

Increase in activated T cells and reduction in suppressor inducer T cells in systemic sclerosis

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Abstract

Blood lymphocytes from 37 patients with systemic sclerosis were characterised using monoclonal antibodies in a two colour flow cytometric (fluorescence activated cell sorter (FACS)) analysis. The ratio of helper CD4⁺ to suppressor/cytotoxic CD8⁺ T cells was raised in patients compared with that in 30 healthy controls owing to decreased CD8⁺ cells. In the patients CD4⁺ and CD8⁺ cells displayed an increased expression of the activation marker HLA-DR. The relative number of CD11b⁺ CD8⁺ lymphocytes (suppressor T cells) was normal, but the calculated absolute counts of this cell type were slightly reduced. The proportions and absolute numbers of suppressor inducer T cells, defined as CD45R⁺ CD4⁺ cells, were on average only half the levels observed in controls. These findings were not related to the inflammatory activity as measured by acute phase plasma proteins or serum immunoglobulins. Activated T cells were seen at all stages of the sclerotic process and especially during the early stages of the disease and in patients who had suffered occupational exposure to silica dust. A high proportion of activated T cells was also linked with impaired small intestine function but not with the degree of skin or lung involvement. A loss of suppressor inducer T cells was more pronounced later in the disease and in patients with the CREST (calcinosis, Raynaud's phenomenon, oesophageal dysmotility, sclerodactyly, telangiectasia) syndrome. These data provide further evidence for an involvement of T cell mediated immunity in the perpetuation of systemic sclerosis.

The extent to which immunological mechanisms are important in the pathogenesis of systemic sclerosis is a widely debated but still largely unresolved question. Much evidence for immunological aberrations in systemic sclerosis has been obtained from a study of autoantibodies, and antinuclear antibodies have been shown in 60-90% of patients with this disease.¹ Although most of these autoantibodies are also found in other chronic inflammatory conditions, the presence of antibodies to centromeres^{2,3} seems to be a specific feature of a subgroup of patients with systemic sclerosis, indicating that specific immune reactions may be implicated in systemic sclerosis. An analysis of local sclerotic lesions in the skin by immunohistological methods, however, showed an infiltration of T lymphocytes and macrophages but no B cells/plasma cells,^{4,5}

suggesting that cell mediated immunity may be more important than humoral immunity in the development of systemic sclerosis.

T and B cell activation or subset markers have so far not been widely studied in systemic sclerosis. Previous studies have indicated that the CD8⁺ suppressor/cytotoxic T cells are decreased and the activated T cells are increased in the disease.⁶⁻⁹ With the availability of new monoclonal antibodies and flow cytometric (fluorescence activated cell sorter (FACS)) analysis it has become possible to phenotype T cells according to functionally distinct subsets. The CD4⁺ subset contains helper/inducer T lymphocytes and the CD8⁺ subset suppressor/cytotoxic T lymphocytes.¹⁰⁻¹² When T cells are activated by specific antigens or mitogens they acquire surface expression of HLA-DR.¹³ By the combined use of different monoclonal antibodies it is also possible to distinguish, for example, between T suppressor effector cells (CD11b⁺ CD8⁺) and T cytotoxic cells (CD11b⁻ CD8⁺).¹⁴ Within the CD4⁺ subset at least two subpopulations are recognised: cells that induce help for B cell immunoglobulin secretion and cells that induce suppression of B and T cell functions.¹⁵ The suppressor inducer T cells can be identified by estimating CD45R⁺ CD4⁺ cells.¹⁶ In an attempt to establish the possible involvement of T cells in systemic sclerosis we used monoclonal antibodies in a two colour FACS analysis to characterise peripheral blood lymphocytes according to levels of activated CD4⁺ and CD8⁺ subsets, suppressor T cells, and suppressor inducer T cells. The results showed that in patients with systemic sclerosis the numbers of phenotypically activated CD4⁺ and CD8⁺ cells were increased, while the number of circulating cells in the suppressor inducer subset was markedly depressed; these changes were related to the duration of the disease. We also searched for possible correlations between alterations in the T lymphocyte subsets and various clinical and laboratory indicators of disease activity.

Patients and methods

Thirty seven patients (20 male, 17 female, mean age 53 years, range 26-75) with progressive systemic sclerosis (27 with diffuse scleroderma and 10 with CREST) were studied. The CREST syndrome variant of systemic sclerosis was characterised by three or more of the syndrome's five signs—that is, subcutaneous calcinosis, Raynaud's phenomenon, oesophageal dysmotility, sclerodactyly, and telangiectasia—and with no scleroderma proximal to the forearm or

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involving the trunk. All patients fulfilled the 1982 American Rheumatism Association preliminary diagnostic criteria for progressive systemic sclerosis.¹⁷ At the time of lymphocyte collection all patients were inpatients of the rheumatology section of the University Hospital, Uppsala. They were all assessed for extent of skin and organ involvement. Skin involvement was scored blindly on a scale of 0 to 3 by a semiquantitative estimation of the degree and extent of skin thickening of 18 anatomic sites. Score 0 indicated uninvolved skin and 3 the most severe thickening and induration of the dermis; the maximum total skin score was 54. Raynaud's phenomenon was scored according to a four graded scale. Measurements of vital capacity, forced expiratory volume in one second, and diffusion capacity for carbon monoxide or transfer factor (single breath test) were performed at the department of clinical physiology of the University Hospital, Uppsala.¹⁸ Evidence of sclerodermal changes in the distal oesophagus was obtained by cine-graphic x ray examination. The absorption of xylose was performed as previously described.¹⁹ Inflammatory arthritis was recorded and muscle weakness and raised serum creatine kinase, suggestive of inflammatory myopathy, was confirmed by electromyography or histology.

CELLS

Mononuclear cells were separated by routine density gradient centrifugation (Ficoll-Paque, Pharmacia AB, Uppsala, Sweden) from 20 ml of heparinised venous blood (collected between 8 and 11 am). After washing, cells were suspended in RPMI 1640 medium (Flow Laboratories, Glasgow, Scotland) supplemented with 20% fetal calf serum (Flow) and 10% dimethyl sulphoxide, followed by storage in liquid nitrogen. Each individual's prospective cell samples were all thawed at the same time for phenotypic and functional studies. The absolute number of blood lymphocytes was determined in a Technicon H1 differential counter.

ANTIBODIES AND FACS ANALYSIS

Mononuclear cells were washed, suspended ($10^6/50 \mu\text{l}$) in phosphate buffered saline supplemented with 2% fetal calf serum plus 0.1% sodium azide and incubated for 15 minutes at room temperature with combinations of optimally diluted fluorescein isothiocyanate labelled and phycoerythrin labelled monoclonal antibodies (Becton Dickinson, Mountain View, CA) to lymphocyte subsets or activation antigens (table 1).^{10 11 14 16 20} After washing, cells were suspended in the same medium supplemented with 1% paraformaldehyde (BDH, Poole, England). Fluorescent cells were analysed in a FACStar (Becton Dickinson) analyser/sorter equipped with a 5 W argon laser emitting at 488 nm and run at 0.2 W. Ten thousand lymphoid cells were gated, and red (phycoerythrin) fluorescence was collected through a 585/42 nm filter and green (fluorescein isothiocyanate) fluorescence through a 530/30 nm filter. Data were processed in a Hewlett-Packard 217 com-

Table 1: Monoclonal antibodies, their specificities, and references

MAbs*	CD*	Cell population defined	Ref
Leu 3	4	Helper/inducer T cells	10
Leu 2	8	Suppressor/cytotoxic T cells	11
HLA-DR+Leu 3	ns+4	Activated Leu 3 cells	20 10 13
HLA-DR+Leu 2	ns+8	Activated Leu 2 cells	20 10 13
Leu 18+Leu 3	45R+4	Suppressor inducer T cells	16
Leu 15+Leu 2	11b+8	Suppressor T cells	14

*MAbs=monoclonal antibodies; CD=cluster of differentiation; ns=CD number not specified.

puter (H-P, Fort Collins, CO) with the Consort 30 software (Becton Dickinson). As controls we investigated 30 healthy individuals of both sexes aged 24–72 years, using the same markers and methods as used for the patients with scleroderma.

Serum concentrations (reference ranges) of the acute phase proteins haptoglobin (0.3–2.0 g/l) and orosomucoid (0.4–1.2 g/l) and the immunoglobulins G (7–18 g/l), A (0.8–4.0 g/l), and M (0.4–2.8 g/l) were measured at the department of clinical chemistry of the University Hospital, Uppsala. Erythrocyte sedimentation rate was read after one hour according to Westergren. Antinuclear antibodies were determined using rat liver sections at the department of bacteriology of the University Hospital, Uppsala. Results were considered positive if fluorescence was present at a titre >1/25.

Statistical differences between groups and correlations were estimated by Wilcoxon's non-parametric tests. This study was performed according to principles of the Declaration of Helsinki and was approved by the local ethical committee.

Results

LYMPHOCYTE ACTIVATION AND SUBCLASS MARKER EXPRESSION IN SYSTEMIC SCLEROSIS

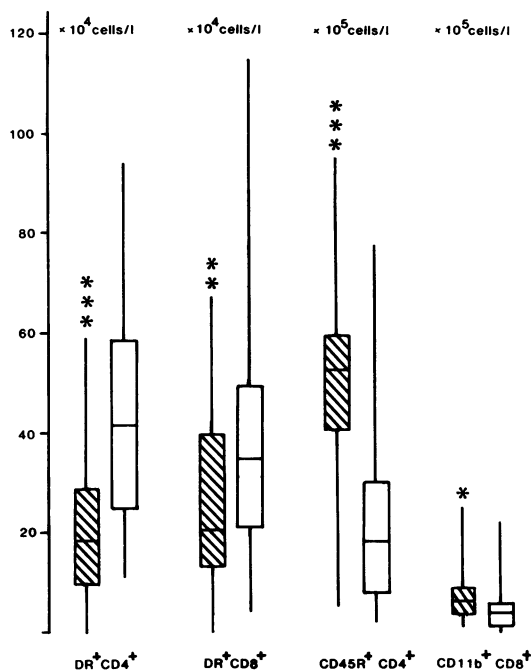
The percentage (mean (SD)) of circulating CD4⁺ lymphocytes in patients with systemic sclerosis was 51 (20)% and similar to that found in the healthy controls (53 (8)%). The CD8⁺ cells (mean (SD) value 21 (8)%) were significantly ($p<0.001$) decreased compared with the controls (29 (5)%). The ratio of circulating CD4⁺ to CD8⁺ T cells was 2.8 (SD 1.9) in the patient group and was significantly ($p<0.01$) greater than that in the control group (1.9 (0.5)). Table 1 presents the lymphocyte activation and subclass marker expression in patients with systemic sclerosis and in controls. The median percentage of circulating HLA-DR⁺ CD4⁺/total CD4⁺ cells was 5% in patients with systemic sclerosis—that is, a 2.5-fold increase compared with the controls ($p<0.001$). Significantly ($p<0.001$) increased percentages of DR⁺ CD8⁺/total CD8⁺ cells were also seen in the patient group. The proportion of circulating CD45R⁺ CD4⁺/total CD4⁺ cells in patients with systemic sclerosis was decreased and was, on average, half that observed in the controls

($p < 0.001$). The lymphocyte subpopulation $CD11b^+ CD8^+$ /total $CD8^+$ cells was similar in patients and controls (table 2).

The median number (first and third quartiles in parentheses) of circulating lymphocytes in the patients was $1.7 (1.5-1.9) \times 10^7/l$ and in the controls $1.8 (1.3-2.2) \times 10^7/l$. The differences in the calculated absolute cell counts of the different lymphocyte subpopulations (figure) between patients and controls were similar to those for the relative cell counts except for the numbers

of $CD11b^+ CD8^+$ cells; the absolute number of these cells in contrast with the relative number was significantly reduced in the patient group. No sex or age dependency of the lymphocyte subpopulations was noted in patients and controls.

In the patient group the percentage of $DR^+ CD4^+$ /total $CD4^+$ cells was clearly related to the proportions of the other lymphocyte subpopulations studied (table 3), while in the controls the proportion of $DR^+ CD4^+$ cells was only related to the proportion of the $DR^+ CD8^+$ cells.



Lymphocyte subsets (activated $CD4^+$ and $CD8^+$ lymphocytes, suppressor inducer T cells ($CD45R^+ CD4^+$), and suppressor T cells ($CD11b^+ CD8^+$)) presented as absolute cell numbers in peripheral blood from 30 healthy controls and 37 patients with systemic sclerosis. The data are presented as box plots (with medians and first and third quartiles) and actual ranges. The open boxes represent data from the patients and the hatched boxes data from the controls. Significant differences between patients and controls are flagged (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

LYMPHOCYTE SUBPOPULATIONS AND DISEASE DURATION

The duration of disease for patients with systemic sclerosis ranged from 0.5 to 25 years with a median duration of five years. Tendencies towards inverse relations between disease duration and percentages of $DR^+ CD4^+$ cells ($r = -0.22$; $p = 0.08$), $DR^+ CD8^+$ cells ($r = -0.27$; $p = 0.06$), or $CD45R^+ CD4^+$ cells ($r = -0.26$; $p = 0.06$) were indicated. When the patients were subgrouped with respect to disease duration those patients who had a disease duration < 2 years ($n = 7$) had significantly increased proportions of $DR^+ CD4^+$ (median 8%; first and third quartiles (4-8) and $DR^+ CD8^+$ cells (13%; 11-13) compared with the patients with a disease duration > 2 years; their corresponding values were 4.5%; 3-6 ($p < 0.05$) and 8.5%; 4-10 ($p < 0.01$) respectively. The percentage of $CD45R^+ CD4^+$ cells was lower in patients with a longer disease duration (25%; 16-35) than in patients with a short disease duration (37%; 17-37), but the difference did not reach statistical significance.

LYMPHOCYTE SUBPOPULATIONS AND CLINICAL PICTURE

Lung function in the patient group was studied by vital capacity (VC), forced expiratory volume

Table 2: Lymphocyte activation and subclass marker expression* in patients with systemic sclerosis and in healthy controls

	Patients (n=37)	Controls (n=30)	p Value†
$DR^+ CD4^+$ /total $CD4^+$ cells (%)	5.0 (3.0-8.0)	2.0 (1.0-3.0)	< 0.001
$DR^+ CD8^+$ /total $CD8^+$ cells (%)	9.0 (5.0-12)	4.5 (3.0-7.0)	< 0.001
$CD45R^+ CD4^+$ /total $CD4^+$ cells (%)	26 (17-42)	55 (47-62)	< 0.001
$CD11b^+ CD8^+$ /total $CD8^+$ cells (%)	10 (5-17)	12 (6-15)	NS

*Medians with first and third quartiles in parentheses.

†Statistical comparison between patients and controls (Wilcoxon's non-parametric test).

Table 3: Correlation coefficients between lymphocyte activation and subclass expression* markers in patients with systemic sclerosis and healthy controls

	$DR^+ CD4^+$ (%)	$DR^+ CD8^+$ (%)	$CD45R^+ CD4^+$ (%)	$CD11b^+ CD8^+$ (%)
Systemic sclerosis				
$DR^+ CD4^+$ (%)	—	0.53***	-0.29*	0.51***
$DR^+ CD8^+$ (%)	—	—	-0.01	0.41***
$CD45R^+ CD4^+$ (%)	—	—	—	-0.10
Controls				
$DR^+ CD4^+$ (%)	—	0.48**	0.04	0.21
$DR^+ CD8^+$ (%)	—	—	-0.07	-0.01
$CD45R^+ CD4^+$ (%)	—	—	—	-0.10

Correlations tested by Wilcoxon's rank sum test: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

(FEV), and transfer factor (TLCO). The mean (SD) VC was 77 (19)% of predicted value, FEV 76 (18)% of predicted value, and TLCO 59 (22)% of predicted value. The lymphocyte subpopulations were not related to the degree of lung affection as measured by these variables.

Most patients underwent a xylose absorption test. In 14 patients abnormal xylose absorption was noted. These patients had significantly increased proportions of DR⁺ CD4⁺ and DR⁺ CD8⁺ cells compared with those patients (n=20) with no signs of malabsorption (table 4).

All patients suffered from skin involvement (scored 0–54) and from Raynaud's disease (scored 0–4). The mean skin score reflecting the skin involvement was 25, range 4–41. The skin or Raynaud's disease scores did not relate to the lymphocyte subpopulations. At the time of the investigation 15 of the patients suffered from ulcerations of fingers or toes, or both. Patients with ulcerations had a significantly (p<0.01) reduced proportion of CD45R⁺ CD4⁺ cells (19%; 10–24) compared with those lacking ulcerations (28%; 19–42). The other lymphocyte subsets were similar in both subgroups (data not shown). Four of the patients had clinical and laboratory signs of myositis and three had inflammatory arthritis. Their lymphocyte subsets did not differ from those of other patients.

Ten of the patients had the CREST syndrome. These patients had a significantly lower proportion of CD45R⁺ CD4⁺ cells than the patients without CREST (table 5).

LYMPHOCYTE SUBPOPULATIONS AND EXPOSURE TO SILICA DUST

Ten of the patients, all men, had been occupationally exposed to silica dust as miners, rock blasters or drillers, foundry workers, or cement factory workers. These patients had significantly increased percentages of DR⁺ CD4⁺ and DR⁺ CD8⁺ cells compared with the non-exposed male patients (table 6).

LYMPHOCYTE SUBPOPULATIONS AND SEROLOGICAL ABNORMALITIES

The inflammatory activity was defined by erythrocyte sedimentation rate (ESR) and the acute phase reactant haptoglobin. The median ESR was 20 mm/h, range 3–95 and the median haptoglobin value was 2.0 g/l, range 1.4–4.1. No relation was seen between the lymphocyte subpopulations and ESR or haptoglobin, either calculated by regression analyses or on patients subgrouped with respect to absence (n=18) or presence (n=19) of inflammatory activity—that is, ESR >15 mm/h or haptoglobin >2.0 g/l.

The antinuclear antibody titre varied between 0 and 1/3200; eight of the patients were antinuclear antibody negative—that is, had an antinuclear antibody titre <1/25. No relation was seen between the lymphocyte subpopulations and the antinuclear antibody titre. The serum concentrations of immunoglobulin G (mean (SD) value 13.4 (4.5) g/l), immunoglobulin A (2.9 (1.7) g/l), or immunoglobulin M (1.6 (1.0) g/l) did not correlate with the lymphocyte subsets.

Table 4: Lymphocyte activation and subclass marker expression* in patients with systemic sclerosis subgrouped with respect to the xylose absorption test

	Normal xylose absorption (n=20)	Reduced xylose absorption (n=14)	p Value
DR ⁺ CD4 ⁺ /total CD4 ⁺ cells (%)	4 (3–6)	7 (4–8)	<0.01
DR ⁺ CD8 ⁺ /total CD8 ⁺ cells (%)	7.5 (3.8–11.5)	10 (9–13)	<0.01
CD45R ⁺ CD4 ⁺ /total CD4 ⁺ cells (%)	26.5 (21.5–39.5)	17.5 (11–42)	NS
CD11b ⁺ CD8 ⁺ /total CD8 ⁺ cells (%)	9.5 (4.3–16)	10.5 (6–16)	NS

*Medians with first and third quartiles in parentheses.

Table 5: Lymphocyte activation and subclass marker expression* in patients with systemic sclerosis subgrouped with respect to the CREST syndrome

	CREST (n=10)	Non-CREST (n=27)	p Value
DR ⁺ CD4 ⁺ /total CD4 ⁺ cells (%)	4.5 (2.5–5)	5 (3–8)	NS
DR ⁺ CD8 ⁺ /total CD8 ⁺ cells (%)	9.5 (2.5–12)	9 (5–11)	NS
CD45R ⁺ CD4 ⁺ /total CD4 ⁺ cells (%)	19 (10–25)	27 (18–42)	<0.001
CD11b ⁺ CD8 ⁺ /total CD8 ⁺ cells (%)	7.5 (5–11)	10 (4.5–16)	NS

*Medians with first and third quartiles in parentheses.

Table 6: Lymphocyte activation and subclass marker expression* in male patients with systemic sclerosis subgrouped with respect to exposure to silica dust

	Silica exposed (n=10)	Not silica exposed (n=10)	p Value
DR ⁺ CD4 ⁺ /total CD4 ⁺ cells (%)	7 (4–8)	3.2 (3–4)	<0.05
DR ⁺ CD8 ⁺ /total CD8 ⁺ cells (%)	10.5 (8–13)	6 (2.5–9.0)	<0.05
CD45R ⁺ CD4 ⁺ /total CD4 ⁺ cells (%)	25 (16–27)	22.5 (4–35)	NS
CD11b ⁺ CD8 ⁺ /total CD8 ⁺ cells (%)	13.5 (8–20)	10.8 (7–13)	NS

*Medians with first and third quartiles in parentheses.

Discussion

Characterisation of peripheral blood T lymphocytes in patients with systemic sclerosis showed an increased ratio of helper CD4⁺ to suppressor/cytotoxic CD8⁺ T cells, primarily due to a reduction in CD8⁺ cells. The suppressor fraction of the CD8⁺ cells, defined as CD11b⁺ cells, was normal. Our results also suggested that patients with systemic sclerosis had higher counts of phenotypically activated, HLA-DR positive CD8⁺ and CD4⁺ cells than the controls but reduced numbers of CD45R⁺ CD4⁺ cells—that is, suppressor inducer T lymphocytes.¹⁶ Previous reports on T lymphocyte subpopulations in systemic sclerosis are partially conflicting, but the results of most studies support an increased T helper/T suppressor ratio in this disease.^{6–9} Recently, the percentage of T lymphocytes bearing activation markers was reported to be increased in patients with systemic sclerosis.⁹ The number of suppressor inducer T cells has not been evaluated before in systemic sclerosis but was recently reported to be reduced among peripheral blood lymphocytes in multiple sclerosis²¹ and among synovial fluid lymphocytes in rheumatoid arthritis.²²

The appearance of activated circulating T cells is a common finding in patients with human autoimmune conditions such as systemic lupus erythematosus,¹³ multiple sclerosis,²³ primary biliary cirrhosis,²⁴ aplastic anaemia,²⁵ and insulin dependent diabetes mellitus.²⁶ In these diseases as well as in systemic sclerosis the presence of activated T cells probably implies a continuing T cell dependent immune reaction somewhere in the body. The nature of the activation process in systemic sclerosis is unknown, but the activating mechanism seems to be present during the early stages of the disease. The observation that patients with prolonged disease and clinical stabilisation of the sclerotic process also expressed HLA-DR on their circulating T cells indicates that the activation is not only present as an initial transient event but is maintained throughout all stages of the disease. Thus most and perhaps all patients with systemic sclerosis presumably during some period of their disease have activated T cells in their circulation. Patients who had been exposed occupationally to silica dust had a significantly higher degree of T cell activation than patients without this exposure, suggesting that silica may somehow, probably via unspecific immunostimulation, enhance T cell activation. The association between silica exposure and subsequent development of scleroderma has been proposed by several investigators,^{27–30} but the pathophysiological link has not been identified. The pattern of activated T cells in systemic sclerosis was not related to the inflammatory activity defined by acute phase plasma proteins or ESR; neither was any relation seen with the plasma immunoglobulin concentrations or the presence of antinuclear antibodies. No consistent pattern was seen between the severity of the disease and T cell activation, though a higher degree of activation was linked to more severe affection of the small bowel. The presence of activated T cells in the circulation at various stages of systemic sclerosis may nevertheless

only be secondary to the hitherto unknown pathogenetic process. It is conceivable that repeated activation of T cells may be critical for the perpetuation of systemic sclerosis, and, if so, interference with this T cell activation might be of benefit particularly in early but also in some late stages of systemic sclerosis. The present direct estimation of activated T cells seems to be the only possible way of identifying stages with continuing T cell activation, as high levels of activated T cells did not correlate with any of the conventionally used activation markers.

Another observation suggestive of T cell involvement in systemic sclerosis was the paucity of the suppressor inducer T cell subset. In contrast with the appearance of activated T cells during the early stages of the disease, loss of suppressor inducer T cells was more apparent later in the sclerotic disease process. In multiple sclerosis a loss of suppressor inducer cells is not observed in patients with the acute form of the disease but only in patients with a chronic progressive disease.²¹ Further, Akbar *et al* recently reported that CD4⁺ cells lose the CD45R (200/220 kD) epitope and acquire expression of a 180 kD surface epitope (detected by the UCHL1 monoclonal antibody) after *in vitro* priming with antigen.³¹ Specific allo-antigen reactive CD4⁺ memory cells possess the CD45R⁻ UCHL1⁺ phenotype. Possibly, chronic stimulation of 'naive' CD45R⁺ T helper/inducer cells by a putative autoantigen in systemic sclerosis might result in accumulation of CD45R⁻ memory cells, though direct evidence at present is lacking. The mechanisms underlying the loss of these potentially important cells in immunoregulation are not known. Viral infection of the cells, lymphocytotoxic antibodies, or extravascular influences on cell surface structure are all potential mediators, however. The biological implications of the observed reduction of the number of suppressor inducer cells are uncertain as it is unknown to what extent they are in an activated state and thus actually operative. The possibility remains that the observed loss of CD45R⁺ CD4⁺ cells in systemic sclerosis results in a decrease of T cell suppression and an activation of T and B cells reacting with certain constituents of the connective tissue. Thus the finding that patients with the CREST syndrome had a more pronounced reduction of suppressor inducer T cells than patients with diffuse scleroderma may be significant.

In conclusion, our present data provide further support for the hypothesis that cell mediated immunity, as indicated by activated T cells and deficiency of suppressor inducer T cells, is involved in the pathogenesis of systemic sclerosis.

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