Annals of the Rheumatic Diseases 1994; **53**: 122–127

Increased frequency of in vivo *hprt* gene-mutated T cells in the peripheral blood of patients with systemic sclerosis

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Abstract

***Objectives*—Activated T lymphocytes are involved in the pathogenesis of scleroderma (systemic sclerosis, SSc); such cells rapidly divide in vivo and are thus theoretically subject to random mutation more frequently than resting cells. To study whether SSc is associated with rapidly expanding T cell clones the frequency was determined of in vivo mutated T cells (MF) at the hypoxanthine guanine phosphoribosyl transferase (*hprt*) gene in the peripheral blood from patients with SSc. Specific clinical or serological associations were also investigated.**

***Methods*—Peripheral blood lymphocytes from 16 healthy individuals and 20 patients with SSc were cultured using an *hprt* clonal assay; mutated and wild T cell clones were established to assess individual values of T cell MF. T cell clones were further expanded in vitro and their phenotype was determined by standard immunofluorescence technique. Enzyme-linked immunosorbent assays were used for simultaneous measurements of plasma levels of soluble Interleukin-2 receptors (s-IL-2R) and Intercellular adhesion molecule-1 (s-ICAM-1).**

***Results*—Mean (SD) value of T cell MF in patients with SSc was 2·5-fold higher than the normal mean (SD) value [10·6 (6·6) $\times 10^{-6}$ *v* [4·4 (2·8) $\times 10^{-6}$, p = 0·0007]. Eleven of 20 patients with SSc (55%) had T cell MF values greater than two SD above the normal mean value. The majority (84%) of mutated T cells had a helper/inducer, memory phenotype while 12% were cytotoxic/suppressor T cells. There was no association between T cell MF and the extent of skin involvement or the duration of Raynaud's phenomenon. High individual T cell MF values were not related to a possible concurrent immune overactivity as assessed by plasma levels of s-IL-2R and s-ICAM-1. Patients with long standing skin disease, however, had almost double T cell MF values than patients with early skin disease [(13·6 (7·4)) $\times 10^{-6}$ *v* (7·5 (4·3)) $\times 10^{-6}$, p = 0·03], suggesting that increased T cell MF in SSc may reflect an ongoing process of chronic in vivo T cell proliferation and/or prolonged survival.**

***Conclusion*—Increased in vivo T cell mutation in patients with SSc suggests that excessive division and/or survival of T cell clones contribute to the pathology in SSc; this approach can be used in further investigations to identify the stimulus that is triggering T cell activation in this disease.**

(*Ann Rheum Dis* 1994; **53**: 122–127)

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Accepted for publication 1 November 1993

Scleroderma (systemic sclerosis, SSc) is a systemic autoimmune disorder of unknown origin.[1 2] T cell infiltration in early sclerodermatous lesions,[3] as well as the presence of activated T cells in the peripheral blood of patients with SSc,[4–6] suggests that T cells may either directly (by reacting to self endothelial cells),[7–9] or indirectly, (through the release of cytokines such as interleukin 2, interleukin 4 and transforming growth factor-β)[10–12] play a central role in the development of vascular damage and fibrosis in patients with SSc. The hypothesis that a T cell-mediated autoimmune process is primarily involved in SSc is also suggested by its HLA class-II association. This hypothesis, however, remains unproven.[1 2]

Cells undergoing rapid division in vivo are subject to a higher rate of spontaneous somatic mutation, presumably as a result of errors in replication, insufficient DNA repair, or fixation of the mutation. An in vivo mutation therefore occurs more frequently in activated T cells compared with resting T cells. T cells carrying mutations at the hypoxanthine guanine phosphoribosyl transferase (*hprt*) gene, that codes for the salvage pathway, enzyme *hprt*, can be clonally selected in culture by virtue of growth in the presence of 6-thioguanine which is deleterious for all cells having intact *hprt* gene.[13 14] By comparing the in vitro cloning efficiency of T cells in the absence or in the presence of 6-thioguanine, it is possible to estimate the frequency of in vivo mutated T cells in peripheral blood lymphocytes.[15 16] Recently, increased frequency of *hprt* mutant T cells in patients with multiple sclerosis[17] and systemic lupus erythematosus (SLE)[18] have been reported. Furthermore, by using the *hprt* clonal assay, it is possible to isolate and expand T cells carrying mutations that have occurred already in vivo, presumably in the presence of self-antigen(s),[17] or environmental muta-

gen(s).[19] Mutant T cell clones from patients with multiple sclerosis demonstrated reactivity to myelin basic protein, a putative self-antigen in this disease.[17]

To study whether SSc is associated with rapidly expanding T cell clones we determined the in vivo frequency of mutated T cells in peripheral blood lymphocytes in patients with SSc, and studied the immunophenotype of mutated, as well as of non-mutated (wild), T cell clones derived from patients with SSc and normal individuals. In addition, we searched for any specific clinical associations, as well as for any possible correlation between circulating levels of immune activation markers, such as soluble Interleukin-2 receptor and Intercellular adhesion molecule-1, and the in vivo frequency of mutated T cells in the peripheral blood of these patients.

Methods

PATIENTS AND CONTROLS

Twenty patients [16 women, four men, mean (SD) age 42 (18) years, range 9–71 years] all of whom fulfilled the American Rheumatism Association criteria for diagnosis of definite SSc[20] were studied. Fifteen of 20 patients had diffuse SSc and five patients had limited skin disease, according to the classification by LeRoy *et al.*[21] Disease duration was calculated from time of onset of the first clinical event, other than Raynaud's phenomenon, that was a clear manifestation of SSc. Skin involvement marked the onset of the disease in all patients. Disease duration of three years was chosen as the dividing point between early and late disease.[22] All patients had Raynaud's phenomenon and its duration was also recorded. None of the patients was receiving any immunomodulatory treatment at the time of the study. Patients who had received immunomodulatory treatment other than low doses of prednisone were excluded. The protocol was approved by the Department of Clinical Investigation, Walter Reed Army Medical Center. Informed consent was obtained from all patients before venepuncture, and review of the medical records.

hprt CLONAL ASSAY

Peripheral blood mononuclear cells (PBMC) were isolated by gradient centrifugation over Ficoll-Hypaque (Sigma, St Louis, MO) of heparinised venous blood. PBMC ($\times 10^6$/ml) were cultured immediately in RPMI-1640 (Gibco, Grand Island, NY), supplemented with 20 mM Hepes Buffer (Gibco), 2 mM L-glutamine (Gibco), penicillin/streptomycin (Gibco), 5% fetal calf serum (FCS) heat inactivated (Sigma), 20% nutrient medium HL-1 (Ventrex, Portland, ME), in the presence of 1 μg/ml phytohemagglutinin (PHA, HA-16/17, Wellcome Diagnostics, Dartford, UK), for 38 hours. This priming period allows the maximum number of cells to acquire the IL-2 receptor but is not sufficient for cell division to occur.[15] Subsequently the cells were suspended and counted using a haemocytometer. Cells were inoculated at 5, 2, 1 and 0·5 cells per well to determine cloning efficiency (CE), in 0·2 ml of non-selection growth medium, in 96-well round bottom microtitre plates (Corning Glass Works, Corning, NY). The limiting dilution protocol was always the same. For each cell concentration, at least 144 wells were plated. Non-selection growth medium contained 65% RPMI-1640 supplemented as described above, 5% FCS, 20% HL-1, 10% human T-STIM-^{tm} without PHA, (an enriched mixture containing IL-2 and other lymphokines, Collaborative Research Inc., Bedford, MA), and 0.1 μg/ml PHA. In parallel, 2×10^4 cell per well were inoculated in selection medium (434 wells at least for each experiment) to determine the mutant frequency (MF). Selection medium was the non-selection growth medium as described above supplemented with 10^{-5} M of 6-thioguanine (2-amino-6-mercapto-purine; Sigma). Only the cells that carry a mutation at the *hprt* gene are 6-thioguanine resistant. As a source of feeder cells the human lymphoblastoid cell line WIL-2 that carries a mutation at the *hprt* gene, designated TK6, was used. Mycoplasma-free TK6 cells (American Type Culture Collection, Rockville, MD) were irradiated with 8000 rads from a ^{137}Cs source and added (15×10^3 cells per well) in all wells. In every experiment 72 wells containing growth medium and irradiated TK6 cells only served as negative control. The microcultures were incubated at 37°C in a humidified 5% CO_2 atmosphere for 11 days to allow colony growth.

EVALUATION OF CLONING EFFICIENCY AND MUTANT FREQUENCY OF T CELLS

The microcultures were scored for colony growth at the end of the incubation period using a phase contrast inverted microscope. Wells were classified as positive or negative for growth. All colonies that are grown under these conditions contain exclusively T cells.[13–19] Assuming a Poisson distribution of clonable cells in limiting dilution studies[23] the cloning efficiency (CE) values for the platings of 5, 2, 1 and 0·5 cells per well were calculated as follows: CE = –ln (fraction of negative wells in non-selection medium)/number of cells per well.

The mutant frequency of T cells (MF) was determined as the ratio of the cloning efficiencies in selection and non-selection medium, as follows: MF = –ln (fraction of negative wells in selection medium)/2×10^4/mean CE, where mean CE is the mean of the CE values for the platings of 5, 2, 1 and 0·5 cells per well.

The CE values for the 5, 2, 1, and 0·5 cell/well platings do not represent the true cloning efficiency of T cells because the initial haemocytometer count does not distinguish between T and B cells or monocytes. However, the ratio of the cloning efficiencies in selection and non-selection medium, that is, the MF represents the true frequency of *hprt* mutant T cells because it compares T cell mutants to total clonable T-cells.

EXPANSION OF T-CELL CLONES FOR FURTHER STUDY

After scoring, T cells from positive wells that had been seeded with 0·5 cell/well (wild clones) and T cells from positive wells cultured in the presence of 6-thioguanine (mutant clones) were replated in 24 well plates (Gibco) and cultured in non-selection or selection medium respectively. Irradiated TK6 cells were added at 5×10^4 cells/ml. After 3–4 days the growing clones were transferred into larger volumes and cultured in growth medium for varying periods (up to two weeks) using neither feeder cells or 6-thioguanine. At the point where each T-cell clone reached a cell number of $3–4 \times 10^6$ cells, cells were harvested and used for further study. Generally, no differences were observed in the growth of clones between mutant and wild clones within each experiment.

IMMUNOFLUORESCENCE ANALYSIS OF T-CELL CLONE PHENOTYPE

One and two colour direct or indirect immunofluorescence was performed using a Becton Dickinson fluorescence-activated cell sorter (FACS) according to standard techniques. The following monoclonal antibodies were used: anti-CD-2 (T11), anti-CD3 (T3), anti-CD4 (T4), anti-CD8 (T8), anti-CD5 (T1), anti-CD19 (B4), anti-CD20 (B1), anti-CD25 (IL-2R1), anti-CD45RA (2H4) and anti-CD29 (4B4) were obtained from Coulter (Hialea, FL), anti-HLA-DR was obtained from Becton Dickinson, (Mountain View, CA), anti-CD45RO (UCHL1) was obtained from Daco, (Glostrup, Denmark). In addition, the monoclonal antibodies to human T cell receptor (TCR), anti-αβTCR (Identi-T Pan-TCRαβ) and anti-δTCR (Identi-T TCRδ 1) obtained from T Cell Sciences, (Cambridge, MA), were used.

DETERMINATION OF SOLUBLE IL-R (S-IL-2R) AND ICAM-1 (S-ICAM-1) LEVELS IN THE PLASMA OF PATIENTS

Plasma levels of s-IL-2R were measured with a sandwich enzyme linked immunosorbent assay (ELISA), using two different monoclonal antibodies against the a-chain (Tac molecule or p55) of the IL-2 receptor (T-cell Sciences). Plasma s-IL-2R values are expressed in units/ml relative to a set of standards supplied with the test kit. Intra-and interassay coefficients of variation were 3·2% and 10%, respectively, and recovery was 95–103%.

Levels of s-ICAM-1 were measured with a sandwich ELISA method (Bender Med Systems, Vienna, Austria) using two different mouse monoclonal antibodies against the extracellular domain D4 (CL203·4) and the domain D2 (R6·5) of the ICAM-1 molecule.[24] This assay is highly specific for the measurement of human s-ICAM-1 and has a minimum sensitivity of 0·625 ng/ml. Intra- and interassay coefficients of variation were 3·8 and 7·8 respectively, and mean recovery was 103%.

Table 1 Assessment of frequency of mutant T cells in serial samples from three normal individuals using the hprt *clonal assay*

Subject	MF* in sample number			Mean (SD)
	1	2	3	
A	7·9	4·2	5·8	6·0 (1·9)
B	2·8	3·2	4·9	3·6 (1·1)
C	4·3	8·5	8·8	7·2 (2·5)

*MF is expressed as number of mutants per million cells.

STATISTICAL ANALYSIS

Differences between proportions were examined using the chi-square test and differences between mean values were determined by Student's *t* test for unpaired variables. Linear regression analysis was used to assess correlation between individual value of MF and plasma s-ICAM-1 or s-IL-2R levels. Data are expressed as mean (1SD). P values greater than 0·05 were considered non-significant.

Results

DETERMINATION OF *hprt* GENE T CELL MF IN PATIENTS WITH SSC

The in vivo T cell MF in the peripheral blood of 20 patients with SSc and 16 healthy control individuals was determined using the *hprt* clonal assay. We studied three normal individuals repeatedly (table 1). The relatively low variance of three determinations in each of the subjects studied enabled us to conclude that the used clonal assay has acceptable reproducibility. The mean (SD) MF of the control group (n = 16) was $[4·4\ (2·8)] \times 10^{-6}$ (range from $0,6 \times 10^{-6}$ to $10,9 \times 10^{-6}$). The T cell MF in the group of patients with SSc ranged from $1·9 \times 10^{-6}$ to $30·2 \times 10^{-6}$ (table 2), while the mean was $10·6\ (6·6) \times 10^{-6}$, that is, 2·5 times higher than the mean value of the control group (p = 0·0007). Eleven of 20 patients had values higher than 10×10^{-6} which is two SD above the mean value of the control group. The CE was almost similar in patients and controls [0·42 (0·24) and 0·44 (0·25) respectively], thus the difference of T cell MF between patients and controls was not due to differences in CE of T cells.

Table 2 Pertinent clinical feature, CE and T cell MF in patients with systemic sclerosis

Patient no	Age/ Sex	Years of Raynaud's/ skin disease	Extent of skin disease*	CE	MF‡
1	52/F	12/11	D	0·15	30·2
2	48/F	22/16	D	0·14	19·6
3	47/F	12/10	D	0·27	16·1
4	46/F	4/3	D	0·35	13·3
5	40/F	4/4	D	0·31	13·1
6	40/F	14/14	D	0·44	13·0
7	30/F	1/1	L	0·33	12·5
8	46/F	40/35	L	0·55	11·8
9	62/M	6/5	L	0·21	11·3
10	34/F	3/3	D	0·22	10·5
11	71/F	33/20	L	0·42	10·4
12	9/F	0·5/0·5	D	0·14	9·3
13	62/F	30/2	L	0·24	9·3
14	52/M	1/1	D	0·64	7·3
15	42/M	7/7	D	0·63	6·6
16	36/F	3·5/3	D	0·95	5·0
17	34/M	6/5	D	0·75	3·5
18	12/F	2/2	D	0·64	3·4
19	13/F	2·5/2·5	D	0·34	2·9
20	70/F	4/2	D	0·75	1·9

*Diffuse (D) or limited (L) disease, ‡MF is expressed as number of mutants per million cells.

Table 3 T cell MF in various clinical subgroups of patients with systemic sclerosis

Group (n)		*MF, mean/(SD) (range)**		*p‡*
Total SSc	(20)	10·6 (6·6)	(1·9–30·2)	0·0007§
Diffuse SSc	(15)	10·4 (7·6)	(1·9–30·2)	NS†
Limited SSc	(5)	11·1 (1·2)	(9·3–12·5)	
Early SSc	(10)	7·5 (4·1)	(1·9–13·3)	0·03¶
Late SSc	(10)	13·6 (7·4)	(3·5–30·2)	
Healthy controls	(16)	4·4 (2·8)	(0·6–10·9)	

*MF is expressed as number of mutants per million cells. ‡Determined by group *t* test, §Versus healthy controls, †Versus limited SSc. ¶Versus Late SSc.

CLINICAL ASSOCIATIONS

The pertinent clinical features and individual CE and T cell MF values are presented in table 2. It is obvious that the age of the patients does not correlate with the T cell MF values. However, because three patients aged nine, 12 and 13 years had a relatively low mean MF value ($5{\cdot}2 \times 10^{-6}$), we studied two normal children of the same age and sex and their T cell MF values were much lower ($0{\cdot}6 \times 10^{-6}$ and $3{\cdot}6 \times 10^{-6}$, respectively). We then searched for possible associations between the extent of skin involvement and disease duration and MF values in patients with SSc. The range and the mean T cell MF of subgroups of patients with diffuse or limited disease, as well as with early or late disease are shown in table 3. The mean MF value in patients with diffuse truncal skin involvement was similar to that of patients with disease limited to the hands and face. Patients with duration of skin disease greater than three years had two-fold higher mean T cell MF value than patients with skin disease duration less than three years ($p = 0{\cdot}03$), table 3). In contrast, the duration of Raynaud's phenomenon did not correlate with T cell MF (table 2).

CORRELATION OF T CELL MF AND CIRCULATING MARKERS OF IMMUNE SYSTEM ACTIVATION

In an attempt to assess whether concomitant immune system overactivity is associated with the presence of increased numbers of mutated T cells in patients with SSc, we measured s-IL-2R and s-ICAM-1 in the plasma of patients that was drawn at the time of the performance of these experiments. Both s-IL-2R and s-ICAM-1 molecules have been found to be useful markers of inflammation and immune system activation in patients with SSc.[25–29] Interestingly, no correlation (p, NS) between either T cell MF values and s-IL-2R levels or s-ICAM-1 levels was found in patients with SSc (table 4), a finding that suggested that an increased T cell MF is not necessarily accompanied by immune system overactivity at a given time during the disease. As expected, both s-IL-2R and s-ICAM-1 levels in patients with SSc were significantly higher ($p<0{\cdot}0001$ and $p<0{\cdot}001$ respectively) than normal levels.

IMMUNOPHENOTYPE OF *hprt* GENE-MUTATED AND WILD T CELL CLONES DERIVED FROM PATIENTS WITH SSc

A total of 86 T cell clones, 25 mutant and 26 wild that were derived from five patients with SSc, as well as 19 mutant and 16 mild clones that were derived from four healthy controls, were studied. All clones expressed the pan-T cell markers CD2 and CD3 on the surface membrane. In addition, all clones were $CD5^+$. Twenty one of 25 (84%) mutant clones that were derived from the SSc patients displayed the helper T cell phenotype ($CD4^+CD8^-$), whereas 3 of 25 (12%) displayed the cytotoxic/supressor T cell phenotype ($CD4^-CD8^+$). One T cell clone was double positive ($CD3^+$ $CD4^+CD8^+$) (table 5). It should be noted that the conditions of the clonal assay that we used to develop the above clone did not enable us to distinguish between doubly marked cells and a mixed population of singly marked cells. Similar results were obtained from the staining of the wild T cell clones that were derived from patients with SSc. A double negative ($CD3^+$ $\alpha\beta^+CD4^-CD8^-$) T cell clone was detected among the wild clones. The distribution of the mutant and wild T cell clones that were derived from healthy controls among various phenotypes did not differ (chi-square test) from the distribution of the SSc patient-derived clones (table 5). All $CD4^+$ clones were activated, helper/inducer, memory T cells ($HLADR^+$, $CD25^+$, $CD29^+$, $CD45RA^-$, $CD4RO^+$).

Discussion

In view of the information supporting an in vivo activation of T cells in SSc patients and the fact that in vivo acquisition of any random mutation occurs preferentially in dividing cells, we assumed that the frequency of mutated T cells is increased in the peripheral blood of patients with SSc. As previously reported, this assay detects mutations that have resulted from independent mutational events.[14] The majority of the patients studied had increased T cell MF, as assessed by the *hprt* clonal assay. Normal MF values in our hands are almost identical to normal values reported by those who developed and optimised the *hprt* clonal assay.[15 16] Eleven patients of a representative

Table 4 Correlation of plasma levels of s-IL2R and s-ICAM-1 with T cell MF values in patients with systemic sclerosis

	Mean (SD)	*Correlation* with MF*		
		(range)	*r*	*p*
s-IL2R (U/ml)	1234 (495)	(702–2286)‡	0·003	NS
s-ICAM-1 (ng/ml)	512 (144)	(373–766)§	0·117	NS

*Using regression analysis. ‡Significantly higher ($p<0{\cdot}0001$) comparing normal mean (SD) value of 698 (185) (n = 22) using group *t* test. §Significantly higher ($p<0{\cdot}001$) comparing normal (SD) mean value of 373 (108) (n = 22) using group *t* test.

Table 5 Immunophenotype of mutant and wild T cell clones derived from healthy individuals and patients with systemic sclerosis

Immunophenotype	*Healthy Individuals*		*Patients with systemic sclerosis*	
	Mutant clones (n = 19)	*Wild clones (n = 16)*	*Mutant clones (n = 25)*	*Wild clones (n = 26)*
$CD4^+CD8^-$	17	12	21	24
$CD4^-CD8^+$	1	3	3	2
$CD4^-CD8^-$	0	1	0	0
$CD4^+CD8^+$	1	0	1	0

group of 20 patients with SSc had MF values higher than two SD above the normal mean value. This proportion is comparable to the proportion of patients with SLE that exhibit high T cell MF (four of eight SLE patients studied, unpublished experiments). As expected, increased numbers of in vivo mutated T cells is not a specific feature of SSc; theoretically, this could occur in any disease featuring chronic T cell activation. This is, however, the first report of such an abnormality of T cells in SSc. These findings expand the evidence that T cells contribute to the pathology in SSc by demonstrating increased in vivo division and/or alternatively, prolonged survival.

Elevated value of T cell MF were not associated with the extent of skin involvement. All five patients with limited skin disease had high MF values. Duration of Raynaud's phenomenon, considered by certain investigators as the beginning of 'true' SSc disease, was also not associated with elevated T cell MF in our patients. However, 10 patients who had long standing skin disease showed significantly higher T cell MF than the 10 patients who had early (<3 years) skin disease. These findings suggest that the presence of Raynaud's phenomenon before the involvement of the skin may not be associated with persistent T cell activation resulting in proliferation, as with increased T cell MF, in SSc patients. Furthermore, to assess whether the increased T cell MF in patients with SSc was associated with concomitant overactivity of the immune system at the time of the study, we simultaneously measured circulating levels of s-IL-2R and s-ICAM-1 in the plasma of the patients studied. We elected these two soluble immunological products because s-IL-2R has been considered to be a useful marker of lymphocyte activation in general[25] and in SSc in particular,[26–28] and we have found that circulating s-ICAM-1 levels are elevated in patients with SSc.[29] As expected, the patients in our study also had elevated plasma levels of both s-IL-2R and s-ICAM-1. No correlation was found between individual values of MF and either s-IL-2R or s-ICAM-1 levels. Lack of correlation indicates that T cell activation at a certain time of the disease does not necessarily result in increased rate of in vivo mutation, that can be detected with the *hprt* clonal assay. Together with the significant difference between long standing and early disease, this finding suggests that increased T cell MF in patients with SSc may reflect an ongoing process of T cell activation and proliferation that started in the past. Whether this process is a cause and effect in SSc or a secondary phenomenon to various other processes, such as, tissue ischaemia, cytokine effect or induction of tissue antigenicity by other processes can not be determined from this study. Serial observations starting from the beginning of the clinical symptoms in a large homogeneous group of patients will be needed to delineate the pathogenetic significance of increased T cell MF in SSc.

Flow cytometric analyses of T cell subsets in patients with SSc have shown reduced $CD8^+$ T cell counts in early diffuse disease and normal $CD8^+$ cell counts in patients with late disease.[6 22] Normal[22] or increased[30] numbers of helper/inducer ($CD4^+Cd29^+$) T cells, and increased[22] or normal[30] $CD4^+$ to $CD8^+$ ratio have also been reported in the peripheral blood of patients with SSc. Most recently it was reported that patients with SSc have increased numbers of double negative ($CD4^-CD8^-$) peripheral blood T cells that displayed a rather restricted T cell receptor Vβ repertoire.[31] To our knowledge, T cell clones from scleroderma patients have not been established previously. We describe the phenotype of such T cell clones derived from peripheral blood lymphocytes. The majority (91%) of the wild T cell clones, as well as 84% of the *hprt* gene-mutated T cell clones that we established from SSc patients were helper T cells. No differences were observed in the distribution of T cell clone phenotype between patients and normal individuals. The apparent preponderance of $CD4^+$ T cell clones may be due to the fact that $CD4^+$ cells can be cloned easier than $CD8^+$ cells. In fact, suppressor T cells from the peripheral blood have been cloned in only a few instances,[32] and although unclear, the majority of self-reactive T cells that have been characterised to date are confined to the subpopulation of helper T cells.[33] Because of the relatively small number of T cell clones that we established from patients with SSc we did not come across any double negative ones. Identification and expansion of such clones may contribute to our understanding of the disease.[31] All the *hprt* gene-mutated T cell clones that were established from both patients with SSc and normal individuals were activated, memory, lymphocytes ($HLADR^+$ $CD25^+CD45RA^-CD45RO^+$). Memory T lymphocytes is the result of the clonal expansion of antigen-reactive cells, are long lived, and selectively traffic from the blood to peripheral tissue.[34] However, some of these clones may not have been derived from in vivo activated memory T cells, because the clones are IL-2 dependent in vitro and it is known that during prolonged culture periods immature ($CD45RA^+$) T cells may express the $CD45RO^+$ antigen on their surface.[35]

One of the potential mechanisms whereby T cells become activated in vivo in SSc may involve a reaction to autoantigenic epitopes.[1] The study of self-reactive T cells in SSc has been hampered by their rarity in the accessible tissues and the difficulty of obtaining them in vitro without antigenic stimulation. Clones that are derived after in vitro antigenic stimulation are of questionable pathophysiological importance because they can also be obtained from normal individuals and they may derive from cells that are otherwise tolerant.[33] The *hprt* clonal assay presents an opportunity to select in vitro T cells that have been activated in vivo, including those that have encountered non-tolerogenic autoantigens. Further experiments to confirm the pathogenic relevance of T cell clones derived using the *hprt* clonal assay in SSc are currently underway. T cells that are more likely to sustain this somatic mutation, such as the

rapidly proliferating in vivo T cells, may be able to preferentially focus on the skin and other involved organs and initiate or perpetuate the pathogenic process that leads to fibrosis.

In conclusion, the frequency of in vivo mutated T cells in the peripheral blood of patients with SSc is increased, suggesting excessive division and/or survival of circulating T cells. Chronic (self)-antigenic stimulation may account for this finding. Selection of mutated T cells from peripheral blood of these patients may therefore be used to identify the stimulus that is triggering T cell activation in this disease. Such studies will improve our understanding of the pathogenesis of SSc and may direct our therapeutic efforts towards the elimination of selected pathogenic T cell subpopulations.[36]

We thank Dr P Panayiotides for helpful discussions and for critical reading of the manuscript and Drs D Monos and B Cizman for their advice and help during the initial phases of the experiments. We are grateful to Mr L Billops for performing the FACS analysis and Mrs L Teale for testing the TK6 cells for mycoplasma. Dr Sfikakis is a recipient of a scholarship from the A Onassis Foundation.

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