Monocyte-induced inhibition of lymphocyte response to phytohaemagglutinin in progressive systemic sclerosis

MICHAEL D. LOCKSHIN, JOSEPH A. MARKENSON, LASZLO FUZESI, SALPI KAZANJIAN-ARAM, CATHERINE JOACHIM, AND MARGORIE ORDENE

From the Division of Rheumatic Diseases, Department of Medicine, Hospital for Special Surgery and the Cornell University Medical College, New York, New York 10021

SUMMARY In patients with progressive systemic sclerosis (PSS) lymphocyte responses to phytohaemagglutinin (PHA) are abnormal ($27.2 \pm 3.5 \times 10^{-3}$ counts per minute (cpm) versus $69.8 \pm 4.4 \times 10^{-3}$ for normal persons, $p < 0.005$). Removal of adherent peripheral blood mononuclear cells improves the response of PSS lymphocytes ($42.3 \pm 3.4 \times 10^{-3}$ cpm, 155% of control) but diminishes the response of normal lymphocytes ($60.3 \pm 5.9 \times 10^{-3}$, 86% of control). Supernatant fluids from cultures of PSS unfractionated and adherent cells depress normal T cell response to PHA (64% and 55% of control respectively), but supernatant fluids from normal unfractionated and adherent cells do not (104% and 101% of control). Supernatant fluids of PSS and normal adherent cells, cultured in the presence of indomethacin, are not inhibitory to normal T cells ($109 \pm 15\%$ and $124 \pm 14\%$ of control respectively). Supernatant fluids from PSS patients are more inhibitory than comparable fluids from patients with systemic lupus erythematosus ($60 \pm 8\%$ of control versus $80 \pm 5\%$ of control). These data support the hypothesis that cellular immunity is abnormal in patients with PSS and indicate that adherent mononuclear cells mediate at least one component of the abnormality via an indomethacin-sensitive mechanism.

In progressive systemic sclerosis (PSS), abnormalities in lymphocyte response to mitogen,¹ proportional distribution of lymphocyte subtypes,²⁻⁴ leucocyte migration inhibition,⁵ antibody-dependent cell-mediated cytotoxicity,⁶ and serum inhibition of mitogen response⁷ have been described. In patients with systemic lupus erythematosus (SLE),^{8,9} Hodgkin's disease,¹⁰ sarcoidosis,¹¹ and tuberculosis¹² poor responses of peripheral blood mononuclear cells to phytohaemagglutinin (PHA) appear to be due at least in part to adherent mononuclear cells or to their soluble products. This report extends these observations to patients with PSS and demonstrates a more severe abnormality than previously seen with SLE.

Materials and methods

Patients. Patients with PSS and normal volunteers were studied. The patients were not selected on the

basis of activity of disease or concurrent therapy. Most were untreated; 2 of 13 in the first set of experiments took corticosteroids (5-10 mg/day of prednisone) and none of 13 in the second set of experiments did. No patient took indomethacin or other nonsteroidal anti-inflammatory drug. SLE patients studied as controls were chosen for low or absent steroid dose.

Cell separation and purification. 50 ml of heparinised blood were obtained by venepuncture (informed consent was obtained), layered on a Ficoll-Hypaque gradient in order to isolate the mononuclear cells¹³; these cells were then washed 3 times in Hanks's balanced salt solution and cultured directly (first series) or processed for adherent cells (second series). T cells were obtained by passage of 2×10^7 mononuclear cells per ml over a G-200 Sephadex column to which purified rabbit antihuman F(ab)₂ was conjugated.¹⁴ The cells were eluted with Roswell Park Memorial Institute solution (RPMI) 1640 containing 5% fetal calf serum (Grand Island Biological Co., Grand Island, New York), 100 µg/ml penicillin, and 100

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Correspondence to Dr Michael D. Lockshin, Hospital for Special Surgery, 535 East 70th Street, New York, New York 10021, USA.

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$\mu\text{g/ml}$ streptomycin. Eluted cells demonstrated $<1\%$ staining for surface immunoglobulin and greater than 90% sheep erythrocyte rosette formation, and are referred to as T cells. Adherent cells were obtained by incubating 1×10^6 mononuclear cells/ml (RPMI 1640 with 20% human AB serum, penicillin, and streptomycin) in glass Petri dishes for 90 minutes at 37°C in 5% CO_2 . The resulting adherent cells were removed with a rubber policeman, resuspended in the above media, and passed over cotton-wool columns¹⁵ to remove any nonviable cells. These cells stained $>90\%$ positive by peroxidase¹⁶ and had greater than 90% viability as determined by trypan blue staining. Nonadherent lymphocytes were obtained by incubating mononuclear cells (1×10^6 cells/ml) on glass Petri dishes for 90 minutes. The medium, including nonadherent cells, was pipetted off. Aspirated cells stained less than 3% peroxidase-positive.

Generation of supernatant fluids. $1 \times 10^6/\text{ml}$ unfractionated mononuclear cells, T cells, or monocytes were suspended in RPMI 1640, 20% AB serum, 2% penicillin, and streptomycin and incubated in Petri dishes for 48 hours at 37°C in 5% CO_2 . After 48 hours of culture greater than 50% plated cells were recovered; these demonstrated $>98\%$ viability by trypan blue dye exclusion. The supernatant fluids were then aspirated, centrifuged to remove cells and debris, and stored at -20°C . In some experiments indomethacin in ethanol to a final concentration of $1 \mu\text{g/ml}$ was added to cultures at the time of plating. Control ethanol did not affect the cultures, and indomethacin added at termination of culture did not alter the results.⁹ In other experiments exogenous prostaglandin E_2 in concentrations of 10^{-5} to 10^{-10} molar (a generous gift of Dr Robert Kimberly) was added to the supernatant fluid immediately prior to assay.

Mitogen cultures. T cells were cultured in microtitre plates according to previously published methods⁹ with RPMI 1640 and human AB serum. Response was measured on day 3 by tritiated thymidine pulse and recorded as the peak of 8 concentrations of PHA (0.5 to 300 $\mu\text{g/ml}$) in the first

series and 3 concentrations (0.5, 5.0, and 50 $\mu\text{g/ml}$) in the second. All cultures were done in duplicate in the first series and quadruplicate in the second.

Assay of supernatants and calculation of responses. For testing supernatant fluids T cells from normal subjects were suspended in supernatant fluids and compared with a medium control. Supernatants from each subject were assayed on various combinations of sets of T cells obtained from 3 normal donors. The ratio cpm in test supernates to cpm in medium control $\times 100$ was calculated; grouped data are presented as the averages of the ratios (mean percentage response).

Results

First series: direct cell responses. *Proportion of monocytes (Table 1).* Unfractionated peripheral blood mononuclear cells (PBMC) from patients with PSS contained proportionately more peroxidase-positive cells than did unfractionated PBMC from normal persons, but the difference was not significant. Recalculating data for effect of dilution of T cells by monocytes does not alter the results. Yields of total PBMC were similar. After passage over anti-F(ab)₂ columns effluent cells from both PSS patients and normals were similar.

Unfractionated PBMC response to PHA (Table 1). Peak response to 8 concentrations of PHA of unfractionated cells of PSS patients was markedly deficient compared with that of normal persons. At suboptimal and supraoptimal concentrations of PHA the same deficient response was seen; there was no apparent change in the shape of the dose-response curve.

T cell response to PHA (Table 1). Peak response of patients' T cells remained deficient compared with that of normal persons. At suboptimal and supraoptimal PHA concentrations the same effect was seen. When T cell response was calculated as a function of unfractionated PBMC response, the normal T cell response *diminished* compared with the unfractionated PBMC (86% of unfractionated), whereas the PSS T cell response markedly *increased* (155% of

Table 1 Peak PHA response of unfractionated peripheral blood mononuclear cells and T cells from normal persons and patients with PSS

Subject	No.	Unfractionated cells		T cells		c/a
		cpm $\pm$ SEM $\times 10^{-3}$ (a)	% monocytes (range) (b)	cpm $\pm$ SEM $\times 10^{-3}$ (c)	% monocytes (range) (d)	
Normal	19	69.8 $\pm$ 4.4*	20 (9-33)	60.3 $\pm$ 5.9‡	5 (2-10)	0.86
PSS	13	27.2 $\pm$ 3.5**†	35 (14-60)	42.3 $\pm$ 3.4†‡	5 (2-9)	1.55

cpm = Counts per minute.

* $t=7.57$, $p<0.001$.

† $t=3.09$, $p<0.005$.

‡ $t=2.64$, $p<0.02$. The means were computed as normal versus PSS and unfractionated versus T.

Table 2 Peak PHA responses of unfractionated peripheral blood mononuclear cells and nonadherent peripheral blood mononuclear cells from normal persons and patients with PSS

Subject	No.	Unfractionated cells		Nonadherent cells		c/d
		cpm $\pm$ SEM $\times 10^{-3}$ (a)	% monocytes (range) (b)	cpm $\pm$ SEM $\times 10^{-3}$ (c)	% monocytes (range) (d)	
Normal	4	36.4 $\pm$ 3.2*	21 (18–26)	30.6 $\pm$ 7.1	1 (0–1)	0.83†
PSS	3	11.3 $\pm$ 1.5*	27 (20–36)	24.5 $\pm$ 5.9	4 (1–8)	2.15†

* $t=7.10$, $p<0.001$.† $t=2.36$, $p<0.05$ for normalised data.

unfractionated cell response), suggesting that an inhibitor cell population had been removed by passage over an anti-F(ab)₂ column.

Unfractionated cell response to PHA after removal of adherent cells (Table 2). To test whether the improvement of PSS T cell response compared with unfractionated PBMC response was the effect of the removal of B cells or of adherent cells by the anti-F(ab)₂ column, unfractionated cells were plated on Petri dishes, and the nonadherent cell population was resuspended and assayed. The response of normal nonadherent cells was lower than that of the unfractionated cells, while in PSS patients the response of nonadherent cells again increased, suggesting that the adherent cell population rather than the B cell population is responsible for the poor unfractionated PBMC response of PSS patients.

Second series (indirect response in supernatant fluids); soluble inhibition from adherent cells (Table 3). To determine whether an inhibitor exists in culture supernates from adherent cell cultures, unfractionated cells, T cells, and adherent cells were cultured for 48 hours and their supernates tested on normal T cells in the presence of PHA. In these experiments supernates from none of the normal cell populations were inhibitory, but supernates from both unfractionated PSS cells and PSS-adherent cells were strongly inhibitory, suggesting that adherent cells do release a soluble inhibitor into the medium. Addition of neither glutamine nor fresh media abolished the inhibition, suggesting that the poor response is not due to depletion of an essential

medium component. The inhibition could not be diluted more than 1:4.

Stability of active supernates. Active supernates stored up to 2 months at -20°C produced similar degrees of inhibition, but responses were erratic after 2 months. Hence all supernates were tested within 2 months.

Effect of indomethacin (Table 4). With normal subjects slightly inhibitory adherent cell supernates became stimulatory when indomethacin was included at the beginning of the culture. Adherent cell supernates from PSS patients were far more inhibitory; inhibitory activity was prevented by indomethacin.

Effect of exogenous PgE₂. To test the hypothesis that exogenous PgE₂ could intensify the inhibition by acting additively or synergistically with a second indomethacin-insensitive inhibitor, varying concentrations of PgE₂ were added to adherent cell supernates and indomethacin-adherent cell supernates. Inhibition was not recovered by addition of PgE₂ to indomethacin-adherent cell supernates or increased in adherent cell supernates (data not shown).

Comparison with SLE (Table 4). In experiments with 13 SLE patients (repeating our prior experience⁹) the inhibition caused by SLE adherent cell supernates was less than the inhibition caused by PSS adherent cell supernates. The effect of indomethacin was similar in SLE and PSS supernates.

Table 3 Inhibition of PHA response of normal T cells cultured in various supernatant fluids results are expressed as mean percentage of response in medium control

Subject	Supernatant fluids from		
	Unfractionated cells %	T cells %	Adherent cells %
Normal*	96 $\pm$ 26 (16)†	104 $\pm$ 33 (3)	101 $\pm$ 10 (25)‡
PSS	64 $\pm$ 12 (3)	88 $\pm$ 10 (5)	55 $\pm$ 4 (5)‡

*Data from reference 9.

†Number tested.

‡ $t=4.27$, $p<0.001$.Table 4 PHA response of normal T cells in the presence of adherent cell supernatant fluids obtained in the presence and absence of indomethacin. Results are expressed as mean percentage of response in medium control $\pm$ SEM

Subject	Supernatant fluid	
	Adherent cells: no indomethacin	Adherent cells: indomethacin
Normal	87 $\pm$ 8 (9)*†	124 $\pm$ 14 (9)
PSS	60 $\pm$ 8 (13)‡	109 $\pm$ 15 (7)‡
SLE	80 $\pm$ 5 (11)	111 $\pm$ 13 (11)

*Number tested.

† $t=2.38$, $p<0.05$.‡ $t=3.31$, $p<0.01$. See footnote to Table 1.

Discussion

Adherent cells have been shown in a variety of diseases to be responsible for hyporesponsiveness of peripheral blood mononuclear cells to PHA.^{8 12 17} In systemic lupus erythematosus this hyporesponsiveness is associated with a cell-free inhibitory product of monocytes.⁹ The current results indicate that a similar quantitatively greater phenomenon occurs in PSS.

Abnormalities of the humoral immune system such as hypergammaglobulinaemia,¹⁸ rheumatoid factors, and autoantibodies¹⁹⁻²¹ are clearly demonstrable in patients with PSS. Abnormalities of the cell-mediated immune system, although reported, have received less attention. Among the abnormalities reported is that patients with PSS have a selective decrease in antibody-dependent cell-mediated cytotoxicity,⁶ a function which may be influenced by products of adherent cells. PSS lymphocytes have been shown to release leucocyte migration inhibition factor when cultured in the presence of autologous lymphocytes, mitochondrial human liver microsomes,⁵ RNA type I human collagen, or muscle antigen.²² Chemotactic factors for human monocytes are produced when white cells from patients with PSS are cultured with collagen.²² Skin changes similar to those seen in PSS have been observed in chronic graft-versus-host disease²³ and suggest that a cell-mediated immune reaction may contribute to the pathogenesis of sclerosis in PSS.

There is also a reduction in the number of peripheral blood T cells⁴ and a decreased response to some mitogens.^{1 7} T μ cells (with Fc receptors for IgM) and T γ (receptors for IgG, suppressor cells)²⁴ have been counted in PSS. The reduced absolute number of T cells as well as a deficiency in T μ cells with an increase in T γ cells were observed by Gupta and workers.³ Inoshita's study² contradicted this finding and reported a decrease in T γ cells. Alterations in numbers of T μ and T γ cells as well as immunoregulatory activity of mononuclear cells have been reported in immunodeficiencies.^{25 26} Although we did not specifically analyse the distribution T subsets in this study, the adherent cells used to process supernates contained less than 10% peroxidase-negative cells; it is therefore unlikely that quantitative aberrations in T subsets in either direction account for the results seen.

Mononuclear cells have the capacity to produce inhibitors of other cell functions.²⁷ In this study, and in others involving inhibition of lymphocyte response to mitogen, prostaglandins of the E series have been presumed to be mediators of the inhibition.^{10 12 17 27 28} Our prior work with SLE-adherent cells suggested (on the basis of failure of indomethacin to prevent

completely the elaboration of an inhibitor) that a second, indomethacin-insensitive inhibitor was present.⁹ The current experiments suggest that the inhibitor in PSS patients is quantitatively greater than that found in SLE patients, and that its elaboration is completely prevented by indomethacin. In parallel experiments with a new group of SLE patients we could not confirm our prior suspicion of an indomethacin-insensitive inhibitor. That we could not reproduce the effect of the inhibitor by adding known quantities of PgE₂ to indomethacin-adherent cell supernates continues to suggest that the inhibitor may not be PgE₂. Our data are, however, consistent with the possibility that other prostaglandins mediate the inhibition.

The present experiments do support the hypothesis that cell-mediated immunity is abnormal in PSS, and suggest that adherent cells at least in part mediate the abnormality. This experience adds PSS to the list of diseases sharing this abnormality. That the abnormality of adherent cell function plays a pathogenetic role in PSS is speculation at present.

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