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Functional and phenotypic analysis of T lymphocytes cloned from the skin of patients with systemic sclerosis

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SUMMARY

Activated T lymphocytes often accumulate in the lower dermis of patients with systemic sclerosis (scleroderma) and may play a role in the development of dermal fibrosis. We propagated and cloned these cells directly from skin biopsies in four of eight cases of early, untreated systemic sclerosis with diffuse scleroderma. The cloning frequency estimates were $f=0{\cdot}20$ and $f=0{\cdot}48$ for T cells derived from the skin of two patients *versus* $f=0{\cdot}68$ and $f=0{\cdot}96$ for autologous blood T lymphocytes. All but one of 24 skin-derived scleroderma clones were $CD4^+$. Clonal analyses performed with $CD4^+$ clones from patients and normal controls showed that all but one skin-derived clones synthesized either interferon-gamma (60%), glycosaminoglycan-stimulatory factor (26%) or both (9%) when induced *in vitro* by a mitogen, concanavalin A, but not by autologous dermal fibroblasts. In contrast, blood-derived clones had a different functional phenotype. All skin-derived clones produced tumour necrosis factor-alpha. Our results demonstrate that T lymphocytes obtained from the skin of patients with systemic sclerosis synthesized cytokines which could modulate functions of human dermal fibroblasts.

Keywords scleroderma (PSS) skin-derived T cells cloned T lymphocytes from skin cytokine production *in vitro* clonal analysis

INTRODUCTION

In systemic sclerosis (scleroderma), the accumulation of connective tissue in the skin and internal organs has been attributed to the activation of fibroblasts, which produce excessive amounts of connective tissue components (Bashey *et al.*, 1977; Buckingham *et al.*, 1978). Cytokines are among the factors known to modulate fibroblast growth and synthetic activities (Freundlich *et al.*, 1986). For example, it has been reported that interleukin-1 (IL-1) stimulates human fibroblast proliferation and glycosaminoglycan (GAG) production in tissue culture (Schmidt *et al.*, 1982; Yaron *et al.*, 1987), and that interferon-gamma (IFN-γ) and tumour necrosis factor-alpha (TNF-α) may selectively inhibit collagen synthesis (Freundlich & Jimenez, 1987). We have demonstrated that supernatants of mitogen or alloantigen-stimulated T lymphocytes or T cell clones contain GAG stimulatory activity (Tsao, Zeevi & Whiteside, 1986). Recently, this activity has been related to a distinct lymphokine, a 67-kD glycoprotein with pI of 5·6 which was purified to homogeneity from supernatants of *in vitro* activated lymphocytes (Tsao *et al.*, 1989).

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While the pathogenesis of scleroderma is unknown, there are indications that interactions between immune cells, lymphocytes, and fibroblasts culminate in the excessive accumulation of connective tissue and fibrosis (Wahl, Wahl & McCarthy, 1978; Jimenez, McArthur & Rosenbloom, 1979). Thus, it is not surprising that dermal mononuclear cell infiltrates often seen in early disease correlate with both the severity and progression of cutaneous sclerosis (Roumm *et al.*, 1984). These infiltrates have been shown to consist mainly of activated T lymphocytes with the helper/inducer ($CD4^+$) phenotype (Roumm *et al.*, 1984).

In order to explore further the role of T lymphocytes in the pathogenesis of scleroderma, we proceeded to outgrow and expand in culture lymphocytes from skin biopsies of patients with active, untreated disease. Our assumption was that T lymphocytes present at the sites of recent skin thickening would be activated *in vivo* and thus able to proliferate in response to recombinant interleukin-2 (rIL-2) in culture. These lymphocytes could then be cloned to allow for functional analysis. In this report, we demonstrate the feasibility of this approach and present evidence for the existence in lower dermis of patients

with scleroderma of T lymphocytes which produce GAG stimulatory factor and other cytokines capable of modulating the behavior of dermal fibroblasts.

MATERIALS AND METHODS

Patients

Skin biopsies were obtained from eight patients with scleroderma (seven women aged 25–60 years and one 39-year-old man). All had early, rapidly progressive untreated systemic sclerosis with diffuse scleroderma.

Skin biopsies

Seven millimetre punch skin biopsies were taken from an area of skin thickening in the dorsal aspect of the forearm, midway between the elbow and wrist, using sterile technique, under local anaesthesia. Freshly excised tissue was delivered to the laboratory in sterile tissue culture medium (TCM, see below) containing 5% human **AB** serum. The tissue was divided into four pieces: one was embedded in OCT medium (Miles Laboratories, Naperville, IL) for immunohistology, and a second was processed for electron microscopy. A third piece was used for establishing scleroderma fibroblasts in culture, according to the procedure previously described by us (Buckingham *et al.*, 1978). The fourth piece was prepared for cloning of T lymphocytes, beginning with mincing into fragments smaller than 1 mm^3 using scalpels. These fragments were partially digested with a mixture of collagenase (2 mg/ml, Cooper Biochemicals, Malvern, PA) and DNAse (100 μg/ml, Sigma Chemical Co., St Louis, MO) in TCM for 30 min at 37°C to 'soften' the tissue, washed twice in TCM, and then placed (1–2 tissue pieces/well) in the wells of a 48-well flat-bottomed Costar plate (No. 3548; Cambridge, MA). The tissue was incubated at 37°C in a humidified atmosphere of 5% CO_2 and air in TCM (RPMI 1640 supplemented with 5% pooled human **AB** serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin; all from GIBCO, Grand Island, NY; except human serum which was purchased from NABI, Miami, FL). rIL-2 from *Escherichia coli*, kindly donated by Cetus Corporation (Emeryville, CA), was used at 500 U/ml.

The wells containing tissue fragments were observed daily using an inverted stage microscope to monitor cells outgrowing from the tissue in the presence of rIL-2. The cultures were supplemented with fresh IL-2-containing TCM every week. After 2 weeks of culture, when slowing of cell growth was generally observed in the positive wells, the cells were recovered, layered over Ficoll–Hypaque gradients, and centrifuged to remove tissue debris. The cells were washed three times in HBSS and cloned by limiting dilution (see below).

Peripheral blood lymphocytes (PBL)

PBL were isolated from heparinized venous blood collected from the patients at the time of skin biopsy and from healthy volunteers when needed. Mononuclear cells (MNC) were recovered from Ficoll–Hypaque gradients, washed three times in HBSS, and counted in a haemocytometer in trypan blue. T lymphocytes were then isolated by a sheep red blood cells (SRBC) rosetting technique, exactly as described by Saxon, Feldhaus & Robins (1976).

Immunohistology

Cryostat sections (5 μm thick) of the skin tissue were cut in an Ames cryostat, air-dried overnight, and fixed in cold acetone for 10 min. The sections were then stained with monoclonal antibodies by an indirect immunoperoxidase method used in this laboratory and described earlier (Chen *et al.*, 1987). The monoclonal antibodies used were T11, T4, and T8 (Coulter Electronics, Hialeah, FL). Slides were examined in a Leitz light microscope for the presence of MNC infiltrates.

Cloning of T lymphocytes

T lymphocytes from the peripheral blood or from PSS skin were cloned in a limiting dilution assay under conditions described by Moretta *et al.* (1983). Briefly, phytohaemagglutinin (PHA) stimulated lymphocytes were plated in wells of 96-well U-bottomed Costar plates under limiting dilution conditions in the presence of autologous or allogeneic irradiated PBL as feeder cells and human rIL-2 (100 U/ml). Cells were plated at a density of 1, 5 or 10 cells/well and incubated in an atmosphere of 5% CO_2 in air at 37°C. The microcultures were supplemented weekly with irradiated feeder cells in fresh TCM and observed for cell growth. Microcultures were expanded in TCM containing IL-2 and split as needed.

Flow cytometry

The phenotype of established T cell clones was measured by flow cytometry as follows: 2×10^5 cells were stained with fluorescein- or phycoerythrin-labelled monoclonal antibodies Leu4, Leu3a and Leu2a (Becton Dickinson, Mountain View, CA) for 30 min at 4°C. Cells were washed twice with a phosphate-buffered saline (PBS) and 0·1% sodium azide buffer, and fixed in 1% paraformaldehyde. A FACStar instrument was used for two-colour analysis.

Preparation of supernatants from T cell clones

Lymphocytes were harvested from wells of Costar plates, washed twice in TCM supplemented with 5% human serum, counted in a haemocytometer in trypan blue, and adjusted to the concentration of 4×10^5 cells/ml. Concanavalin A (Con A, Miles Laboratories, Elkhart, IN) was added at a concentration of 20 μg/ml, and the lymphocytes were incubated for 24 h at 37°C in a CO_2 incubator. Con A was then inactivated by treatment with 0·1 M alpha-methyl-D-mannoside (Sigma), and the supernatants were harvested and stored at −70°C to be tested for cytokine activities. Titration experiments with T cell clones obtained from the blood of healthy volunteers were used to determine the optimal concentration of Con A and time for induction of $CD4^+$ clones. Supernatants from lymphocytes incubated overnight in TCM served as negative controls. Supernatants from selected clones were also generated during the incubation of 4×10^5 cells/ml with confluent monolayers of autologous irradiated (5000 rad) fibroblasts.

Cytokine assays

IFN-γ was quantified by a radioimmunoassay (Centocor, Philadelphia, PA). The sensitivity of the assay was 0·01 U/ml, and a coefficient of variation was less than 5% as calculated on the basis of an internal standard used in > 50 assays.

TNF-α was measured by an ELISA described elsewhere (Wing *et al.*, 1989). Monoclonal antibodies to two different epitopes on human recombinant TNF-α were obtained from Dr

A. Moller, courtesy of Knoll Pharmaceuticals. Plates (96-well, flat-bottomed; Immulon) were coated with 50 μl/well of the first anti-human TNF antibody No. 199) at a concentration of 5 μg/ml in $NaHCO_3$ buffer (0·05 M, pH 9·5) for 16 h at 4°C. The wells were washed and then coated with 0·2 ml/well of 1% BSA (Sigma) in PBS for 1 h at room temperature. Test samples or recombinant TNF-α standard were applied to the plates (50 μl/well) in two-fold dilutions and incubated for 2 h at room temperature. Plates were washed with PBS containing 0·05% Tween, and 100 μl/well of biotin-conjugated monoclonal mouse anti-human TNF antibody (No. 195) at a concentration of 2·5 μg/ml were added for 2 h at room temperature. After extensive washing with PBS containing 0·05% Tween, streptavidin–peroxidase complex (BRL) diluted 1:2000 in 0·1% BSA was applied for 30 min at room temperature. The plates were washed again with PBS containing Tween and 100 μl/well of the substrate solution, 2′2′2′2′–AZINO–bis (3-ethylbenzthiazoline-6-sulfonic acid; ABTS; Sigma), were added. The substrate solution was prepared immediately prior to use by adding 1 ml of 0·5% ABTS in distilled water to 9 ml of 0·1 M sodium citrate buffer, pH 4, followed by the addition of 0·1% (v/v) H_2O_2. Absorption was measured after 30 min on a Dynatech MR700 at a reference wavelength of 490 nm and test wavelength of 410 nm. Optical density (OD) readings obtained with the test samples were compared with a standard curve obtained with human recombinant TNF and the TNF values expressed in ng/ml. The assay detects ≥0·1 ng/ml of TNF and is specific for TNF-α based on neutralization experiments performed by Dr A. Moller (personal communication).

GAG-stimulatory activity in supernatants was measured as described by us earlier (Whiteside *et al.*, 1985). Briefly, normal dermal fibroblasts were seeded at 40000 cells per well (24-well Falcon plates) on the day the experiment was initiated (day 0). Culture medium (minimum essential medium, GIBCO, Grand Island, NY; supplemented with 10% FCS, 2 mM L-glutamine, proline, 100 U/ml penicillin and 100 μg/ml streptomycin) was changed on days 1 and 4. By day 4, all cultures reached confluence, as judged by microscopic observations. At the time of medium change on day 4, 50-μl aliquots of the supernatants of control and Con A-induced T cell clones were added to triplicate wells. Forty-eight hours later, 1 μCi of D[1,6-^{3}HN]-glucosamine (NEN, Boston, MA; specific activity, 44·8 Ci/mmole) was added to each well 12 h prior to harvest. Determinations of GAG in the cetyl pyridinium chloride (CPC) precipitable material were performed as described earlier (Whiteside *et al.*, 1985), and the assay was calibrated using an internal standard (Tsao *et al.*, 1989).

Statistical analysis

Minimal estimates of the proliferating T cell clones were obtained by the minimum χ^2 method from the Poisson distribution relationship between the responding cell number and the logarithm of the percentage of non-responding (negative) cultures, as described by Taswell (1981). Differences between the mean values obtained with control and experimental cultures were determined by paired Student's *t*-test.

RESULTS

Immunohistology

Cryostat sections of skin biopsies were made, stained with monoclonal antibodies using the immunoperoxidase technique and examined for the presence of MNC infiltrates in the dermis. Half of the patients (four out of eight) had MNC infiltrates in the dermis. In agreement with previous reports (Roumm *et al.*, 1984), these infiltrates were usually found in a perivascular distribution in the mid and lower dermis and consisted almost entirely of $CD4^+$ lymphocytes.

Table 1. Surface marker pattern of skin-derived and blood-derived T cell clones

	Number positive clones/total tested clones		
	CD3	CD4	CD8
Scleroderma patients			
Skin	24/24	23/24 (96%)	1/24 (4%)
Blood	22/22	15/22 (68%)	7/22 (32%)
Normal controls blood	23/23	19/23 (83%)	4/23 (17%)

T cell clones from the skin biopsies of two systemic sclerosis patients (cloning frequencies were $f = 0{\cdot}2$ and 0·48), and from the blood of one patient ($f = 0{\cdot}68$) and one normal control ($f = 0{\cdot}77$) were labelled with monoclonal antibodies Leu4, Leu3a and Leu2a and evaluated for their surface phenotypes by two-colour flow cytometry.

In vitro *culture of lymphocytes from PSS skin*

Fragments (< 1 mm^3) of skin biopsies were placed in TCM and cultured in rIL-2 over a period of 2–3 weeks. Upon microscopic examination, a small exudate of lymphoid cells was detectable next to and outgrowing from the biopsy specimen generally within 2–3 days; the lymphocytes then grew over the ensuing 10–14 days in the presence of IL-2. Lymphocytes outgrew only from those four skin biopsies noted above that contained MNC infiltrates on histologic and immunohistologic examination. This indicated that the cells proliferating in the presence of IL-2 were actually skin-infiltrating lymphocytes.

Slowing of cell growth occurred usually after 2 weeks; at that time, the total number of lymphocytes recovered ranged from $2{\cdot}5 \times 10^3$ to 3×10^4.

Cloning frequencies

The skin-derived and autologous blood T lymphocytes were cloned by limiting dilution. The cloning frequencies were estimated to be 0·20 and 0·68, respectively, for the first pair and 0·48 and 0·96 for the other. The cloning frequencies for T lymphocytes obtained from the peripheral blood of three healthy volunteers and cloned at the same time as the patient cells were 0·62, 0·92 and 0·77. These results indicate that the tissue-derived T lymphocytes had a somewhat lower cloning frequency than autologous blood T lymphocytes.

Surface phenotype of T cell clones

T cell clones obtained from skin biopsies of two patients with systemic sclerosis and from the blood of one of the patients and one normal control, were labelled with monoclonal antibodies Leu4, Leu3a and Leu2a and analysed for surface phenotype (Table 1). All the clones studied were $CD3^+$. As expected from

Table 2. Lymphokine production by CD4$^+$ clones derived from the skin of patients with PSS

Clone		GAG stimulatory factor (ct/min × 10^3 per 10^6 cells)	IFN-γ (U/ml)	TNF-α (ng/ml)
A	Control	23±3	ND	—
	+Con A	23±4	ND	1·7
G10	Control	18±3	—	—
	+Con A	66±2*	—	0·3
F11	Control	17±3	—	—
	+Con A	19±4	380	1·6
A11	Control	20±4	—	—
	+Con A	177±10*	—	2·7
F2	Control	23±7	—	ND
	+Con A	65±8*	—	ND
B11	Control	30±3	—	—
	+Con A	145±4*	—	1·0
C12	Control	22±2	—	—
	+Con A	23±4	26	2·0
E3	Control	31±5	—	—
	+Con A	26±6	208	1·25
C6	Control	22±4	—	—
	+Con A	79±19*	—	1·7
E12	Control	27±6	—	—
	+Con A	46±5*	50	1·7
F6	Control	16±2	—	ND
	+Con A	20±4	5	ND
E2	Control	21±1	—	ND
	+Con A	26±3	17	ND
E1	Control	17±2	—	ND
	+Con A	17±2	74	ND
E7	Control	21±3	—	—
	+Con A	14±2	124	1·3
G1	Control	19±2	—	ND
	+Con A	68±13*	—	ND
E5	Control	19±4	—	ND
	+Con A	33±13	10	ND
F5	Control	20±2	—	ND
	+Con A	19±3	100	ND
C1	Control	26±3	—	ND
	+Con A	23±5	140	ND
G4	Control	29±9	—	ND
	+Con A	32±7	48	ND
A4	Control	55±16	—	ND
	+Con A	109±12*	47	ND
C1	Control	43±3	—	ND
	+Con A	44±4	94	ND
A2	Control	147±34	—	ND
	+Con A	247±86	50	ND
B7	Control	154±29	—	ND
	+Con A	243±62	78	ND

Twenty-three CD4$^+$ clones from the skin of two scleroderma patients were tested for the production of glycosaminoglycan (GAG) stimulatory factor, interferon-gamma (IFN-γ) and tumour necrosis factor-alpha (TNF-α) without (control) and with stimulation with concanavalin A (Con A). GAG stimulatory factor was measured as described in Materials and Methods. ND, not done; "—", undetectable levels of cytokines.

* $P<0.05$ *versus* control.

Table 3. Cytokine production profile of T cell clones derived from scleroderma skin and blood and normal blood

	Number positive clones/total tested clones (% positive)			
	GAG-SF only	IFN-γ only	GAG-SF + IFN-γ	TNF-α
Systemic Sclerosis				
Skin	6/23 (26)	14/23 (60)	2/23 (9)	10/10 (100)
Blood	2/15 (13)	1/15 (7)	11/15 (73)	14/15 (93)
Healthy controls blood	0/15 (0)	8/15 (53)	6/15 (40)	13/15 (87)

Clones were considered positive for GAG-SF when the production by Con A-stimulated clones of GAG stimulatory activity, evaluated as described in Materials and Methods, was significantly different from that of unstimulated controls at $P<0.05$.

GAG-SF, glycosaminoglycan stimulatory factor; IFN-γ, interferon-gamma; TNF-α, tumour necrosis factor-alpha; Con A, concanavalin A.

the histologic findings, all the skin-derived clones but one (96%) were also CD4$^+$, while the frequencies of blood-derived CD4$^+$ clones for the systemic sclerosis patient and the normal control were 68% and 83%, respectively.

Lymphokine production by skin-derived T cell clones

Twenty-three CD4$^+$ T cell clones from the skin biopsy of two patients with systemic sclerosis were studied for the spontaneous and Con A-induced production of GAG stimulatory factor, IFN-γ and TNF-α (Table 2). Of the 23 CD4+ T cell clones obtained from sclerodermatous dermis, eight (35%) produced significantly greater quantities ($P<0.05$) of GAG stimulatory factor and 16 (70%) produced IFN-γ after activation by Con A (Table 2); only two clones (9%) synthesized both these lymphokines (Table 3). All the skin-derived clones that were studied (10/10) produced TNF-α after stimulation with Con A; in addition to TNF-α, these clones also released either GAG stimulatory factor or IFN-γ (Tables 2 and 3).

Lymphokine production by blood-derived T cell clones

Fifteen CD4$^+$ clones obtained from the blood of one of the systemic sclerosis patients, and 15 clones obtained from the blood of one healthy individual were also studied to compare their cytokine profile with that of the skin-derived clones (Tables 4 and 5). Upon stimulation with Con A, 13 out of 15 clones from the scleroderma patient produced increased quantities of GAG stimulatory factor (87%) and 12 (80%) IFN-γ (Table 4). T cell clones obtained from the skin produced higher levels of IFN-γ than did those derived from peripheral blood. As shown in Table 3, two (13%) blood-derived scleroderma clones produced exclusively GAG stimulatory factor, while only one made IFN-γ alone; 11 out of 15 (73%) synthesized both of these cytokines. In contrast, none of the clones obtained from normal blood produced GAG stimulatory factor alone, although six out of 15 (40%) were induced to synthesize both GAG stimulatory factor and IFN-γ. Also, one normal clone made neither of the cytokines we measured (Table 5).

No correlation could be demonstrated between the amount of GAG stimulatory factor and that of IFN-γ produced by either systemic sclerosis or normal blood-derived clones.

Table 4. Lymphokine production by CD4$^+$ clones derived from the blood of a patient with systemic sclerosis

Clone		GAG stimulatory factor (ct/min × 10^3 per 10^6 cells)	IFN-γ (U/ml)	TNF-α (ng/ml)
C4-1	Control	45±12	—	—
	+Con A	72±10*	2	2·0
D11-1	Control	34±13	—	—
	+Con A	66±1*	—	0·7
C9-2	Control	36±4	—	—
	+Con A	62±6*	100	3·4
D2-1	Control	30±1	—	—
	+Con A	87±2*	2	1·4
A8-1	Control	64±7	—	—
	+Con A	81±5	2	2·6
E8-2	Control	36±5	—	—
	+Con A	88±3*	4	3·7
G8-;2	Control	41±3	—	—
	+Con A	76±1*	51	0·8
B2-1	Control	20±3	—	—
	+Con A	37±5*	44	2·7
B3-1	Control	18±1	—	—
	+Con A	30±5	—	1·7
G9-1	Control	21±3	—	—
	+Con A	37±4*	3	—
G8-1	Control	18±1	—	—
	+Con A	34±3*	—	3·1
F8-1	Control	12±2	—	—
	+Con A	37±1*	32	3·1
F11-1	Control	11±1	—	—
	+Con A	22±3*	48	1·6
D7-1	Control	14±2	—	—
	+Con A	30±7*	60	0·4
C12-1	Control	14±4	—	—
	+Con A	24±2*	5	1·3

Fifteen CD4+ clones from the blood of one slceroderma patient were tested for the production of glycosaminoglycan (GAG) stimulatory factor, interferon-gamma (IFN-γ) and tumour necrosis factor-alpha (TNF-α) without (control) and with stimulation with concanavalin A (Con A). GAG stimulatory factor was measured as described in Materials and Methods. ND, not done; —, undetectable levels of cytokines.

* $P < 0·05$ *versus* control.

Table 5. Lymphokine production by CD4$^+$ clones derived from the blood of a healthy volunteer

Clone		GAG stimulatory factor (ct/min × 10^3 per 10^6 cells)	IFN-γ (U/ml)	TNF-α (ng/ml)
E6-1	Control	33±1	—	—
	Con A	40±5	36	1·6
B12-1	Control	34±2	—	—
	Con A	71±1*	0·4	1·0
G6-2	Control	17±1	—	—
	Con A	57±2*	19	0·9
F8-1	Control	23±1	—	—
	Con A	57±5	4	0·5
D5-1	Control	16±1	—	—
	Con A	70±4*	50	2·3
B10-2	Control	20±1	—	—
	Con A	31±4	40	1·5
C6-1	Control	19±2	—	—
	Con A	19±1	39	2·3
D9-2	Control	16±1	—	—
	Con A	41±4	0·4	—
B10-1	Control	21±2	—	—
	Con A	22±4	16	0·6
D11-2	Control	16±3	—	—
	Con A	42±2	11	0·6
B11-1	Control	23±4	—	—
	Con A	79±1*	0·4	1·3
A9-2	Control	16±1	—	—
	Con A	39±6	—	—
E7-1	Control	15±1	—	—
	Con A	48±1*	50	4·3
D6-1	Control	18±2	—	—
	Con A	40±2	2	0·7
C5-1	Control	19±4	—	—
	Con A	53±1*	14	0·8

Fifteen CD4+ clones from the blood of one normal individual were tested for the production of glycosaminoglycan (GAG) stimulatory factor, interferon-gamma (IFN-γ) and tumour necrosis factor-alpha (TNF-α) without (control) and with stimulation with concanavalin A (Con A). GAG stimulatory factor was measured as described in Materials and Methods. ND, not done; "—", undetectable levels of cytokines.

* $P < 0·05$ *versus* control.

Unlike the skin-derived clones, not all the blood-derived clones tested released TNF-α when stimulated with Con A (Table 3): three clones (one out of 15 for systemic sclerosis and two of 15 for normal blood-derived clones) made no TNF-α.

Effect of the autologous dermal fibroblasts on the production of lymphokines by systemic sclerosis clones

In order to evaluate the capability of autologous fibroblasts to stimulate lymphokine production by scleroderma clones, 22 skin-derived T cell clones from two scleroderma patients and 15 blood-derived clones from one scleroderma patient were incubated overnight with irradiated autologous dermal fibroblasts; the supernatants were then collected and tested for GAG stimulatory factor, IFN-γ and TNF-α activities. None of the supernatants contained detectable amounts of these lymphokines (data not shown).

DISCUSSION

Dermal MNC infiltrates are a common pathologic finding in early scleroderma (Roumm *et al.*, 1984). To provide a rational explanation for the accumulations of activated T lymphocytes at the site of early skin thickening, it has been postulated that these cells initiate or participate in the pathogenesis of the disease (Roumm *et al.*, 1984). Indeed, this hypothesis appears reasonable, since it is now known that activated T lymphocytes modulate fibroblast growth and synthetic activities through the release of soluble mediators (Wahl *et al.*, 1978; Jimenez *et al.*, 1979).

In order to investigate the role of T lymphocytes in the pathogenesis of dermal sclerosis, we developed a procedure for outgrowing, expanding, and cloning these cells from skin biopsies of scleroderma patients. Similar approaches have been undertaken in other pathologic conditions characterized by the presence of infiltrating lymphocytes, such as tumours (Whiteside *et al.*, 1986; Miescher *et al.*, 1987), renal allografts (Mayer *et al.*, 1985; Moreau *et al.*, 1986), and autoimmune thyroid glands (Londei, Bottazzo & Feldmann, 1985). Cultured tissue-derived lymphocytes can then be examined *in vitro* for their phenotypic and functional characteristics and abilities to modulate the behaviour of tissue cells in the microenvironment.

The clonal analysis offers a powerful tool for defining the functional profile of tissue-infiltrating lymphocytes. However, this experimental approach is not easy and is potentially open to errors. The recovery of lymphocytes requires substantial amounts of fresh tissues and yields relatively few cells (Whiteside *et al.*, 1986). Enzymatic treatments needed for dispersion of tissues may alter functional and phenotypic features of the recovered cells or impair their viability. Expansion of these cells *in vitro* depends on IL-2, which activates lymphoid cells and thus alters their properties. If cloning of these cells is achieved, the frequency of clones obtained must be high enough to assure a fair representation of the infiltrating population.

One of the major difficulties with culturing and cloning of tissue-infiltrating cells is a possible contamination with blood lymphocytes. There are several indications that the cells we cultured were actually skin-infiltrating lymphocytes: in cases for which there was no histologic evidence of MNC infiltration, lymphocytes proliferation was not observed; and skin-derived and blood-derived T cell clones from one of the patients showed a clearly different pattern with regard to their surface markers and a synthetic profile. These findings provide a reasonable assurance that we cloned skin-infiltrating cells. Moreover, the population of IL-2-expanded T cells that we cloned should be representative of that present at the site of skin involvement as judged by cloning frequencies: the estimated cloning frequencies for skin-derived lymphocytes were somewhat lower but close to those obtained with peripheral blood T lymphocytes. These cloning frequencies were much higher than those reported in the literature for other tissue infiltrating lymphocytes (Whiteside *et al.*, 1986; Moreau *et al.*, 1986; Miescher *et al.*, 1987). On the basis of the preliminary data reported here, this approach appears to be valid for the assessment of the phenotypic and functional characteristics of *in vivo* activated T lymphocytes from scleroderma skin biopsies. It is possible, however, that T cells outgrowing from these biopsies in the presence of a relatively high dose of rIL-2 may not be specific or related to the pathology of the disease. Since clones of skin-derived $CD4^+$ T cells had a functional repertoire distinct from that of blood-derived $CD4^+$ clones, it may be tentatively concluded that in scleroderma, the skin becomes infiltrated by T cells that differ functionally from circulating T cells.

Our laboratory has previously demonstrated that supernatants of mitogen or alloantigen-stimulated T lymphocytes and T cell clones contain a GAG stimulatory activity (Tsao *et al.*, 1986). We now extend this observation to mitogen-stimulated T cell clones from skin biopsies and peripheral blood of patients with systemic sclerosis. Both normal and systemic sclerosis clones producing the GAG stimulatory factor were $CD3^+$, $CD4^+$, and in addition to this lymphokine, they also produced IFN-γ and TNF-α. The fact that skin-infiltrating lymphocytes may produce IFN-γ is of particular interest, because it has been reported that this lymphokine is capable of inhibiting collagen synthesis by scleroderma fibroblasts, and, therefore, a defect in the production of IFN-γ by MNC from scleroderma patients has been suggested (Rosenbloom *et al.*, 1988). It has been generally assumed that TNF-α is produced by activated macrophages, whereas T lymphocytes are a source of lymphotoxin (TNF-β) (Mannel, Moore & Mergerhagen, 1980). However, recent studies indicate that some T lymphocytes could also produce TNF-α (Steffen, Ottmann & Moore, 1988). Our observations of the Con A-induced production of TNF-α by $CD4^+$ lymphocyte clones are consistent with these reports. Although the amounts of TNF-α produced by these clones were small, compared with those released by lipopolysaccharide-activated monocytes isolated from the blood of healthy volunteers under the same experimental conditions, the presence of TNF-α in the clone supernatants was a consistent finding. Antibodies used in our ELISA do not cross-react with TNF-β (Dr A. Moller, personal communication). Experiments are in progress to probe for the TNF-α message in the systemic sclerosis and normal $CD4^+$ clones.

In regard to the *in vitro* synthesis of lymphokines, the skin-derived clones showed a distinct synthetic pattern. While more than half of the clones obtained from the scleroderma peripheral blood released both GAG stimulatory factor and IFN-γ only 9% of the skin-derived clones made both the cytokines and most (87%) showed exclusive synthesis of either one or the other lymphokine. However, these results have to be interpreted with caution. T cell clones from skin were selected by the initial culture (2 weeks) in rIL-2, while those from blood were cloned directly after isolation. In the skin biopsy, the number of infiltrating lymphocytes was small, and their outgrowth from tissue prior to cloning was necessary. If these data are confirmed by more extensive clonal analyses, it may be possible to predict that skin-infiltrating activated T lymphocytes make soluble products with selective or restricted effects on dermal fibroblasts (Freundlich *et al.*, 1986) and are responsible for or contribute to the functional alterations found in scleroderma fibroblasts. In our hands, the clonal analysis of scleroderma fibroblasts demonstrated a heterogeneous phenotype with regard to the production of collagen and GAG, as well as a loss of regulation in the synthesis of these two connective tissue components (Whiteside *et al.*, 1988), rather than general activation of these cells. Cytokines produced by activated T cells could certainly contribute to a loss of regulatory control by scleroderma fibroblasts.

Finally, we showed that in our system, cultured scleroderma fibroblasts were not able to stimulate autologous T cell clones to

produce GAG stimulatory factor and other cytokines. This is not surprising, since even if scleroderma is an autoimmune disease, the autoantigen(s) has not yet been identified. It is, therefore, possible that the putative autoantigen was simply not expressed in the fibroblast cultures; alternatively, the conditions used *in vitro* may not be comparable to those operative *in vivo*. For instance, it has been reported that scleroderma fibroblasts express class II MHC antigens *in vivo* (Pearson *et al.*, 1988). We could not, however, find evidence for the class II MHC antigen expression in our fibroblast cultures (personal observations), even though this expression was inducible by treatment with IFN-γ, as also reported for normal fibroblasts in culture (Pober *et al.*, 1983).

A feasible method for outgrowing, expanding and cloning of skin-infiltrating T lymphocytes from the skin and blood of systemic sclerosis patients was developed and evaluated. These $CD4^+$ cells upon *in vitro* stimulation, released cytokines capable of mediating fibroblast synthetic activities. It remains to be determined whether T cells isolated from skin biopsies from other conditions, e.g., psoriasis or contact sensitivity, are also able to make GAG stimulatory activity similar to that made by scleroderma T lymphocytes. Further studies are needed to confirm these observations and to elucidate the complex interactions between the skin-derived T lymphocytes and their target dermal fibroblasts.

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