

Abnormalities of T lymphocyte subsets in systemic sclerosis demonstrated with anti-CD45RA and anti-CD29 monoclonal antibodies

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Abstract

T cell subpopulations were assessed by two colour flow cytometry with phycoerythrin conjugated anti-CD45RA and anti-CD29 and fluorescein conjugated anti-CD4 and anti-CD8 monoclonal antibodies, on peripheral blood lymphocytes from 12 patients with systemic sclerosis and from nine control subjects. The percentage of CD4+CD29+ cells was significantly higher in patients with systemic sclerosis than in controls (mean (SEM) 68.8 (3.1) v 47.9 (4.1) respectively). CD4+CD45RA+ cells were not significantly different in patients and controls. CD8+CD29+ and CD8+CD45RA+ subpopulations were significantly higher in patients with systemic sclerosis than in controls (83.0 (3.2) v 58.7 (6.8) and 80.2 (3.0) v 66.9 (3.2) respectively). The increase in the percentage of CD29+ cells suggests an activation of memory cells in patients with systemic sclerosis, which may play an important part in the pathogenesis of the disease.

The cause and the pathogenesis of systemic sclerosis have not been well elucidated. Systemic sclerosis is characterised by vascular and microvascular abnormalities, excessive fibroblastic activity, and collagen deposition in numerous organs, which may be induced by immunological defects.^{1 2}

A wide range of B and T cell abnormalities has been described in this disease, including antinuclear antibodies, immune complexes, decreased number of circulating T lymphocytes, increased CD4+/CD8+ ratio with decreased number of CD8+ cells,³ and defective Epstein-Barr virus specific T cell regulation.⁴ Moreover, c-myc and c-myb proto-oncogenes expression, using RNA hybridisation techniques, is significantly increased in T but not in B lymphocytes from patients with systemic sclerosis compared with control subjects, suggesting an in vivo activation of T cells.⁵

Studies have shown that CD4+ T cells are both phenotypically and functionally heterogeneous and can be clearly distinguished by monoclonal antibodies to CD29 and CD45RA antigens.^{6 7} The CD29 and CD45RA antigens are present on T lymphocytes, B lymphocytes, and some monocytes; about 40% of peripheral blood lymphocytes from normal subjects are positive for each antigen. Within the CD4+ subset anti-CD29 and anti-CD45RA antibodies identify reciprocal subpopulations. CD4+CD45RA+ cells, although not directly suppressive, induce activation of CD8+ cells and suppress immunoglobulin production by

B cells in response to pokeweed mitogen and specific antigen; thus they are designated as a suppressor inducer subset.⁶ CD4+CD45RA+ cells are preferentially activated through interactions with self class II major histocompatibility complex in the autologous mixed lymphocyte reaction,^{7 8} but poorly activated by soluble antigen stimulation.^{6 7}

CD4+CD29+ cells can provide help to B cells in both a pokeweed mitogen stimulated immunoglobulin synthesis and in an antigen specific antibody production system and are thus designated as a helper-inducer subset. The helper-inducer subset corresponds functionally to T lymphocytes that are identified by the 4B4⁷ and WR19⁹ monoclonal antibodies (CD29), and the suppressor-inducer subset corresponds to those previously defined by the leucocyte common antigen (CD45RA) and identified by the 2H4⁶ and WR16 monoclonal antibodies.¹⁰

We used monoclonal antibodies to CD29 (4B4) and CD45RA (2H4), in combination with monoclonal antibodies to CD4 and CD8, to enumerate T lymphocytes in blood from patients with systemic sclerosis and in control subjects. Single and two colour immunofluorescence and flow cytometry techniques were used.

Patients and methods

PATIENTS

Twelve patients with systemic sclerosis with diffuse scleroderma (10 women, two men; mean (SD) age 46 (13) years; mean (SD) disease duration 9 (7) years) and nine control subjects (four women, five men; age 49 (7) years) were studied. All patients satisfied the American Rheumatism Association preliminary criteria for classification of definite systemic sclerosis.¹¹ Table 1 shows the clinical characteristics of the patient with active (five patients) and inactive (seven patients) systemic sclerosis. All patients had stopped taking slow acting therapeutic agents for at least six months before the study. Control subjects had sciatica (seven patients) or osteoporosis (two patients); six of them were taking non-steroidal anti-inflammatory drugs.

CELL PREPARATIONS

Mononuclear cells from heparinised blood were separated by Ficoll-Paque (Pharmacia Laboratories, Uppsala, Sweden) density gradient centrifugation. Cells were washed twice and adjusted to 5×10^6 /ml, and 200 μ l of this cell suspension was exposed to 5 μ l of fluorescein isothiocyanate (FITC) conjugated anti-CD4 or anti-CD8 (Coulter Immunology, Hialeah, Florida) and 5 μ l

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Accepted for publication
4 July 1990

Table 1 Clinical characteristics of patients with active or inactive systemic sclerosis. Values are the number of patients or the mean (SD)

Characteristic	Active disease (n=5)	Inactive disease (n=7)
Female:male	5:0	5:2
Age (years)	48 (8)	44 (17)
Duration of disease (years)	5 (3)	11 (8)
Raynaud's phenomenon	5	7
History of digital ulcers	3	3
Oesophageal dysmotility*	4	5
Pulmonary disease†	3	4
Sjögren's syndrome	3	0
Taking non-steroidal anti-inflammatory drugs	2	1
Taking prednisone (<10 mg/day)	3	3
ESR‡ (mm/h)	20 (21)	10 (11)
WBC‡ ($\times 10^{-9}/l$)	7.8 (2.0)	6.6 (2.2)
Peripheral lymphocytes ($\times 10^{-9}$ cells/l)	2.2 (0.6)	1.9 (0.5)
Serum creatinine ($\mu\text{mol/l}$)	74 (7)	79 (11)
Positive ANA‡	3	4

*Oesophageal dysmotility assessed by oesophageal manometry.

†Forced vital capacity or single breath diffusing capacity for carbon monoxide <80% of predicted normal value, or interstitial markings on chest radiographs.

‡ESR=erythrocyte sedimentation rate; WBC=white blood cells; ANA=antinuclear antibodies.

of phycoerythrin (PE) conjugated anti-CD45RA (2H4) or anti-CD29 (4B4) (Coulter Immunology) monoclonal antibodies for 30 minutes at 4°C. The final combinations were the following: anti-CD4-FITC and anti-CD45RA-PE, anti-CD4-FITC, and anti-CD29-PE, anti-CD8-FITC and anti-CD45RA-PE, anti-CD8-FITC and anti-CD29-PE. Control samples consisted of cells stained with monoclonal mouse isotype IgG-FITC or monoclonal IgG-PE. In some experiments with purified CD4+ and CD8+ subpopulations PE conjugated anti-CD45RA (2H4) and FITC conjugated anti-CD29 (4B4) monoclonal antibodies were simultaneously used. The cells were washed twice in phosphate buffered saline and fixed with 1% formaldehyde in phosphate buffered saline for analysis by flow cytometry.

COMPLEMENT DEPENDENT LYSIS OF LYMPHOCYTES WITH MONOCLONAL ANTIBODIES

T cells were separated from non-T cells by rosetting with sheep red blood cells treated with 2-aminoethylisothiouonium bromide. T lymphocytes were then treated with anti-CD4 or anti-CD8 monoclonal antibodies and rabbit complement (Behring AG, Marburg, W Germany). Briefly, 10×10^6 cell aliquots were incubated with 50 μl of antibody for one hour at 4°C, then 250 μl of rabbit complement was added. The mixture was incubated for another hour in a 37°C shaking water bath and washed three times in RPMI 1640 medium (Dutch modification, Flow Laboratories, Rockville, MD). The procedure was repeated twice. After lysis with anti-CD4 and complement more than 95% of residual cells were CD8+ and less than 2% were CD4+. After lysis with anti-CD8 and complement more than 90% of the remaining cells were CD4+ and less than 5% were CD8+. These two populations will be referred to as CD4+ and CD8+ respectively.

FLOW CYTOMETRY

Flow cytometry was performed with EPICS CS cell sorter (Coulter Electronics) equipped with argon laser with single and two colour analysis.

For some experiments a FACScan (Becton-Dickinson, Palo Alto, CA) was used. Cells were gated using forward and right angle scatter for lymphocyte characteristics. Five thousand cells were analysed on a log fluorescent scale. Background green and red fluorescence was determined with the control samples using monoclonal mouse isotype IgG-FITC or monoclonal IgG-PE. Positive cells were determined by setting a region with reference to this relevant control. As CD29 is expressed on all T lymphocytes the cut off point was set between the CD29 dim and CD29 bright cells, the last being considered as CD29+ cells. The same setting was used for lymphocytes from patients with systemic sclerosis and controls.

For two colour analyses red fluorescence \times green fluorescence dot plots were generated with mutual exclusive counting regions set to measure fluorescence negative, red fluorescence positive, green fluorescence positive, and red and green fluorescence positive events. The cells were then analysed by the Quad Stat software package for calculation of percentages of single and dual staining populations. The flow cytometer was run on a fixed laser power during the whole study.

STATISTICAL ANALYSIS

Data were compared for significance with Student's unpaired *t* test and Wilcoxon's rank sum test. *p* Values <0.05 were considered significant.

Results

The differences in absolute lymphocyte counts were not statistically significant between control subjects (mean (SEM) $2.288 (0.269) \times 10^9$ cells/l) and all patients with systemic sclerosis ($2.035 (0.163) \times 10^9$ cells/l), or patients with active ($2.196 (0.284) \times 10^9$ cells/l) and inactive ($1.92 (0.2) \times 10^9$ cells/l) systemic sclerosis. Thus the results are given as the percentage of peripheral blood lymphocytes and of the CD4+ or the CD8+ cells which expressed the CD45RA or the CD29 antigens. Table 2 shows these results.

SINGLE FLUORESCENCE ASSAYS

The percentage of CD4+ cells was significantly lower in patients with systemic sclerosis (mean (SEM) $43.6 (3.0)$) than in controls ($52.0 (1.6)$) ($p < 0.05$). The differences in absolute numbers of CD4+ cells between patients with systemic sclerosis (mean (SEM) $0.905 (0.105) \times 10^9$ cells/l) and controls ($1.206 (0.168) \times 10^9$ cells/l) did not reach significance, however. No significant differences were found for CD8+ cells and the CD4+:CD8+ ratio between patients with systemic sclerosis and controls (table 2).

The percentage of peripheral blood lymphocytes expressing the CD45RA antigen was not significantly different in patients with systemic sclerosis ($67.0 (3.6)$) and controls ($57.1 (3.9)$). In contrast, the percentage of CD29+ lymphocytes was significantly higher in patients with systemic sclerosis ($68.7 (2.9)$) than in controls ($49.9 (4.8)$) ($p < 0.01$) (table 2).

Table 2 Cell surface characteristics of lymphocytes from patients with systemic sclerosis and from controls. Values are means (SEM)

% Positive subset × 100	Controls (n=9)	Total systemic sclerosis (n=12)	Active systemic sclerosis (n=5)	Inactive systemic sclerosis (n=7)
CD4+	52.0 (1.6)	43.6 (3.0)*	43.2 (6.5)	44.6 (2.8)
CD8+	23.1 (1.5)	27.0 (2.9)	28.7 (5.3)	25.3 (3.2)
CD4+:CD8+	2.3 (0.2)	1.9 (0.3)	1.7 (0.5)	2.1 (0.5)
CD45RA+:PBL†	57.1 (3.9)	67.0 (3.6)	66.4 (5.5)	67.5 (5.4)
CD29+:PBL	49.9 (4.8)	68.7 (2.9)**	66.8 (6.3)	70.0 (2.8)
CD4+CD45RA+:PBL	20.8 (2.1)	19.9 (2.5)	17.5 (6.1)	21.3 (2.5)
CD4+CD45RA+:CD4+	40.2 (4.0)	46.2 (5.7)	42.8 (7.5)	48.6 (8.5)
CD4+CD29+:PBL	24.9 (2.3)	27.5 (2.4)	25.8 (5.6)	28.4 (2.7)
CD4+CD29+:CD4+	47.9 (4.1)	68.8 (3.1)**	70.1 (6.1)	67.5 (3.6)
CD8+CD45RA+:PBL	15.2 (0.8)	22.3 (2.9)	24.0 (5.8)	21.0 (3.2)
CD8+CD45RA+:CD8+	66.9 (3.2)	80.2 (3.0)**	76.4 (3.9)	83.3 (4.2)
CD8+CD29+:PBL	13.2 (1.6)	22.4 (2.2)**	25.5 (4.6)	20.6 (2.4)
CD8+CD29+:CD8+	58.7 (6.8)	83.0 (3.2)**	81.3 (3.5)	83.6 (5.1)

*p<0.05; **p<0.001.

†PBL=peripheral blood lymphocytes.

TWO COLOUR FLUORESCENCE CYTOMETRY

The percentage of peripheral blood lymphocytes expressing both CD4 and CD45RA antigens was not significantly different between patients with systemic sclerosis and controls (19.9 (2.5) v 20.8 (2.1)). The percentage of CD4+ cells expressing the CD45RA antigen was also not significantly different in patients and controls (46.2 (5.7) v 40.2 (4.0)) (table 2).

In contrast, the percentage of CD4+ cells expressing the CD29 antigen was significantly higher in patients with systemic sclerosis (68.8 (3.1)) than in controls (47.9 (4.1)) (p<0.01) (table 2).

The percentage of peripheral blood lymphocytes expressing both the CD8 and CD45RA antigens tended to be higher in patients with systemic sclerosis than in controls (22.3 (2.9) v 15.2 (0.8)); the difference reached significance for the percentage of CD8+ cells expressing the CD45RA antigen (80.2 (3.0) v 66.9 (3.2)) (p<0.01).

The percentage of peripheral blood lymphocytes expressing both the CD8 and CD29 antigens was significantly higher in patients with systemic sclerosis than in controls (22.4 (2.2) v 13.2 (1.6)) (p<0.01). The percentage of CD8+ cells expressing the CD29 antigen was also significantly higher in patients than in controls (83.0 (3.2) v 58.7 (6.8)) (p<0.01) (table 2).

Phenotypic characteristics were analysed in the two clinical subgroups of patients with systemic sclerosis. No significant difference was found between patients with active or inactive disease (table 2).

The mean (SEM) CD45RA/CD29 'calculated overlap' in CD4+ cells (defined as the sum of the percentages of CD4+ cells expressing either the CD45RA or the CD29 antigens) was significantly increased in patients (15.4 (5.9)) compared with controls (3.1 (2.6)) (p<0.05). The mean (SEM) calculated overlap in CD8+ cells (defined as the sum of the percentages of CD8+ cells expressing either the CD45RA or the CD29 antigens) was significantly higher in patients (62.3 (4.5)) than in controls (28.9 (6.7)) (p<0.01). Figures 1 and 2 show characteristic histograms of CD45RA+ and CD29+ cells in gated CD4+ and CD8+ subpopulations respectively in a control subject and in a patient with systemic sclerosis. The last profiles show mainly bright CD45RA and CD29 cells.

To analyse further the significance of the calculated overlap CD4+ and CD8+ cells from three patients with systemic sclerosis and four controls were purified by complement dependent cytotoxicity. These purified subpopulations were treated simultaneously with PE conjugated anti-CD45RA and FITC conjugated anti-CD29 monoclonal antibodies.

In purified CD4+ cells the mean (SEM) percentage of double positive CD45RA+CD29+ cells was 9.2 (2.2) (range 3–13) in controls and 20.7 (2.2) (range 15–24) in patients with systemic sclerosis.

In purified CD8+ cells the mean (SEM) percentage of double positive CD45RA+CD29+ cells was 25.2 (7.0) (range 5–37) in controls and 39.0 (6.3) (range 31–52) in patients with systemic sclerosis. Figure 3 shows the double positive CD45RA+CD29+ cells (upper right quadrant) in the purified CD4+ and CD8+ subpopulations from a patient with scleroderma.

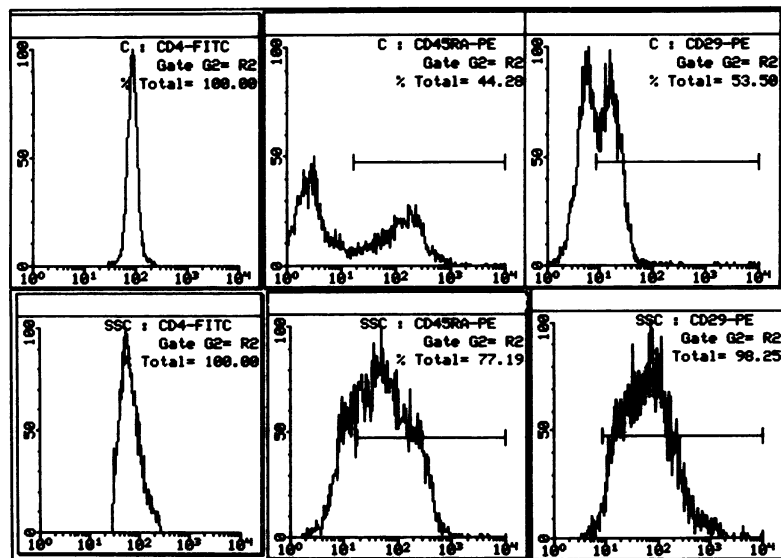


Figure 1 Two colour immunofluorescence flow cytometry analysis. Each profile represents fluorescence data from list mode files after gating CD4+ cells (G2): PE conjugated anti-CD45RA and PE conjugated anti-CD29 monoclonal antibodies were used. The sum of percentages of CD45RA+ and CD29+ cells was 97.78 in a control patient (top) and 175.44 in a patient with systemic sclerosis (bottom). FITC=fluorescein isothiocyanate; PE=phycoerythrin; C=control; SSC=systemic sclerosis.

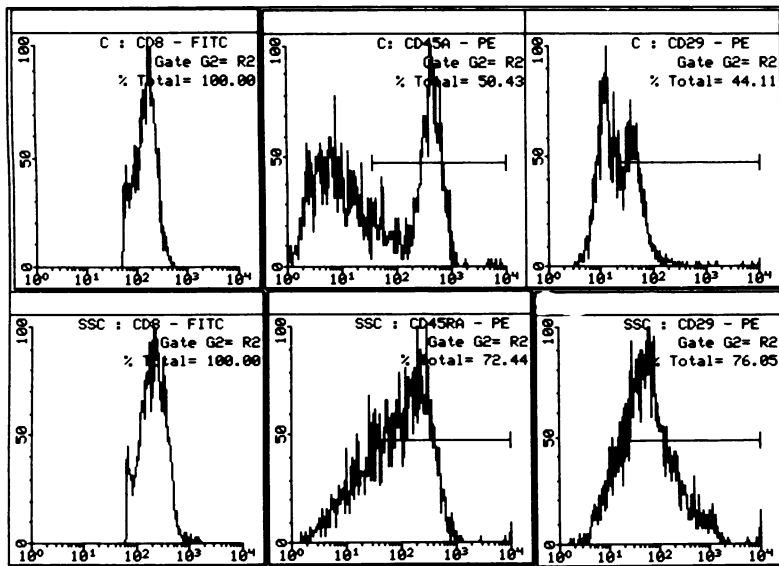


Figure 2 Two colour immunofluorescence flow cytometry analysis. Each profile represents fluorescence data from list mode files after gating CD8+ cells (G2): PE conjugated anti-CD45RA and PE conjugated anti-CD29 monoclonal antibodies were used. The sum of percentages of CD45RA+ and CD29+ cells was 94.54 in a control patient (top) and 148.49 in a patient with systemic sclerosis (bottom).

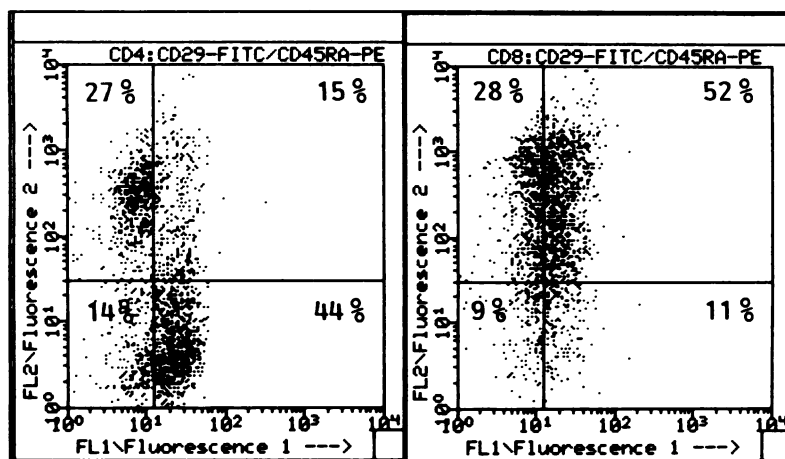


Figure 3 Dot-plot analysis of purified CD4+ (left) and CD8+ (right) cells using PE conjugated anti-CD45RA and FITC conjugated anti-CD29 monoclonal antibodies in a patient with systemic sclerosis. Positive cells were determined by setting a region with reference to the negative relevant control. Each profile was divided into four quadrants: upper left: CD45RA+CD29- cells; upper right: CD45RA+CD29+ cells; lower left: CD45RA-CD29- cells, and lower right: CD45RA-CD29+ cells. Double positive CD45RA+CD29+ cells were 15% in CD4+, and 52% in CD8+ purified subpopulations.

Discussion

In systemic sclerosis activated T cells *in vivo* have been demonstrated in the dermis and subcutaneous tissue,¹² and in peripheral blood lymphocytes,¹³ by means of activation markers (Ia, Tac, and transferrin receptor). Moreover, the increased early response of autologous mixed lymphocyte reaction,¹⁴ increased interleukin-2 production by phytohaemagglutinin stimulated peripheral blood lymphocytes,¹⁵ and increased helper activity¹⁶ are different functional aspects of *in vivo* activation of T cells from patients with scleroderma. Our previous studies showing an increased expression of c-myc and c-myb proto-oncogenes in T lymphocytes from patients with systemic sclerosis are also consistent with an *in vivo* early activation of T cells.⁵

Which T lymphocyte subsets are involved in

this *in vivo* activation remains to be determined. The use of monoclonal antibodies to the two functional subsets recently described as helper-inducer (CD29) and suppressor-inducer (CD45RA) might help to answer this question.

Our results show a significant increase in the CD29+ subset in patients with systemic sclerosis compared with controls. The higher percentage of CD29+ cells concerns both CD4+ and CD8+ cells. The CD8+CD45RA+ population is also increased in patients with scleroderma. This increase is not related to the absolute number of circulating lymphocytes, which was similar in patients and controls, nor to the activity of the disease.

In contrast, CD4+CD45RA+ cells are not significantly different in patients with systemic sclerosis and controls. This is in marked contrast with recent data which showed decreased percentage of CD4+CD45RA+ cells in systemic lupus erythematosus, especially in patients with active disease and renal involvement.¹⁷⁻¹⁹ In other systemic autoimmune diseases, such as rheumatoid arthritis²⁰ and progressive multiple sclerosis,²¹ a selective decrease in the percentage of circulating CD4+CD45RA+ cells was also described. Nevertheless, synovial T cells are predominantly CD4+CD29+ in chronic synovitis from rheumatoid^{22,23} or non-rheumatoid patients,²⁴ but also in pleural and peritoneal inflammatory effusions,²⁴ suggesting that a high percentage of CD29+ cells occurs locally in different chronic inflammatory sites.

The function of these CD4+ lymphocyte subsets is under discussion. Some assume that they represent different lineages of T cells,^{6,7} whereas others suggest that they represent different maturational stages²⁵⁻²⁷: CD4+CD45RA+ are considered as naive cells, CD4+CD29+ as memory cells. The CD4+CD29+ subset proliferates when restimulated with an antigen to which the donor has been previously primed *in vivo*. The poor response of CD4+CD45RA+ T cells to soluble antigens seems to reflect an absence of memory cells rather than an intrinsic defect as they proliferate and produce interleukin-2 when stimulated with lectins. Moreover, in neonates CD4+CD45RA+ are predominant and CD4+CD29+ are rare.^{26,27} The proportion varies with age: in normal adults the percentages are 20% for both CD4+CD45RA+ and CD4+CD29+ in peripheral blood lymphocytes; in elderly subjects the percentage of CD4+CD45RA+ decreases significantly, while the percentage of CD4+CD29+ increases slightly.^{28,29} Thus the increased number of CD4+CD29+ cells seems to reflect a conversion from the naive to memory state due to the continuing immunological response.²⁸ The functions of CD8+CD45RA+ and CD8+CD29+ subsets are unknown; however, CD8+CD29+ are assumed to be memory cells also.²⁶

In vitro CD4+CD45RA+ as well as CD8+CD45RA+ cells can convert into the CD29+ phenotype after stimulation with phytohaemagglutinin, and the two markers can be found on the same cell early after activation²⁹; experiments done with bulk cultures were confirmed by clonal analysis.²⁹

We found a significant increase in the calculated overlap in T lymphocytes from patients with systemic sclerosis, in CD4+ as well as in CD8+ cells, as compared with control subjects. Furthermore, in our experiments with purified CD4+ and CD8+ subpopulations from patients with scleroderma the presence of both CD29 bright and CD45RA molecules on the same cell was shown in about 20 and 39% of cells respectively, suggesting an in vivo conversion of naive cells into memory cells.

Thus in patients with systemic sclerosis the CD29 molecule may be a useful marker of stable activation of T cells—that is, memory T cells, while interleukin-2 receptor and transferrin receptor are markers of early and transitory activation.¹³ As CD29+ cells express higher density of adhesion molecules (LFA1, LFA3, and CD2) than CD45RA+ cells in peripheral blood lymphocytes^{26, 27} and in synovial T cells,³⁰ CD29+ cells may have a greater ability to adhere to vascular endothelium and participate in other adhesion dependent interactions, such as antigen response or cytotoxicity. One could speculate on the importance of such cells in the pathogenesis of microvascular lesions and fibrosis in scleroderma.

In conclusion, in patients with systemic sclerosis the CD29+ subset of T cells is significantly increased, suggesting an in vivo conversion of naive cells into memory cells, which may play an important part in the pathogenesis of the disease.

We are indebted to Mrs Martine Nedelec for excellent technical assistance. Supported by the French National Institute of Health and Medical Research (INSERM).

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