Natural killer cells and $\gamma\delta$ T cells in scleroderma: relationship to disease duration and anti-Scl-70 antibodies

Randall F Holcombe, Bruce A Baethge, Robert E Wolf, Kenneth W Betzing, Ruby M Stewart

Abstract

Objectives—To examine the expression of the natural killer (NK) antigen CD56, and T cell receptor δ chain antigen (TCR δ), expressed on the $\gamma\delta$ T cell subset, in patients with scleroderma, and to correlate levels of expression with clinical characteristics.

Methods—Peripheral blood mononuclear cells (PBMCs) from 15 patients with scleroderma and 11 controls were obtained from heparinised blood on a ficoll/hypaque gradient, stained with monoclonal antibodies, and examined by flow cytometry for expression of CD56 and TCR δ .

Results—Overall, the proportion of PBMCs expressing CD56 in the patient group (14.4 (SEM 2.6)%) was not significantly different from controls (8.75 (2.6)%). The greatest levels of expression were found in patients late (more than three years) in their disease course (18.1 (3.3)%) and in patients who did not express anti-Scl-70 antibodies (17.1 (3.5)%). The proportion of $\gamma\delta$ T cells was significantly lower in the patient group (1.61 (0.52)% *v* control 2.61 (0.46)%) ($p < 0.05$). Patients early in their disease or with anti-Scl-70 antibodies accounted for the reduction in $\gamma\delta$ T cells (0.71 (0.29)% and 0.96 (0.41)%) ($p < 0.01$ and $p < 0.05$, respectively).

Conclusions—This study emphasises that NK and $\gamma\delta$ T cell numbers vary depending upon patient characteristics and may help explain prior contradictory reports. Decreased numbers of $\gamma\delta$ T cells were seen in scleroderma patients, especially those with anti-Scl-70 antibodies and a disease duration of less than three years.

(*Ann Rheum Dis* 1995; 54: 69–72)

Scleroderma is a collagen-vascular disease characterised by abnormalities in cellular and humoral immunity.¹ Natural killer (NK) cells have been postulated to be important in the pathogenesis of scleroderma but have been variably reported to be present in peripheral blood in increased, decreased, and normal numbers.^{2–6} Similarly, the activity of NK and lymphokine-activated killer (LAK) cells has been reported as increased, decreased and

normal.^{3–5 7–9} Specific subtypes of $\gamma\delta$ T cells, which exhibit NK activity and LAK activity, have also been reported to be increased in patients with scleroderma.¹⁰

Natural killer cells are able to kill target cells without major histocompatibility (MHC) restriction and without the requirement for prior sensitisation. LAK cells are a heterogeneous population of peripheral blood mononuclear cells (PBMCs) including NK and T cells which, upon stimulation with interleukin-2 (IL-2), acquire the ability to lyse tumour cells in a non-MHC-restricted fashion. $\gamma\delta$ T cells comprise approximately 5% of CD3 PBMCs and their function *in vivo* is unknown. Freshly isolated $\gamma\delta$ T cells exhibit NK-like activity, and clones of $\gamma\delta$ T cells cultured with IL-2 *in vitro* exhibit LAK activity.

In this study, we have examined the proportion of PBMCs expressing NK and T cell receptor (TCR) $\gamma\delta$ antigens in patients with scleroderma. Correlations were made with clinical characteristics and the presence of anti-topoisomerase I (Scl-70) antibodies. The literature relating to NK cells in scleroderma is also briefly reviewed.

Materials and methods

PATIENT POPULATION

Fifteen patients with scleroderma all met American College of Rheumatology guideline criteria for systemic sclerosis.¹¹ Disease duration was determined from the onset of first symptoms referable to scleroderma and cutaneous involvement was characterised as limited or diffuse as defined by Medsger.¹² Patients were receiving no medication, non-steroidal anti-inflammatory medication, or low dose prednisone (<10 mg/day). No patients were receiving penicillamine or immunosuppressive chemotherapeutic agents. Controls were healthy individuals with no evidence of rheumatological disease. They were sex matched with the patient group, but were slightly younger (mean 34 years *v* 49 years). Seven control subjects were white and four were black.

SCL-70 ANTIBODIES

The presence of antibodies to topoisomerase I (anti-Scl-70 antibodies) was determined on patient serum (Theratest Laboratories, Inc., Chicago, IL).

Department of
Medicine,
LSU Medical Center,
Shreveport,
LA, USA
R F Holcombe
B A Baethge
R E Wolf
K W Betzing
R M Stewart

Correspondence to:
Dr Holcombe,
Section of
Hematology/Oncology,
LSU Medical Center,
1501 Kings Highway,
Shreveport,
LA 71130, USA.

Accepted for publication
1 September 1994

Figure 1 Percent of PBMCs staining positively for NK antigen CD56 in controls (C), scleroderma patients (Sd), and subgroups of scleroderma patients having (Scl-70⁺) or not having (Scl-70⁻) anti-topoisomerase I antibodies. <3 yr, >3 yr = disease duration from first symptoms. Mean values with SEM bars. * $p < 0.05$ v control.

CELL SURFACE STAINING

Heparinised blood was obtained from patients with their informed written consent and PBMCs were obtained by centrifugation on a ficoll/hypaque gradient (LSM, Organon Technika, NC). Cells were washed, resuspended in phosphate buffered saline (PBS) with 50 mg/dl bovine serum albumin, and incubated at 4°C for 30 minutes with a murine monoclonal antibody specific for the NK-associated antigen CD56 (AMAC, ME), and an antibody directed against the δ chain of the T cell receptor (TCR δ 1) (T-Cell Sciences, MA). The latter antibody recognises an invariant epitope on the δ polypeptide chain and therefore recognises both disulphide-linked and non-disulphide-linked $\gamma\delta$ T cells. After additional washes, cells were incubated at 4°C for 30 minutes with fluorescein isothyo-

Figure 2 Percent of PBMCs staining positively with antibody TCR δ 1, identifying $\gamma\delta$ T cells, in controls (C), scleroderma patients (Sd), and subgroups of scleroderma patients having (Scl-70⁺) or not having (Scl-70⁻) anti-topoisomerase I antibodies. <3 yr, >3 yr = disease duration from first symptoms. Mean values with SEM bars. Significant differences compared with control: * $p < 0.05$, ** $p < 0.01$.

cyanate conjugated goat-anti-mouse antibody (AMAC, ME), washed again, and fixed in PBS/1% formaldehyde. Second antibody only and unstained samples were run concomitantly for all patients and controls. Fluorescence was determined with an Epics 753 cell sorter by analysis of a minimum of 10 000 cells for each sample. Gates were established based upon experiments with negative controls, with the gate drawn at 0.2%–0.5% positive events.

STATISTICAL ANALYSIS

Comparisons between patient and control groups were performed using a non-parametric two-tailed Mann-Whitney test for independent variables.

Results

Five of the 15 patients had evidence of anti-Scl-70 antibodies.

NATURAL KILLER CELLS

The proportion of PBMCs expressing the NK antigen CD56 in this population of scleroderma patients was greater than, but not significantly different from controls (fig 1). The increase in NK cells was related primarily to large numbers in patients without anti-Scl-70 antibodies and patients with a disease duration greater than three years. Patients without anti-Scl-70 antibodies had an increased proportion of NK cells compared with control which reached marginal significance ($p = 0.069$). Similarly, NK cell numbers were increased in patients with disease duration greater than three years ($p < 0.05$ v control), while patients earlier in their disease course had NK cell numbers which were not statistically different from control.

Absolute numbers of CD56 and TCR δ 1 cells were available for all patients. Values for CD56 were greater for patients with a disease duration greater than three years (377 (SEM 119) v 158 (67) cells/mm³), those without anti-Scl-70 antibodies (358 (113) v 153 (49) cells/mm³) and those with limited rather than diffuse disease (454 (273) v 230 (52) cells/mm³). These results are all consistent with data analysed for percent CD56 cells.

$\gamma\delta$ T CELLS

The proportion of PBMCs expressing TCR δ was decreased in patients with scleroderma compared with controls ($p < 0.05$) (fig 2). Control values in this study corresponded with values reported previously.¹³ Patients with anti-Scl-70 antibodies and patients with shorter disease duration had smaller numbers of $\gamma\delta$ T cells compared with controls ($p < 0.05$ and $p < 0.01$, respectively). Shorter disease duration and the presence of anti-Scl-70 antibodies were independent variables by multivariate analysis.

Absolute values of TCR δ 1 cells were smallest for patients with a disease duration less than three years (14.2 (5.9) v 37.5 (12.5)

cells/mm³), those with anti-Scl-70 antibodies (23.9 (10.4 v 30.3 (11.5) cells/mm³) and those with diffuse disease (23.7 (7.2) v 40.3 (25.1) cells/mm³). Again, all these results are consistent with data for percent TCR δ 1 cells. There was no significant difference in the proportion or absolute number of CD56 cells or TCR δ 1 T cells between patients receiving or not receiving steroid medications.

Discussion

As summarised in the table, NK cells have been reported to be present in increased, decreased and normal numbers in patients with scleroderma. Previous studies have utilised antibodies directed against CD56,^{2,3} CD16,³⁻⁶ and CD57,^{2,5} each of which identifies potentially different populations of PBMCs with NK activity. Differences in the antibodies utilised to identify NK cells contribute to the significant variations between studies. Additionally, control groups in earlier studies have been reported to have NK cells ranging from as few as 5.7% of PBMCs to 21.0%.² Values for controls in the current study lie within this range, though the control group was not age matched to the patient group. Differences in reported values for control groups and the use of controls not closely matched to the patient group studied also contribute to the apparent contradictions in the literature as to the proportion of NK cells present in scleroderma patients. Most importantly, differences in patient characteristics in the study groups have a critical influence on the reported numbers of NK cells.

Anti-Scl-70 antibodies are typically associated with diffuse cutaneous disease. The comparatively smaller numbers of CD56 cells seen here in patients with anti-Scl-70 antibodies are consistent with several previous reports demonstrating the smaller proportions of NK cells in patients with diffuse disease.^{3,5,14} The greater proportions of NK cells in patients with a disease duration greater than three years is also consistent with previous reports which have demonstrated lower levels in patients early in the course of their disease.^{3,5} Lower NK activity has also been reported to be

correlated with early and diffuse disease.^{5,8} In one previous report, 29 of 40 scleroderma patients with decreased NK activity had evidence of anti-Scl-70 antibodies.¹⁵ Diminished LAK activity in patients early in their disease who express anti-Scl-70 antibodies may result from diminished numbers of specific subpopulations of NK cells or smaller numbers of NK cells and $\gamma\delta$ T cells.^{3,7} The normal LAK activity seen by Gonzalez *et al*⁹ may reflect a different patient group composition.

The decreased proportion of $\gamma\delta$ T cells found in this study contrasts with the only previous investigation of these cells in patients with scleroderma, which revealed a statistically significant increase in non-disulphide linked $\gamma\delta$ T cell receptor polypeptide chains, a small but non-significant decrease in the expression of the disulphide linked form, and overall normal levels.¹⁰ All seven of the patients studied had a disease duration less than five years. The extent of cutaneous involvement and the presence or absence of anti-Scl-70 antibodies were not reported. The current study is the first to report a statistically significant decrease in $\gamma\delta$ T cells in a subgroup of patients with scleroderma. Tissue recruitment of $\gamma\delta$ T cells in patients with active disease may explain the reduction of these cells in the circulation.

Overall, the proportion of NK cells and $\gamma\delta$ T cells in the peripheral blood of scleroderma patients depends upon the specific characteristics of the patient group studied and the specific monoclonal antibodies utilised to define a population of PBMCs as NK cells. Conclusions regarding increases or decreases in these cell types also depend in part upon values determined for a particular control group. Based on this study and a review of other studies reporting NK numbers and activities, it appears that the smallest proportions are seen in patients early (less than three years) in the course of their disease and in patients with diffuse cutaneous disease, especially if they express anti-Scl-70 antibodies. Decreased numbers of $\gamma\delta$ T cells are also seen in patients early in disease and in those with anti-Scl-70 antibodies. The decrease in NK and LAK activity variably reported in scleroderma patients may reflect a relative decrease in NK or $\gamma\delta$ T cell number in specific subgroups, or may reflect an as yet undefined defect in the function of PBMCs mediating NK and LAK activity.

Supported in part by NHLBI grant 1KO8-02467 (RFH), Louisiana Board of Regent's grant LEQSF-RD-A-18 (RFH), and the Centers for Excellence in Cancer Research and Arthritis & Rheumatology, LSUMC, Shreveport.

Flow cytometric analysis of the proportion of PBMCs expressing natural killer antigens: comparison of current study with previously reported values for scleroderma patients and controls

MAb	Controls		Patients		Conclusion ^b	Reference
	mean (SEM) % ^a	n	mean (SEM) % ^a	n		
CD56+	21.0 (2.1)	8	9.5 (1.5)	20	Decreased	2
	8.75 (2.6)	11	14.4 (2.6)	15	NS ^c	Current study
			290 (80) ^a	15		
CD16+	193 (14) ^a	71	197 (16) ^a	34	NS	3
	7.4 (0.5)	21	20.8 (1.2)	16	Increased	4
	12.7 ^d	18	10.7 ^d	50	NS	5
	15.9 (1.2)	23	10.4 (1.0)	24	Decreased	6
CD56+/CD16-	171 (18) ^a	41	147 (15) ^a	34	NS	3
	218 (17) ^a	29	91 (9) ^a	34	Decreased	3
	5.7 (0.5)	14	10.5 (1.5)	15	Increased	2
CD57+	10.7 ^d	18	8.7 ^d	50	NS	5

MAb = Monoclonal antibody; n = number of subjects; NS = not significantly different from control.

^aExcept those indicated thus, which are absolute number of cells/mm³.

^bConclusion of authors as to level of (+) staining cells in scleroderma patients v control.

^cAll scleroderma patients; increased in certain subgroups

^dVariance from mean not reported.

1 Korn J H. Immunologic aspects of scleroderma. *Curr Opin Rheum* 1991; 3: 947-52.

2 Frieri M, Angadi C, Paolano A, *et al*. Altered T-cell subpopulations and lymphocytes expressing natural killer cell phenotypes in patients with progressive systemic sclerosis. *J Allergy Clin Immunol* 1991; 87: 773-9.

3 Kantor T V, Whiteside T L, Friberg D, Buckingham R B, Medsger T A Jr. Lymphokine-activated killer cell and natural killer cell activities in patients with systemic sclerosis. *Arthritis Rheum* 1992; 35: 694-9.

4 Grazia Cifone M, Giacomelli R, Famularo G, *et al*. Natural killer activity and antibody-dependent cellular cytotoxicity in progressive systemic sclerosis. *Clin Exp Immunol* 1990; 80: 360-5.

- 5 Miller E B, Hiserodt J C, Hunt L E, Steen V D, Medsger T A Jr. Reduced natural killer cell activity in patients with systemic sclerosis. *Arthritis Rheum* 1988; **21**: 1515–23.
- 6 Freundlich B, Jiminex S A. Phenotype of peripheral blood lymphocytes in patients with progressive systemic sclerosis: activated T lymphocytes and the effect of D-penicillamine therapy. *Clin Exp Immunol* 1987; **69**: 375–84.
- 7 Silver R M. Lymphokine activated killer (LAK) cell activity in the peripheral blood lymphocytes of systemic sclerosis (SSc) patients. *Clin Exp Rheumatol* 1990; **8**: 481–6.
- 8 Majewski S, Blaszczyk M, Wasik M, Jablonska S. Natural killer cell activity of peripheral blood mononuclear cells from patients with various forms of systemic scleroderma. *Br J Dermatol* 1987; **116**: 1–8.
- 9 Gonzalez-Amaro R, Alcocer-Varela J, Alarcon-Segovia D. Natural killer cell activity in the systemic connective tissue diseases. *J Rheumatol* 1988; **15**: 1223–8.
- 10 Gerli R, Agea E, Bertotto A, *et al.* Analysis of T cells bearing different isotypic forms of the $\gamma\delta$ T cell receptor in patients with systemic autoimmune diseases. *J Rheumatol* 1991; **18**: 1504–10.
- 11 Masi A T, Rodnan G P, Medsger T A Jr, *et al.* Preliminary criteria for the classification of systemic sclerosis (scleroderma). *Arthritis Rheum* 1980; **23**: 581–90.
- 12 Medsger T A Jr. Systemic sclerosis (scleroderma), localized scleroderma, eosinophilic fasciitis, and calcinosis. In: D J McCarty, ed. *Arthritis and allied conditions*, 11th edn. Philadelphia: Lea & Febiger, 1989.
- 13 Parker C M, Groh V, Band H, *et al.* Evidence for extrathymic changes in the T cell receptor gamma/delta repertoire. *J Exp Med* 1990; **171**: 1597–612.
- 14 Weiner E S, Earnshaw W C, Senecal J-L, Bordwell B, Johnson P, Rothfield NF. Clinical associations of anti-centromere antibodies and antibodies to topoisomerase I. *Arthritis Rheum* 1988; **31**: 378–85.
- 15 Majewski S, Jablonska S. NK cells in systemic sclerosis. *Br J Dermatol* 1987; **116**: 400–2.