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Coexpression of CD4 and CD8 on peripheral blood T cells and lamina propria T cells in inflammatory bowel disease by two colour immunofluorescence and flow cytometric analysis

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Abstract

Using two colour immunofluorescence with fluorescein isothiocyanate and phycoerythrin labelled monoclonal antibodies and multiparameter flow cytometry, we investigated the coexpression of CD4 and CD8 antigens on peripheral blood lymphocytes and lamina propria lymphocytes of patients with ulcerative colitis and Crohn's disease and normal control subjects. Both the absolute number and the proportion of peripheral blood CD4+, CD8+ cells in inflammatory bowel disease were small but significantly increased compared with those in normal control subjects. Peripheral blood lymphocytes activated with phytohaemagglutinin showed appreciably increased coexpression of CD4+, CD8+. These CD4, CD8 positive cells were large and granular. Thus the increased number of peripheral blood CD4+, CD8+ cells in inflammatory bowel disease suggests that chronic immune activation occurs not only in the active state of the disease but also in remission. The proportion of CD4+, CD8+ cells in the lamina propria was greater than in peripheral blood in normal subjects, suggesting chronic immune stimulation of the local immune system. This was also seen in patients with Crohn's disease or inactive ulcerative colitis. The proportion of CD4+, CD8+ cells was, however, significantly less in the lamina propria of patients with active ulcerative colitis. Whether this implies a possible defect in mucosal immunoregulation in active ulcerative colitis cannot be determined from these results.

The expression of CD4 and CD8 seems to be associated with maturation of T cells. Both CD4 and CD8 antigens are first expressed on the cell surface of stage II thymocytes.[1-6] The most abundant thymocyte subset is CD3+, CD4+, CD8+, representing 52% of total lymphocytes.[7] With further thymic differentiation, either CD4 or CD8 is suppressed to generate the reciprocal subsets of CD4+, CD8- and CD4-, CD8+ mature peripheral T cells.

Recently, monoclonal antibodies to cell surface antigens and new techniques in highly sensitive multiparameter flow cytometry have been developed.[8 9] Thus it is possible to examine directly the expression of multiple antigens on the surface of lymphoid cells.

In this study we investigated the coexpression of CD4 and CD8 on peripheral blood lymphocytes and lamina propria lymphocytes in patients with ulcerative colitis and Crohn's disease by using two colour immunofluorescence staining and multiparameter flow cytometric analysis.

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Methods

PATIENTS

Peripheral blood was obtained from 58 patients with ulcerative colitis, 26 patients with Crohn's disease, and 17 normal control subjects. The disease activity of ulcerative colitis was assessed by the method of Truelove and Witts.[10] The colitis was active in 40 patients (13 mild, 18 moderate, nine severe) and inactive in 18. The criteria of deDombal *et al* were used to determine the disease activity of Crohn's disease.[11] Twelve patients had active disease (five mild, four moderate, three severe), while in 14 patients the disease was quiescent. No patients had been treated with steroids in the month before the study and no other immunosuppressive treatment had been used within six months.

For CD4+, CD8+ lamina propria lymphocytes, colonic or ileal mucosa was obtained from patients with inflammatory bowel disease undergoing resection. Twelve patients had ulcerative colitis, and nine had Crohn's disease. All the patients with ulcerative colitis were receiving intravenous hydrocortisone at the time of surgery. Three of them had distal disease which allowed cells to be isolated from the uninflamed portion of the colon as well as from the affected part. All the patients with Crohn's disease except one were receiving intravenous hydrocortisone at the time of operation. The diagnosis of both diseases was based on clinical, endoscopic, histological, and radiological features. As control samples, two normal ileal and 14 colonic mucosal samples were obtained from intestine resected for tumour, one for lipoma of the transverse colon, and one for diverticulosis of the sigmoid colon. The mucosa was obtained at least 5 cm from the tumour and was macroscopically and histologically normal. All the resected specimens were examined histologically and the diagnosis confirmed. The study was approved by the local ethics committee.

ISOLATION OF PERIPHERAL BLOOD MONONUCLEAR CELLS

For phytohaemagglutinin activation, peripheral blood mononuclear cells were isolated from healthy donors by Ficoll-Paque (Pharmacia, Uppsala, Sweden) gradient centrifugation.

TABLE I *Percentage of peripheral blood CD4+, CD8+ cells*

Disease	*No of cases*	*Mean (SD) % of total lymphocytes*
Normal control subjects	17	1·2 (1·0)
Ulcerative colitis:		
Active	40	2·4 (2·1)*
Remission	18	2·6 (1·4)†
Crohn's disease:		
Active	12	2·3 (1·7)*
Inactive	14	2·1 (1·1)*

*p<0·05; †p<0·01.

ISOLATION OF LAMINA PROPRIA MONONUCLEAR CELLS

Lamina propria mononuclear cells were isolated from normal and inflamed colonic and ileal specimens by a modification of the ethylenediamine tetra-acetic acid collagenase technique of Bull and Bookman,[12] as previously described.[13] Briefly, after washing, strips of mucosa were dissected and incubated in 1 mmol/l dithiothreitol solution (DTT; Sigma, Poole, Dorset, UK) for 20 minutes at room temperature. To remove the epithelial cells, mucosal strips were shaken with 5 mmol/l ethylenediamine tetra-acetic acid (BDH, Poole, Dorset, UK) at 37°C for half an hour on three occasions. After the final wash the mucosa was minced into 1 mm pieces and digested in RMPI 1640 (Gibco, Paisley, Scotland) containing collagenase (from *Clostridium histolyticum*, Boehringer Mannheim GmbH, Germany) at a concentration of 1 mg/ml as well as 10% fetal calf serum at 37°C for three hours. The digested tissue was passed through a nylon mesh (Henry Simon, Stockport, Cheshire, UK) to obtain the cells. After thorough washing lamina propria mononuclear cells were obtained by centrifugation on Ficoll-Paque.

MONOCLONAL ANTIBODIES

To detect T cells subsets, T helper/suppressor test (Leu-3a fluorescein isothiocyanate (FITC) + Leu-2a phycoerythrin (PE)), LeucoGATE (HLe-1 FITC + Leu-M3 PE) for the optimum lymphocyte gate, and SimulSET control reagent (lgG1 FITC + lgG2 PE) for setting the quadrant study were used. All monoclonal antibodies were prepared at the Becton Dickinson Immunocytometry Systems (Mountain View, CA). They were all directly conjugated with fluorescein isothiocyanate or phycoerythrin.

TWO COLOUR DIRECT IMMUNOFLUORESCENCE STAINING

For peripheral blood cells, 100 µl of whole blood collected by venepuncture with ethylenediamine tetra-acetic acid was placed into a 12×75 mm polystyrene tube (Falcon Plastics, Oxnard, CA); 20 µl of each monoclonal reagent was added to each tube. The tubes were incubated for 15 minutes at room temperature in the dark; 2 ml of 1X FACS Lysing Solution (Becton Dickinson) were added to each tube. The samples were then agitated and incubated for 10 minutes. The samples were washed once in DulBeccos' modified phosphate buffered saline without calcium and magnesium (Gibco, UK) containing 0·1% sodium azide. The cell sediment was resuspended in 0·5% paraformaldehyde in phosphate buffered saline.

For intestinal mononuclear cells the suspension of the mononuclear cells was adjusted to 2×10^7 cells/ml: 20 µl of each monoclonal reagent was placed into a polystyrene tube; 50 µl of the cell suspension (1×10^6 cells) was added to the tube. The tubes were incubated for 30 minutes on ice in the dark. The samples were washed once in phosphate buffered saline containing 0·1% sodium azide. The cell sediment was resuspended in 0·5% paraformaldehyde in buffer. The fixed cells were stored at 4°C in the dark until analysis.

TWO COLOUR FLOW CYTOMETRY

Analysis by two colour flow cytometry was performed with a FACScan (Becton Dickinson Immunocytometry Systems, Mountain View, CA). Data acquisition and analysis were performed using SimulSET software (Becton Dickinson). Full details are described elsewhere.[21]

PHYTOHAEMAGGLUTININ ACTIVATION

A solution of 1×10^6 cells in 1 ml of RPMI 1640 containing 10% fetal calf serum was incubated with 10 µg/ml phytohaemagglutinin (Wellcome Reagents, Beckenham, UK) at 37°C in a humid atmosphere of 95% air and 5% CO_2. Incubation was performed in 24 flat bottom well tissue culture plates (Flow Laboratories, UK).

STATISTICAL METHODS

Statistical analysis of results was performed by using Student's paired or unpaired *t* test, and a probability less than 5% was considered to be significant.

Results

The proportion of peripheral blood CD4+, CD8+ T lymphocytes in normal control subjects was 1·2 (1·0)% (mean (SD)). The reproducibility of this value in samples of peripheral blood taken at different times was less than 10%. Although CD4+, CD8+ cells were also a small population in patients with ulcerative colitis and Crohn's disease, the proportion of CD4+, CD8+ cells in inflammatory bowel disease was significantly increased over that in normal subjects (ulcerative colitis: active 2·4 (2·1), inactive 2·6 (1·4); Crohn's disease: active 2·3 (1·7), inactive 2·1 (1·1)) (Table I). Absolute numbers of CD4+,

TABLE II *Absolute numbers of white blood cells, lymphocytes, and CD4+, CD8+ cells in peripheral blood of patients with ulcerative colitis or Crohn's disease and in control subjects (mean (SD))*

	Normal subjects	*Ulcerative colitis*		*Crohn's disease*	
		Active	*Inactive*	*Active*	*Inactive*
White blood cells ($\times10^6$/l)	6224 (1826)	7839 (1975)*	7042 (1425)	9367 (2074)†	7437 (2190)
Lymphocytes ($\times10^6$/l)	2125 (610)	1792 (628)	1848 (563)	1940 (843)	1895 (674)
CD4+, CD8+ cells ($\times10^6$/l)	26 (7)	43 (15)†	48 (15)†	45 (19)*	40 (14)*

Comparisons with the results of normal control subjects: *p<0·01; †p<0·001.

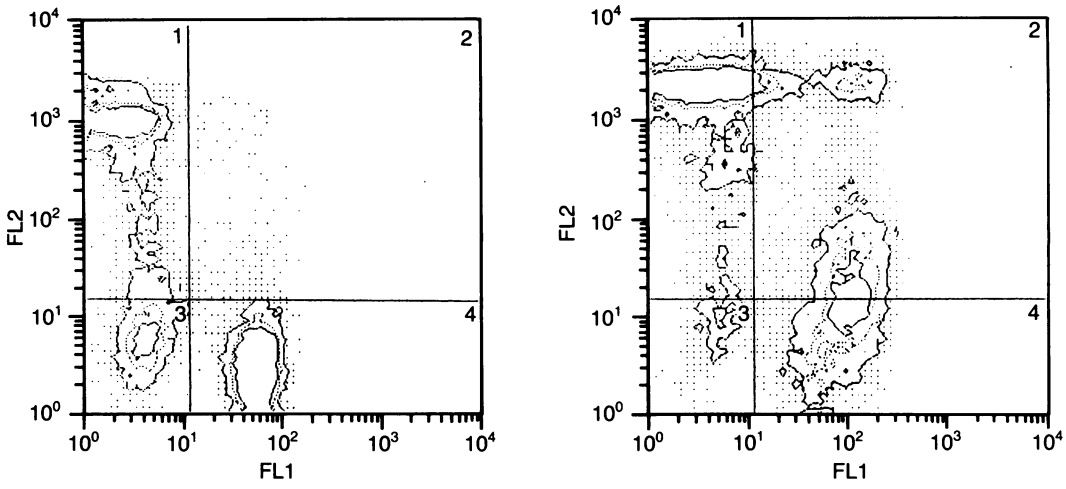

Figure 1: Coexpression of CD4 and CD8 antigens on human healthy peripheral blood lymphocytes with (right) and without (left) phytohaemagglutinin activation by two colour analysis. Peripheral blood lymphocytes were stained with CD4 fluorescein isothiocyanate and CD8 phycoerythrin. Samples were analysed by FACScan, and data were displayed as two dimensional contour plots (FL1 for CD4 and FL2 for CD8).

CD8+ were also significantly raised, even in patients in remission, when compared with control subjects (Table II).

The number of CD4+, CD8+ cells was strongly increased when peripheral blood lymphocytes were cultured with phytohaemagglutinin (Fig 1). Coexpression of CD4 and CD8 antigens appeared after three days of phytohaemagglutinin activation and was maximal on day 5 (Fig 2). The cytogram of forward light scatter, which is related to cell size, and side light scatter, which provides a measure of cell granularity, clearly showed that stimulated peripheral blood lymphocytes were larger and higher in granularity than unstimulated cells (Fig 3). The cytogram of forward light scatter and side light scatter, after three, four, or five days of stimulation with phytohaemagglutinin, showed that small and large size cells were present (Fig 3). Using set gates for small and large cells in the cytogram of forward and side light scatter, it was found that most of the large cells coexpressed CD4 and CD8 antigens on the surface (Fig 4). In contrast, the small cells expressed either CD4+ or CD8+ cells. Monocyte contamination in the gate of large cells was generally less than 2% when assayed by the LeucoGATE panel (HLe-1 fluorescein isothiocyanate + Leu-M3 phycoerythrin).

The CD4+, CD8+ phenotype was also found in lamina propria lymphocytes (Table III). Interestingly, the proportion of CD4+, CD8+ cells from inflamed mucosa of ulcerative colitis patients was significantly decreased compared with that from uninflamed ulcerative colitis mucosa or from that of control subjects ($p<0.05$). The percentage of CD4+, CD8+ T lymphocytes in the normal intestine (mean (SD) 4·7 (3·3)) was significantly increased over that in the circulation (1·2 (1·0), $p<0.001$). Although the proportion of CD4+, CD8+ cells in inflamed mucosa of Crohn's disease (4·9 (3·6)) was significantly higher than that in the circulation of active Crohn's disease (2·3 (1·7), $p<0.05$), there was no difference in the proportion of CD4+, CD8+ cells in active ulcerative colitis between inflamed mucosa (2·6 (1·3)) and the circulation (2·4 (2·1)).

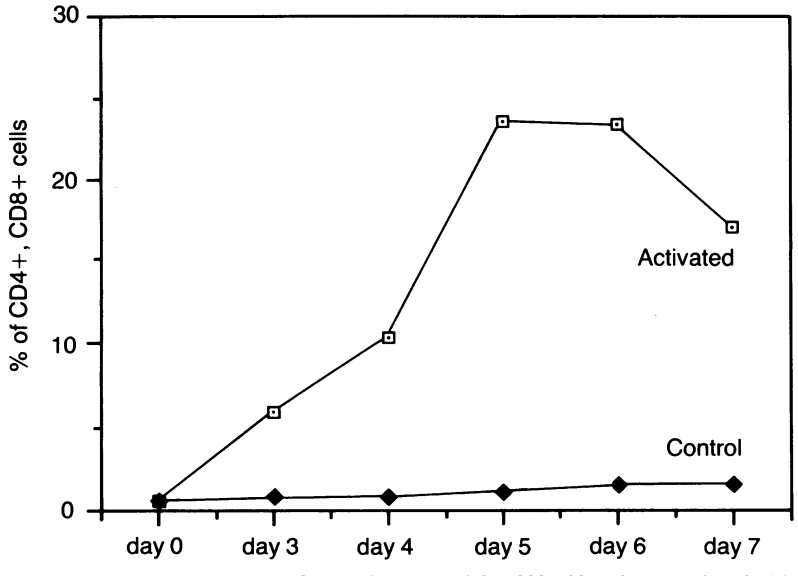

Figure 2: Expression of CD4 and CD8 antigens on peripheral blood lymphocytes cultured with phytohaemagglutinin or in medium alone. The data from a single experiment are representative among five similar experiments.

Discussion

Using a recently developed system for simultaneous two colour immunofluorescence staining and highly sensitive multiparameter flow cytometry (FACScan), we investigated the existence of CD4+, CD8+ T cells in peripheral blood lymphocytes and lamina propria lymphocytes of patients with ulcerative colitis and Crohn's disease as well as normal control subjects.

Although most peripheral blood T lymphocytes express either CD4 or CD8 antigen,[3] we have shown coexpression of CD4 and CD8 antigens on peripheral blood lymphocytes not only in control subjects but also in patients with ulcerative colitis and Crohn's disease. Although the percentage of CD4+, CD8+ lymphocytes was small in both control subjects and patients with inflammatory bowel disease, the proportion was significantly higher in the latter than in

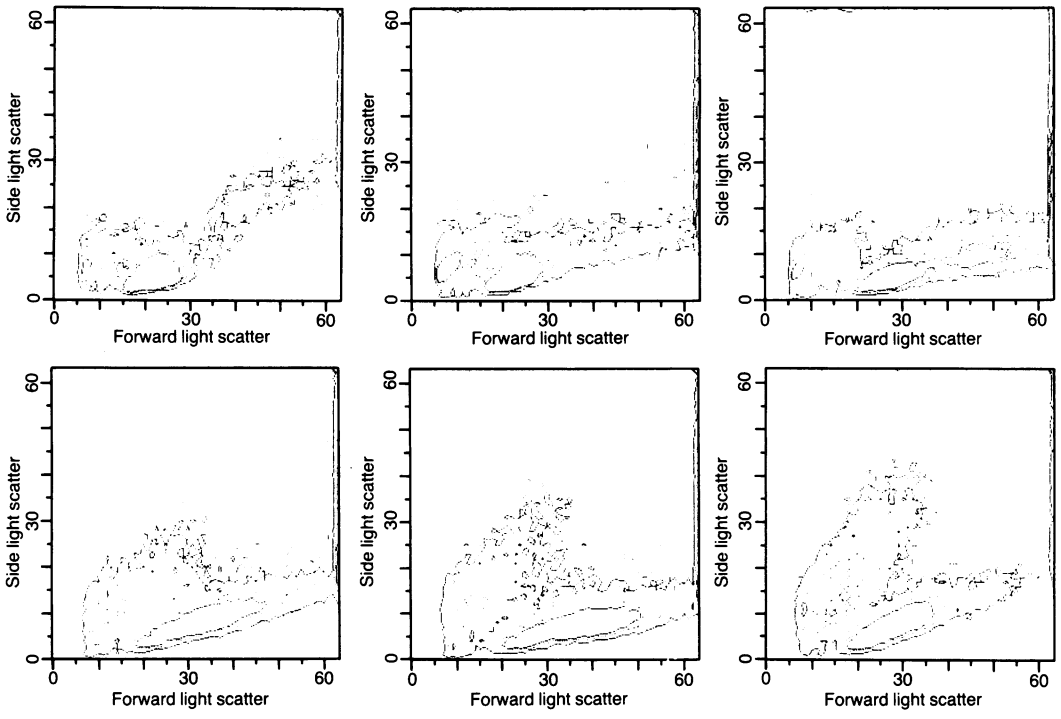

Figure 3: The two dimensional (forward and side light scatter) contour plots of peripheral blood lymphocytes on day 0 (upper left), and cultured lymphocytes on day 3, day 4, day 5, day 6, and day 7. Data were displayed as two dimensional contour plots (forward light scatter for cell size and side light scatter for cell granularity). On day 4 small and large cells were clearly shown by contour plots of forward and side light scatter (upper right).

control subjects. This increase in CD4+, CD8+ cells was found not only in active ulcerative colitis and Crohn's disease but also in patients with quiescent disease. Using gate analysis by FACScan, these CD4+, CD8+ cells were mainly found to be large and granular cells.

The present study also showed that coexpression of CD4 and CD8 antigens could be induced in peripheral blood lymphocytes by phytohaemagglutinin stimulation in vitro. Using concanavalin A with T cell growth factor with normal peripheral blood T cells or normal human thymocytes, Blue *et al* have obtained similar results.[5 6 14 15] In active Crohn's disease, an increased proportion of peripheral blood T cells express the early activation markers, transferrin receptor (T9), 4F2,[16-18] and interleukin-2 receptor (CD25).[19] The increased proportion of CD4+, CD8+ cells confirms activation but it cannot be inferred from the present data that T9, 4F2, or CD25 antigens are expressed only in the CD4+, CD8+ population.

In the lamina propria the proportion of CD4+, CD8+ cells was higher than that in peripheral blood, both for uninflamed and inflamed mucosa. This is further evidence that lamina propria T cells are activated even in healthy mucosa and is consistent with previous studies showing increased T cell expression of 4F2, OKT9, HLA-DR, and CD25 antigens.[18 20 21]

The decrease in the proportion of T cells expressing CD4, CD8 in the lamina propria lymphocytes isolated from active ulcerative colitis was unexpected and requires explanation. One possibility is that this is an artifact since there are problems in interpreting proportions in cell suspensions isolated from the colonic mucosa. There is no precise method for determining the absolute number of mononuclear cells in the segment of colon used for cell isolation, thus it is not possible to obtain meaningful data concerning the percentage recovery of cells nor is it possible to express the CD4+, CD8+ cells in absolute numbers. Double staining using tissue sections also presents problems of absolute quantitation when the proportion of cells under study is present in such low numbers.

Nevertheless, the reduction of CD4+, CD8+ cells in active ulcerative colitis may be relevant to the pathogenesis of mucosal inflammation. In vitro studies have shown that for coexpression of CD4 and CD8 antigens to occur, interaction between CD4+ and CD8+ cells is required and factors released by activation of CD8+ cells may also be involved.[15 22] Thus the failure of T cells to coexpress CD4 and CD8 antigens in the colonic mucosa of patients with active ulcerative colitis may suggest impairment of these mechanisms. Whether these changes in ulcerative colitis are important for local immunoregulatory control can only be determined by functional assays.

We are grateful to Dr Frank Hulstaert and Dr James Lowder (Becton Dickinson, Immunocytometry Systems Europe, Erembodegem, Belgium) for helpful discussion and for the supply of antibodies.

TABLE III *Percentage of lamina propria CD4+, CD8+ cells*

Disease	*No of cases*	*Mean (SD) % of total lymphocytes*
Control subjects	16	4·7 (3·3)
Ulcerative colitis:		
Inflamed	12	2·6 (1·3)*
Uninflamed	3	6·4 (4·3)
Crohn's disease	12	4·9 (3·6)

*p<0·05.

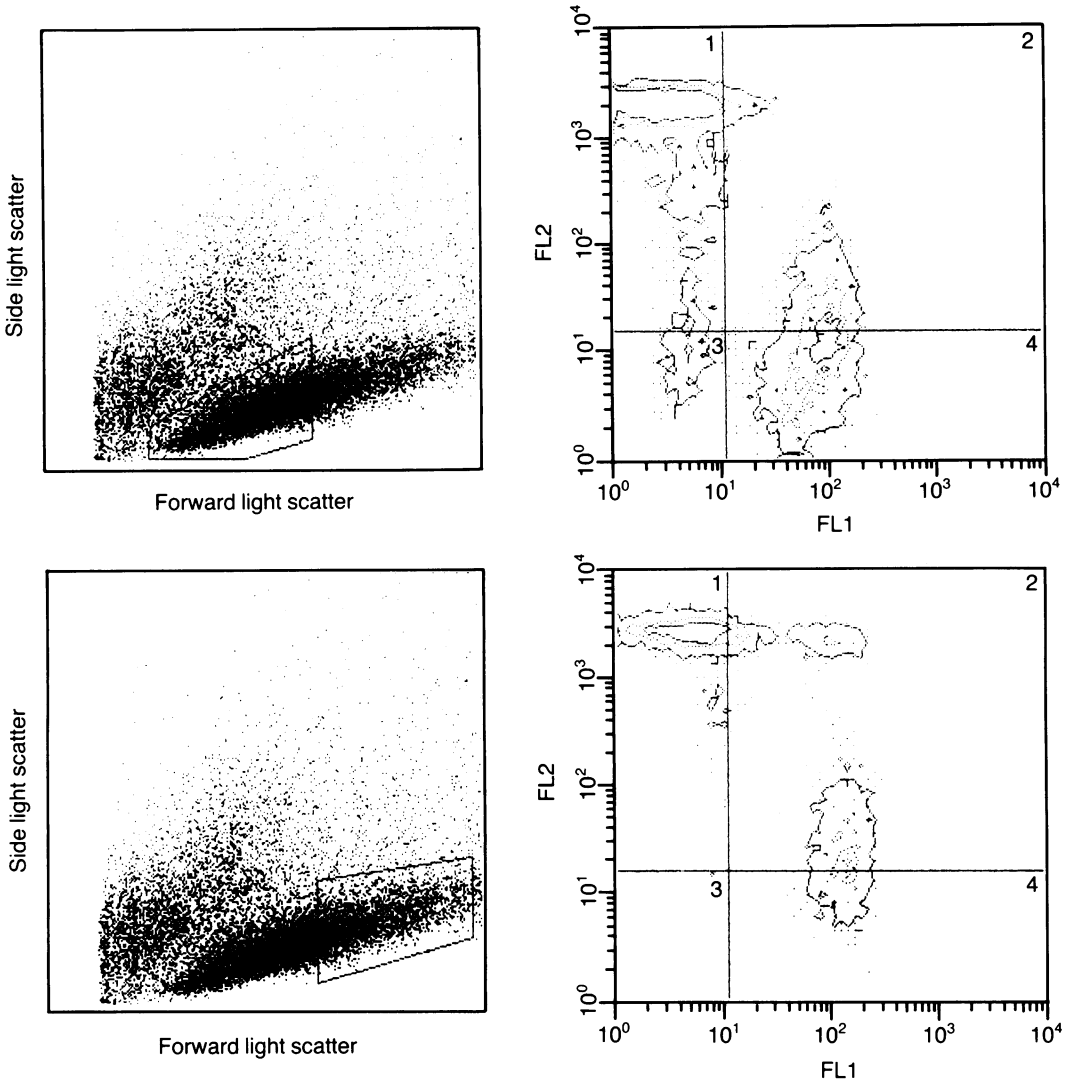

Figure 4: Distribution of CD4+, CD8+ cells among small and large cells activated with phytohaemagglutinin on day 5. The gate for small cells (upper left) and for large cells (lower left) was set on the cytogram of forward and side light scatter. Contour plots (FL1 for CD4 and FL2 for CD8) of small cells (upper right) and large cells (lower right) were displayed.

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