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Polymorphonuclear neutrophil function in systemic sclerosis

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SUMMARY In vitro functions of polymorphonuclear (PMN) neutrophils were studied in 20 patients with progressive systemic sclerosis (PSS). An increase in the basal chemiluminescence (CL) activity of peripheral blood PMNs was found, suggesting that these cells had been preactivated in vivo. Patients with more extensive skin disease or signs of disease progression tended to have higher basal CL values. Active oxygen products during the respiratory burst may increase the extent of inflammatory and fibrotic processes and could be involved in the endothelial injury in PSS. The stimulatory capacity of CL response was normal in our study. No alterations were found in the opsonised yeast phagocytic activity of granulocytes when compared with control values. The binding of erythrocyte-antibody particles was found also to be normal. A depressed chemotactic activity of PMN cells against zymosan activated serum was also shown. The cause of the decreased chemotaxis of PMNs remains to be elucidated.

Key words: chemiluminescence, chemotaxis.

Progressive systemic sclerosis (PSS) is characterised by fibrotic, degenerative, and inflammatory changes involving the skin and some internal organs. Although the cause of PSS is unknown, the microvascular involvement and abnormalities in the immune system are well characterised in patients with scleroderma.[1 2]

The mechanism(s) which lead to endothelial injury, inflammatory processes, and fibrosis are poorly understood in PSS. In these complex pathological events the polymorphonuclear neutrophils (PMNs) and their products may be involved in either endothelial injury or inflammatory and fibrotic processes. Spisani *et al* found normal yeast phagocytosis, spontaneous migration, and chemotactic activity of PMNs in nine patients with PSS.[3]

In our study we investigated the granulocyte functions of 20 patients with systemic sclerosis.

Patients and methods

PATIENTS

Twenty female patients with systemic sclerosis were investigated. Their mean age was 47·5 (SD 6·8) years (range 34–61). All patients fulfilled the preliminary diagnostic criteria for scleroderma.[4] Clinical data of the patients are given in Table 1. Twelve patients received 30–50 mg nifedipine daily. D-Penicillamine was administered in six cases, prednisone in three. All drug administration was stopped 48 hours before blood collection. There were no clinical signs of infection in patients with PSS.

Controls were fifteen women matched by age.

Table 1 *Clinical profile of 20 patients with systemic sclerosis*

Age (mean (SD), years)	47·5 (6·8)
Duration of disease (mean (SD), years)	8·4 (6·9)
Number of patients with:	
'Proximal' scleroderma	13
Sclerodactyly	7
Joint symptoms	18
Raynaud's phenomenon	19
Lung involvement	11
Oesophageal dysfunction	12
Sicca syndrome	4
Myositis	5
Cardiac involvement	5
Renal manifestation	—
Calcinosis	2
Exposure to organic solvents	5

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MEASUREMENT OF CHEMILUMINESCENCE

The chemiluminescence (CL) of peripheral PMNs was investigated in heparinised whole blood, diluted fourfold with colourless, phenol red free Hanks's solution to a final volume of 1 ml. Each sample contained 10 units heparin. To 0·5 ml of the diluted blood was added 0·5 mg/0·5 ml of zymosan or 0·5 ml of Hanks's solution (controls) at 37°C for 10 min. Luminol at a concentration of 10^{-4} mol/l was added to each sample. The chemiluminescence of cells was measured in glass vials by a Nuclear Chicago Isocap/300 liquid scintillation counter (Searle Industries, USA) in the off coincidence mode.[5 6] The total number of photons in counts per minute (cpm) was measured five times at five-minute intervals, and the result was regarded as the characteristic value for each sample. The chemiluminescence values were related to the concentrations of PMNs in blood. The CL of blood unstimulated and stimulated by zymosan was determined and their rate was expressed by the stimulation index (SI).

$$SI=CL(B+Z)/CL(B)$$

where B=the basal value and Z=the zymosan stimulated value.

MEASUREMENT OF YEAST PHAGOCYTOSIS BY GRANULOCYTES

PMNs from blood were isolated by sequential density centrifugation in Ficoll-Uromiro. Dextran (5%) diluted in phosphate buffered saline (PBS) was used for the sedimentation of the granulocyte-erythrocyte pellet. The time of incubation was 45 min at room temperature. After hypotonic lysis of residual erythrocytes the twice washed cells contained about 97% viable granulocytes. The neutrophils were suspended in PBS at 5×10^6 cells/ml.

A monolayer technique was used with cells adherent to a glass slide in a plastic collet fixed to glass by wax. Cells (5×10^6) were pipetted onto the slides, incubated at room temperature for 30 min, and washed. Twenty five microlitres of bakers' yeast suspension (*Saccharomyces cerevisiae*) containing 5×10^6 particles in 0·5 ml Parker's medium was added. After incubation at 37°C for 60 min in 5% CO_2 and at 100% humidity the cells were washed and stained with 0·01% crystal violet or Wright's stain. Two hundred PMN cells in two parallel samples were evaluated, and the number of ingested particles was counted. The number of ingested yeast particles was divided by the total number of cells giving the phagocytic index (PI).

PHAGOCYTOSIS OF C3b COATED YEAST PARTICLES

The monolayer of granulocytes was incubated as described above at 37°C for 60 min with 25 µl of 2×10^8/ml yeast particles opsonised with 1 ml of human AB serum at 37°C for 60 min and washed. After incubation the cells were washed, stained, and the PI was determined.

BINDING OF ERYTHROCYTE-ANTIBODY (EA) CELLS BY GRANULOCYTES

The binding of sheep red blood cells (SRBCs) sensitised with a subagglutinating amount of IgG fraction isolated on Sephadex G–200 from a rabbit anti-SRBC serum (EA) was determined. The granulocytes were incubated with 0·2 ml of 2% sensitised SRBCs in 0·3 ml of Parker's medium at 37°C for 30 min. After washing and staining the percentage of neutrophils binding or ingesting three or more SRBC particles (the granulocyte uptake of EA cells) was determined (EA %).

CHEMOTAXIS ASSAY

A chemotactic test was performed by means of the micropore leading front assay. A modification of the Boyden chamber technique was used.[7] Granulocytes ($0·5\times10^6$) in 0·5 ml medium with zymosan (Sigma, GFR) activated, complement derived chemotactic factor and 5 µm pore size Millipore filter (Sartorius Membranfilter, GFR) were used in each sample. When the chambers were filled they were incubated at 37°C for 90 min. A two cell leading front assay was used. The filters were stained with haematoxylin-eosin and evaluated microscopically by measuring the distance travelled by the two leading PMNs in five microscopic fields. The chemotactic test was carried out in triplicate.

The polyethylene glycol precipitation method[8] was used to determine the immune complex levels of sera. The values for the samples were expressed as the actual percentage of the sample compared with the mean value for healthy controls.

STATISTICAL ANALYSIS

The mean values of the data and the standard deviation (SD) were calculated. Differences between groups were evaluated by a paired Student's *t* test.

Results

Twenty female patients with PSS were investigated. The basal (unstimulated) chemiluminescence activity of phagocytes in diluted whole blood was found to be higher in the patients with PSS than in the controls ($p<0·001$; Fig. 1). In six patients with clinical signs of disease activity (recorded progression of skin or pulmonary symptoms, or both; active myositis) the basal chemiluminescence value was

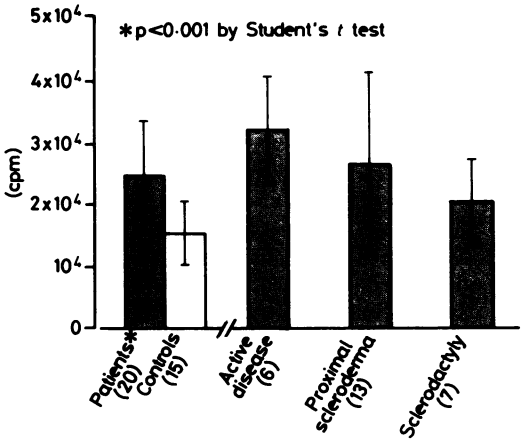

Fig. 1 *The basal chemiluminescence of PMNs in patients with systemic sclerosis. The chemiluminescence values are expressed as the total number of photons in cpm measured five times at five-minute intervals. The number of cases are in parentheses.*

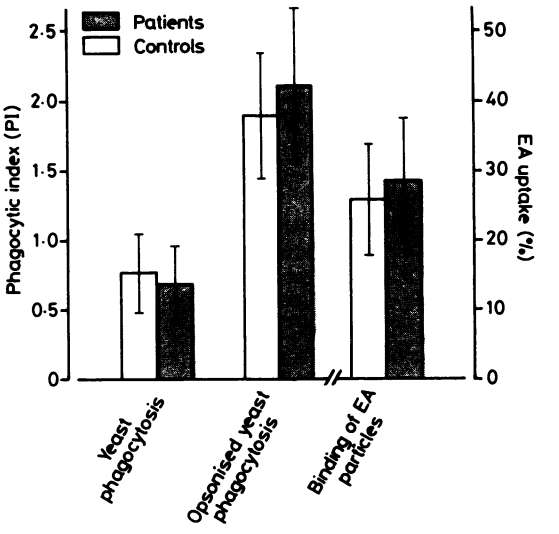

Fig. 2 *Phagocytic function of PMNs in 20 patients with systemic sclerosis. The phagocytic index is expressed as the number of ingested yeast particles per total number of neutrophils. EA uptake is the percentage of neutrophils binding or ingesting three or more SRBC particles.*

greater than that for the other 14 patients, though the difference between the two groups was not statistically significant (Fig. 1). No differences were found when the eight patients with less than three years' disease duration were compared with the other 12 cases with a longer disease history. Patients with 'proximal' scleroderma tended to show higher basal chemiluminescence values than patients with acrosclerosis, but the difference between the two groups was not statistically significant (Fig. 1). The phagocytic functions of the five patients who had been previously exposed to organic solvents[9] were indistinguishable from those of the other patients (data not shown).

The PMN chemiluminescence response of patients after stimulation by zymosan did not differ from that of the controls (154 (SD 48)% of the basal chemiluminescence mean value *v* 149 (61)%).

The yeast phagocytosis of granulocytes and the EA cell binding of PMNs were found to be normal. Similar results were obtained when the phagocytosis of C3b coated yeast particles was studied (Fig. 2).

In our study a reduced chemotactic activity of PMN neutrophils was found against zymosan activated particles (Fig. 3). No correlations with clinical symptoms were found.

The patients' sera showed a slightly increased immune complex level by the polyethylene glycol precipitation method (158 (SD 92)% of the control values). There was no relation between immune complex levels and chemotactic, phagocytic functions of neutrophils (data not shown).

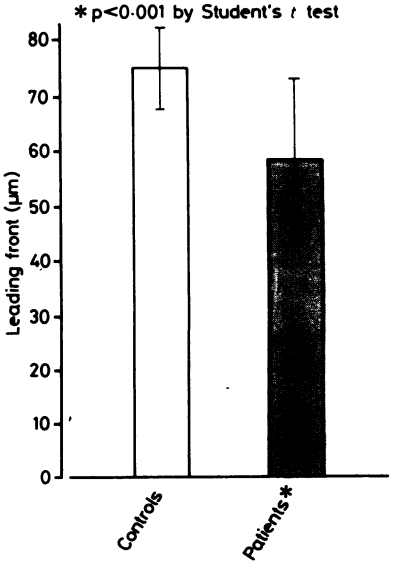

Fig. 3 *Chemotaxis of PMNs against zymosan activated serum in 20 patients with PSS. The distance travelled by cells is expressed in micrometres (μm) in the two cell leading front assay.*

Discussion

The complex pathological events which lead to vascular endothelial injury, inflammation, and fibrosis remain to be elucidated in patients with PSS. There are some data which suggest a possible role for PMN neutrophils in some of these pathological events. An increased ratio of neutrophils was found at the site of active fibrosis as the bronchoalveolar lavage studies showed in scleroderma.[10–12]

Measurement of the CL of phagocytic cells is a sensitive method for the characterisation of metabolic activity of phagocytes during phagocytosis.[5] It is generally accepted that the CL measured in whole blood is derived almost completely from neutrophils.[13] In our study a marked increase in the basal chemiluminescence activity of the blood phagocytes was found (Fig. 1), suggesting that the neutrophils from patients with PSS had been preactivated in vivo.

The cause of the increased basal neutrophil chemiluminescence value in patients with PSS remains to be elucidated. It is tempting to speculate that hypoxia near the microvessels could be one of the PMN activating factors. This hypothesis is supported by the fact that patients with more extensive skin disease or with signs of disease progression tended to have higher basal chemiluminescence values (Fig. 1). In these cases the microvascular injury and the hypoxia near the microvessels could be more pronounced. Recent evidence suggests that oxygen derived free radicals may be abundantly produced in ischaemic tissues.[14]

There is growing evidence that the intermediates of oxygen reduction products during the respiratory burst may increase the extent of inflammatory and fibrotic processes. Active oxygen products from normal stimulated granulocytes can damage the vascular endothelium in vitro.[15] It has recently been suggested that endothelial cell cytotoxicity is mediated via a proteolytic mechanism involving the depression of antiprotease activities in scleroderma sera.[16] One of the possibilities of functional defects in serum protease inhibitors is the direct damage from reactive oxygen species generated by phagocytosing neutrophils.[17] Endothelial cells can also be destroyed indirectly through the lipid peroxidation as a result of neutrophil activation.[18 19] Oxygen radicals can mediate endothelial cell damage by complement stimulated granulocytes.[20] PMN cells incubated with soluble immune complexes or IgG aggregates generate a dose dependent chemiluminescence response, indicating a membrane activation and production of oxygen radicals.[21] In our study the basal chemiluminescence value of granulocytes was not significantly influenced by patients' sera. Platelet or mast cell exposure to oxidants or free radicals may also influence the subsequent inflammatory and haemostatic reactions.[22 23]

There are few data about the behaviour of granulocytes in patients with PSS. Phagocytotoxic autoantibodies were found in scleroderma sera.[24] An increased eosinophil chemotactic activity was shown in 30% of patients with PSS.[25] Spisani *et al* found a normal in vitro yeast phagocytic activity in nine patients with PSS.[3] The random migration of granulocytes was also normal. Our results are consistent with these findings considering the normal rate of yeast phagocytosis by PMN cells. This method is less sensitive than the chemiluminescence assay.

In our study the uptake of EA cells was found to be normal in PSS. In contrast with Spisani's findings,[3] we found a reduced chemotactic activity of PMNs. The discrepancy could be attributed to the discrepancies in methods and demographic differences or to the relatively small number of patients investigated. The cause of the depressed chemotaxis of PMNs in PSS is unknown and further investigations are needed. Granulocytotoxins, other serum factors, and chemotaxis inhibitors may influence the chemotaxis of neutrophils.[24 26] The effect of lymphokines or the impaired leucocyte adherence may also cause a decreased chemotactic response of neutrophils.[27] Either reduced number or down regulation of membrane chemoattractant receptors may also be involved in the depressed chemotactic activity of PMNs in PSS.

Normal zymosan phagocytosis was found in patients with systemic lupus erythematosus, but findings are inconsistent in patients with rheumatoid arthritis.[28–30] In patients with malignancies the basal chemiluminescence activity was raised.[31]

Alterations in the functions of PMN cells could, in a complex way, influence the vascular, immunological, and fibrotic processes in patients with PSS.

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