Early phenotypic activation of circulating helper memory T cells in scleroderma: correlation with disease activity

Ugo Fiocco, Mara Rosada, Luisella Cozzi, Claudio Ortolani, Giustina De Silvestro, Amelia Ruffatti, Emanuele Cozzi, Cristina Gallo, Silvano Todesco

Abstract

Objectives—The differential expression of several accessory/activation molecules (CD26, CD29, CD45RA, CD25, MLR4, HLA-DR) on peripheral blood CD4+ and CD8+ T lymphocytes in patients with scleroderma was compared with that in controls and patients with other connective systemic diseases to look for evidence of the involvement of T cells in the disease process of scleroderma.

Methods—The two colour expression of surface molecules by circulating T cells was analysed with a panel of monoclonal antibodies and flow cytometry in 17 patients with scleroderma, 10 patients with systemic lupus erythematosus, and five patients with rheumatoid arthritis, and the results compared with those for 10 normal controls. The two colour T CD4+ phenotype was further compared between patients with active and quiescent disease in these patients with scleroderma. The coexpression of surface molecules by CD4+ T cells was also analysed by three colour flow cytometry in eight patients with scleroderma.

Results—Patients with scleroderma showed increased CD4+CD26+ and CD4+CD25+ percentages and absolute numbers and decreased CD8+CD29+ percentages compared with controls. Moreover, a significant correlation between the higher CD4+CD26+ T cell percentage and absolute cell numbers with disease activity was observed. Most of the CD4+ peripheral blood T cells from patients with scleroderma showed the CD26+CD45RA- phenotype by three colour flow cytometry analysis.

Conclusions—The distinctive pattern of early helper memory T cell activation in these patients with rapidly evolving scleroderma supports the role of a T cell mediated mechanism in the progression of scleroderma.

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features of the affected skin. In recent onset disease the presence of HLA class II (DR) positive CD4 T cells² in perivascular areas, together with the high density expression of intercellular adhesion molecule 1 (ICAM-1), β 1 (CD29) and β 2 (CD18) integrin subunits on lymphocytes, endothelial cells and adjacent fibroblasts,^{3,4} suggests the early appearance of an abnormal cell mediated immune response to vascular or extracellular matrix components.⁵

The surface expression of lymphocyte adhesion proteins, also known as accessory molecules, may account for the different activation requirements and effector functions of CD4+ T cell subclasses. Reciprocal subpopulations of peripheral blood T cells, naive and memory, are defined by coordinate expression of multiple adhesion/activation molecules^{6,7} and different isoforms of CD45 Ag.⁸ Naive and memory cells, at least in CD4+ T cells, correspond to the 'helper inducer' and 'suppressor inducer' T cell subsets⁸ previously identified by monoclonal antibodies (MoAbs) 4B4 (directed against CD29)⁹ and 2H4 (directed against CD45R).¹⁰ The two CD4+ subsets show a distinct recirculatory pathway, with preferential migration of helper memory cells into the site of inflammation.¹¹ Transition from the naive to the memory phenotype after antigen stimulation is accompanied by alterations in the surface density of adhesion molecules. The increased expression of lymphocyte receptors for major extracellular matrix proteins, such as the integrin very late antigens (VLA) and CD26, plays an important part in local cell positioning and differentiation.¹²

Although the role of T cell hyperactivity is widely accepted in scleroderma, few data have been reported on peripheral blood T cell abnormalities in different stages of the disease or on the distinctive pattern of T cell activation with respect to other systemic connective tissue diseases.

In this study we examined the differential expression of several accessory/activation molecules (CD29, CD45RA, CD26, CD25, HLA-DR, and MLR4) on peripheral blood T cell fractions in patients with scleroderma and compared them with controls using two colour flow cytometry. We also investigated the distribution of these surface molecules on peripheral blood T cell subsets in two other groups of patients with systemic

Division of Rheumatology, Institute of Internal Medicine, University of Padova, Padova, Italy

U Fiocco
M Rosada
L Cozzi
A Ruffatti
E Cozzi
C Gallo
S Todesco

Laboratory of Clinical Pathology, Hospital of Venice, Venice, Italy
C Ortolani

Immunohematology and Blood Transfusion Service, Hospital of Padova, Padova, Italy
G De Silvestro

Correspondence to:
Dr Ugo Fiocco,
Istituto di Medicina Interna,
Cattedra e Divisione di Reumatologia,
Policlinico Universitario,
Via Giustiniani 2,
35128 Padova, Italy.

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Scleroderma is a connective tissue disease of unknown aetiology which is expressed in several clinical forms.¹ Altered immune regulation of collagen metabolism has been suggested in view of the immunohistological

lupus erythematosus (SLE) and rheumatoid arthritis (RA).

The two colour T CD4+ phenotype was further studied in two subgroups of patients with rapidly evolving and quiescent disease for possible correlations between T cell subclass abnormalities and clinical stages of scleroderma. Lastly, the coexpression of surface molecules by CD4+ T cells was also analysed by three colour flow cytometry in eight patients with scleroderma to characterise better the phenotypic activation of this subset. A selective activation of CD4+ T cells expressing a helper memory phenotype was found to be confined to patients with scleroderma and to correlate significantly with the rapidly evolving phase of the disease.

Patients and methods

PATIENT SELECTION

Thirty two patients attending the division of rheumatology were selected. Thirteen met the preliminary diagnostic criteria for systemic sclerosis¹³ and four had localised scleroderma.¹⁴ Ten patients had SLE according to the American Rheumatism Association (ARA) criteria,¹⁵ showing moderately active disease, and five with RA as defined by the revised ARA criteria,¹⁶ all with acute knee joint effusion. Nine patients with SLE and two patients with RA were treated with prednisone (<5 mg/day). Ten healthy subjects matched for age and sex made up a control group.

According to cutaneous disease and serological patterns in the early stage of the disease, nine patients were classified as having diffuse systemic scleroderma and four as having limited systemic scleroderma.¹⁷ The four patients with localised scleroderma had the generalised morphoea form and none had Raynaud's phenomenon.¹⁸ Table 1 gives the prominent clinical features. At the time of our study nine patients had never been treated for scleroderma. Five had been treated with D-penicillamine and three with prednisone (<5 mg/day) (table 1). When possible, the patients with scleroderma were divided into two groups according to the clinical course of cutaneous or visceral disease, or both, in the six months before our study. Group 1 included eight patients with active or rapidly progressive scleroderma (mean disease duration 2.6 years;

seven subjects with a mean disease duration of 1.2 years, and one in whom the disease had lasted 12 years).

Group 2 included seven patients with inactive or quiescent scleroderma (mean disease duration 4.4 years) (table 1). The stage of disease activity was established by repeated clinical examinations. Patients were considered to have active disease when the area of sclerotic skin had increased by 50% and new ischaemic digital infarct or new visceral disease, or both, or deterioration of previously affected organs as detected by clinical investigations had appeared. Two of 17 patients with scleroderma who entered the study, one with diffuse scleroderma and one with localised scleroderma, treated with prednisone and D-penicillamine respectively, did not meet the disease activity criteria for inclusion in group 1 (active) or group 2 (quiescent) (table 1). Their clinical and laboratory features are reported under the heading of very low activity (group 3) in table 1.

ANTIBODIES

The following MoAbs were used: anti-monomorphic HLA-DR Ag (OKDR), anti-MLR4 T cell Ag (Tec-MLR4), anti-CD4 (OKT4; anti-Leu3), anti-CD8 (OKT8; anti-Leu2), anti-CD26 (Ta1, now identified with the ectoenzyme dipeptidylpeptidase IV),¹⁹ anti-CD25 (Tec-IL-2-R), anti-CD29 (4B4, integrin β 1 subunit of the VLA heterodimeric protein), and anti-CD45RA (2H4, the high molecular weight isoform of the leucocyte common antigen family). Monoclonal antibodies of the OK series were purchased from Ortho Diagnostics Systems (Raritan, NJ, USA) and the Leu series from Becton Dickinson (Milan, Italy). Monoclonal antibodies Ta1, 2H4, and 4B4 were from Coulter Immunology (Hialeah, FL, USA); Tec-MLR4 and Tec-IL-2-R were from Technogenetics (Milan, Italy). Monoclonal antibodies were conjugated with fluorescein isothiocyanate (FITC), phycoerythrin or biotin according to the staining protocols required by one, two, or three colour analysis.

Ta1 has been considered a marker of Ag primed T cells in vivo and of previously activated T cells (CD26+),^{7 20} which includes a group responding to recall antigens.^{7 21}

Table 1 Prominent clinical and laboratory features in various subgroups of patients with scleroderma

	Overall (n=17)	Active disease (group 1) (n=8)	Quiescent disease (group 2) (n=7)	Very low activity disease (group 3) (n=2)
Sex (M/ F)	3/14	1/7	1/6	1/1
Mean (range) age (years)	49.8 (25-68)	47.9 (25-63)	47.7 (34-68)	65 (62-68)
Mean (range) disease duration (years)	3.9 (0.5-13)	2.6 (0.5-12)	4.4 (1-9)	5.7 (3.5-8)
Subsets of scleroderma				
Diffuse systemic scleroderma	9	5	3	1
Limited systemic scleroderma	4	2	2	-
Localised scleroderma	4	1	2	1
Treatment				
D-Penicillamine	5	2	2	1
Prednisone (<5 mg/day)	3	1	1	1
Antinuclear antibodies on HEp-2 cell line	15	8	6	1
Anti-topoisomerase I	9	4	4	1
Anti-centromere	4	2	2	-

The Tec-MLR4 MoAb recognises a 130 kilodalton membrane protein expressed by mixed leucocyte reaction activated T cells, not yet CD classified.²² Peripheral blood resting T cells reacting with MLR4 MoAb (MLR4+) showed helper activity for pokeweed mitogen driven B cell differentiation, but did not proliferate to soluble Ags.²³

CELL STAINING AND FLOW CYTOMETRY

Two and three colour analysis was performed on peripheral blood lymphocytes from whole blood samples by the standard direct immunofluorescence technique in 17 and eight patients with scleroderma respectively. For three colour measurements an indirect technique was adopted, first with incubation with FITC, phycoerythrin and biotin conjugated MoAbs, followed after washing by a second incubation with avidin-Duochrome (Becton Dickinson, Mountain View, CA, USA), a tandem fluorochrome emitting at 633 nm. In some instances the first incubation with purified MoAb was followed by a second incubation with polyclonal biotinylated F(ab) goat antimouse immunoglobulin (γ and light chains) antibody (Tago, Burlingame, CA, USA). After saturation with mouse serum (Dako), avidin-Duochrome and conjugated MoAbs were added. Flow cytometry analysis was performed, after lysis of red blood cells, with a FacScan cell analyser (Becton Dickinson). Statistical analysis was performed using Student's *t* test for unpaired data.

Results

Table 2 gives the findings of two colour peripheral blood T CD4+ cell phenotypes in patients with scleroderma, SLE, and RA compared with controls. Higher CD4+CD26+ ($p<0.01$) and CD4+CD25+ ($p=0.01$) T cell percentages in patients with scleroderma and CD4+DR+ cell percentages ($p<0.001$) in

patients with RA were found, together with reduced CD4+ ($p<0.02$) and CD4+MLR4+ ($p<0.001$) and increased CD4+DR+ cell percentages ($p<0.01$) in patients with SLE. Table 3 shows the results of the two colour flow cytometry study of the distribution of accessory/activation molecules on CD8+ peripheral blood lymphocytes performed in 17 patients with scleroderma and compared with 10 healthy subjects. The patients with scleroderma showed reduced CD8+CD29+ cell percentages ($p<0.05$). The differences in the calculated absolute cell numbers of the several lymphocyte subsets between patients and controls were similar to those for relative cell counts, except for the absolute cell numbers of the CD8+CD29+ cells which were not significantly decreased compared with controls (data not shown).

Similar proportions of CD4+CD45RA+/CD4+ were found in eight patients with scleroderma (21%) (mean age 43.4 years), four of whom had active disease, and eight healthy controls (25%) (mean age 43.8 years) (data not shown).

Table 4 shows the results of the two colour expression of five activation markers by CD4+ peripheral blood cells in patients with scleroderma with active versus quiescent disease, each with respect to 10 healthy controls. The active group showed increased CD4+CD26+ T cell percentages and absolute cell numbers ($p<0.02$, $p<0.05$ respectively) and CD4+CD25+ absolute cell numbers ($p<0.02$), compared with the quiescent group. Compared with controls, the patients with active scleroderma showed higher CD4+ ($p<0.05$) cell percentages and higher CD4+CD26+ and CD4+CD25+ T cell percentages ($p<0.001$, $p=0.001$ respectively) and T cell absolute numbers ($p=0.001$, $p<0.001$ respectively). CD4+CD26+/CD4+ and CD4+CD25+/CD4+ ratios were also increased in patients with active scleroderma compared with controls ($p<0.01$, $p<0.01$

Table 2 Two colour flow cytometry analysis (FacScan) of CD4+ peripheral blood lymphocytes in patients with scleroderma, systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA), and in healthy controls. Results are mean (SD) percentages of positive fluorescent lymphocytes

	CD4+	CD4+CD26+	CD4+CD29+	CD4+DR+	CD4+CD25+	CD4+MLR4+
Scleroderma (n=17)	48.9 (7.8)	39.9 (6.8)‡	30.5 (7.6)	3.6 (1.4)	6.0 (2.1)†	6.5 (4.6)
SLE (n=10)	34.6 (12.5)*	25.2 (12.8)	28.0 (7.5)	5.1 (2.2)‡	5.3 (2.8)	1.7 (1.1)§
RA (n=5)	43.7 (10.5)	31.8 (7.0)	31.8 (10.3)	4.5 (0.6)§	3.8 (1.3)	5.1 (2.4)
Controls (n=10)	46.7 (5.9)	33.4 (3.2)	29.7 (6.5)	3.0 (0.6)	3.9 (1.4)	6.1 (1.5)

* $p<0.02$ v controls.

† $p=0.01$ v controls.

‡ $p<0.01$ v controls.

§ $p<0.001$ v controls.

Table 3 Two colour flow cytometry analysis (FacScan) of CD8+ peripheral blood lymphocyte phenotype in 17 patients with scleroderma and 10 healthy controls. CD8+ percentages are reported for comparison. Results are mean (SD) percentages of positive fluorescent lymphocytes

	CD8+	CD8+CD26+	CD8+CD29+	CD8+DR+	CD8+CD25+	CD8+MLR4+
Patients with scleroderma	26.1 (7.4)	10.1 (4.2)	16.7 (4.4)*	4.9 (2.5)	1.4 (1.9)	5.6 (3.3)
Controls	31.6 (8.2)	10.4 (4.9)	21.6 (6.2)	4.0 (1.5)	0.3 (0.1)	5.7 (1.3)

* $p<0.05$ v controls.

Table 4 Two colour flow cytometry analysis (FacScan) of CD4⁺ peripheral blood lymphocytes from patients with active and quiescent disease and healthy controls. Percentages and ratios of positive subsets are reported in part (a) and absolute numbers in part (b). Results are mean (SD) percentages (a) and absolute values (b) of positive cells reacting to the monoclonal antibody

(a) Percentages and ratio of positive subsets

	CD4 ⁺ (%)	CD4+CD26 ⁺		CD4+CD29 ⁺		CD4+DR ⁺		CD4+CD25 ⁺		CD4+MLR4 ⁺	
		%	/CD4 ⁺	%	/CD4 ⁺	%	/CD4 ⁺	%	/CD4 ⁺	%	/CD4 ⁺
Active disease (n=8)	52.7 (5.6)*	45.0 (6.0)§	85.4 (7.3)†	31.2 (5.7)	62.8 (9.0)	3.8 (1.4)	7.2 (2.7)	7.5 (2.5)‡	14.1 (4.1)†	9.1 (7.3)	17.0 (12.7)
Quiescent disease (n=7)	45.0 (8.4)	35.7 (6.3)§	79.6 (6.5)	29.0 (9.3)	66.5 (15.1)	3.6 (1.4)	8.1 (3.1)	5.2 (1.5)	11.6 (3.3)	5.5 (6.1)	11.2 (10.2)
Controls (n=10)	46.7 (5.9)	33.4 (3.2)	72.1 (7.8)	29.7 (6.5)	63.3 (10.5)	3.0 (0.6)	7.2 (1.7)	3.9 (1.4)	9.2 (1.9)	6.1 (1.5)	15.5 (4.4)

*p<0.05; †p<0.01; ‡p=0.001; §p<0.001 v controls.

||p<0.02 active v quiescent disease.

(b) Absolute values of positive subsets

	CD4 ⁺	CD4+CD26 ⁺	CD4+CD29 ⁺	CD4+DR ⁺	CD4+CD25 ⁺	CD4+MLR4 ⁺
Active disease (n=8)	1093.5 (214.7)	933.7 (197.1)*‡	644.6 (144.9)	76.5 (26.2)	152.8 (46.4)‡§	186.6 (137.5)
Quiescent disease (n=7)	854.2 (263.4)	676.4 (210.9)‡	583.5 (228.9)	69.7 (37.2)	95.4 (32.7)§	87.5 (81.0)
Controls (n=10)	906.3 (245.7)	640.3 (127.5)	579.7 (193.4)	56.5 (13.6)	64.0 (20.6)	101.8 (24.3)

*p=0.001; †p<0.001 v controls.

‡p<0.05; §p<0.02 active v quiescent disease.

respectively). No significant differences were found between patients with quiescent disease and controls.

Three colour analysis of the combined expression of CD26 and CD29 or CD45RA Ags performed on CD4⁺ peripheral blood T cells of five patients with scleroderma clearly showed a dissociation among these molecules. Direct comparison of CD26 expression with CD29 expression showed CD26⁺ and CD26⁻ cells in both CD29⁺ and CD29⁻CD4⁺ T cell subsets. Twenty to thirty per cent of CD26⁺ bright cells overlapped the area of CD26⁺ high fluorescent cells (fig 1A) and most were also

CD45RA negative (fig 1B). The MLR4⁺ cells were mostly included in the fluorescence area of CD45RA Ag (data not shown). The CD4⁺CD26⁺ cells again showed low levels of CD25 expression, and only a few cells coexpressed DR Ag (fig 2).

Discussion

Using the two colour flow cytometry technique we found normal CD4⁺CD29⁺ and CD4⁺CD45RA⁺ immunoregulatory peripheral blood T cell subsets and reduced CD8⁺CD29⁺ T cells in a random group of patients with scleroderma (table 3). Up to now, conflicting data have been reported about the imbalance of naive and memory helper T cell subclasses in scleroderma as defined by 2H4 and 4B4 MoAbs.²⁴⁻²⁶ An expansion of T CD4⁺CD29⁺, including helper memory T cells, has been reported to be associated with more prolonged disease.²⁷ According to other studies,²⁴⁻²⁶ the normal proportions of CD4⁺2H4⁺ and CD4⁺4B4⁺ peripheral blood T cell subsets may be due to the shorter disease duration of the patients (table 1). Two colour flow cytometry examination of the differential expression of several activation molecules by peripheral blood T cells in patients with scleroderma showed selective activation of the CD4⁺ fraction, with higher numbers of T CD4⁺ expressing CD25 and CD26 Ag (table 2). Moreover, when rapidly evolving versus quiescent disease was compared in our patients, CD26 expression by T CD4⁺ cells was significantly correlated with disease activity (table 4).

CD26 Ag represents an accessory pathway of T cell activation associated with the interleukin 2 (IL-2) autocrine pathway²⁸⁻²⁹ and has proved to be a marker of high IL-2 producing cells.³⁰ Thus the higher CD25 and CD26 expression by circulating CD4⁺ cells is in good agreement with the enhanced IL-2 production by T CD4⁺, already reported in vitro in scleroderma.³¹ At the single cell level the T CD4⁺ phenotype obtained with three colour flow cytometry showed a bright CD26 Ag associated with a weak IL-2R positivity and

Figure 1 Flow cytometry analysis (FacScan) of peripheral blood lymphocytes of patients with scleroderma. Upper panels, fluorescence histogram; lower panels, dot plot displays of CD4 gated cells. Panels show staining profiles with monoclonal antibodies. (A) CD29 (4B4) (light) and CD26 (Ta1) (dark); (B) CD45RA (2H4) (light) and CD26 (Ta1) (dark). Fluorescence intensity is plotted on a logarithmic scale (x axis) v cell number (y axis).

Figure 2 Flow cytometry analysis (FACS) of peripheral blood lymphocytes of patients with scleroderma. Dot plot displays of CD4 gated cells. (A) CD26 (Ta1) v OKDR monoclonal antibody. (B) CD26 (Ta1) v Tec-IL-2R. Each figure is divided into four quadrants: lower right, CD4CD26+ peripheral blood lymphocytes negative for DR (A) or IL-2R (B) Ag; upper right, peripheral blood lymphocytes simultaneously carrying CD4, CD26, and DR (A) or IL-2R (B) markers.

with the coexpression of only a low level of HLA-DR Ag (fig 2). This incomplete T CD4+ activation seems to be typical of low grade persistent stimulation of circulating helper memory cells.³² Furthermore, we found only a partial overlap between CD26 and CD29 Ags on CD4+CD45RA- T cells (fig 1A). The CD4+CD26+ subset may represent more recently activated cells than the T CD4+ coexpressing the very late CD29 Ag, as an increase in CD26 expression was already evident 48 hours after in vitro stimulation.⁷ In our patients with scleroderma, the T CD4+ two colour phenotype was different from that obtained in patients with active SLE or RA (table 4), suggesting that peripheral blood helper memory T cell hyperactivity is a distinctive feature of scleroderma.³¹ Enhanced lymphocyte-endothelial cell interaction has been reported in scleroderma, as shown by increased lymphocyte adhesion or proliferation to endothelial cells and in cell mediated endothelial cell cytotoxicity.³³ The in vitro collagen stimulation of peripheral blood CD4+ T cells in patients with scleroderma has been found to induce higher IL-2 production, confined to early onset disease.⁵ Interestingly, CD26 Ag is now known to include CD4 surface structures mediating binding activity and cell proliferation to collagen.³⁴ Therefore, CD26 upregulation on peripheral blood T CD4+ cells in patients with scleroderma may affect vascular or extracellular matrix positioning and further activation of helper memory cells at the site of tissue inflammation.¹² Moreover, the CD26 pathway may selectively regulate helper memory cell functions, as the CD26 MoAb can mediate T cell proliferation and cytolytic activity.²⁸

The high serum IL-2 level associated with the extent of skin disease in scleroderma,³⁵ and our evidence of early in vivo CD4 activation correlated with disease activity, previously unreported, support the role of a T cell mediated mechanism in the progression of the disease.

The phenotypic abnormality of a distinctive peripheral blood T cell subclass in the rapidly evolving phase of scleroderma may be of value in monitoring and managing the disease and may also indicate more specific targeting for direct immunotherapeutic intervention in scleroderma.³⁶

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