Lymphocyte cytotoxicity in systemic sclerosis: no increase on short-term culture with established human cell lines

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SUMMARY Lymphocyte cytotoxicity towards 4 established human epithelial and fibroblast cell lines was investigated in 18 patients with systemic sclerosis by means of both whole blood and peripheral blood mononuclear cells in short term (18 h) ^{51}Cr -release assays. No difference in the levels of cytotoxicity was found in patients compared with 25 normal controls, even when allowance was made for the severity of the disease and the sex of patients and controls. It is suggested that long-term (3–6 days) culture may be necessary to demonstrate the increased lymphocyte cytotoxicity originally described in the disorder.

A high incidence of both autoantibodies^{1,2} and rheumatoid factor³ is a well recognised feature of systemic sclerosis. Interest in the possibility of an immunological pathogenesis for the disorder, however, has come principally from the observations of Trayanova *et al.*⁴ and Currie *et al.*⁵ that lymphocytes from patients with the disorder showed elevated levels of cytotoxicity towards cultured target cells. Additional impetus for this concept was provided by the recognition that graft-versus-host disease, initially in experimental animals⁶ and later in man,^{7,8} might be complicated by sclerodermatous lesions. However, an element of controversy has been introduced into the literature by rather limited subsequent studies of lymphocyte cytotoxicity^{9,10} which have failed to confirm the original reports of Trayanova *et al.*⁴ and Currie *et al.*⁵ The present investigation of lymphocyte cytotoxicity in systemic sclerosis, which utilised 4 widely differing established human cell lines, was undertaken, therefore, as part of a further series of studies of cytotoxic mechanisms in the disorder.

Materials and methods

Patients. Eighteen patients with systemic sclerosis (16 women, 2 men; mean age 53.56 ± 4.04 years) were studied. Only 2 of the patients were receiving

treatment with immunosuppressive drugs (prednisolone 5–10 mg daily). Raynaud's phenomenon and acrosclerosis were constant features, and all patients were assessed for the extent of systemic involvement by the disorder as previously described¹¹ and were thus categorised as having 'severe' or 'mild' disease.

Controls. These consisted of 25 normal healthy volunteers (19 women, 6 men; mean age 34.96 ± 2.72 years).

Cell lines. Three established human epithelial cell lines (Girardi heart, Intestine 407, L132) and one established human fibroblast line (MRC 5) were obtained from Flow Laboratories (Irvine, Scotland) and maintained in 75 cm² glass culture bottles on Eagle's basal medium supplemented with 10% fetal bovine serum, nonessential amino acids, and antibiotics. Single cell suspensions were obtained by treating monolayers with 0.1% trypsin (Sigma Chemical Co., London) in calcium magnesium-free Hanks's balanced salt solution with 20 mmol Hepes buffer pH 7.2 for 10 min at 37°C.

Cytotoxicity assays. Target cells were labelled with ^{51}Cr as previously described¹¹ and incubated in Eagle's basal medium with 10% fetal bovine serum at a concentration at 2×10^4 cells with either 5×10^5 peripheral blood mononuclear cells or 200 μl whole blood. Triplicate cultures were maintained for 18 hours at 37°C and the 'percentage specific cytotoxicity' was then calculated from the ^{51}Cr release, as previously described.¹¹

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Statistical methods. Comparison of the cytotoxicity data in patients and control groups was made by the Mann-Whitney ranking test.

Results

The results of the cytotoxicity assays against the 4 established cell lines using both whole blood and peripheral blood mononuclear cells from patients with systemic sclerosis and controls are summarised in Table 1. Although levels of cytotoxicity were generally higher in whole blood assays, neither effector system revealed any difference between the patient and control groups. Although the data are not presented, analysis of the patients with systemic sclerosis in terms of 'severe' and 'mild' disease also failed to reveal any significant difference in levels of cytotoxicity from that found in controls. In view of reports that men have higher levels of spontaneous lymphocyte mediated cytotoxicity than women,¹² a further comparison was made between female patients and the corresponding female controls, as shown in Table 2. Although there now appeared to be a trend for whole-blood assays from patients with

systemic sclerosis to show higher levels of cytotoxicity against the Intestine 407 and L132 cell lines than the controls, this did not reach statistical significance. Overall there was, once more, no difference in the levels of cytotoxicity between patients and control groups for any of the 4 established cell lines.

Discussion

This investigation, like the earlier attempts of Kondo *et al.*⁹ and Cooper and Friou,¹⁰ has failed to confirm the observations of both Trayanova *et al.*⁴ and Currie *et al.*⁵ that lymphocytes from patients with systemic sclerosis show increased cytotoxicity towards cultured target cells. As the range of target cells used by us was larger than in either of the other 2 attempts at confirmation, it is perhaps relevant to consider possible factors underlying the conflicting reports.

The origin and type of target cells used in all these studies is very likely to be of vital importance. Although Currie *et al.*⁵ used target cells that were predominantly of rat origin, the possibility that discrepant findings could have arisen as a result of species incompatibility is unlikely, as Trayanova *et al.*⁴ certainly used human target cells, as have all the subsequent attempts at confirmation.^{9 10} The use by us of established cell lines, in contrast to the primary fibroblasts used by Trayanova *et al.*⁴ cannot account for the differences, for both Kondo *et al.*⁹ and Cooper and Friou¹⁰ also used primary human fibroblast targets.

The period of incubation of lymphocytes with target cells, however, may also be very important in explaining the conflicting results. Both the original studies used morphological criteria to detect cytotoxicity, which was maximal after 3 to 6 days of culture in most instances. All the subsequent studies, however, have used shorter term ⁵¹Cr-release assays, and this difference may well underlie the differing pattern of results, as target cell sensitivity may differ in long- and short-term culture.¹³ The conflicting patterns of cytotoxicity observed in prolonged and short-term assays may also be a reflection of the differing lymphocyte subpopulations exerting such cytotoxic effects. Short-term cytotoxicity assays are likely to show a considerable component of NK cell activity, whereas prolonged incubation may allow the development of activated T lymphocyte killing.¹⁴ In view of the fact that both experimental and human graft-versus-host disease can be complicated by the development of sclerodermatous lesions,⁶⁻⁸ continuing investigation of lymphocyte cytotoxicity would certainly be justified by prolonged periods of culture not only with primary and established cell lines but also with fractionated and enriched lymphocyte subpopulations.

Table 1 Spontaneous lymphocyte cytotoxicity for established human target cells in patients with systemic sclerosis (SS) and controls

Target cell	Mean specific cytotoxicity ($\pm$ SEM) in			
	WB assays		PBM assays	
	Controls (n=25)	Total SS (n=18)	Controls (n=25)	Total SS (n=18)
Girardi	14.7 (1.8)	14.7 (2.3)	9.1 (0.9)	9.4 (1.4)
Intestine-407	12.0 (1.8)	14.3 (2.7)	5.7 (0.9)	6.8 (1.2)
L-132	16.9 (2.7)	19.9 (3.9)	9.9 (1.5)	9.7 (1.6)
MRC-5	12.9 (1.9)	8.5 (1.6)	7.4 (1.7)	8.1 (1.7)

WB: whole blood 200 μ l. PBM: peripheral blood mononuclear cells 5×10^5 . All target cells: 2×10^4 .

Table 2 Spontaneous lymphocyte cytotoxicity for established human target cells in female patients with systemic sclerosis (SS) and female controls

Target cell	Mean specific cytotoxicity ($\pm$ SEM) in			
	WB assays		PBM assays	
	Female controls (n=19)	Female SS (n=16)	Female controls (n=19)	Female SS (n=16)
Girardi	13.7 (2.1)	14.8 (2.4)	9.1 (0.9)	9.5 (1.5)
Intestine-407	10.2 (1.9)	14.3 (2.9)	4.9 (0.9)	6.9 (1.3)
L-132	14.1 (2.9)	20.2 (4.2)	9.6 (2.0)	9.7 (1.5)
MRC-5	12.7 (2.2)	8.1 (1.6)	8.1 (2.2)	7.6 (1.7)

WB: whole blood 200 μ l. PBM: peripheral blood mononuclear cells 5×10^5 . All target cells: 2×10^4 .

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