

## ENDOTHELIAL INJURY IN SCLERODERMA\*

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Scleroderma, which follows rheumatoid arthritis and systemic lupus erythematosus as the third most prevalent rheumatic disorder, is poorly understood. Connective tissue abnormalities have been explored extensively (1); recently, vascular involvement has been emphasized as a unifying pathogenetic concept (2, 3). The vascular features in scleroderma include Raynaud's phenomenon; an early, edematous phase of the disorder; telangiectasia; capillary abnormalities as seen by nailfold and ultrastructural microscopy; and widespread vascular pathology noted in all involved organs. The most striking histological abnormalities occur in small arteries and arterioles and consist of distinctive intimal proliferation of cells arranged concentrically in a matrix of ground substance; the cells are thought to originate from medial smooth muscle and to migrate toward the intima after injury to the endothelium (4). Evidence for endothelial injury includes: (a) the disappearance of endothelium in association with thrombosis or fibrinoid necrosis in ultrastructural studies; (b) the absence of endothelial cells within the thickened intima (4, 3) the duplication of basement membrane, a common observation in scleroderma and known to occur after endothelial perturbation in other settings.

The ability to isolate, characterize, and maintain endothelial cells *in vitro* provides a target-cell population to study endothelial damage in scleroderma. The present report describes the effect of scleroderma serum on endothelial, smooth muscle, and fibroblast cell types. Sera from patients with scleroderma (31/52) and Raynaud's syndrome (11/19) contain cytotoxic activity specific for endothelial cells which is nondialyzable, heat-stable, and elutes with albumin on gel-filtration chromatography.

### Materials and Methods

#### *Cell Cultures*

**ENDOTHELIAL CELL (EC)<sup>1</sup> CULTURE.** Cells were obtained from a human umbilical cord vein by adapting the method of Jaffe (5). The vein was washed with balanced salt solution (BSS, pH 7.4) and digested with collagenase (*Clostridium histolyticum*, grade B, Worthington Biochemical Corp., Freehold, N. J., 0.2% in BSS) for 15 min at 37°C and rinsed with BSS. The cells, collected by centrifugation at 250 *g* for 5 min, were washed and cultured in F12K medium supplemented with 20% fetal calf serum (FCS, Grand Island Biochemical Co., Grand Island, N. Y.), 100 U-ml penicillin and 50 µg/ml gentamicin in plastic Petri dishes (Falcon Labware,

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<sup>1</sup> Abbreviations used in this paper: BSS, balanced salt solution; EC, endothelial cell(s); FB, fibroblast(s); FCS, fetal calf serum; <sup>3</sup>HTdR, tritium-labeled thymidine; SMC, smooth muscle cell.

Div. of Becton, Dickinson, & Co., Oxnard, Calif.) at 37°C in 5% CO<sub>2</sub>. Cells were fed twice weekly, transferred at confluence, and used for experiments in the first subpassage.

**SMOOTH MUSCLE CELL (SMC) CULTURE.** Cells were obtained from an umbilical cord vein by modification of the technique for EC culture. Before collagenase perfusion, the cord was traumatized by repeated clamping with a hemostat. The usual procedure was then followed. The cultured, effluent cells yielded a mixture of endothelial and long, spindle-shaped cells. Within 2–3 wk in culture, a homogeneous population of spindle-shaped cells grew in multiple layers.

**FIBROBLAST (FB) CULTURE.** FB were derived from a newborn human foreskin obtained at circumcision or from an umbilical cord as follows: Initial sample was trimmed (to remove the dermis from skin and the vessels from cord interstitium) and incubated with 0.2% collagenase in BSS for 5 h at 37°C, washed three times, resuspended in culture medium, and plated in 35-mm Petri dishes. The cells grew out from tissue fragments within 2 wk and reached confluence by 4 wk.

**MONONUCLEAR CELL PREPARATION.** Mononuclear cells were isolated by density sedimentation of heparinized blood over Ficol (Sigma Chemical Co., St. Louis, Mo.) and sodium diatrizoate (Hypaque Sodium, 50%, Winthrop Laboratories, N. Y.) by modification of the technique of Boyum (6). Mononuclear cells were resuspended in culture medium at 10<sup>6</sup> cells/ml.

**Immunofluorescence Studies.** Cells were grown on glass cover slips for 2–4 d, fixed in acetone, air-dried, washed with BSS for 15 min, incubated with rabbit anti-human factor VIII antigen (Behring Diagnostic, American Hoechst Corp., Summerville, N. J.) for 30 min at 37°C, and stained with fluorescein-conjugated goat anti-rabbit gammaglobulin.

#### *Assay of Cell Growth and Survival*

**CELL COUNT.** 4 × 10<sup>4</sup> cells were placed in 35-mm Falcon dishes (Falcon Labware, Div. of Becton, Dickinson, & Co.) in F12K culture medium supplemented with 2% FCS and allowed to attach overnight. The medium was then replaced with 2 ml F12K medium supplemented with test serum. After 72 h, cells were harvested with 0.25% trypsin-0.01% EDTA and counted in a hemocytometer.

**[<sup>3</sup>H]THYMIDINE (<sup>3</sup>HTdR) UPTAKE.** 1 × 10<sup>4</sup> cells were seeded in Falcon microtest plates (Microtest 2, Falcon Labware, Div. of Becton, Dickinson, & Co.) in F12K medium-2% FCS and left overnight; the medium was then replaced with F12K medium supplemented with test serum. 48 h later, <sup>3</sup>HTdR (6 Ci/mmol, Schwartz/Mann Div., Becton, Dickinson, & Co., Orangeburg, N. Y.) was introduced at a 1-μCi/ml concentration. 24 h later, medium was shaken off the plate, one drop of 0.25% trypsin-0.01% EDTA was added to each well for 2 min. The cells were harvested on glass-fiber filters (Reeve Angel & Co., Inc., Clifton, N. J.) and washed with distilled water, using a semi-automatic cell harvester (Otto Hiller Company, Madison, Wis.). Filter disks were counted in Aquasol-2 (New England Nuclear, Boston, Mass.) on a scintillation counter (Beckman LS-345, Beckman Instruments, Inc., Fullerton, Calif.).

**MICROCYTOTOXICITY ASSAY.** Using a one-stage microcytotoxicity technique (7), cells were harvested, placed in standard microcytotoxicity wells (Microtest, Terasaki Falcon Company) at 30–40 cells/well in 5 μl F12K medium-2% FCS, and left overnight to attach. The medium was then replaced with 5 μl of test serum. All trays were gently stirred and incubated for 3 h at 37°C. 5 μl of dye (Eosin-Y [Fisher Scientific Co., Pittsburgh, Pa.], 5% in BSS) was added to each well. After 10 min, cytotoxicity was determined by counting stained cells; toxicity of 20% or greater was considered significant.

**Patient Population.** Sera were obtained from 52 patients with scleroderma,<sup>2</sup> 19 patients with Raynaud's syndrome,<sup>3</sup> 24 patients with active connective tissue disease other than scleroderma (8 patients with systemic lupus erythematosus, 9 patients with rheumatoid arthritis, 5 patients with dermatomyositis, 2 patients with polyarteritis nodosa) and 21 healthy controls. Sera were heat inactivated (56°C, 30 min) and stored at -70°C.

<sup>2</sup> Scleroderma was defined as taut skin proximal to the metacarpophalangeal joints.

<sup>3</sup> Raynaud's syndrome was defined as episodic pallor or cyanosis followed by suffusion, erythema, fullness, or pain (without other evidence of rheumatic or connective tissue disease).

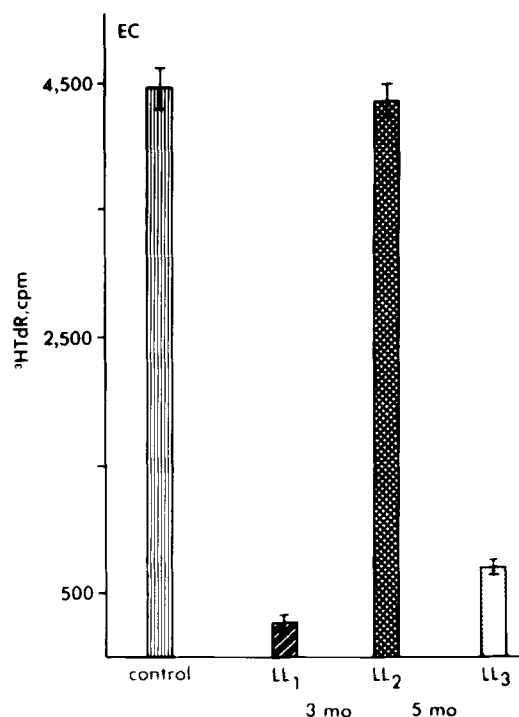


FIG. 1.  $^3\text{HTdR}$  uptake by EC after a 72-h exposure to control and index case serum (LL): LL<sub>1</sub>, serum obtained on presentation; LL<sub>2</sub>, 3 mo after presentation; and LL<sub>3</sub>, 5 mo after LL<sub>2</sub>. Results are expressed as mean CPM  $\pm$  SD of quadruplicate experiments.

**Serum Fractionation.** 3 ml of serum equilibrated with Tris(Fisher Scientific Co.)-saline buffer (pH 7.6) was fractionated on a gel-filtration column (Sephadex G-200, Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.); fractions were collected, dialyzed, lyophilized, reconstituted to original volume and used to supplement culture medium.

## Results

**Cell Identification.** EC were identified and distinguished from SMC and FB by morphological criteria. Cultured EC grew as monolayers of flat, polygonal cells, required higher than usual concentration of serum for growth, and were contact inhibitable (8). In contrast, SMC and FB grew as multilayered arrays of spindle-shaped cells. In addition, SMC grew in a characteristic hills and valleys pattern which was strikingly different from FB and EC (9). In the present experiments, EC cultures contained >99% factor VIII-positive cells (EC are the only cell type known to produce factor VIII antigen in culture (10) and can be definitely identified by immunofluorescent staining with anti-human factor VIII antisera).

**Index Case.** Serum obtained from a 40-yr-old white male (LL<sub>1</sub>) with a 1 yr history of scleroderma with associated myositis, induced 95% inhibition of EC  $^3\text{HTdR}$  uptake compared to uptake in the presence of an age-, sex-, and race-matched control serum (Fig. 1). 3 mo later, after prednisone therapy, the patient's serum (LL<sub>2</sub>) showed no inhibition of  $^3\text{HTdR}$  uptake. At that time, the myositis was inactive clinically. 5 mo later, myositis recurred in association with worsening of the involved organs (skin, lung, and esophagus). At that time, serum (LL<sub>3</sub>) showed 85% inhibition of EC  $^3\text{HTdR}$

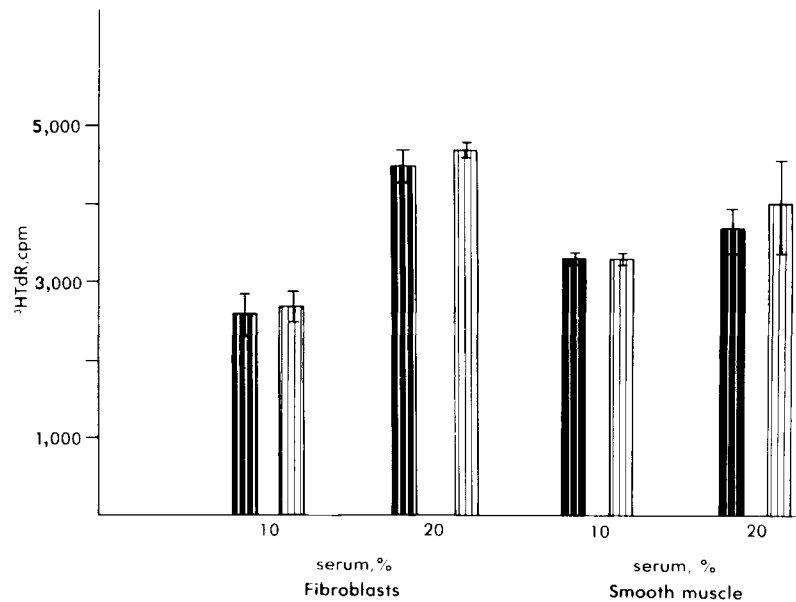


FIG. 2. <sup>3</sup>HTdR uptake by FB and SMC after exposure to control and scleroderma (Scl) sera at 10 and 20% concentrations. ▨, control serum; ■, Scl serum.

uptake. When SMC and FB were grown in this patient's serum, no difference was observed in the <sup>3</sup>HTdR uptake between the LL1 serum and control serum at 10 and 20% concentrations (Fig. 2).

Direct cell counts after 72 h of exposure to LL1 serum were 30% fewer than the original inoculum, whereas control serum produced a 100% increase in cell count. When SMC and FB were used as target cells, there was no difference in the cell count between scleroderma serum and control serum (data not shown).

When serum concentration in culture medium was increased from 2–30%, <sup>3</sup>HTdR uptake by EC exposed to control serum increased gradually (Fig. 3), whereas uptake by EC exposed to scleroderma serum remained low. Differences between control and scleroderma sera were most apparent at 10–30% concentrations. When sera were mixed in equal parts, the resulting curve was almost identical to the one induced by scleroderma serum alone. The low uptake in the beginning of the curve is a result of low serum concentration (EC require high serum concentrations for proliferation, 15% or greater); thus, to avoid the effects of serum deprivation on cells, serum concentrations consisting of varying proportions of test serum and FCS were held constant at 20% in all dishes (Fig. 4). <sup>3</sup>HTdR uptake increased with increasing control serum concentrations whereas the reverse was observed with scleroderma serum.

**Cytotoxicity Studies.** Five cell types were exposed to scleroderma or control sera (Table I). Significant cellular injury occurred only when EC were exposed to scleroderma serum. No significant toxicity was seen with four other target cell types. Control sera showed no cytotoxicity. The cytotoxic activity of scleroderma sera was stable at 56°C for 30 min; similar levels of cytotoxicity were observed in scleroderma plasma and serum (data not shown).

**Serum Fractionation.** Serum was fractionated by gel-filtration chromatography

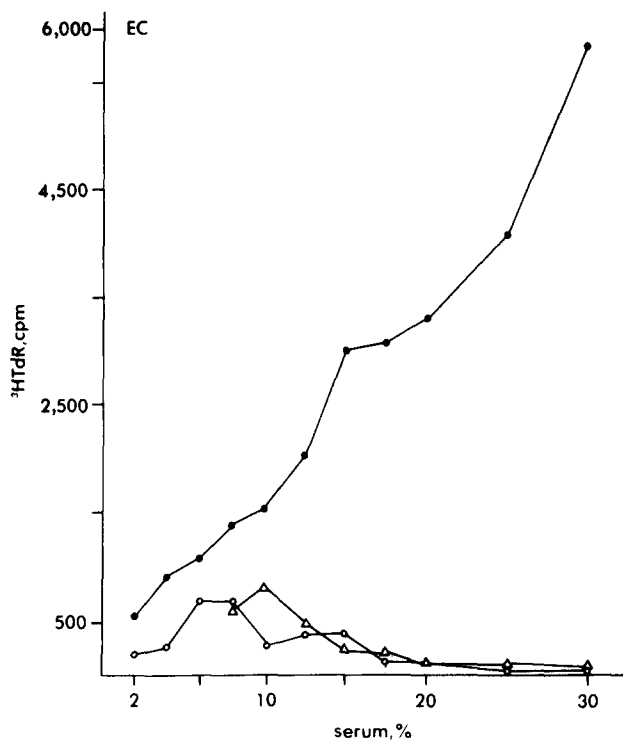


FIG. 3.  $^3\text{HTdR}$  uptake by EC after exposure to increasing concentration of control, scleroderma, and 1:1 mixed sera; each point represents the mean uptake of quadruplicate determinations. ●, control serum; ○, Scl serum; △, 1:1 mixed serum.

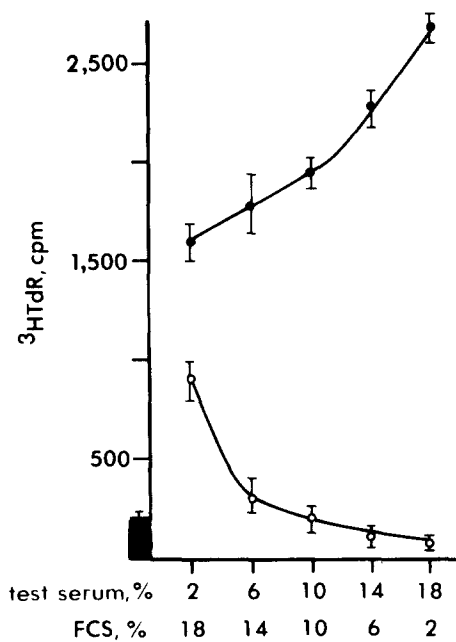


FIG. 4.  $^3\text{HTdR}$  uptake by EC after exposure to increasing relative concentration of control or scleroderma serum (test serum) and decreasing relative concentration of FCS. Total serum concentration is kept constant at 20%. Each point represents the mean uptake of quadruplicate wells. ●, control serum; ○, Scl serum; ■, 2% FCS.

TABLE I  
Microcytotoxicity of Control and Scleroderma Sera

Cells	Serum	
	Control	Scleroderma
EC	6 ± 2	52 ± 14
SMC	12 ± 1.4	12 ± 1.4
FB	13 ± 3.5	14 ± 4
3T3	9.5 ± 1	11 ± 2.3
Mononuclear	6 ± 0.1	6.5 ± 0.5

Results are expressed as mean percent cells containing dye ± SD in quadruplicate determinations. Cytotoxicity of 20% or greater is considered significant.

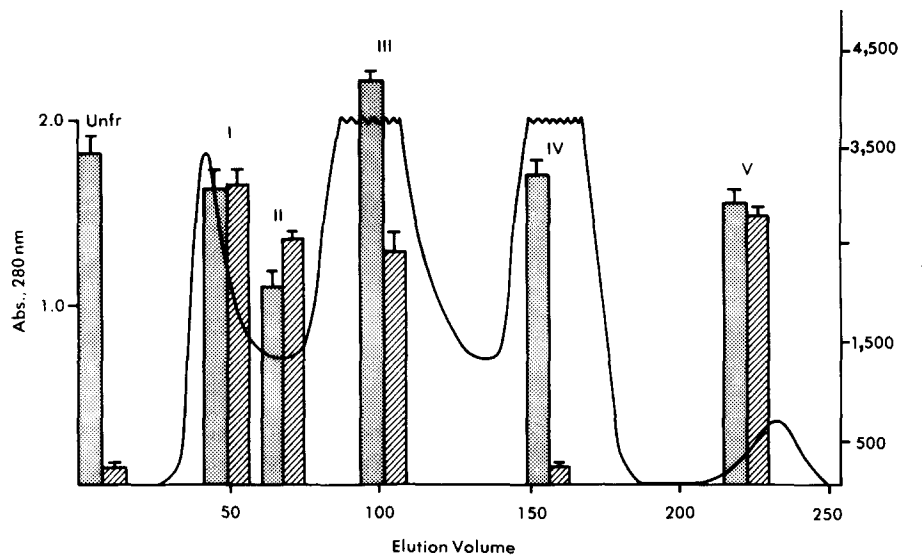


FIG. 5. Serum fractionation by gel filtration chromatography. Column dimension, 1.5 × 175 cm. Flow rate, 5–10 ml/h. Eluant, 50 mM Tris-HCl (PH 7.6), 5 mM CaCl<sub>2</sub>. Four peaks were observed and five fractions were pooled. Fraction I, 30–60 ml, II, 61–75 ml, III, 76–140 ml, IV, 141–180 ml, and V, 200–250 ml. Bars represent mean <sup>3</sup>HTdR uptake by EC after exposure to 15% dialyzed, lyophilized but unfractionated (unfr.) sera (scleroderma and control) and to individual fractions. ▨, Scl serum; ▩, control serum.

(Sephadex G-200); four major peaks were observed and all aliquots were pooled into five fractions (Fig. 5); each was dialyzed, lyophilized, reconstituted to original volume, and used to supplement culture medium at 15% concentration. <sup>3</sup>HTdR uptake by EC exposed to 15% unfractionated, dialyzed, and lyophilized serum showed that the inhibitory activity is nondialyzable and stable to lyophilization. Cytotoxic activity was observed in fraction 4 and, to a lesser degree, in adjacent fraction 3 of scleroderma serum (Fig. 5).

*EC Cytotoxic Activity in Connective Tissue Disease.* <sup>3</sup>HTdR uptake by the three cell types, EC, SMC, and FB, was determined after exposure to sera at 15% concentration from 21 healthy volunteers, 24 patients with active rheumatic disease other than scleroderma, 52 patients with scleroderma, and 19 patients with Raynaud's syndrome alone.

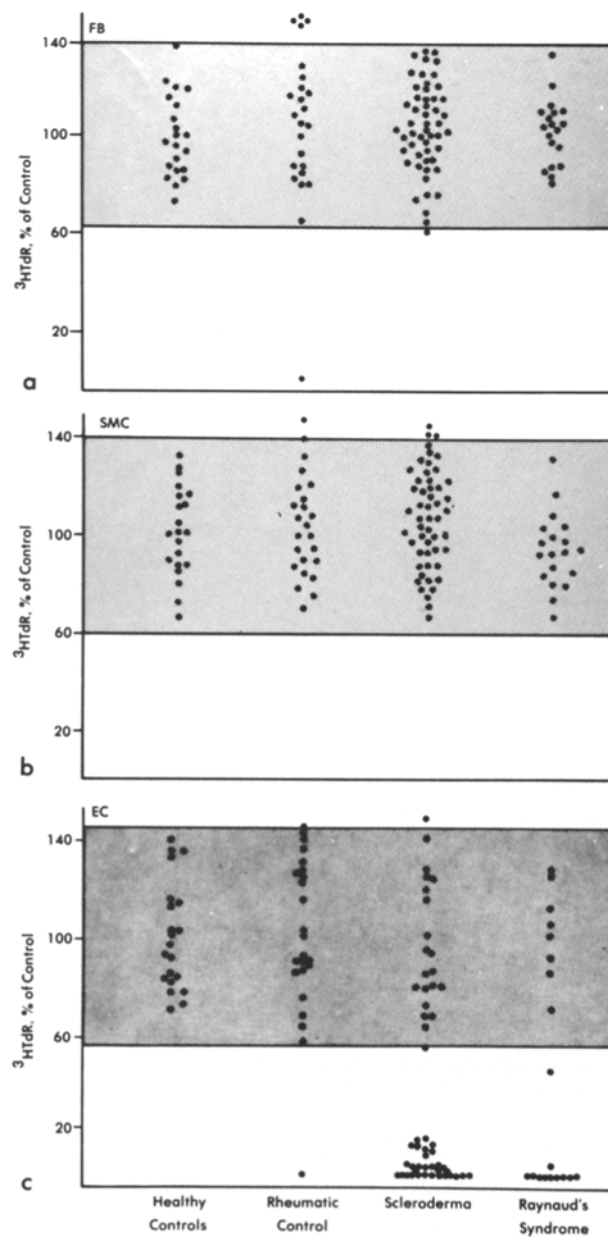


FIG. 6. <sup>3</sup>HTdR uptake by three cell types. (a) FB, (b) SMC, and (c) EC. Cells were cultured in the presence of 15% test serum. Test sera consisted of 21 healthy volunteers, 24 rheumatic controls (8 patients with systemic lupus erythematosus, 9 with rheumatoid arthritis, and 5 with dermatomyositis, 2 polyarteritis), 52 patients with scleroderma, and 19 with Raynaud's syndrome. Shaded area represents mean uptake by healthy control sera  $\pm$  2 SD. Uptake  $<$ 2 SD below the mean is considered inhibitory.

With FB as the target cell (Fig. 6a), inhibition was seen in the serum from one patient with active polyarteritis; the remaining sera showed no inhibitory activity. Using SMC, no sera contained inhibitory activity (Fig. 6b). With EC, one serum from the rheumatic disease control group inhibited <sup>3</sup>HTdR uptake (the patient with

dermatomyositis); in the scleroderma group, 21 sera did not manifest inhibitory activity, whereas the remaining 31 sera markedly inhibited EC  $^3\text{HTdR}$  uptake (Fig. 6c). Serum inhibitory activity has been observed to fluctuate directly with disease activity that was determined clinically in a few patients followed prospectively (e.g. patient LL, Fig. 1). In the Raynaud's group, 8 sera did not inhibit  $^3\text{HTdR}$  uptake, although 11 did. One of the 11 patients with serum-inhibiting activity developed scleroderma 6 mo after the initial serum determination.

### Discussion

Functional and structural vascular lesions have been observed in the organs involved in scleroderma, including reduction in the number of capillaries, telangiectatic vessels, thickening of arterial walls, and intimal fibrosis of small arteries (11). The cause of these changes is not clear. In the present study, using *in vitro* techniques, EC-specific cytotoxic activity has been observed in the plasma and sera of patients with scleroderma and Raynaud's syndrome. The cytotoxicity observed is not a result of a deficiency of nutrient factors, as shown by serum-mixing experiments. Partial characterization indicates a heat-stable, nondialyzable activity which migrates on gel filtration with albumin.

The endothelial and smooth muscle cells, and, more recently, fibroblasts, used in this study were obtained from the same tissue source and, presumably, share common transplantation and other surface antigens. The selective cytotoxicity observed in this study is cell type-specific (EC) and not tissue-specific.

Arteriolar and capillary endothelia may be the primary target cell populations in scleroderma; the cells used in this study are venous in origin because techniques for isolating endothelial cells in quantity from capillaries or small arterioles are not satisfactory. Differences may exist among venous, arteriolar, and capillary endothelia. Subtle morphological, functional, and permeability differences have been described. Huttner (12) demonstrated that arterial endothelium, in contrast to capillary endothelium, is linked together by highly interdigitated cell junctions that appear to exclude molecules of 40,000 daltons or larger. Whether or not the present cytotoxic activity would show differential effects on different vascular EC remains to be determined. Scleroderma vascular disease involves certain sites in the vascular tree, suggesting the importance of local tissue factors for propagation of the disease.

The arterial lesion in scleroderma is similar to those seen in malignant hypertension, thrombotic microangiopathies, and renal allograft rejection. In malignant hypertension, endothelial injury and increased permeability may play a role in the pathogenesis of the vascular lesion (13). Anti-endothelial-cell antibodies have been demonstrated and are considered to play a role in the pathogenesis of thrombotic microangiopathies (hemolytic-uremic syndrome, postpartum renal failure, and thrombotic thrombocytopenic purpura) (14). Endothelial injury to the graft by anti-endothelial-cell antibody, in addition to cellular immunity, has been implicated as a mechanism of renal allograft rejection (15). In disorders characterized by endothelial damage and vascular repair, the particular disease entity might depend more on the severity and chronicity of the vascular insult than the specific injurious agent.

Many theories exist regarding the etiology and pathogenesis of scleroderma: endocrine dysfunction, nervous disorder, infection, physical trauma of various types, and immune factors (16). Many, if not all, of the manifestations of scleroderma can be explained on the basis of functional and structural vascular compromise after repeated



vascular insults, subsequent healing of vascular walls with proliferative vascular response, and luminal narrowing. The coagulation cascade may be triggered by the intimal lesion, leading to fibrin deposition, reduced blood flow, and local ischemia.

The initial events that occur after endothelial injury involve platelet adherence, aggregation, and release at the site of exposed subendothelium. These events are observed after acute (17, 18) or chronic endothelial injury (19). Release of vasoactive amines leads to increased microvascular permeability as a result of EC contraction (20). Caplan (21), using dye labeling and tritiated thymidine incorporation, showed that sites of increased permeability are usually associated with sites of increased thymidine labeling. The puffy, or edematous, phase of scleroderma may be the clinical expression of increased vascular permeability. Platelets also release a factor which promotes proliferation of smooth muscle cells *in vitro* (22). In experimental EC injury, SMC respond to the injury by migration to the intima followed by proliferation (23). In scleroderma vascular lesions, ultrastructural studies characterize the cell involved in intimal thickening as having smooth muscle-like features (4).

A variety of substances are known to induce endothelial damage: cholesterol (24), bile acids (25), angiotensin (26), homocystine (19), endotoxin (27), and immune complexes (28). It is important to identify the factor in scleroderma serum that injures endothelial cells; it is equally important to identify factors that promote endothelial cell proliferation. In this manner, a rational approach to the therapy of scleroderma might be designed.

### Summary

Functional and structural vascular lesions have been observed in the organs involved in scleroderma. The etiology of these vascular changes is poorly understood. The ability to isolate, characterize, and maintain endothelial cells *in vitro* provides a target cell population to study endothelial damage in scleroderma. The present report describes the effect of scleroderma serum on endothelial, smooth muscle, and fibroblast cell types. Sera from patients with scleroderma (31/52) and Raynaud's syndrome (11/19) contain cytotoxic activity, specific for endothelial cells, which is nondialyzable, heat-stable, and elutes with albumin on gel-filtration chromatography.

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