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Natural killer activity and antibody-dependent cellular cytotoxicity in progressive systemic sclerosis

M. GRAZIA CIFONE, R. GIACOMELLI*, G. FAMULARO*, R. PAOLINI†, C. DANESE‡, T. NAPOLITANO, A. PROCOPIO, A. M. PEREGO‡, A. SANTONI & G. TONIETTI* *Departments of Experimental Medicine, *Internal Medicine and Public Health, University of L'Aquila, †Department of Experimental Medicine and ‡Medical Clinic II, University La Sapienza of Rome, Italy*

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SUMMARY

Enhanced natural killer (NK) activity and normal lymphocyte antibody-dependent cellular cytotoxicity (ADCC) were observed in 16 patients with a diagnosis of progressive systemic sclerosis (PSS). Higher NK activity levels were observed against NK-sensitive K562 target cells, while the NK-resistant P815, Daudi and Raji cell lines were not lysed. Cytofluorimetric studies and morphological analysis of peripheral blood lymphocytes (PBL) showed an increased number of CD16 positive cells and large granular lymphocytes (LGL), indicating that the enhancement observed was probably attributable to an increase in the number of circulating NK cells.

Keywords progressive systemic sclerosis large granular lymphocytes NK activity ADCC

INTRODUCTION

Progressive systemic sclerosis (PSS) is a disease of unknown aetiology characterized by connective tissue abnormalities and unexplained over-accumulation of collagen, resulting in fibrosis in the skin and often in the internal organs. The presence of various immunological abnormalities, such as hypergammaglobulinaemia, circulating autoantibodies, cryoglobulins (Rothfield & Rodnan, 1968; Bernstein, Steigerwald & Tan, 1982) and clinical overlapping with diseases such as systemic lupus erythematosus (Kirkland, 1964) have led to the characterization of PSS as an autoimmune disease. The polyclonal activation of B lymphocytes as a major immunologic feature of PSS is also supported by our previous observations of an increase of both total and activated B lymphocytes concomitantly with higher IgG and IgA levels in the supernatants of peripheral blood lymphocytes (PBL) from PSS patients (Famularo *et al.*, 1989).

Several studies suggest that cell-mediated immunity is involved in the pathogenesis of PSS, including decreased total number of T cells, alterations of T cell subsets, and decreased T cell response to mitogens (Gupta *et al.*, 1979; Inoshita *et al.*, 1981; Whiteside *et al.*, 1983).

Natural killer (NK) cells are CD3-Ti complex negative large granular lymphocytes (LGL) easily identified morphologically by the presence of azurophil granules in their cytoplasm when stained with Giemsa (Timonen, Ortaldo & Herberman, 1981). In humans, they commonly express certain cell surface markers, such as CD16 (Fc receptor III) (Perussia *et al.*, 1984) and CD56 (NKH-1) (Griffin *et al.*, 1983; Lanier *et al.*, 1986). NK cells appear to play a leading role in host defense against tumours and viral infection, as shown in many experimental systems (Herberman, 1982). They mediate non-MHC-restricted cytolytic reactions without prior sensitization and are the main cellular effectors of antibody-dependent cell cytotoxicity (ADCC) through the expression of low affinity IgG-specific Fc receptors (CD16) (Herberman *et al.*, 1979; Bolhuis, 1980). They also mediate antigen presentation (Scala *et al.*, 1985) and are involved in the B cell responses (Arai *et al.*, 1983; Abbruzzo, Mullen & Rowley, 1986; Kuwano *et al.*, 1986; Robles & Pollack, 1986).

Involvement of NK cells in the pathophysiology of PSS has been suggested, although both diminished and enhanced responses have been reported (Wright, Hughes & Rowell, 1982; Valentini *et al.*, 1984; Freundlich & Jimenez, 1987; Majewski *et al.*, 1987; Miller *et al.*, 1988).

In this study, we have investigated both NK cell number and activity (natural killing and ADCC) in an homogeneous group of PSS patients with mild progression and a duration of disease >7 years (max. 30 years).

MATERIALS AND METHODS

Patients and controls

The patients were 16 women ranging in age from 28 to 67 years (mean age 57·7 years) diagnosed according to the criteria of the American Rheumatism Association for diffuse PSS (Masi *et al.*,

Correspondence: Dr M. Grazia Cifone, Department of Experimental Medicine, University of L'Aquila, Collemaggio, 67100 L'Aquila, Italy.

Table 1. Laboratory data in PSS patients (normal value)

Patients	ESR (<15)	RF (neg.)	ANA* IF (neg.)	IC† (neg.)	C3 (55–120 mg/dl)	C4 (20–50 mg/dl)	IgG (800–1500 mg/dl)	IgA (90–325 mg/dl)	IgM (45–150 mg/dl)
DGA	10	–	–	+	95	29	1214	95	130
NE	7	–	+ (d)	++	85	31	1256	83	210
CA	18	–	++ (d)	+	132	32	1342	121	254
BR	20	+	++ (r)	++	90	34	1219	86	74
BF	25	–	+++ (d)	–	72	26	1230	67	280
FM	19	++	+++ (d)	–	161	41	1220	184	223
DGT	29	–	++ (s)	–	120	30	1723	284	280
DM	9	–	–	+	85	37	1088	480	192
SE	15	–	++ (d)	–	101	35	1040	378	131
LP	13	+	+ (s)	+	90	34	1211	220	115
CT	16	–	–	+	76	28	1310	190	200
GR	9	–	–	–	104	39	1398	229	190
RM	12	–	–	–	79	41	1278	311	228
FA	20	+	+ (d)	++	69	30	1257	284	130
RG	13	–	+ (r)	+	59	37	1420	150	191
LS	8	–	–	–	100	40	1365	248	160

ESR, Erythrocyte sedimentation rate; RF, rheumatoid factor; ANA, anti-nuclear autoantibodies; IF, indirect fluorescence; IC, immune complexes.

*ANA d, diffuse; r, rim; s, speckled.

†Clq binding.

1980). Cutaneous biopsies showed proliferation and extension of collagen from dermis to subcutaneous tissue, infiltration of mononuclear cells at the border of the lesions, thickening of the intima of the capillaries with narrowing and/or occlusion of the lumen in all patients. All but one exhibited oesophageal dismotility, evaluated by manometry, and Raynaud's phenomenon (94·1%). Pulmonary fibrosis diagnosed by conventional chest X-ray and/or by the demonstration of a chronic restrictive lung disease using pulmonary function tests were always present (100%). One patient showed symptoms (malabsorption syndrome with steatorrhoea and weight loss) referable to intestinal involvement. All patients showed a mild evolution of the disease, with a disease duration ranging from a minimum of 7 to a maximum of 30 years. None of patients were receiving drugs for the duration of the study. Only three patients, who were receiving steroids (prednisone, 10 mg/day), suspended it 15 days before performing the immunological assays. Laboratory data of the PSS patients are summarized in Table 1.

Fifteen patients with systemic lupus erythematosus (SLE) (mean age 41·5 years) diagnosed according to the criteria proposed by the American Rheumatism Association for the diagnosis of SLE (Tan *et al.*, 1982) have been included in this study as a disease control group for the determination of $CD16^+$ cells.

Twenty-one age- and sex-matched healthy volunteers were used as controls.

Effector cells

PBL were isolated from heparinized blood drawn from PSS patients or controls using differential centrifugation over Ficoll–Hypaque, washed twice and finally resuspended in culture medium (RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 100 U penicillin and 100 μg streptomycin/ml). The cells were counted and their viability, as assessed by trypan blue dye exclusion, was routinely greater than 98%.

Target cells

K562 cells from a patient with chronic myelogenous leukaemia in blastic crisis were used for the NK assay. The target cells for ADCC assay were P815 cells (a mouse mastocytoma of DBA/2 origin) sensitized with a rat anti-P815 serum. P815 cells and two Burkitt lymphoma lines, Raji and Daudi, were used as NK-resistant cells. All cell lines were maintained in a suspension culture at 37°C in a humidified atmosphere of 5% CO_2 in RPMI 1640 supplemented with 10% FBS.

Cytotoxic assays

Various concentrations of effector cells were incubated with 5×10^3 ^{51}Cr (Amersham, Buckinghamshire, UK) (100 μCi/5×10^6 cells at 37°C for 1 h) as labelled target cells (K562 for NK and antibody-coated P815 for ADCC) at 100:1, 50:1, and 25:1 effector:target (E:T) ratios, for 4 h at 37°C in round-bottomed 96-well plastic microtitre plates. The NK-resistant cell lines were utilized for certain experiments. The supernatant was removed and counted in a gamma-scintillation counter. All combinations were performed in triplicate. Baseline ^{51}Cr release, was 5–10% of the total ^{51}Cr incorporated into the target cells. Standard deviations were less than 5% and are not included in the figures. The per cent cytotoxicity was calculated as follows:

$$\frac{(^{51}\text{Cr released from experimental group} - \text{Baseline } ^{51}\text{Cr release})}{(\text{Total } ^{51}\text{Cr incorporated} - \text{Baseline } ^{51}\text{Cr release})} \times 100$$

Cytochemical staining

For morphological analysis of the LGL populations, 2×10^5 cells were centrifuged for 7 min at 900 rev/min onto slides with a Cytospin (Shandon Instruments, Sewickley, PA) centrifuge.

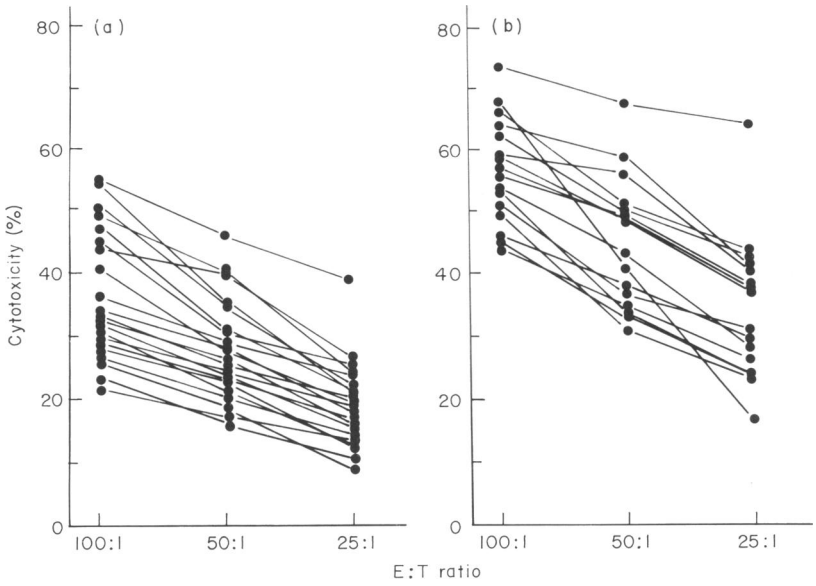

Fig. 1. NK activity of PBL from control subjects (a) and PSS patients (b) measured *in vitro* against K562 targets in a 4-h chromium release test. The points represent the means of triplicate determinations (s.d. < 5%). The differences between the means for the 2 groups were significant ($P < 0{\cdot}001$) at all E:T ratios as evaluated by Student's *t*-test.

Table 2. Persistence of high NK cell number and activity levels in PSS patients

			NK activity		
Case no.	% LGL	% CD16$^+$	100:1	50:1	25:1
1 (a)	22±0·2	17±0·3	49·9±1·2	31·4±0·9	24·5±1·1
(b)	27±0·9	24±2·3	52·8±2·4	43·3±2·8	28·5±0·5
(c)	20±1·9	18±2·1	50·6±1·1	46·3±1·6	30·0±1·1
2 (a)	33±1·8	29±1·7	56·8±1·25	49·3±0·7	38·1±1·4
(b)	28±1·5	20±2·1	57·6±2·2	48·1±0·9	36·6±1·8
(c)	30±2·3	21±0·6	61·1±3·0	50·4±1·3	39·6±2·7
3 (a)	27±1·6	26±1·7	58·6±1·8	50·0±1·1	37·9±1·9
(b)	20±0·9	15±2·9	49·9±1·9	44·1±1·0	32·4±1·0
(c)	25±1·3	23±1·8	57·9±2·3	56·2±2·0	41·2±1·3

All values represent the means±s.d. of triplicate determinations.
The assays were performed at the onset of the study (a), at 6 months (b), and at 1 year (c).

Air-dried slides were fixed for 10 min in methanol and stained for 25 min with Giemsa (Fisher Scientific Co., Failawn, NJ) in PBS, pH 7·4. The percentage of LGL was determined after counting at least 400 cells.

Phenotypic analysis by cytofluorimetry

1×10^6 PBL incubated for 30 min at 4°C with anti CD16 (Leu-11; Becton–Dickinson, Mountain View, CA). They were then washed and analysed by gating lymphocytes, with a Becton–Dickinson cytofluorimeter.

Statistical analysis

Statistical analysis was performed by Student's *t*-test.

RESULTS

NK activity

This was significantly enhanced in PSS at all E:T ratios ($P < 0{\cdot}001$) (Fig. 1). One-year-long serial studies in individual patients showed persistently high NK number and lytic activity (Table 2). The possibility that this enhanced activity was mediated by activated cells was tested by using NK-resistant cells that are lysed by either IFN- or IL-2-activated effectors. These cells were not lysed by PSS patient cells in the same way as control cells (data not shown).

ADCC assay

Patient ADCC levels were not significantly different from the controls (Fig. 2).

NK cell number

Cytospin preparations showed the LGL from PSS patients to be morphologically similar to controls. The percentages of PBL exhibiting LGL morphology and reacting with the monoclonal antibody Leu 11 (anti-CD16) by cytofluorimetric analysis are shown in Fig. 3. The percentage of CD16$^+$ cells was increased in PSS patients (mean±s.d. 20·8±4·6) when compared with controls (mean±s.d. 7·4±2·1). The percent of CD16$^+$ cells was lower in the SLE subjects (mean±s.d. 5·9±2·2).

In parallel, the percentage of LGL as determined by light microscopy was significantly higher in PSS patients (mean±s.d. 23·2±7·0) than controls (mean±s.d. 9·6±3·3).

Thus, there was a strong link between the high levels of circulating NK cells and percentage of cytotoxicity against K562 cells. On the other hand, there is an evident lack of correlation between CD16$^+$ cell percentage and ADCC levels.

DISCUSSION

The pathogenesis of PSS is unclear, though there is increasing evidence that defective immunoregulation is important. NK cell

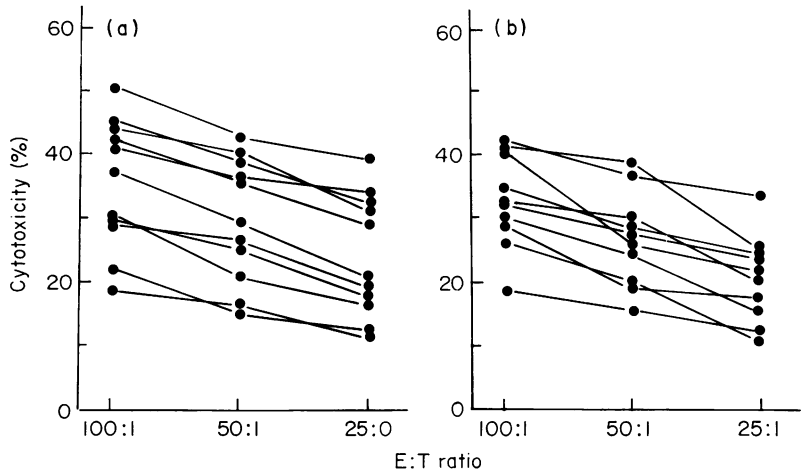

Fig. 2. ADCC against antibody-coated P815 cells in PBL from normal subjects (a) and PSS patients (b), as measured *in vitro* in a 4-h chromium release test. The points represent the means of triplicate determinations (s.d. < 5%). No significant differences were observed.

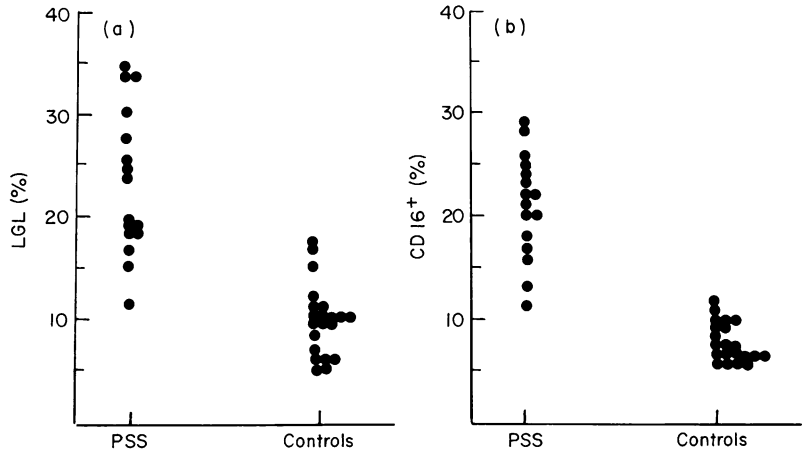

Fig. 3. (a) Percentage of LGL in PSS patients and controls, as determined by light microscopy. (b) Percentage of CD16$^+$ cells in PSS patients and controls as determined by flow cytometry. Each point represents the mean of triplicate determinations. The differences were significant ($P < 0{\cdot}001$).

abnormalities have been described in several rheumatic diseases (Minato *et al.*, 1982; Reinitz, Neighbour & Graycel, 1982; Katz *et al.*, 1982; Goto, 1985; Ichikawa *et al.*, 1985; Gonzalez-Amaro *et al.*, 1987), as well as in PSS, although the findings are often conflicting, mostly due to differences between disease subsets (Wright *et al.*, 1982; Majewski *et al.*, 1987; Miller *et al.*, 1988). In PSS patients, we have found a persistent, significant increase of NK activity against NK-sensitive K562 cells. Our data are fully consistent with a previous paper reporting an absolute increase of Leu 11b$^+$ cells in patients with diffuse scleroderma (Valentini *et al.*, 1984).

Several factors indicate that the observed increase in NK activity could be due to a high number of circulating effector cells. Enumeration of the main effectors of NK activity by morphology and phenotype showed significantly augmented numbers of LGL and CD16$^+$ cells. Enhanced NK activity is often associated with greater susceptibility of target cells to lysis (Timonen, Ortaldo & Herberman, 1982; Ortaldo, Mason & Overton, 1986; Phillips & Lanier, 1986; Herberman *et al.*, 1987). However, our experiments with a panel of NK-resistant tumour cells (P815, Daudi, and Raji), revealed that the spectrum of target sensitivity to PSS patient cells was unchanged when compared with controls, indicating that enhancement may depend on an increase in the number of circulating NK cells rather than their intrinsic activation. Both NK cell number and activity were enhanced in three PSS patients for 1 year, suggesting that this is a persistent alteration.

It is noteworthy that in PSS patients ADCC against the P815 mastocytoma line was normal, while NK activity was increased. These functions are generally assumed to be exerted by largely overlapping lymphoid subsets (Santoni, Herberman & Holden, 1979; Timonen *et al.*, 1981; Bradley & Bonavida, 1982). The lack of correlation between NK and ADCC activities could stem from the fact that these two types of cytotoxicity are mediated to some extent by distinct cell subsets, or overlapping populations of LGL with distinct biological characteristics. There are several lines of evidence to support this contention (Koren & Williams, 1978; Neville, 1980; Bardos *et al.*, 1981; Saxena, Saxena & Adler, 1982; Wahlin *et al.*, 1984; Fukui *et al.*, 1987). In one study, interferon was shown to increase NK activity, but not ADCC (Trinchieri, Santoli & Koprowski, 1978). Also, normal NK activity and absent ADCC have been described in agammaglobulinemia (Koren, Amos & Buckley, 1978).

Increased NK activity in PSS may be either a primary event

or an effect of a subclinical condition. The first hypothesis is supported by the observation that viral infection may be an aetiologic factor. In some systemic diseases (e.g. systemic lupus erythematosus), and, though less strikingly, PSS, the presence of antibodies against RNA-dependent DNA polymerase of baboon endogenous virus has led to the hypothesis that a retrovirus or retroviruses are involved in the pathogenesis of PSS (Okamoto, Tamura & Takano, 1983). Increased NK cell number and turnover have, in fact, been reported during viral infections (Herberman *et al.*, 1977; Welsh, 1978; Biron, Turgiss & Welsh, 1983).

Further detailed investigation of both immunological abnormalities in PSS patients and their association with the clinical and serological features of the disease in a prospective study is clearly needed.

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