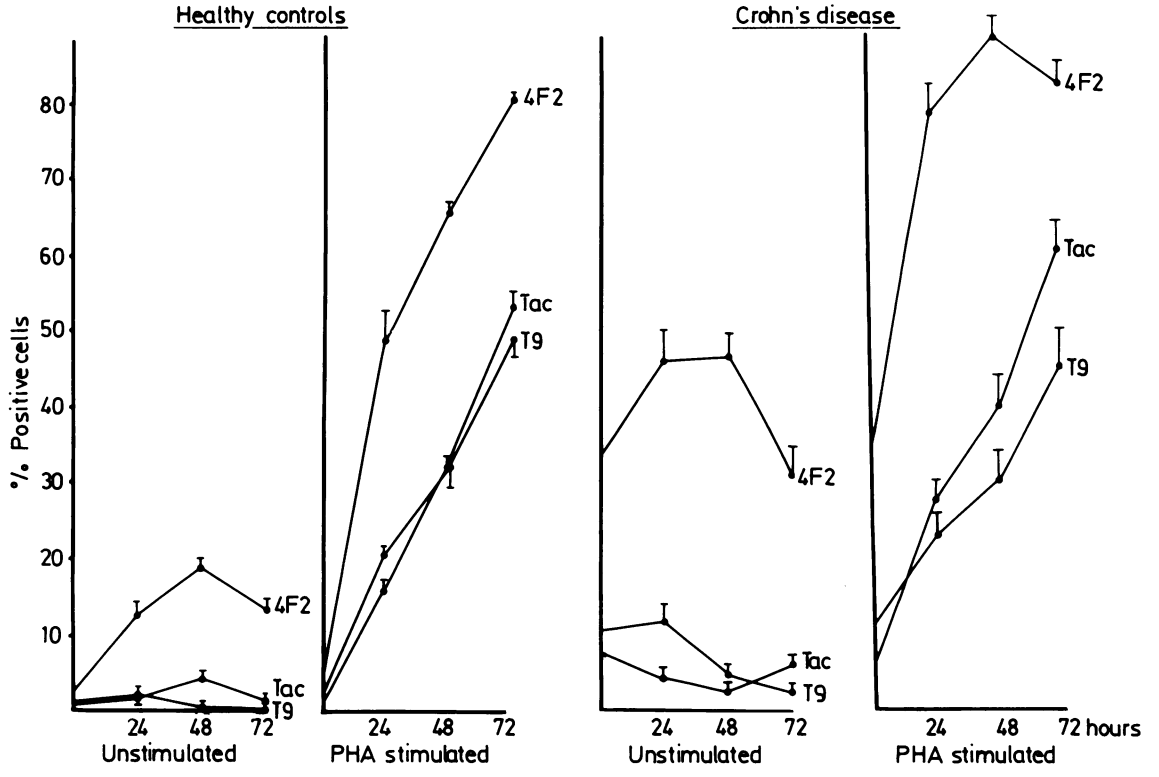

Fig. 2 *Kinetics of appearance of early activation antigens (EAA) on cultured peripheral blood mononuclear cells (PBMNC) of 12 patients with Crohn's disease and 11 controls with and without mitogen phytohaemagglutinin (PHA) stimulation. Each point in the curves is the mean ± SEM (vertical bars) of the counts in each group.*

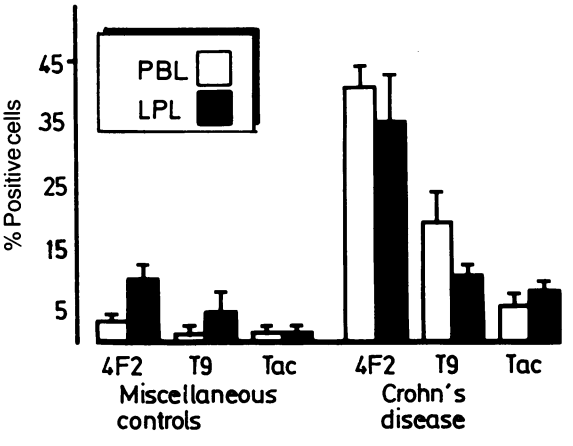

Fig. 3 *Percentages of early activation antigens bearing lamina propria mononuclear cells (LPMNC) and autologous peripheral blood mononuclear cells (PBMNC) in the miscellaneous controls and in patients with Crohn's disease. Vertical bars are SEM.*

ages of lamina propria mononuclear cells expressing the 4F2 (35±16), the T9 (10±4), and the Tac (7±2) antigens were significantly ($p<0.01$) higher than those of the miscellaneous control group (4F2: 13± 9%, T9: 5±4%, Tac: 3±3%). When the counts of early activation antigens bearing lamina propria mononuclear cells were compared with those of the autologous peripheral blood mononuclear cells no significant difference was found between peripheral blood mononuclear cells and lamina propria mononuclear cells in patients with Crohn's disease whereas in the control group the lamina propria mononuclear cells showed a percentage of 4F2 positive cells higher ($p<0.05$) than that of the autologous peripheral blood mononuclear cells. Experiments with adherent cell depleted cells suspensions of lamina propria mononuclear cells gave results similar to those of peripheral blood mononuclear cells.

KINETICS OF MITOGEN INDUCED EARLY ACTIVATION ANTIGENS EXPRESSION BY LAMINA PROPRIA MONONUCLEAR CELLS

Lamina propria mononuclear cells from eight

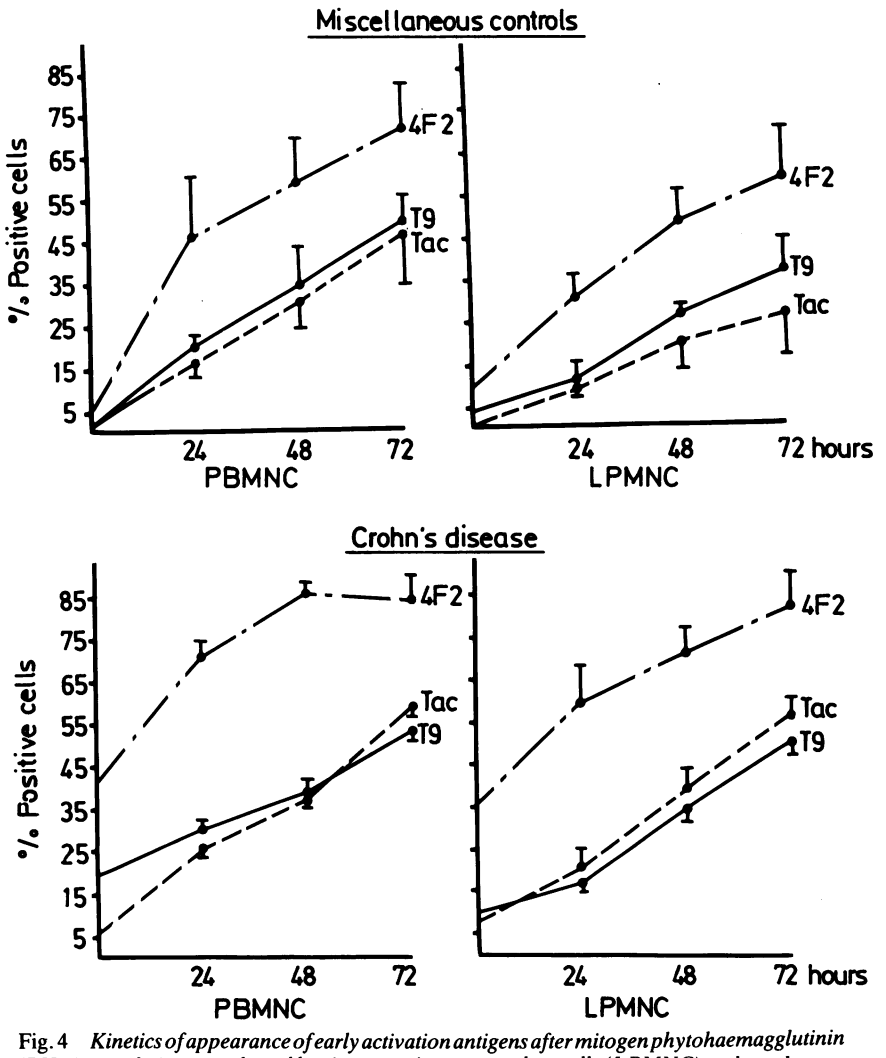

Fig. 4 *Kinetics of appearance of early activation antigens after mitogen phytohaemagglutinin (PHA) stimulation on cultured lamina propria mononuclear cells (LPMNC) and autologous peripheral blood mononuclear cells (PBMNC) of the miscellaneous control patients and of patients with Crohn's disease. Each point in the curves is the mean ± SEM (vertical bars) of the counts in each group.*

Crohn's disease patients and six patients from the miscellaneous control group were cultured for three days and stimulated with purified phytohaemagglutinin. The autologous peripheral blood mononuclear cells were also cultured in the same way. Percentages of lamina propria mononuclear cells bearing each of the early activation antigens increased significantly after phytohaemagglutinin stimulation in comparison with the unstimulated cultures. As shown in Figure 4 the percentages of 4F2, T9 and Tac positive cells increased significantly after 24, 48, and 72 hours. There was no significant difference between Crohn's disease and controls cultures in terms of relative increase of phytohaemagglutinin induced early activation antigens at 24, 48, and 72 hours. The counts at 72 hours tended to be higher in the cultures of Crohn's disease lamina propria mononuclear cells than in those of control lamina propria mononuclear cells. The response to phytohaemagglutinin of lamina propria mononuclear cells from either Crohn's disease patients and controls proved to be quite similar to that of the autologous peripheral blood mononuclear cells (Fig. 4). There was no difference between ileal and colonic

Crohn's disease in terms of early activation antigens expression either *in vivo* and *in vitro* by lamina propria mononuclear cells.

Discussion

The data that emerged from the present investigation confirm and extend previous preliminary results from our laboratory showing that patients with Crohn's disease have an increased proportion of circulating lymphocytes expressing early activation antigens.[19 20] This observation has been recently confirmed by others.[26] The rise in the proportion of early activation antigens bearing peripheral blood mononuclear cells was not restricted to patients with Crohn's disease as shown by the comparison with disease control groups. In Crohn's disease, we have shown that the expression of 4F2 and T9 antigen in the peripheral blood was not only highly increased in patients with clinically active disease but also in patients with quiescent disease who had a significantly raised proportion of 4F2 and T9 antigens bearing peripheral blood mononuclear cells. Only in those patients with no evidence of macroscopic disease (namely those resected without recurrence) early activation antigens bearing peripheral blood mononuclear cells counts proved to be no different from those of the healthy controls. Longitudinal observations in individual patients have confirmed these findings. The expression of 4F2 and T9 antigens in the peripheral blood seems therefore to reflect in Crohn's disease the occurrence of inflammation in the gut rather the 'clinical activity'. Longitudinal determinations of these markers may be of value in monitoring resected patients and possibly in identifying patients who are at risk of an early recurrence of the disease.

In the present study a highly increased proportion of *in vivo* early activation antigens bearing mononuclear cells was also found in the intestinal lamina propria in Crohn's disease. As for peripheral blood mononuclear cells the number of IL2 receptor positive cells was much smaller than those cells expressing the 4F2 and T9. This confirms previous immunohistochemical observations.[27] These observations raise the question of whether different factors interfering with the sequence of events of the lymphocyte activation process operate *in vivo* in these patients or if there is an intrinsic defect of IL-2 receptor generation and expression by these cells in Crohn's disease. The results of our *in vitro* kinetics experiments showed that in Crohn's disease either peripheral blood mononuclear cells and lamina propria mononuclear cells from the actively inflamed mucosa are capable of generating and fully expressing the IL2 receptor in response to a mitogen stimulation. These experiments have shown that the sequence of early activation antigens expressed by either Crohn's disease peripheral blood mononuclear cells and Crohn's disease lamina propria mononuclear cells is both qualitatively and quantitatively normal, the 4F2 antigen being the first to appear followed by the transferrin receptor and then the IL-2 receptor.[13 28] This is further supported by the observation that phytohaemagglutinin stimulation induces a high IL-2 activity in cultures of lamina propria mononuclear cells from patients with Crohn's disease and controls.[29] Thus, in contrast with a recent report[30] data in the present investigation show that no intrinsic cellular defect of growth factor receptors, (including IL-2 receptor) generation and expression by either peripheral blood mononuclear cells or lamina propria mononuclear cells is a feature of Crohn's disease. Whether the relatively low IL-2 receptor bearing cells counts *in vivo* are related to a low number of binding sites[31 32] or to factors interfering with the mechanisms of activation such as corticosteroids,[33] prostaglandins[34-37] or soluble serum inhibitors[38] is not clear.

We have shown here that in patients with Crohn's disease there is a divergence in the expression of early activation antigens *in vivo* and after *in vitro* activation either in the peripheral blood or in the intestinal lamina propria. This would suggest that the increased population of 4F2 and T9 bearing peripheral blood mononuclear cells and lamina propria mononuclear cells in Crohn's disease consists of preactivated rather than fully activated mononuclear cells. The high proportion of these cells either in the peripheral blood or in the intestinal lamina propria seems to indicate that a chronic immune activation is present in these patients outside as well as within the affected bowel. It is likely that in CD factors modulating *in vivo* such as immune activation contribute to the pattern of lymphocyte activation defined by the expression early activation antigens. The origin and function in Crohn's disease of preactivated cells as defined by the expression of early activation antigens is unknown. On the basis of the data presented here it can be speculated that this is an heterogeneous population as suggested by functional and morphological observations.[39 40] These cells may not be committed to proliferation and may include, among others, T cells secondarily recruited to the circuits of the *in vivo* cell mediated response. Further studies are in progress to define the functional properties of preactivated lymphocytes both in the peripheral blood and in the intestinal lamina propria of patients with Crohn's disease.

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