Monocytes of Patients with Systemic Sclerosis (Scleroderma) Spontaneously Release *In Vitro* Increased Amounts of Superoxide Anion

Paola Sambo, Laura Jannino, Marco Candela, Aldo Salvi, Marta Donini,* Stefano Dusi,* Michele M. Luchetti, and Armando Gabrielli

Institute of Internal Medicine, Hematology and Clinical Immunology, University of Ancona, Ancona, Italy; *Institute of General Pathology, University of Verona, Verona, Italy

It has been suggested that toxic oxygen free radicals can be involved in the pathogenesis of systemic sclerosis (scleroderma) (SSc). Because the cells that contribute to the generation of free radicals are not known, our aim was (i) to evaluate the ability of unmanipulated and phorbol 12-myristate 13-acetate-stimulated monocytes and polymorphonucleate neutrophils of SSc patients to generate superoxide anion ($O_2^{\cdot-}$); and (ii) to investigate whether the $O_2^{\cdot-}$ produced by these cells involved the activation of nicotinamide-adenine dinucleotide diphosphate oxidase biochemical pathway. Employing the superoxide dismutase-inhibitable reduction of cytochrome c to evaluate the generation of $O_2^{\cdot-}$, unmanipulated monocytes of SSc patients generated more $O_2^{\cdot-}$ than primary Raynaud's phenomenon patients and normal control monocytes (p = 0.0001), and the release was higher in patients with diffuse cutaneous involvement and 5 y or less disease duration (p = 0.02). The involvement of nicotinamide-adenine dinucleotide diphosphate oxidase in the enhanced $O_2^{\cdot-}$ production was demonstrated by the finding that the cytosolic components of the enzyme, p47[phox] and p67[phox], were both translocated to the plasma membrane of enriched but otherwise unmanipulated monocytes of SSc patients. The involvement of mitochondrial oxidases was excluded by the lack of inhibition of $O_2^{\cdot-}$ production when monocytes were incubated in the presence of rotenone, a mitochondrial oxidase inhibitor. Upon stimulation with phorbol 12-myristate 13-acetate, monocytes of SSc patients produced more $O_2^{\cdot-}$ than controls. In SSc patients untreated polymorphonucleate neutrophils generated significantly less $O_2^{\cdot-}$ than monocytes (p = 0.0001) and only slightly more than polymorphonucleate neutrophils of primary Raynaud's phenomenon patients and normal controls (p = 0.03). In conclusion, we demonstrate that in patients with scleroderma, unmanipulated and phorbol 12-myristate 13-acetate-stimulated monocytes release *in vitro* increased amounts of superoxide anion through the activation of nicotinamide-adenine dinucleotide diphosphate oxidase and, thus, contribute to the oxidative stress found in this disease. *Key words: connective tissue disease/free radicals/monocyte/scleroderma. J Invest Dermatol 112:78–84, 1999*

S ystemic sclerosis (scleroderma) (SSc) is the clinical mani-festation of several interacting factors that culminate in abnormal fibrosis of the skin and internal organs (Krieg and Meuer, 1988; Black and Stephens, 1993). The high prevalence of Raynaud's phenomenon (Belch, 1989; Belch, 1991) and of nailfold capillaroscopy abnormalities (Maricq and LeRoy, 1979), and histologic evidence of microvascular injury (Fleischmajer *et al*, 1976; Rodnan *et al*, 1980; Prescott *et al*, 1992), suggest that vascular involvement is one of the main events in the pathogenesis of scleroderma. The factors implicated in vascular derangement in SSc patients are unknown. Likely candidates are considered to be the soluble mediators secreted by cells of the immune system, IL-1, IL-2, tumor necrosis factor (TNF)-α, transforming growth factor (TGF)-β, platelet derived growth factor (PDGF), and leukotrine B4, reported to be present in high amounts in SSc patients, directly or indirectly could alter functions of vascular cells (White, 1996). Although supported by little evidence to date, it has also been suggested that antibodies might contribute to endothelial cell alteration or damage through direct binding (Salojin *et al*, 1997) or antibody-dependent cellular cytotoxicity (Marks *et al*, 1988; Holt *et al*, 1989). Lastly, granzyme A, a serine protease released by cytolytic CD8^{+} T cells and found in SSc skin, may be the factor in SSc serum that is cytotoxic to the endothelial cells (Kahaleh and LeRoy, 1983).[1,2] With regard to nonimmune mechanisms, the main mediators of cytotoxicity of endothelial cell

[1]Kahaleh MB, Yin T: The molecular mechanism of endothelial cell (EC) injury in scleroderma (SSc): identification of granzyme 1 (a product of cytolytic T cell) in SSc sera. *Arthritis Rheum* 33:S21, 1990 (abstr.)

[2]Kahaleh MB, Fan PS: Cytotoxic T cell involvement in scleroderma (SSc): detection of granzyme A gene expression in SSc skin. *Arthritis Rheum* 35:S38, 1992 (abstr.)

Table I. Clinical characteristics of patients

Group	No.	M/F	Median age, y (range)	Median duration of Raynauld's phenomenon, mo (range)[a]	Median duration of disease, mo (range)[b]	Subset lSSc/dSSc[c]
SSc	29	7/22	57 (21–77)	96 (24–360)	48 (12–322)	21/8
PRP	10	3/7	39 (14–61)	70 (32–40)	70 (32–40)	–
Healthy controls	21	7/14	42 (26–67)	0	0	–

[a]From onset of symptoms.
[b]From diagnosis of systemic sclerosis (scleroderma).
[c]lSSc, limited scleroderma; dSSc, diffuse scleroderma (LeRoy et al, 1988).

injury are considered to be free radicals (Murrel, 1993), because microvascular endothelial cells have an impaired capacity to synthesize the enzyme catalase (Shingu et al, 1985), a free radical scavenger, and are thus particularly prone to reactive oxygen species–mediated injury.

Excessive oxidative stress has been implicated in SSc as determined by increased plasma levels of malondialdehyde (a measure of free radical activity) (Lau et al, 1992) and higher urinary concentration of the bioactive F2-isoprostanes (peroxidation products of arachidonic acid) (Stein et al, 1996). Bruckdorfer et al (1995) showed that low density lipoproteins from scleroderma patients were more susceptible to oxidation than those from healthy controls and concluded that free radical damage is involved in SSc. Additionally, low serum levels of selenium and ascorbic acid have been described in patients with scleroderma and with primary Raynaud's phenomenon (Herrick et al, 1994). These findings suggest that a concurrent antioxidant deficiency could contribute to the oxidative damage observed in scleroderma.

It is therefore conceivable that an enhanced production of reactive oxygen intermediates, a decreased defence against the oxygen free radicals, or both, can play an important role in the pathogenesis of scleroderma; however, it has not been elucidated which cell type and which biochemical reactions are involved in the production of such radicals in SSc patients.

Free radicals are generated during many biologic processes by mitochondria, and enzymatic reactions of xanthine oxidase, aldehyde oxidase, flavin dehydrogenase generate superoxide. A possible source of toxic oxygen derivatives in blood vessels is the nicotinamide-adenine dinucleotide diphosphate (NADPH) oxidase that is expressed in phagocytic cells. This enzymatic system is composed of various proteins, some located on the cell membranes (the subunits of the cytochrome b_{558}, gp91^{phox} and p22phox) and others in the cytosol (p47^{phox} and p67^{phox}) (De Leo and Quinn, 1997). The activation of NADPH oxidase involves the translocation of p47^{phox} and p67^{phox} from cytosol to the plasma membrane, where they interact with the cytochrome b_{558} to form an electron transport chain that transfers electrons from intracellular NADPH to extracellular oxygen (Clark et al, 1990; De Leo and Quinn, 1997).

In this study we present data demonstrating an increased generation of oxygen free radicals by both unmanipulated and phorbol 12-myristate 13-acetate (PMA)-stimulated monocytes of SSc patients. Here we also show that the NADPH oxidase of monocytes is involved in the production of oxygen free radicals in scleroderma patients.

MATERIALS AND METHODS

Patients Twenty-nine nonsmoking scleroderma patients (seven men and 22 women) with a median age of 57 y (range 21–77) were studied. The prevalence of female SSc patients is in agreement with the female to male ratio between 3 and 8:1 already reported (Silman, 1997). The clinical features of the study populations are presented in **Table I**. Diagnosis was made following the ACR criteria (Masi et al, 1980) and the patients were classified into the diffuse SSc and limited SSc subset according to LeRoy et al (1988). Moreover, patients within the diffuse SSc subset were divided into patients with early disease (5 y or less of disease duration) and patients with late disease (more than 5 y of disease duration) (Steen and Medsger, 1990).

At the time of the investigation the patients, who had never been on imunosuppressive therapy, had not received any treatment for the previous 6 wk.

Because Raynaud's phenomenon may precede by years the development of scleroderma, 10 patients with primary Raynaud's phenomenon (PRP) (three men and seven women), were included in the study (**Table I**). Diagnosis of PRP was made according to the criteria reported elsewhere (LeRoy and Medsger, 1992).

Twenty-one age, sex, and race matched, normal, nonsmoking, healthy volunteers were also evaluated and constituted the control population.

Blood was taken from patients and controls after acclimatization at 21°C for 30 min.

Cells Monocytes and polymorphonucleate neutrophils (PMN) were enriched from 100 ml of freshly drawn venous blood collected into sterile, pyrogen-free, vacuum blood collection tubes using heparin (10 U per ml blood) as anticoagulant, as described elsewhere (Bertani et al, 1989). Aliquots of 15 ml of heparinized blood diluted 1:2 with pyrogen-free phosphate-buffered saline were placed on 15 ml of Ficoll–Hypaque (Sigma, St Louis, MO) for centrifugation at $400 \times g$ for 25 min at room temperature, to allow separation of mononuclear cells from PMN and red blood cells. Mononuclear cells collected at the interface were washed twice in phosphate-buffered saline at $200 \times g$ to remove platelets and separated into lymphocytes and monocytes by 47% Percoll (Pharmacia, Uppsala, Sweden) gradient centrifugation at $600 \times g$ for 30 min at 4°C. Under the described experimental conditions electron microscopy study of monocytes excluded internalization of Percoll particles that could be responsible for nonspecific cell activation (data not shown). PMN recovered from the top of the red cell pellet after Ficoll–Hypaque centrifugation were enriched by a 67% Percoll gradient centrifugation at $400 \times g$ for 20 min at 4°C. Monocyte and PMN populations were >95% pure as assessed by nonspecific esterase staining and May-Grunwald-Giemsa staining, respectively. The monocyte and PMN fractions were placed into separate 15 ml test tubes, diluted in ice-cold Krebs-Ringer phosphate buffer with glucose, prepared as described (De la Harpe and Nathan, 1985), and kept in ice until use.

Superoxide anion assay Superoxide anion release, from unmanipulated and PMA-activated cells, was estimated using the superoxide dismutase-inhibitable cytochrome c reduction (Weening et al, 1975; Markert et al, 1984). Briefly, 1.5×10^6 cells were placed in 1.5 ml polypropylene microfuge test tubes and incubated in Krebs-Ringer phosphate buffer with glucose, pH 7.4, containing 80 µM of ferricytochrome c (type III; Sigma), with and without superoxide dismutase (Sigma) (300 U per ml, final concentration), in the presence or absence of PMA (Sigma) (100 ng per ml, final concentration), in a total volume of 1 ml. After incubation for 30 min at 37°C, the reaction was stopped by placing the tubes in melting ice and the absorbance of the supernatant was read at 550 nm. An extinction coefficient of 21.0×10^3 per M per cm was used for oxidized *versus* reduced cytochrome c. In selected experiments, to determine whether a functional NADPH-oxidase complex was involved in the generation of $O_2{\cdot}^-$, monocytes were treated with 20 µM of diphenylene iodonium, a potent and highly selective flavoprotein inhibitor, for 30 min before evaluation of superoxide anion generation. To investigate whether mitochondrial oxidase had any role in the production of $O_2{\cdot}^-$, monocytes were incubated with 50 µM of rotenone, a mitochondrial oxidase inhibitor, for 30 min. Results are expressed as nmol $O_2{\cdot}^-$ per 30 min per 10^6 cells.

Preparation of monocyte cell membranes and cytosols To analyze the membranes of monocytes of individual patients and controls, monocytes, separated as described above, were pretreated with 1 mM di-isopropyl fluorophosphate (Sigma) for 5 min at 4°C, washed, resuspended in Krebs-Ringer phosphate buffer with glucose and activated with 100 ng PMA per ml or treated with the solvent alone (0.01% dimethylsulfoxide) for 30 min at 37°C. The cells were then frozen and kept in liquid nitrogen until use.

The membranes of monocytes were isolated as previously described (Abo *et al*, 1994; Dusi *et al*, 1996). Briefly, unmanipulated and PMA-activated monocytes were thawed, washed with RPMI 1640, and resuspended in 0.4 ml of ice-cold relaxation buffer [100 mM KCl, 3 mM NaCl, 3.5 mM MgCl$_2$, 1.25 mM ethyleneglycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid, 10 mM Pipes pH 7.3] containing 5 μg leupeptin per ml, 5 μg pepstatin per ml, 10 μM phenylarsine oxide, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 5 mM sodium fluoride (all from Sigma). Cells were then disrupted by two cycles of sonication for 30 s at 100 W using a Labsonic 1510 sonicator. Unbroken cells and nuclei were pelleted by centrifugation at 500 × *g* for 10 min at 4°C. The supernatant was carefully layered on the top of a discontinuous sucrose gradient of 1.5 ml of 15% (wt/wt) sucrose on 1.5 ml of 34% (wt/wt) sucrose made up in relaxation buffer, and centrifuged at 100,000 × *g* for 40 min at 4°C in a Beckman L5–50B ultracentrifuge using a SW50 rotor. The light membrane fractions were collected at the 15%–34% sucrose interface, washed in cold relaxation buffer, and pelleted by ultracentrifugation at 100,000 × *g* for 30 min at 4°C. Membranes were resuspended in electrophoresis sample buffer [60 mM Tris/HCl, 20% (vol/vol) glycerol, 4% (wt/vol) sodium dodecyl sulfate (SDS), 2% (vol/vol) 2-mercaptoethanol, pH 6.8] and boiled for 5 min at 100°C. The protein content was measured with the Bio-Rad Protein assay (Bradford, 1976).

Electrophoresis and immunoblotting   Aliquots of monocyte membranes of individual patients and controls containing the same amount of protein (25 μg) were subjected to SDS/polyacrylamide gel electrophoresis on 12% gels, according to standard procedures. Proteins were transferred from the gels to nitrocellulose membranes (Bio-Rad, Milan, Italy) using a Bio-Rad Trans Blot Cell apparatus. Blotting was performed at 70 V for 90 min in 25 mM Tris, 192 mM glycine, 20% (vol/vol) methanol, 3.5 mM SDS, pH 8.3 at 4°C. To ensure that comparable amounts of proteins had been transferred to the nitrocellulose membranes, proteins were revealed on the nitrocellulose membranes by staining with 0.05% (vol/vol) Ponceau S (Sigma) for 1 min. The blots were then rinsed in TBS [50 mM Tris, 170 mM NaCl, 0.2% (vol/vol) Tween 20, pH 7.5] and incubated for 90 min in TBS containing 5% bovine serum albumin (blocking buffer), before incubation overnight at 4°C with rabbit anti-p47^{phox} antibodies (kindly provided by Dr. F. Wientjes, Department of Medicine, University College, London, U.K.), diluted 1:500 in TBS containing 1 mg bovine serum albumin per ml. The blots were rinsed with several changes of TBS and then incubated for 60 min at room temperature in a horseradish-labeled donkey anti-rabbit IgG (Amersham, Little Chalfont, U.K.) diluted 1:15,000 in TBS containing 1 mg bovine serum albumin per ml. After further washing, bound antibodies were detected using enhanced chemiluminescence, western blotting detection reagents (Amersham). The blots were rinsed with TBS and stripped for 30 min at 50°C in a solution containing 100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl pH 6.7. This treatment removed both primary and secondary bound antibodies, and allowed us to reuse the blots with a different primary antibody. After washing with an excess of TBS, the blots were incubated with rabbit anti-p67^{phox} antibodies (also provided by Dr. F. Wientjes), diluted 1:500 in TBS containing 1 mg bovine serum albumin per ml. All the subsequent steps were performed as described for the anti-p47^{phox} antibody.

Flow cytometry analysis   Monocytes were enriched from peripheral blood as described above, washed with phosphate-buffered saline, and incubated at 4°C for 30 min with monoclonal antibody against CD11a, CD11b, and CD11c β2 integrins, followed by washing and labeling with an FITC-conjugated rabbit F(ab)′$_2$ anti-mouse IgG (Dakopatts, Glostrup, Denmark). Samples were analyzed by flow cytometry in a FACScan cytofluorometer (Becton Dickinson).

Statistical analysis   Due to the non-normal distribution of data, the Mann–Whitney U and the Wilcoxon test were employed for the comparison of unpaired and paired data, respectively. Values of p < 0.05 are considered statistically significant.

RESULTS

Unmanipulated monocytes of SSc patients release increased amounts of superoxide anion   **Figure 1** shows the amounts of superoxide anion generated by unmanipulated monocytes of normal, PRP, and SSc patients expressed as nmol of O$_2^{·-}$ per 30 min per 10^6 cells. The median amount of superoxide released by unmanipulated monocytes of SSc patients (1.63 nmol per 30 min per 10^6 cells; 95% confidence interval, 1.0–3.2) is significantly
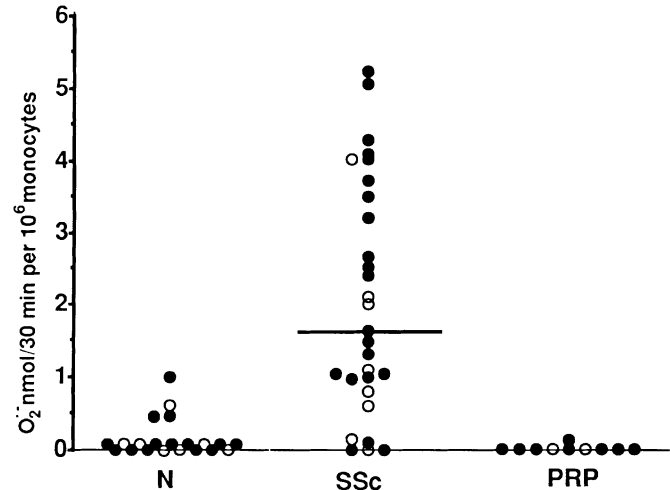

Figure 1. Unmanipulated monocytes of scleroderma patients generate higher amounts of superoxide than PRP patients and normal controls. N, healthy controls. Solid and open symbols indicate female and male patients, respectively. The horizontal bars indicate the median values.

Table II. Phenotypic expression of β2 integrins is similar on monocytes from SSc patients and normal controls[a]

Group	No of patients	CD11a	CD11b	CD11c
SSc	4	155.8 ± 18.3	103 ± 12.7	190.7 ± 68.2
Healthy controls	4	206.4 ± 16.8	110.8 ± 32.8	182.9 ± 32.5

[a]Expressed as mean ± SD. All the comparisons are not statistically significant.

greater (p = 0.0001) than the amount generated by cells of PRP patients (median value, 0; 95% confidence interval, 0–0.12) and by normal monocytes (median value, 0; 95% confidence interval, 0–0.47). The difference between patients with PRP and normal controls is not significant (p = 0.4). Using the ninetieth percentile as the upper limit of normal values, 82.7% of scleroderma patients but only 9.5% of healthy subjects and none of PRP patients display elevated levels of O$_2^{·-}$ release. No significant difference is found when male SSc patients are compared with female SSc patients (data not shown) and no correlation is demonstrated between the extent of organ involvement and O$_2^{·-}$ production (data not shown).

To exclude the possibility that the generation of oxygen free radicals measured was due to cell adhesion to the wall of the test tubes, the release of superoxide anion was also evaluated in test tubes pretreated with silicone. The amount of superoxide released by SSc monocytes in siliconized tubes is comparable with that obtained in untreated tubes (data not shown). Furthermore, the expression of β$_2$-integrins by monocytes of SSc patients is not significantly different from that detected in control cells (**Table II**). This finding rules out a potential mechanism of homotypic cell aggregation in the generation of superoxide anion (Dusi *et al*, 1996).

We then investigated the relationships between the amount of superoxide anion generated by unmanipulated monocytes and the clinical features of scleroderma patients. As **Table III** indicates, no difference is detected when all patients are divided into the limited or the diffuse subset, or into early (5 y or less) or late (over 5 y) disease. In contrast, within the diffuse subset there is a significant difference between patients with early disease and those with late disease (p = 0.02).

In scleroderma patients unmanipulated polymorphonucleate neutrophils release less superoxide anion than monocytes   When unmanipulated PMN are analyzed, the median amount of superoxide released from scleroderma cells is 0.06 nmol O$_2^{·-}$ per 30 min per 10^6 cells (95% confidence interval, 0–0.29)
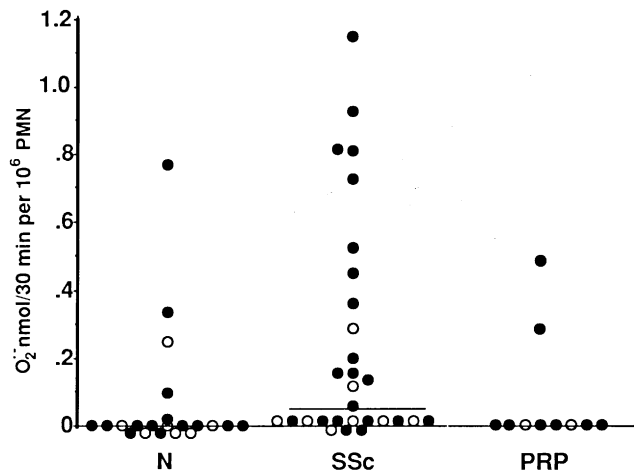
Figure 2. Unmanipulated PMN of scleroderma patients generate higher amounts of superoxide than healthy controls. The difference between scleroderma and PRP monocytes was not statistically significant. Scleroderma PMN generate significantly less $O_2^{\cdot-}$ than monocytes. This is evident once the different scale employed to plot PMN data is taken into account. N, healthy controls. Solid and open symbols indicate female and male patients, respectively. The horizontal bars indicate the median values.

Table III. Monocyte production of superoxide is higher in SSc patients with early, diffuse cutaneous SSc

Subset	No of patients	$O_2^{\cdot-}$ per 30 min per 10^6 monocytes median (range)	p
lSSc[a]	21	2.00 (0–52)	0.73
dSSc	8	1.27 (0–4.07)	
Early SSc[b]	17	2.5 (0–5.2)	0.13
Late SSc	12	1.08 (0–3.9)	
Early dSSc[c]	4	3.6 (1.05–4.07)	0.02
Late dSSc	4	0.8 (0–1.05)	

[a]lSSc, limited scleroderma; dSSc, diffuse scleroderma (LeRoy *et al*, 1988).

[b]All SSc patients are divided in early (5 y or less of disease duration) and late (more than 5 y of disease duration) SSc.

[c]Patients with diffuse cutaneous SSc (LeRoy *et al*, 1988) were divided into patients with early (5 y or less of disease duration) or late (more than 5 y of disease duration) disease.

and the only difference found is between SSc and healthy controls (p = 0,03) (**Fig 2**). Nine per cent of SSc patients and 2% of PRP and of normal subjects are above the ninetieth percentile. In SSc patients, PMN generated significantly less $O_2^{\cdot-}$ than monocytes (p = 0.0001), and no correlation was found between the amounts of superoxide anion produced by monocytes and the amounts of $O_2^{\cdot-}$ generated by PMN.

NADPH oxidase is activated in unmanipulated monocytes of scleroderma patients To understand whether NADPH oxidase is involved in the production of superoxide anion by unmanipulated monocytes of SSc patients, we have analyzed the translocation of the cytosolic components p47phox and p67phox to the plasma membrane of these cells, a step that is essential for the activation of the enzyme (Clark *et al*, 1990; De Leo and Quinn, 1997). For this purpose we have fractionated resting monocytes from five consecutive normal controls and 10 consecutive SSc patients releasing high levels of superoxide, and we have immuno-blotted the light membrane fractions of these cells with antibodies raised against p47phox and p67phox. Two representative experiments are shown in **Figs 3** and **4**. As compared with monocytes of control donors, an increased translocation of both p47phox and p67phox is evident in unmanipulated cells from scleroderma patients.

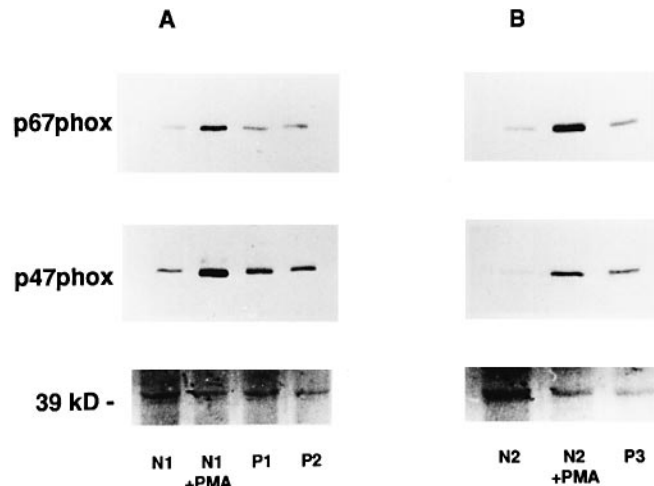To further assess the involvement of NADPH oxidase in the

Figure 3. Activation of NADPH oxidase in unmanipulated SSc patient monocytes is shown by the translocation of NADPH oxidase cytosolic components p47phox and p67phox to the light membrane fractions. Resting monocytes from normal controls (N) and from scleroderma patients (P) were fractionated, the light membranes were subjected to SDS 12% polyacrylamide gel electrophoresis and then immunoblotted with anti-p47phox and anti-p67phox specific antibodies. Monocytes of normal subjects stimulated with PMA are shown as positive control. (*A*) and (*B*) show two experiments performed with cells from distinct patients and controls. Normal loading of proteins was assessed by staining with 0.05% (vol/vol) Ponceau S. See *Materials and Methods* for details.
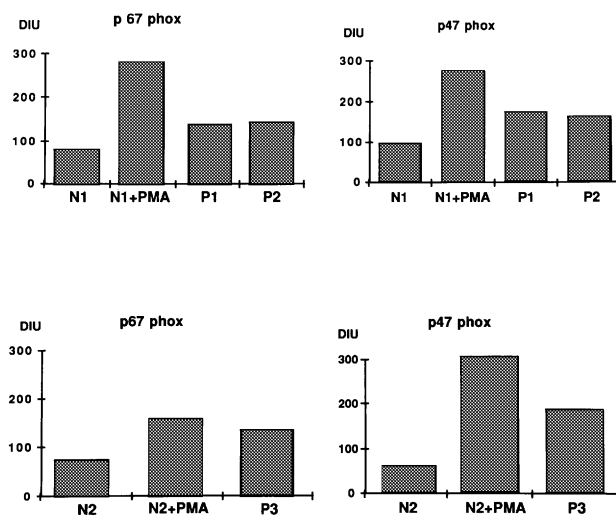

Figure 4. Enhanced translocation of p47phox and p67phox to the light membrane fractions of monocytes of SSc patients is confirmed by densitometric analysis. The figure shows the densitometric analysis performed by laser densitometric scanning on the film of the immunoblotting of **Fig 3**. DIU, densitometric image units.

spontaneous generation of superoxide anion, monocytes of sclero-derma patients were incubated with 25 μM of diphenylene iodonium. The generation of superoxide, estimated using the cytochrome c reduction method, is completely inhibited (**Table IV**). Treatment with 50 μM of rotenone (Sigma), a mitochondrial oxidase inhibitor, failed to reduce the superoxide production, indicating that mitochondrial respiration oxidases have no role in the generation of $O_2^{\cdot-}$.

PMA-stimulated monocytes of SSc and PRP patients gener-ate higher amounts of superoxide than control monocytes It is known that the production of oxygen free radicals by phagocytes in response to a stimulant is enhanced if the cells are previously exposed to a substimulatory dose of an
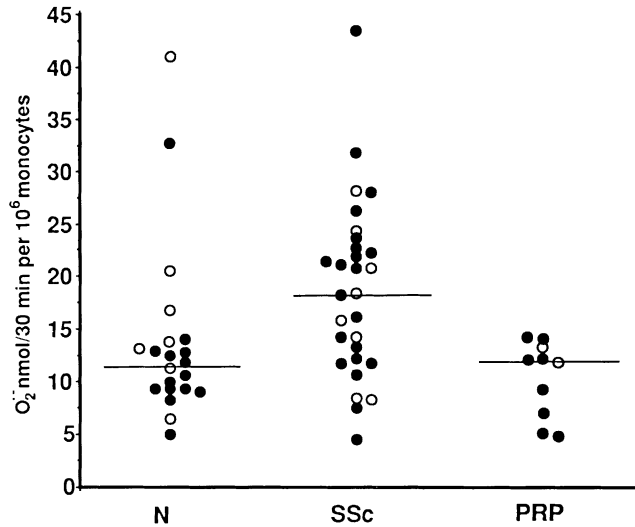
Figure 5. Scleroderma monocytes stimulated with PMA generated higher amounts of superoxide than control monocytes. N, healthy controls. The difference between healthy control and PRP monocytes is not statistically significant. Solid and open symbols indicate female and male patients, respectively. The horizontal bars indicate the median values.

Figure 6. Scleroderma PMN stimulated with PMA generate higher amounts of superoxide than healthy controls. N, healthy controls. The difference between scleroderma and PRP monocytes was not statistically significant. Solid and open symbols indicate female and male patients, respectively. The horizontal bars indicate the median values.
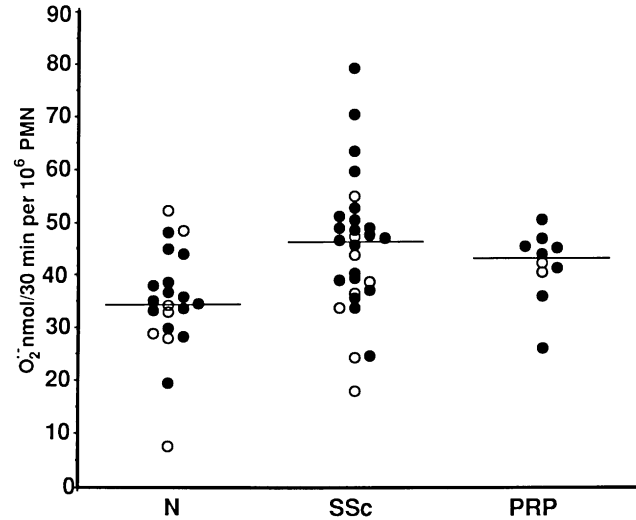
Table IV. Incubation of SSc monocytes with diphenylene iodonium but not with Rotenone inhibits the generation of superoxide (expressed as nMol $O_2^{\cdot-}$ per 30 min per 10^6 monocytes)

	Untreated	w/superoxide dismutase 300 U per ml	w/diphenylene iodonium 20 μM	w/Rotenone 50 μM
SSc1	3.5	0	0	3.4
SSc2	5.0	0	1	5.1
SSc3	4.7	0	1.2	4.2

heterologous agonist (Rossi, 1986; Bastian and Hibbs, 1994). Because SSc patient monocytes are in a state of preactivation, we have investigated whether in these cells the superoxide anion production in response to PMA was enhanced. **Figure 5** illustrates that upon stimulation with PMA the median amount of $O_2^{\cdot-}$ generated by SSc patient monocytes (18.30 nmol per 30 min per 10^6 cells; 95% confidence interval, 13.30–22.09) is higher than that released by PRP patient cells (11.85 nmol $O_2^{\cdot-}$ per 30 min per 10^6 cells; 95% confidence interval, 5.18–14.01) (p = 0.004) and by normal controls (11.90 nmol $O_2^{\cdot-}$; 95% confidence interval, 9.40–14) (p = 0.01). No difference is evident when controls are compared with PRP patients (p = 0.4).

As for PMA-stimulated PMN, cells of patients with SSc and PRP produce a significant higher amount of superoxide than controls (median values, 46.5 nmol $O_2^{\cdot-}$ per 30 min per 10^6 cells, 43 nmol $O_2^{\cdot-}$ per 30 min per 10^6 cells, and 34.3 nmol $O_2^{\cdot-}$ per 30 min per 10^6 cells, respectively; SSc *versus* normals, p = 0.003; PRP *versus* normals, p = 0.04; SSc *versus* PRP, p = not significant) (**Fig 6**).

DISCUSSION

In this report we show that (i) monocytes of scleroderma patients release *in vitro*, albeit unmanipulated, amounts of superoxide anions higher than controls; (ii) in scleroderma patients monocytes release more $O_2^{\cdot-}$ than PMN; (iii) the release *in vitro* of $O_2^{\cdot-}$ occurs through the activation of NADPH oxidase; and finally (iv) $O_2^{\cdot-}$ generation by monocytes is greater in patients with diffuse SSc and a disease duration of 5 y or less.

In recent years it has been proposed that free radicals may play a role in the pathogenesis of scleroderma (Murrel, 1993). This

assumption is mainly based upon indirect evidence. Firstly, a good proportion of people exposed to agents that generate free radicals, like bleomycin, L-5-hydroxytryptophan, rapeseed oil, silicone, and silica dust, may develop scleroderma-like lesions (Rodnan *et al*, 1967; Finch *et al*, 1980; Sternberg *et al*, 1980; Kilbourne *et al*, 1983; Varga *et al*, 1989). Secondly, data suggestive for increased oxidative damage in scleroderma have been reported by several groups (Lau *et al*, 1992; Herrick *et al*, 1994; Bruckdorfer *et al*, 1995; Stein *et al*, 1996), although no experimental explanation has been provided regarding the cellular and the biochemical source of the oxygen free radicals involved. In this respect our findings are consistent with the hypothesis that in SSc patients monocytes contribute, surprisingly more than PMN, to the abnormal oxidative stress documented in this disorder.

In SSc patients, activation of monocyte respiratory burst may be induced by the chain reaction that follows ischaemia-reperfusion events, likely to occur in these patients suffering from Raynaud's phenomenon (see Bulkley, 1994 for more details). If it is ischaemia that leads to superoxide production by circulating monocytes, however, it has to be explained why (i) monocytes of patients with primary Raynaud's phenomenon, who suffer from a vasospastic disorder of the extremities, did not show increased generation of superoxide; (ii) generation of superoxide by unmanipulated PMN of scleroderma patients was considerably less than that by monocytes of the same patients, despite both cell types were suffering from the same ischaemic insult. In view of these considerations, it can be speculated that monocyte NADPH of SSc patients is directly activated *in vivo* by factor(s), other than ischaemia.

Monocyte activation is a complex phenomenon regulated through enhancing and suppressive signals and the phenotype therefore reflects the net sum of activation and deactivation. In SSc patients, monocyte activation may be maintained by one or more enhancing signals, such as IL-1, IL-6, IL-8, and TNF-α, which are increased in SSc patients (White, 1996) and are known to trigger the activation of NADPH oxidase (Bastian and Hibbs, 1994). Alternatively, it can be conceived as a default of deactivating mechanisms of monocytes. Because TGF-β and IL-10 markedly suppress the release of reactive oxigen species (Tsunawaki *et al*, 1988; Bogdan *et al*, 1991), in preliminary experiments we have evaluated the amounts of TGF-β and IL-10 generated *in vitro* by monocytes of SSc patients and found no difference in comparison with controls (data not shown). The physiologic relevance of this finding, however, in particular whether the amount of TGF-β and

IL-10 produced is inadequate to suppress reactive oxygen species production, is currently under investigation.

The complexity of monocyte activation is further suggested by the fact that different cytokines, though sharing the same signaling pathway, are able to induce different patterns of gene expression and that distinct monocyte functions (e.g., growth and respiratory burst) may be differently modulated by a single citokine (Gordon et al, 1995). This is in keeping with our findings that an increased respiratory burst in monocytes of SSc patients is not paralleled by an increase in integrin expression (**Table II**) and TNF-α production (data not shown).

In this study we also demonstrate that, in monocytes of SSc patients, superoxide anion generation is sustained through the activation of the NADPH oxidase. In fact, in unmanipulated monocytes of scleroderma patients the amount of the cytosolic NADPH oxidase components p47^{phox} and p67^{phox} translocated on the plasma membrane is increased, indicating that the enzyme is in an active state (Clark et al, 1990; De Leo and Quinn, 1997). This demonstrates that, as observed for PMN, p47^{phox} and p67^{phox} translocate to the plasma membrane of activated human monocytes.

A very interesting phenomenon, which is relevant for the mechanism of NADPH oxidase activation, is the potentiation of the production of oxygen free radicals in response to a stimulant by prior exposure of the cells to a substimulatory dose of an heterologous agonist (Rossi, 1986; Bastian and Hibbs, 1994). Such a "priming" effect could have a physiologic role in inflammation and defenses against infections. In fact, cells that have been in contact with very low concentrations of inflammation mediators or with bacterial products, may be predisposed to an higher release of toxic oxygen derivatives. Interestingly, in this study we report that SSc patients monocytes are in a primed state, as demonstrated by the increased $O_2 \cdot^-$ production upon stimulation with phorbol esters. Immune complexes, bacteria, cytokines such as IL-1, IL-6, IL-8, and TNF-α, and Fc fragments of immunoglobulins, are all capable of inducing this primed condition in which subsequent stimulation produces an enhanced NADPH activity (Bastian and Hibbs, 1994).

Thus, in scleroderma, unknown agonist(s), probably the same responsible for the NADPH oxidase activity observed in unmanipulated phagocytes of SSc patients, predispose monocytes to respond to a second stimulation with an augmented NADPH oxidase activation that increases the oxidative damage in cells of SSc patients.

The molecular mechanisms of the priming phenomenon remain to be established. The finding that in untreated monocytes of SSc patients the translocation of p47^{phox} and p67^{phox} to the plasma membrane is increased, however, may suggest that a preassociation of a fraction of cytosolic NADPH oxidase components with the cytochrome b$_{558}$ could, in some way, predispose the enzyme to perform a more efficient electron flow through the plasma membrane in response to a subsequent cell stimulation. Obviously, further investigations are required to test this hypothesis.

Finally, patients with diffuse cutaneous involvement and a disease duration of 5 y or less showed an increased production of superoxide when compared with patients with longer disease duration. If this is confirmed in a larger cohort of patients it would suggest that monocyte activation may be an early event in the natural history of the disease and pathologically relevant at least in this subset of patients.

As discussed by Murrel (1993), oxidative stress can explain the tissue lesions occurring in scleroderma, as free radicals can induce endothelial cell injury through peroxidation of lipid components of cell membrane (Del Maestro et al, 1980; Inaunen et al, 1989) and may stimulate fibroblasts to proliferate (Murrel et al, 1990) and to produce increased amounts of collagen (Chojkier et al, 1989). Furthermore, recently, the autoantigens targeted in diffuse scleroderma have been found susceptible to cleavage by reactive oxygen species, in a metal-dependent manner, providing an explanation for the appearance of serum antibodies against topoisomerase I and emphasizing the importance of metal metabolism in the formation of free radical damage (Halliwell, 1994; Casciola-Rosen et al, 1997).

In conclusion, our findings provide evidence that, through the activation of an NADPH enzymatic system, monocytes and not neutrophils contribute to the oxidative stress found in scleroderma.

REFERENCES

Abo A, Webb MR, Grogan A, Segal AW: Activation of NADPH oxidase involves the dissociation of p21^{rac} from its inhibitory GDP/GTP exchange protein (rhoGDI) followed by its translocation to the plasma membrane. *Biochem J* 298:585–591, 1994

Bastian RN, Hibbs JB Jr: Assembly and regulation of NADPH oxidase and nitric oxide synthase. *Curr Opin Immunol* 6:131–139, 1994

Belch JJF: Raynaud's phenomenon. *Curr Opin Rheumatol* 1:490–498, 1989

Belch JJF: Raynaud's phenomenon: its relevance to scleroderma. *Ann Rheum Dis* 50:839–845, 1991

Bertani A, Polentarutti N, Sica A, Rambaldi A, Mantovani A, Colotta F: Expression of c-jun protoncogene in human myelomonocytic cells. *Blood* 74:1811–1816, 1989

Black CM, Stephens C. Systemic sclerosis (scleroderma) and related disorders. In: Maddison PJ, Isenberg DA, Woo P, Glass DN (eds). *Oxford Textbook of Rheumatology.* Oxford: Oxford University Press, 1993, pp. 771–787

Bogdan C, Vodovotz Y, Nathan C: Macrophage deactivation by interleukin 10. *J Exp Med* 174:1549–1555, 1991

Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of proteins utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254, 1976

Bruckdorfer KR, Hillary JB, Bunce T, Vancheeswaran R, Black CM: Increased susceptibility to oxidation of low-density lipoproteins isolated from patients with Systemic Sclerosis. *Arthritis Rheum* 38:1060–1067, 1995

Bulkley GB: Reactive oxygen mediated and reperfusion injury: aberrant triggering of reticuloendothelial function. *Lancet* 344:934–936, 1994

Casciola-Rosen L, Wigley F, Rosen A: Scleroderma autoantigens are uniquely fragmented by metal-catalyzed oxidation reactions: implication for pathogenesis. *J Exp Med* 185:71–79, 1997

Chojkier M, Houglum K, Solis-Herruzo J, Brenner DA: Stimulation of collagen gene expression by ascorbic acid in cultured human fibroblasts. A role for lipid peroxidation. *J Biol Chem* 264:16957–16962, 1989

Clark RA, Volpp BC, Leidal KG, Nauseef WM: Two cytosolic components of the human neutrophil respiratory burst oxidase translocate to the plasma membrane during cell activation. *J Clin Invest* 85:714–721, 1990

De la Harpe J, Nathan CF: A semi-automated micro-assay for H$_2$O$_2$ release by human blood monocytes and mouse peritoneal macrophages. *J Immunol Meth* 78:323–336, 1985

De Leo FR, Quinn M: Assembly of the phagocyte NADPH oxidase: molecular interaction of oxidase proteins. *J Leukoc Biol* 60:677–691, 1997

Del Maestro RF, Thaw HH, Bjork J, Blanker M, Arfors KE: Free radicals as mediators of tissue injury. *Acta Physiol Scand* 492:43–57, 1980

Dusi S, Della Bianca V, Donini M, Nadalini KA, Rossi F: Mechanisms of stimulation of the respiratory burst by TNF in nonadherent neutrophils. Its independence of lipidic transmembrane signaling and dependence on protein tyrosine phosphorylation and cytoskeleton. *J Immunol* 157:4615–4623, 1996

Finch WR, Rodnan GP, Buckingham RB, Prince RK, Winkelstein A: Bleomycin-induced scleroderma. *J Rheumatol* 7:651–659, 1980

Fleischmajer R, Perlish SH, Jay H, Shaw KV, Pirozzi DJ: Skin capillary changes in early systemic scleroderma. *Arch Dermatol* 111:1553–1557, 1976

Gordon S, Clarke S, Greaves D, Doyle A: Molecular immunobiology of macrophages: recent progress. *Curr Opin Immunol* 7:24–33, 1995

Halliwell B: Free radicals, antioxidants, and human disease: curiosity, cause or consequence? *Lancet* 344:721–724, 1994

Herrick AL, Rieley F, Schofield D, Hollis S, Braganza JM, Jayson MIV: Micronutrient antioxidant status in patients with Primary Raynaud's Phenomenon and Systemic Sclerosis. *J Rheumatol* 21:1477–1483, 1994

Holt CM, Lindsey N, Moult J, et al: Antibody-dependent cellular cytotoxicity of vascular endothelium: characterization and pathogenic associations in systemic sclerosis. *Clin Exp Immunol* 78:359–365, 1989

Inaunen W, Payne DK, Kvietys PR, Granger DR: Hypoxia/reoxygenation increases the permeability of endothelial cell monolayers: role of oxygen radicals. *Free Radic Biol Med* 1990:219–223, 1989

Kahaleh MB, LeRoy EC: Endothelial injury in scleroderma. A protease mechanism. *J Lab Clin Med* 101:553–560, 1983

Kantor TV, Friberg D, Medsger TA Jr, Buckingham RB, Whiteside TL: Cytokine production and serum levels in Systemic Sclerosis. *Clin Immunol Immunopathol* 65:278–285, 1992

Kilbourne EM, Rigau-Perez JG, Heath CW Jr, Zack MM, Falck H, Martin-Marcos M, De Carlos A: Clinical epidemiology of toxic–oil syndrome: manifestations of a new illness. *N Engl J Med* 309:1408–1414, 1983

Krieg T, Meuer M: Systemic scleroderma. Clinical and pathophysiological aspects. *J Am Acad Dermatol* 18:457–481, 1988

Lau CS, Bridges AB, Muir A, Scott N, Bancroft A, Belch JF: Further evidence of increased polymorphonuclear cell activity in patients with Raynaud's phenomenon. *Br J Rheumatol* 31:375–380, 1992

LeRoy EC, Medsger TA: Raynaud's phenomenon: a proposal for classification. *Clin Exp Rheumatol* 10:485–488, 1992

LeRoy EC, Black CM, Fleischmajer R, *et al*: Scleroderma (systemic sclerosis): classification, subsets and pathogenesis. *J Rheumatol* 12:217–223, 1988

Maricq HR, LeRoy EC: Progressive systemic sclerosis: disorders of the microcirculation. *Clin Rheum Dis* 5:81–101, 1979

Markert M, Andrews PC, Babior BM: Measurement of $O_2^{\cdot-}$ production by human neutrophils. The preparation and assay of NADPH oxidase-containing particles from human neutrophils. *Meth Enzymol* 105:358–365, 1984

Marks RM, Czerniecki M, Andrews BS, Penny R: The effects of scleroderma serum on human microvascular endothelial cells. Induction of antibody-dependent cellular cytotoxicity. *Arthritis Rheum* 31:1524–1534, 1988

Masi AT, Rodnan T, Medseger TA Jr, *et al*: Preliminary criteria for the classification of systemic sclerosis (scleroderma). *Arthritis Rheum* 23:581–590, 1980

Murrel DF: A radical proposal for the pathogenesis of Scleroderma. *J Am Acad Dermatol* 28:78–85, 1993

Murrel GAG, Francis MJO, Bromley L: Modulation of fibroblast proliferation by oxygen free radicals. *Biochem J* 265:259–265, 1990

Prescott RJ, Freemont AJ, Jones CJP, Hoyland J, Fielding P: Sequential dermal microvascular and perivascular changes in the development of scleroderma. *J Pathol* 166:255–263, 1992

Rodnan GP, Benedek TG, Medsger TA Jr, Cammarata RJ: The association of PSS with coal miners' pneumoconiosis and other forms of silicosis. *Ann Intern Med* 6:323–324, 1967

Rodnan GP, Myerowitz RC, Justh GO: Morphologic changes in the digital arteries of patients with progressive systemic sclerosis (scleroderma) and Raynaud's phenomenon. *Medicine* 59:393–408, 1980

Rossi F: The $O_2^{\cdot-}$ forming NADPH oxidase of phagocytes: nature, mechanisms of activation and function. *Biochim Biophys Acta* 853:65–89, 1986

Salojin KV, Le Tonqueze M, Saraux A, Nassonov EL, Dueymes M, Piette JC, Youinou PY: Antiendothelial cell antibodies: useful markers of systemic sclerosis. *Am J Med* 102:178–185, 1997

Shingu M, Yoshioka K, Nobunaga M, Yoshida K: Human vascular smooth muscle cells and endothelial cells lack catalase activity and are susceptible to hydrogen peroxide. *Inflammation* 9:309–320, 1985

Silman AJ: Scleroderma-dermographics and survival. *J Rheumatol* 24:58–61, 1997

Steen VD, Medsger JA Jr: Epidemiology and natural history of systemic sclerosis. *Rheum Dis Clin North Am* 16:1–10, 1990

Stein CM, Tanner SB, Awad JA, Roberts LJ, Morrow JD: Evidence of free-radical-mediated injury (isoprostane overproduction) in Scleroderma. *Arthritis Rheum* 39:1146–1150, 1996

Sternberg EM, van Woert MH, Young SN, Magnussen I, Baker H, Gauthier S, Osterland K: Development of a scleroderma-like illness during therapy with L-5-hydroxytryptophan and carbidopa. *N Engl J Med* 303:782–787, 1980

Tsunawaki S, Sporn N, Ding A, Nathan C: Deactivation of macrophages by transforming growth factor-B. *Nature* 334:260–262, 1988

Umehara H, Kumagai S, Murakami M, Suginoshita E, Imura H: Enhanced production of interleukin 1 and tumor necrosis factor α by cultured peripheral blood monocytes from patients with scleroderma. *Arthritis Rheum* 33:893–897, 1990

Varga J, Schumacher RH, Jimenez SA: Systemic Sclerosis after augmentation mammoplasty with silicone implants. *Ann Intern Med* 111:377–383, 1989

Weening RS, Wever R, Roos D: Quantitative aspects of the production of superoxide radicals by phagocytizing human granulocytes. *J Lab Clin Med* 85:245–252, 1975

White B: Immunopathogenesis of systemic sclerosis. *Rheum Dis Clin North Am* 22:675–694, 1996