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Lymphocyte subpopulations and reactivity to mitogens in patients with scleroderma

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SUMMARY

T lymphocyte subpopulations were studied in 40 patients with scleroderma (PSS), 26 of whom were studied simultaneously for lymphoproliferative responses to phytohaemagglutinin (PHA), concanavalin A (Con A) and pokeweed mitogen (PWM). PSS patients exhibited a reduction relative to 42 age- and sex-matched controls in the absolute number and percentage of early E rosettes, late E rosettes and E rosettes formed with aminoethylisothiouronium bromide (AET) treated sheep red blood cells. There was no difference between patients and controls in the proportions of B lymphocytes. PSS patients exhibited normal lymphocyte transformation responses to PHA and Con A and an augmented response to PWM. The mitogen responses did not correlate with the absolute number or percentage of lymphocytes or T and B lymphocyte subpopulations. No correlation was observed between any immunological variable studied and the extent of skin or organ involvement, disease duration or therapy.

INTRODUCTION

The participation of cell-mediated hypersensitivity in patients with scleroderma (PSS) has been supported by reports of lymphocyte infiltration in sclerodermatous skin (Fleischmajer, Perlish & Reeves, 1977), *in vitro* cytotoxicity of muscle, epithelial cells and fibroblasts by lymphocytes (Currie, Saunders & Knowles, 1970) and lymphokines released to a variety of antigens (Hughes, Holt & Powell, 1974; Stuart, Postlethwaite & Kang, 1976; Kondo, Rabin & Rodnan, 1976; Johnson & Ziff, 1976). Despite these findings, there is little agreement as to cellular immune function in patients with PSS.

In most studies to date (Hughes *et al.*, 1976; Horwitz & Garrett, 1977; Horwitz & Tuul-Nielsen, 1977; Gupta *et al.*, 1979), with one exception (Winkelstein, Rodnan & Heilman, 1972), an absolute lymphopenia has been confirmed. The proportions of T lymphocytes, however, have been shown to be normal (Horwitz & Garrett, 1977; Horwitz & Tuul-Nielsen, 1977; Lockshin *et al.*, 1975) or reduced (Carapeta & Winkelmann, 1975; deJesus & Clancy, 1975) in the face of normal numbers of B lymphocytes. Studies of lymphocyte proliferation to mitogens have also been inconsistent since both normal (Horwitz & Garrett, 1977; Winkelstein *et al.*, 1972; Lockshin *et al.*, 1975; deJesus & Clancy, 1975) and diminished responses (Salem & Morse, 1976; Hughes *et al.*, 1977) have been reported. Moreover, few investigators have studied both lymphocyte number and lymphoproliferative function simultaneously (Horwitz & Garrett, 1977; Lockshin *et al.*, 1975; deJesus & Clancy, 1975; Hughes *et al.*, 1977). In addition, in the majority of studies the number of subjects has been too

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small to provide definitive results (Horwitz & Garrett, 1977; Horwitz & Tuul-Nielsen, 1977; Lockshin *et al.*, 1975; Carapeta & Winkelmann, 1975; deJesus & Clancy, 1975; Salem & Morse, 1976).

In view of the shortcomings of previous studies and the contradictory results published to date, the present investigation was undertaken to examine T-lymphocyte subpopulations and lymphoproliferative function in a large population of patients with PSS.

MATERIALS AND METHODS

Patient selection. Forty patients, 37 females and three males with a mean age of 54·3 years (range 30–75) and a mean disease duration of 7·8 years (range 1–30) were studied. All patients fulfilled the criteria for definite scleroderma (Subcommittee for Scleroderma, 1980) and were followed in the scleroderma clinic of the Rheumatic Disease Unit of the Wellesley Hospital as part of an on-going clinical study. All patients were evaluated by a standard clinical and laboratory protocol. A detailed historical and physical examination was performed and laboratory testing carried out to include a complete blood count, urinalysis, ESR, latex fixation test, ANA, CPK, LDH, protein electrophoresis, renal function tests, chest X-ray, EKG, barium oesophageal studies, barium enema and pulmonary function testing. Seven patients were receiving prednisone (5 mg or less) and one was receiving 10 mg daily. No patient was being treated with penicillamine or a cytotoxic agent and none were azotaemic. Seven patients were receiving cimetidine in conjunction with prednisone. Eight patients were receiving between 20 and 40 grains of enteric-coated salicylate which was discontinued 24–36 hr prior to the study. Two patients were receiving vasodilators and two, non-steroidal anti-inflammatory agents other than salicylate. In total, 15 patients were receiving medications either singly or in combination. Patients were selected for specific studies solely on the basis of their availability at the time the tests were being performed.

The extent of disease in individual patients was quantitated by allocating points for organ systems involved by the disease according to the method of Hughes *et al.* (1977) with minor modifications. The disease score for each patient was used to ascertain possible correlations between the immune variables studied and severity of disease.

Controls. These consisted of 42 age- and sex-matched healthy volunteers from hospital personnel.

There were 38 females and four males with a mean age of 50·7 years (range 28–64). Informed consent was obtained from both patients and controls.

Cell separation procedure. Peripheral blood mononuclear cells (PBM) were isolated by Ficoll–Hypaque gradient centrifugation (Perper, Zee & Nickelson, 1968). The separated cells were washed twice with alpha medium (National Cancer Institute, Toronto) containing 50 units/ml penicillin, 50 μg/ml streptomycin and 2 mM L-glutamine.

Quantitation of lymphocyte populations

Total lymphocyte count. A total leucocyte count was determined by an automated Coulter counter technique. The proportion of lymphocytes was determined using a Wright–Giemsa-stained blood smear and the absolute lymphocyte count thereby calculated from this proportion.

E Rosettes. E rosettes were performed by resuspending PBM, isolated as described, in phosphate-buffered saline, pH 7·4 (PBS), in a concentration of 3×10^6 cells/ml. One hundred microlitres of cell suspension were added to 100 μl of a 1% sheep red blood cell (SRBC) suspension and incubated at 37°C for 15 min. Early rosettes were enumerated immediately following centrifugation of the cell mixture at 1,000 r.p.m. for 5 min at room temperature. Late rosettes were prepared in a similar fashion except that the resultant pellet was incubated overnight at 4°C and resuspended prior to counting. For detection of the total E rosetting population, 1 vol of 5% SRBCs was preincubated for 20 min at 37°C with 4 vol of 0·14 M.

In all rosette assays the pellet was gently resuspended and a drop of the suspension transferred to a haemocytometer chamber for counting. Two hundred cells were counted and the proportion of PBM binding three or more SRBC was determined.

Immunoglobulin (Ig) bearing lymphocytes were detected using techniques of anti-immunoglobulin fluorescent staining. Briefly, PBM in a concentration of 4×10^6 cells/ml were resuspended in HBSS and 5% fetal calf serum (FCS). Fifty microlitres of the cell suspension were incubated with 50 μl of FITC-conjugated F(ab')$_2$ goat anti-human Ig (Cappel Laboratories, Toronto, Ontario) for 30 min at 4°C. Following centrifugation at 500 r.p.m. for 5 min at 4°C the supernatant was removed and the cells washed twice with HBSS and 5% FCS mixed with 50% glycerine.

The suspension was transferred to slides, incubated for 10 min in the cold and read with a Leitz–Ortholux microscope with fluorescence vertical and phase contrast illuminators. Two hundred cells were counted and the percentage of fluorescing cells determined.

Mitogen-induced lymphocyte transformation. PBM were resuspended in alpha media (as described) containing 25% pooled human AB serum. Triplicate 200-μl cultures each containing 5×10^4 were set up in flat-bottomed Linbro microtitre wells. Fifty microlitres of mitogens were added to each culture in varying concentrations as follows: phytohaemagglutinin-P (PHA) (Wellcome reagents, England) 2·8, 11·25, 22·5, 70 and 700 μg/ml; concanavalin A (Con A) (Calbiochem, USA) 2·5, 5, 10 and 50 μg/ml and pokeweed mitogen (PWM) (GIBCO BIOCULT, USA) 3·2, 12·5, 5, 25 and 500 μg/ml. PBM incubated with PHA and Con A were cultured for 5 days and those incubated with PWM were cultured for 7 days. Tritiated thymidine (0·2 μCi/culture, sp. act. 18·4 Ci/mmol) was added 4 hr prior to harvest. Tritiated thymidine incorporation was determined by liquid scintillation counting. The mitogenic response was calculated by selecting the concentration of mitogen giving the greatest reactivity.

Statistical analyses of the differences between groups and the significance of the correlation coefficient were determined by Student's *t*-test.

RESULTS

Lymphocyte subpopulations

Results for the absolute lymphocyte counts revealed that patients with PSS had a lower total lymphocyte count (1,653 ± 125 cells/mm^3) (mean ± 1 s.e.) when compared with controls

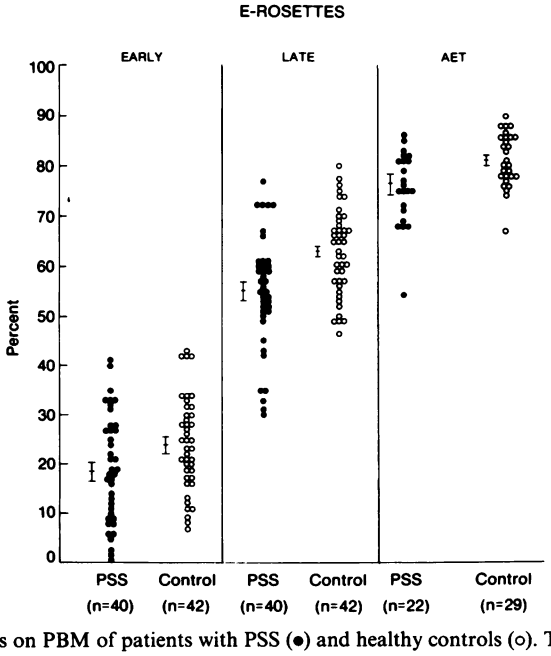

Fig. 1. E rosette markers on PBM of patients with PSS (•) and healthy controls (o). The mean percentage for each group is depicted by the bar and the error bars represent ± 1 s.e.

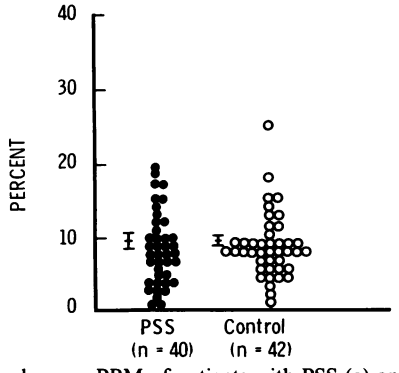

Fig. 2. Surface membrane Ig markers on PBM of patients with PSS (•) and healthy controls (o). The mean percentage for each group is depicted by the cross-bar and the error bars represent ±1 s.e.

(2,339±151 cells/mm^3) ($P<0{\cdot}05$). Analysis of T cell subpopulations revealed that patients with PSS exhibited a reduction relative to controls in the percentage of early rosettes (18·5±1·7 *vs* 24·0±1·5%), late rosettes (55·4±1·8 *vs* 62·9±1·2%) and AET rosettes (75·6±1·6 *vs* 82·5±1·1%) (Fig. 1). The percentage of B lymphocytes (surface membrane immunoglobulin-bearing cells) was normal relative to controls (9·6±0·8 *vs* 9·2±0·6%) (Fig. 2). No statistical correlation was observed between any T lymphocyte subpopulation and the extent of skin or organ involvement, disease duration or therapy. There were no statistically significant differences in the T or B lymphocyte populations between those patients who exhibited systemic involvement, ANA, rheumatoid factor or hypergammaglobulinaemia and those not exhibiting such features.

Results of the lymphoproliferation studies revealed no significant differences in response to PHA or Con A stimulation between patients and controls whether the results were analysed as the $\log_{10}$ mean peak counts per minute (±s.e.) (PHA: 4·81±0·03 *vs* 4·87±0·04 c.p.m.; Con A: 4·55±0·04 *vs* 4·46±0·05 c.p.m. for patients and controls respectively) (Fig. 3) or by the area under the dose–response curve (data not shown). No differences were detected to either mitogen between the patient and control groups at the suboptimal doses tested. In contrast, the $\log_{10}$ peak response of patients' PBM to PWM (4·69±0·04 c.p.m.) was significantly greater than that of controls (4·57±0·04 c.p.m.) ($P<0{\cdot}05$). In addition, the peak responses generally were achieved at lower PWM concentrations in the patient groups relative to controls (data not shown). There were no

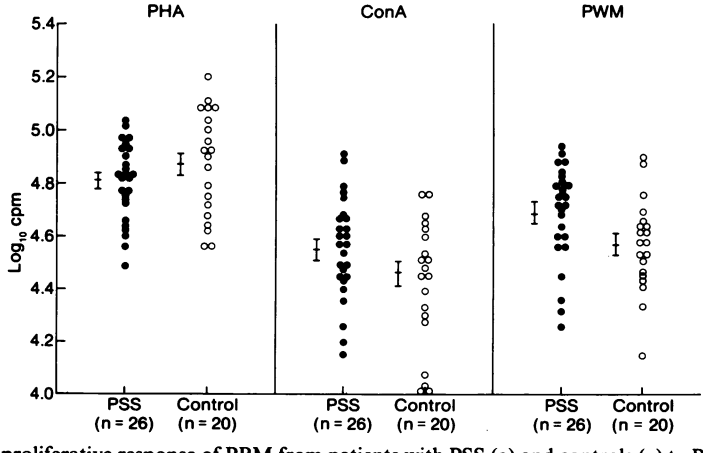

Fig. 3. Lymphoproliferative response of PBM from patients with PSS (•) and controls (o) to PHA, Con A and PWM. The $\log_{10}$ mean c.p.m. for each group is depicted by the cross-bar and the error bars represent ±1 s.e.

Table 1. Immunological variables in PSS patients not receiving drug therapy

Immunological variable	No. of controls	Healthy controls (±s.e.)	No. of patients	PSS patients (±s.e.)
Absolute lymphs/mm^3	27	2,200±160	25	1,669±170*
Early E rosettes (%)	27	26·4±2·4	25	14·8±2·4*
Late E rosettes (%)	27	61·6±2·3	25	55·8±2·1*
AET rosettes (%)	22	81·1±1·5	15	77·4±2·4
Surface membrane Ig-bearing cells (%)	27	9·2±1·2	25	9·0±0·9
PHA (log c.p.m.)	20	4·88±0·05	14	4·82±0·03
Con A (log c.p.m.)	20	4·51±0·06	14	4·58±0·04
PWM (log c.p.m.)	20	4·59±0·03	14	4·66±0·03*

* Significant difference between PSS patients and controls with $P<0·05$.

significant correlations between the mitogen responses and skin or organ involvement or disease duration. The mitogen responses did not correlate with the proportion or absolute number of lymphocytes or T and B lymphocyte subpopulations studied. There were no differences in mitogen responses between patients who exhibited systemic involvement, ANA, rheumatoid factor or hypergammaglobulinaemia and those not exhibiting such features.

Since 15 of the PSS patients were receiving some form of medication at the time of the study, the immune aberrations observed might be ascribed to the drug therapy. To exclude this possibility, the data were reanalysed deleting those patients (and matched controls) receiving the medications outlined in the Methods section. As may be seen in Table 1, differences between PSS patients and controls were still demonstrated with the exception of AET rosettes for which a borderline significant difference was observed with $P=0·1$.

DISCUSSION

The present study demonstrates that patients with PSS have immunological aberrations in their peripheral blood lymphocyte populations relative to healthy controls of comparable age and sex. PSS patients exhibited a reduction in the absolute numbers of peripheral blood lymphocytes and proportion of T lymphocytes. In this study only two patients had CREST syndrome as defined by skin tethering limited to the face, neck and hands. On this account a comparison of lymphocyte variables between CREST and diffuse scleroderma was not possible.

The results of the lymphocyte population studies lend credence to several previous reports demonstrating reduced absolute numbers of peripheral blood lymphocytes (Hughes *et al.*, 1976; Horwitz & Garrett, 1977; Horwitz & Tuul-Nielsen, 1977; Gupta *et al.*, 1979) and reduced proportions of T lymphocytes (Carapeta & Winkelmann, 1975; deJesus & Clancy, 1975). The data, however, are in conflict with a number of studies demonstrating normal proportions of T lymphocytes (Horwitz & Garrett, 1977; Horwitz & Tuul-Nielsen, 1977; Gupta *et al.*, 1979). The reasons for the discrepancies are unclear, but may be a reflection of the small numbers of patients

analysed in these latter studies (Horwitz & Garrett, 1977; Horwitz & Tuul-Nielsen, 1977; Lockshin *et al.*, 1975).

The results of the lymphoproliferation studies are in agreement with several previous reports of normal PHA and Con A responsiveness (Horwitz & Garrett, 1977; Winkelstein *et al.*, 1972; Lockshin *et al.*, 1975; deJesus & Clancy, 1975). The studies demonstrating impaired PHA responsiveness utilized whole blood (Hughes *et al.*, 1977) or unseparated cell suspensions (Horwitz & Garrett, 1977). The reduced PHA responsiveness in these assays likely reflects, at least in part, the lymphopenia observed in PSS patients. Since a lymphocyte transformation assay involving enriched populations of lymphocytes would not reflect the lymphopenia, subtle alterations in the proportion of T lymphocytes might not be detected in this assay system.

The mechanism(s) accounting for the T lymphopenia remains unclear. Although anti-lymphocyte antibodies have been implicated in the T lymphopenia in patients with SLE, there is little evidence implicating this mechanism in patients with PSS (Horwitz & Tuul-Nielsen, 1977; Inoshita, Whiteside & Rodnan, 1980). It is possible that the decreased percentage of T cells identified in PSS patients might be due to an increase in the proportion of monocytes in the mononuclear cell fraction. An increase in the proportion of monocytes would be expected to affect the lymphocyte populations to a similar extent resulting in proportionate decreases in each of the subpopulations within a given patient. However, no statistical correlation could be identified between the various lymphocyte subsets within the PSS group (data not shown), militating against the concept that T cells were diluted out by monocytes. Moreover, the data are consistent with previous studies demonstrating a T lymphopenia when monocytes were either pre-labelled (Horwitz & Garrett, 1977) or removed (Hughes *et al.*, 1976).

In the present study patients were compared with normal, healthy controls. While it is tempting to conclude that differences are related to PSS *per se*, it is conceivable that the small differences between PSS patients and controls could be due to chronic illness irrespective of the nature of the disease. In the present patient population, all patients were ambulatory without serious functional disability and could be considered comparable to a functional class I–II rheumatoid patient. In this context, it is unlikely that debility contributed to the immune deficiencies observed.

The role of T lymphopenia in the pathogenesis of PSS is unknown. Currently, autoimmune disorders are thought to arise from excessive B cell hyperreactivity as a result of defective suppressor T cell function. To date, however, there is little evidence to support this concept in patients with PSS. Indeed, preliminary evidence favours the possibility of excessive helper T cell activity (Inoshita *et al.*, 1980). Recent evidence has suggested a role for cell-mediated hypersensitivity in PSS (Fleischmajer *et al.*, 1977; Currie *et al.*, 1970; Hughes *et al.*, 1974; Stuart *et al.*, 1976; Kondo *et al.*, 1976; Johnson & Ziff, 1976). Suppressor T lymphocytes play a role in regulating cell-mediated immune responses and may be different from those suppressor cells regulating antibody synthesis. It is conceivable then, that subpopulations of T lymphocytes regulating cell-mediated immunity in PSS patients may be selectively reduced. Additional studies of immunoregulatory cell function in patients with PSS are clearly warranted.

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