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The relationship of defective cell-mediated immunity to visceral disease in systemic sclerosis

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SUMMARY

Phytohaemagglutinin-induced lymphocyte transformation and circulating thymus-dependent (T) lymphocyte numbers were studied in twenty-eight patients with systemic sclerosis (SS), in fifty normal controls and in eleven elderly controls.

Patients with SS were found to have impaired lymphocyte transformation responses which showed a positive correlation with both the number of circulating T lymphocytes and the extent of visceral involvement by the disease. This defect of cell-mediated immunity could not be attributed to the effects of increasing age, corticosteroid treatment, iron and folate deficiency or inhibitory serum factors and may have pathogenetic implications for the disorder.

INTRODUCTION

The aetiology of systemic sclerosis (SS) remains uncertain but the multisystem nature of the disorder (Rodnan, 1965) and the occasional association or clinical 'overlap' with disorders and as Sjögren's syndrome (Shearn, 1960; Bunim, 1961) or systemic lupus erythematosus (Tuffanelli & Winkelmann, 1962) suggest that autoimmune processes may be involved. Humoral abnormalities such as hyperglobulinaemia (Šťáva, 1958) and a high incidence of both rheumatoid factors (Rothfield & Rodnan, 1968) and autoantibodies (Beck *et al.*, 1963; Alarcón-Segovia *et al.*, 1975) are well documented but cell-mediated immunity has received comparatively little attention. Although there have been isolated reports of leucocyte migration inhibition by tissue antigens (Hughes, Holt & Rowell, 1973) and lymphocyte cytoxicity to cultured human target cells (Currie, Saunders & Knowles, 1971), cellular immune competence, as indicated by the lymphocyte transformation response to phytohaemagglutinin (PHA), has been reported to be normal (Winkelstein, Rodnan & Heilman, 1972). We have recently described a deficiency of thymus-dependent (T) lymphocytes in SS which showed a correlation with the extent of visceral disease (Hughes *et al.*, 1976) and the present report confirms and extends these observations with a parallel study of lymphocyte-transformation responses to PHA.

MATERIALS AND METHODS

Patients. Twenty-eight patients with SS, two of whom were male with a mean age of 51·7± 12·8 years were investigated. Four of these patients were receiving treatment with prednisolone (7·5–10·0 mg/day). The duration of the disease varied from 1 year to 38 years (mean 11 years) and Raynaud's phenomenon and acrosclerosis were invariably present. All patients were investigated by a standard set of investigations, i.e. electrocardiograph, chest radiograph, estimation of carbon monoxide transfer factor, barium swallow, urinalysis and creatinine clearance. The extent of the disease in individual patient was assessed without prior knowledge of any immunological abnormality by one of us (NRR) using a combination of investigational abnormalities on the standard tests and a clinical estimate of the extent of cutaneous sclerosis. Additional investigations, such as biopsies, muscle enzyme estimations and further gastrointestinal contrast radiology were only performed in individual patients if indicated by the clinical features. Points were allocated for organ involvement by the disease process,

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TABLE 1. Criteria of systemic involvement in patients with systemis sclerosis

System involved	Criteria	Disease score (points allotted for involvement)
Skin	Sclerosis—face	1
	hands	1
	trunk	1
GI tract	Radiological changes	3
Lungs	Radiological changes and/or abnormal CO transfer factor	3
Heart	ECG changes	3
Kidneys	Creatinine clearance < 60 ml/min and/or proteinuria	3
Other	Sjögren's syndrome, myositis etc.	3

as shown in Table 1, and the resulting 'disease score' for each patient was used to examine the possible correlations between lymphocyte transformation responses, absolute T lymphocyte counts and the severity of the disease. Three grades of disease severity, i.e. mild (disease score 0–4), moderate (disease score 5–8) and severe (disease score 9–15) were also used to explore the correlation between cellular immune status and the extent of visceral disease.

Controls. These consisted of two groups of healthy volunteers. The first group of fifty normal controls, nineteen of whom were male, had a mean age of 42·1 ± 14·5 years. The second group of selected elderly controls consisted of eleven subjects, three of whom were male, with a mean age of 62·2 ± 4·1 years.

Lymphocyte transformation. This was performed in both autologous plasma and human AB serum by a modification of the whole blood micro method of Junge *et al.* (1970). For tests in autologous plasma, 1 part of whole blood, anticoagulated with preservative-free heparin (Sigma Chemical Co., St. Louis, U.S.A.) 20 u/ml, was diluted with 9 parts of RPMI 1640 (Flow Laboratories, Irvine) and aliquots (1 ml) of this mixture were then cultured in triplicate at 37°C for 72 hr with PHA (K7950 Wellcome Laboratories Ltd., Beckenham) at 0, 0·1 and 1·0 μg/ml in an atmosphere of 5% CO_2–95% air.

For studies in human AB serum, 1 ml of heparinized blood was washed three times with 10 ml of RPMI 1640 before finally being diluted 1 part in 9 with RPMI 1640 enriched with 5% human AB serum. Aliquots (1 ml) were then similarly cultured and stimulated with PHA. ^{3}H-labelled methyl thymidine, 2 μCi (sp. act. 5 Ci/millimole, Radiochemical Centre, Amersham), was added to the tubes for the last 6 hr of culture and, after preliminary lysis of red blood cells by 3% acetic acid, de-oxyribonucleic acid was then precipitated by 5% trichloroacetic acid. The washed precipitate was solubilized by 1 M alcoholic sodium hydroxide and incorporated into NE 260 scintillator fluid (Nuclear Enterprises, Edinburgh). The samples were counted in an Intertechnique liquid scintillation counter using a quench correction curve relating automatic external standard to percentage counting efficiency on a basis of colour quenching by varying amounts of added red blood cells. The results were expressed in 'absolute' terms as $\log_{10}$ of the average disintegrations per minute (d/min) of triplicate cultures and, as whole blood leucocyte culture makes no allowance for variation in leucocyte counts, also as a 'derived' count based on 1×10^5 lymphocytes, again expressed as $\log_{10}$ of the average d/min.

The investigation was designed so that normal control samples were routinely included in each batch of lymphocyte transformation tests performed on patients.

T lymphocytes. These were estimated by the method of Hughes *et al.* (1976).

Serum iron and total iron binding capacity. These were estimated by autoanalyser.

Serum and red blood cell folic levels. These were estimated by the method of Millbank *et al.* (1970).

Autoantibodies. These were detected using a standard immunofluorescence method in the University Department of Immunology, Hallamshire Hospital, Sheffield S10 2JF.

Statistical methods. Analysis of the transformation responses, T lymphocyte numbers and serum iron and folate levels in patient and control groups was made by Student's *t*-test.

RESULTS

Patients with SS

These were found to have depressed lymphocyte transformation response to PHA in both autologous

TABLE 2. Lymphocyte transformation responses in patients with systemic sclerosis (SS) and controls

Group studied	No.	Age (years) (Mean ± s.d.)	Lymphocyte transformation responses: 'Absolute' (log_{10}) (mean ± s.d.) — Autologous plasma PHA concentrations (μg/ml)			Human AB serum PHA concentrations (μg/ml)			'Derived' (log_{10}) (mean ± s.d.) — Autologous plasma PHA concentrations (μg/ml)			Human AB serum PHA concentrations (μg/ml)		
			0	0·1	1	0	0·1	1	0	0·1	1	0	0·1	1
SS*														
Severe	7	51·3 ± 15·3	2·6163 ± 0·1326	3·4096 ± 0·4747	4·3705 ± 0·3919	2·7970 ± 0·2198	3·8201 ± 0·4171	4·5380 ± 0·3265	2·7512 ± 0·3318	3·5064 ± 0·2597	4·4687 ± 0·2774	2·9114 ± 0·2471	3·9169 ± 0·2019	4·6375 ± 0·2412
Moderate	14	54·6 ± 8·1	2·7200 ± 0·1047	3·8290 ± 0·4148	4·9405 ± 0·1800	2·6301 ± 0·0857	4·1962 ± 0·3169	4·8506 ± 0·2047	2·5067 ± 0·1661	3·6850 ± 0·3313	4·7258 ± 0·1844	2·4164 ± 0·1416	3·9970 ± 0·3249	4·6472 ± 0·2044
Mild	7	46·1 ± 17·5	2·7016 ± 0·1429	4·3943 ± 0·3159	5·1743 ± 0·1747	2·6255 ± 0·1792	4·5647 ± 0·1938	5·1095 ± 0·1772	2·3672 ± 0·2897	4·0756 ± 0·4046	4·8498 ± 0·2356	2·3115 ± 0·3072	4·2413 ± 0·2412	4·7861 ± 0·2327
SS-iron/folate deficient														
Before treatment	6	53·0 ± 9·7	2·6122 ± 0·1761	3·6915 ± 0·4723	4·9017 ± 0·2728	2·5284 ± 0·2119	4·0354 ± 0·4542	4·8010 ± 0·1785	2·5674 ± 0·1555	3·5920 ± 0·4601	4·8021 ± 0·1986	2·4678 ± 0·1836	3·9360 ± 0·4289	4·7022 ± 0·1390
After treatment	6	—	2·6915 ± 0·0939	3·8747 ± 0·4454	4·7608 ± 0·2307	2·6856 ± 0·0989	4·0691 ± 0·4466	4·6941 ± 0·2738	2·6001 ± 0·2521	3·7924 ± 0·2280	4·6120 ± 0·2859	2·5942 ± 0·2556	3·9680 ± 0·4139	4·5499 ± 0·4160
Normal controls	50	42·1 ± 14·5	2·7491 ± 0·3104	4·6131 ± 0·3931	5·1509 ± 0·2457	—	—	—	2·4013 ± 0·3156	4·2670 ± 0·4036	4·8062 ± 0·2683	—	—	—
Normal controls														
Replicate cultures	17	36·5 ± 9·1	2·7613 ± 0·1623	4·5465 ± 0·3032	5·1170 ± 0·2767	—	—	—	2·5040 ± 0·2556	4·3097 ± 0·2735	4·8654 ± 0·3169	—	—	—
Elderly controls	11	62·2 ± 4·1	2·7490 ± 0·1334	4·4265 ± 0·3997	4·9609 ± 0·2483	—	—	—	2·3501 ± 0·1344	4·0315 ± 0·4312	4·5660 ± 0·3044			
One normal control														
Replicate cultures	7	23	2·8609 ± 0·0721	4·7841 ± 0·3004	5·3101 ± 0·1387	2·8018 ± 0·0541	4·5784 ± 0·3907	5·1784 ± 0·0985	—	—	—	—	—	—
Replicate cultures enriched with 5% SS serum	7	—	2·8175 ± 0·0566	4·5076 ± 0·2019	5·1322 ± 0·2279	—	—	—	—	—	—	—	—	—

* Grades of severity as defined in text.

TABLE 3. The difference in lymphocyte transformation responses between patients with systemic sclerosis (SS) and controls

			Lymphocyte transformation	
			Absolute* d/min	Derived* d/min
Disease group	Serum	PHA conc. (μg/ml)	*P*	*P*
Severe SS	Autologous	0·1	< 0·001	< 0·001
		1·0	< 0·001	< 0·01
	AB	0·1	< 0·001	< 0·05
		1·0	< 0·001	n.s.
Moderate SS	Autologous	0·1	< 0·001	< 0·001
		1·0	0·01	n.s.
	AB	0·1	0·001	< 0·05
		1·0	< 0·001	< 0·05
Mild SS	Autologous	0·1	n.s.	n.s.
		1·0	n.s.	n.s.
	AB	0·1	n.s.	n.s.
		1·0	n.s.	n.s.

* Defined in text.

TABLE 4. The correlation between 'disease score' and PHA lymphocyte transformation responses in patients with systemic sclerosis

		Lymphocyte transformation (Absolute* d/min)		Lymphocyte transformation (Derived* d/min)	
	PHA conc. (μg/ml)	Correlation coefficient	*P*	Correlation coefficient	*P*
In autologous plasma	0·1	−0·60	< 0·001	−0·48	< 0·01
	1·0	−0·77	< 0·001	−0·57	< 0·001
In AB serum	0·1	−0·61	< 0·001	−0·35	0·05
	1·0	−0·70	< 0·001	−0·29	n.s.

* Defined in text.

plasma and AB serum irrespective of whether the results were expressed in 'absolute' or 'derived' counts (Tables 2 and 3). The transformation data showed strong correlations with the severity of the disease process which were most evident with 'absolute' responses in autologous plasma and AB serum and 'derived' responses in autologous plasma (Table 4). The 'absolute' transformation responses also showed a strong correlation with the previously noted deficiency of circulating T lymphocytes (Hughes *et al.*, 1976) (Fig. 1, Table 5) but this relationship was not observed when 'derived' responses were compared (Table 5). Transformation responses and circulating T lymphocyte numbers were reduced, therefore, in both severely and moderately affected patients, particularly in the former sub-group, but were indistinguishable from normal control values in patients with only mild disease.

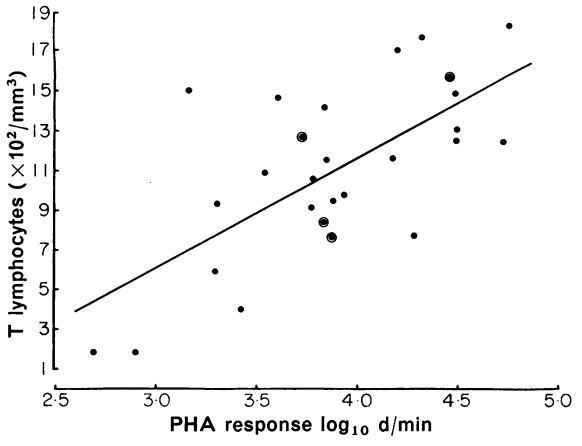

FIG. 1. The relationship between circulating T lymphocytes and lymphocyte transformation response (absolute d/min); PHA 0·1 μg/ml) in patients with SS. ○ Patients receiving treatment with prednisolone. $r = 0{\cdot}68$; $t = 4{\cdot}57$; $P < 0{\cdot}001$.

Visceral involvement by the disease also showed some correlation with both the ESR and circulating autoantibodies, more severely affected patients having both a higher mean ESR and a greater incidence of autoantibodies than mildly affected cases (Tables 6 and 7). There was a similar association with low serum folate levels, both moderately and severely affected patients having subnormal values ($P < 0{\cdot}05$) (Table 6). Serum iron levels, however, were depressed in all categories of the disease ($P < 0{\cdot}05$) (Table 6).

TABLE 5. The correlation between circulating T lymphocytes and PHA lymphocyte transformation responses in patients with systemic sclerosis

	PHA conc. (μg/ml)	Lymphocyte transformation (absolute* d/min)		Lymphocyte transformation (derived* d/min)	
		Correlation coefficient	*P*	Correlation coefficient	*P*
In autologous plasma	0·1	0·68	< 0·001	0·47	< 0·01
	1·0	0·76	< 0·001	0·31	n.s.
In AB serum	0·1	0·65	< 0·001	0·17	n.s.
	1·0	0·68	< 0·001	0·01	n.s.

* Defined in text.

Iron and folate deficiency

The results for six severely affected patients with SS before and after correction of their iron and folate deficiency are shown in Tables 2 and 8. Lymphocyte transformation was initially markedly depressed at both concentrations of PHA in both autologous plasma and AB serum and did not increase after correction of the two deficiencies.

Serum factors

Three sera from patients with SS with depressed lymphocyte transformation consistently produced some impairment of transformation when added to cultures of normal lymphocytes (Fig. 2). A further

TABLE 6. T lymphocyte numbers, serum iron and folate levels and ESR in patients with systemic sclerosis (SS) and controls

Group	No.	T lymphocytes/mm³ (mean±s.d.)	ESR (mm/hr) (mean±s.d.)	Serum iron (μ.mol/l) (mean±s.d.)	Serum folate (μ.mol/l) (mean±s.d.)
Severe SS	7	619±352	55·1±43·1	7·9±4·6	3·7±0·7
Moderate SS	14	1154±281	33·8±21·1	11·7±4·2	4·8±2·4
Mild SS	7	1501±293	21·6±13·6	11·0±4·2	5·7±2·4
Normal control	50	1726±583	—	19·0±6·7	6·8±2·9
Elderly control	11	1876±447	—	15·2±4·0	6·8±2·7

TABLE 7. Autoantibodies in patients with systemic sclerosis (SS)

SS Group	No.	ANF	DNA	Others*	Total
Severe	6	5	2	4	11
Moderate	14	12	0	3	15
Mild	7	4	0	3	7

* Includes antimitochondrial, smooth muscle, anti-thyroid and gastric parietal cell antibodies.

TABLE 8. Pre- and post-treatment iron and folate status of six patients with systemic sclerosis with depressed lymphocyte transformation responses

	Serum iron (μ.mol/l) (mean±s.d.)	Total iron binding capacity (μ.mol/l) (mean±s.d.)	Serum folate (μ.mol/l) (mean±s.d.)	Red cell folate (μ.mol/l) (mean±s.d.)
Pre-treatment	7·9±2·2	59·9±14·8	3·7±1·9	191±107
Post-treatment	14·2±2·8	45·6±7·9	>24·0	1000±245

seven sera, also from patients with impairment of lymphocyte transformation, did not produce any significant depression of transformation when added to cultures of normal lymphocytes (Table 2).

Normal controls

These showed good reproducibility of lymphocyte transformation responses in both 'absolute' and 'derived' forms. Seventeen controls were examined serially over a period of 10 months and produced responses that did not differ from those found in the whole of the original control group (Table 2).

Elderly controls

These showed some reduction of lymphocyte transformation responses, especially at the lower concentration of PHA, when compared with younger controls (Table 2). The reduction was, however, never of the magnitude seen in patients with SS at any age.

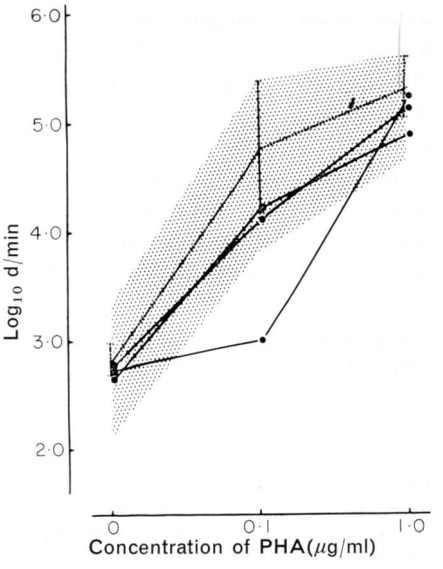

FIG. 2. Modification of PHA-induced lymphocyte transformation (absolute d/min) by 5% SS serum. Depressed transformation of lymphocytes from one normal control produced by three SS sera (●——●) compared with the response (mean ± 2 s.d.) of (a) seven replicate cultures from the normal control (- - -) (b) fifty normal controls (hatching).

DISCUSSION

This investigation has produced evidence of defective cell-mediated immunity in patients with SS who have extensive visceral disease. The close correlation in patients between the impaired 'absolute' PHA transformation responses and the numbers of circulating T lymphocytes not only confirms our earlier report of a T cell deficiency in SS (Hughes *et al.*, 1976) but also emphasizes the relationship of this defective cell-mediated immunity to the severity of the disease process. The persisting reduction of transformation responses in their 'derived' form suggests that there is not only a quantitative deficiency but also a qualitative defect of T lymphocytes in these severely affected patients. This finding contrasts with the earlier failure of Winkelstein *et al.* (1972) to note any reduction of PHA transformation responses in scleroderma; a discrepancy which may be explained partly by the failure of these authors to use appropriate suboptimal stimulatory doses of PHA on a dose-response basis and partly by their probable failure to study severely affected patients.

The possibility that our findings could have resulted from the effect of inhibitory serum factors has also been examined. Although three sera from severely affected patients certainly did produce some inhibition of the PHA transformation response of normal lymphocytes a further seven sera, again from patients with depressed transformation responses, failed to impair the response of normal cells. Further support for the overall absence of inhibitory serum factors is provided by the transformation responses in AB serum which, in severely affected patients, were just as depressed as the corresponding responses in autologous plasma. Similarly, we have found no evidence that either iron and/or folate deficiency, both of which can impair cell-mediated immunity (Joynson *et al.*, 1972; Gross *et al.*, 1975), could have produced the observed immunological abnormality even though the patients studied were, to some extent, deficient in both these substances. Finally, our results have not been influenced by the immunodepressive effects of corticosteroids as the few patients receiving such treatment were not among those showing the greatest T cell impairment (Fig. 1).

The significance of the observed defect in cell-mediated immunity remains uncertain. It may represent nothing more than a secondary effect of severe disease analogous to the impaired cell-mediated immunity found in patients with advanced malignancy (Chretien *et al.*, 1973; Anthony *et al.*, 1975). However, it is possible that the defect may be of some pathogenetic importance. There is evidence from

experimental host-*vs*-graft disease that chronic antigenic stimulation of the host leads to a progressive depletion of T lymphocytes and that the associated loss of 'suppressor' T cells is responsible for the development of the hyperglobulinaemia and immune complex deposition which are features of this model (Hard, 1975). Scleroderma is also a disorder which is characterised by hyperglobulinaemia (Šťáva, 1958) and autoantibodies (Beck *et al.*, 1963; Alarcón-Segovia *et al.*, 1975) especially in those patients with visceral involvement (Jablonska, Blaszczyk & Glinski, 1974), while, conversely, scleroderma-like changes have also been observed to develop in the skin of animals suffering from graft-*vs*-host disease (Statsny, Stembridge & Ziff, 1963). Our findings, therefore, could support the view that autoimmune processes, such as the lymphocyte cytotoxicity described by Currie *et al.* (1971), may play a part in the pathogenesis of SS.

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