Neutrophils From Patients After Burn Injury Express a Deficiency of the Oxidase Components p47-phox and p67-phox

By Joseph Rosenthal, Gail Thurman, Nancy Cusack, Verlyn M. Peterson, Harry L. Malech, and Daniel R. Ambruso

Infection is a major cause of morbidity and mortality in patients after thermal injury. This predisposition to infections is related, in part, to abnormal polymorphonuclear leukocyte (PMN) function and a diminished respiratory burst. To evaluate the biochemical basis for the defective respiratory burst after major burns, the status of the oxidase enzyme system and its components was investigated. PMNs were isolated from 24 patients with 12% to 62% burns. Oxidase activity of intact PMNs, measured as superoxide anion (O$_2^-$) generation or oxygen consumption, was decreased in burn compared with healthy controls. Subcellular fractions from patient PMNs generated less O$_2^-$ in the sodium dodecyl sulfate cell-free system, and this was related to a diminished contribution by cytosol but not by plasma membrane. Subsequently, cytosol was separated with CM-Sepharose, yielding two fractions; one contained the p47-phox and p67-phox (47/67 mix) and the other contained the remaining cytosolic components (run through [RT]). Although the contribution to oxidase activity made by RT from patient cytosol was similar to that of control, the activity of p47/67 mix from PMNs of burn patients was deficient. Quantitative assays using an immunoblotting technique showed a consistent, but significant decrease in both p47-phox and p67-phox. The addition of purified or human recombinant p47-phox but not p67-phox corrected the diminished oxidase activity of cytosol from burn patients. Thus, decreased respiratory burst activity found in PMNs from individuals with thermal injury was associated with a specific, quantitative deficiency of p47-phox.

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Infection is a major cause of morbidity and mortality in patients after thermal injury. Patients surviving the resuscitation phase of burn trauma frequently exhibit defects in many aspects of host defense mechanisms. The polymorphonuclear leukocyte (PMN), a critical component of the host’s response to bacterial invasion, has been intensively studied in this group of patients. After thermal injury, various PMN function defects, including altered chemotaxis, adhesion, phagocytosis, and decreased microbial activity, have been documented. These defects in phagocyte function represent a breach in host defense and provide one cause for the predisposition to infection exhibited by patients with burn injury.

The process of microbial activity by PMNs is complex and requires both oxygen-dependent and oxygen-independent mechanisms. Initiation of the oxygen-dependent system occurs with the activation of the respiratory burst, a complex sequence of metabolic changes resulting in an increase in oxygen consumption and production of superoxide anion (O$_2^-$) and other toxic oxygen metabolites. This process occurs under the direction of an enzyme system, the NADPH:O$_2$ oxidoreductase or oxidase. The clinical significance of the oxidase system is shown in patients with chronic granulomatous disease (CGD), a syndrome representing a group of inherited disorders characterized by an inability of phagocytes to generate toxic oxygen metabolites and an increased incidence of bacterial infections.

In recent years, investigations have documented that the oxidase is a complex enzyme system consisting of several components located in various sites within the resting PMN. Defective contribution of plasma membrane or cytosol components to oxidase activity in cell-free systems for oxidase activity allows for classification of CGD syndromes into several distinct types. The majority of patients have a deficiency in cytochrome b$_{558}$ that is found in the plasma membrane and specific granules and is inherited either more commonly by an X-linked recessive mode (deficiency of the β subunit of cytochrome b$_{558}$, gp91-phox) or more rarely by an autosomal recessive mode (deficiency of the α subunit of cytochrome b$_{558}$, p22-phox). In contrast, other patients exhibit a deficiency in either one of two cytosolic oxidase components (p47-phox and p67-phox, respectively), each inherited through an autosomal recessive mode.

Previous studies have shown diminished oxidase activity in PMNs from burn patients. However, the status of the various oxidase components in PMNs from these patients has not been completely evaluated. The present study confirms that the activity of the NADPH:O$_2$ oxidoreductase system in PMNs from individuals after burn trauma is diminished compared with healthy controls. A detailed analysis shows that the decrease in respiratory burst activity is related to a deficiency in p47-phox and p67-phox and can be reversed in vitro with the addition of purified or recombinant proteins. Thus, an acquired deficiency of cytosolic components is the cause of defective oxidase activity in neutrophils from patients with burn injury.

MATERIALS AND METHODS

Patients. Twenty-four adult patients (4 women and 20 men) who were 19 to 64 years of age (mean, 40 years of age) and who were

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admitted to the Burn Unit with 12% to 62% total body surface area (all but 2 had >30% burns) with partial or full-thickness burns were studied. Patients with electrical or chemical burns were excluded from the study as were patients with confirmed sepsis. After written, informed consent approved by the Human Subject Committee of the University of Colorado Health Sciences Center was given, blood samples were obtained within 3 to 7 days after the burn in all patients. In 5 of these patients, additional samples were collected between days 10 and 15 after the burn.

*Reagents.* Phenylmethyl sulfonl fluoride (PMSF), HEPES, PIPES, 2-[N-Morpholino]ethane sulfonic acid, phosphatidylcholine, phosphatidylserine, superoxide dismutase (SOD), acetyl-leu-leu-arginine-AL (leupeptin), Tris, glycine, phenol myristate acetate (PMA), adenosine triphosphate, cytochrome c, guanosine 5'-O-(3-thio) thiphosphate (GTP-γ-S), NAPDH, EGTA, diisopropyl fluorophosphate (DFP), dimethyl sulfoxide, reagents for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), plater-activating factor (PAF), FMLP, polyethylene-sorbitan monolaurate (Tween 20), and Kodak X-Omat AR films were purchased from Sigma Chemical Co (St Louis, MO). Protein kinase C purified from rat brain was obtained from Lipidex, Inc (Middleton, WI). Carboxymethyl Sepharose (CM Sepharose) was obtained from Pharmacia (Uppsala, Sweden). 125I protein G was purchased from Amersham (Arlington Heights, IL). Methanol and Tween were from J.T. Baker Chemical Co (Phillipsburg, NJ). Horseradish peroxidase-conjugated rabbit-antimouse and rabbit-antigoat IgG antibodies were purchased from Axell (Westbury, NY). Peroxidase substrate solutions were purchased from Kirkegaard & Perry Laboratories (Gaithersburg, MD). Protein assay reagents and standards were obtained from Pierce (Rockford, IL). Prestained molecular weight standards for SDS-PAGE were from Bio-Rad (Richmond, CA).

Monoclonal antibodies to the α subunit (p22-phox) and the β subunit (gp91-phox) proteins of the cytochrome b558 were provided by Drs A. Verhoeven and D. Roos (Central Laboratory of the Netherlands). Goat antisera to human recombinant p47-phox and p67-phox produced in an insect vector and purified as previously described.

*Isolation of neutrophils.* Heparinized (10 U/mL) whole blood was drawn at the same time from patients and healthy controls. Human neutrophils were prepared by standard techniques of dextran sedimentation, Ficoll-Hypaque gradient procedure, and hypotonic lysis, resulting in preparations that contained 98% to 99%. Although some patient samples exhibited a modest increase in immature neutrophils (ie, bands) in peripheral blood and isolated neutrophil preparations, these differences could not account for the much larger alterations documented in oxidase activity and components. PMNs were adjusted to concentration of 10^6 cells/mL.

*Disruption of neutrophils and preparation of subcellular fractions.* Cells suspended in Krebs Ringers phosphate with dextrose were incubated in 2 mmol/L DFP for 10 minutes at 4°C. After centrifugation at 250g for 10 minutes at 4°C, PMNs were resuspended in cold cavitation buffer and were disrupted in a Parr cavitation bomb (Parr Instrument Co, Moline, IL) under the conditions described previously. The cavitation was collected in 1.2 mmol/L EGTA and centrifuged at 800g at 4°C for 10 minutes to remove nuclei and undisrupted cells. The postnuclear supernatant was then layered over a continuous/discontinuous sucrose gradient containing 1.2 mmol/L EGTA, 0.1 mmol/L PMSF, and 1 µg/mL leupeptin. The gradients were centrifuged at 100,000g at 4°C for 90 minutes, and the fractions containing cytosol, plasma membrane, specific granules, and azurophilic granules were collected and stored at −70°C. Plasma membrane and cytosol activity in the SDS cell-free system remained stable during storage for 6 to 8 weeks and assays were completed within 1 month of collection.

*Fractionation of cytosol.* Fractionation of neutrophil cytosol was completed by the method of Bolescher et al. After dialysis against application buffer (1 mmol/L EGTA, 1 mmol/L PMSF, 10 mmol/L MES, pH 6.8) at 4°C for 60 minutes, the cytosol was layered onto a column of CM Sepharose equilibrated with the application buffer. After washing with the application buffer, the column was eluted with buffer containing 0.5 mol/L NaCl. Fractions with maximal absorbance after washing with application buffer contained proteins not retained on the sepharose column and were designated run through (RT). The fractions that were eluted with salt were pooled, contained p47-phox and p67-phox, and were designated 47/67 mix. In some experiments, p47-phox was separated from p67-phox by eluting the column with a gradient of 0 to 0.2 mol/L NaCl in buffer after eluting the RT. The content of p47-phox and p67-phox in collected fractions was assayed by Western blot (technique described below). Fractions eluting early at lower NaCl concentrations were identified as containing only p67-phox, whereas fractions eluting at higher NaCl concentration contained increasing only p47-phox. These purified preparations of p67-phox or p47-phox were used in reconstitution experiments. Human recombinant p47-phox and p67-phox produced in an insect vector and purified as previously described.

Measurement of superoxide production and oxygen consumption by intact neutrophils. Generation of O_2^- was measured as the SOD inhibitable reduction of cytochrome c in neutrophils using standard methods, as previously described. Neutrophils (1.5 × 10^6 cells) in KRP-D were preincubated with 75 µmol/L cytochrome c to a final assay volume of 300 µL at 37°C for 5 minutes. Each cuvette was paired with a reference cuvette with identical content, except for the addition of 16.7 µmol/L of SOD. Cells were stimulated with the addition of 200 ng/mL of PMA or, alternatively, were preincubated with 2 µmol/L PAF for 3 minutes before stimulation with 1 µmol/L FMLP. Changes in optical density at 550 nm were measured in a single-beam spectrophotometer (Response II; Gilford Systems, Ciba Corning Diagnostics Corp, Oberlin, OH), and the maximum rate of cytochrome c reduction was determined for the first 5 minutes using an extinction coefficient of 21.1 µmol/L·cm^-1. For the last 4 patients, O_2^- production by PMNs was measured in 96-well microplates using a kinetic microplate reader (Molecular Devices, Palo Alto, CA). The concentration of various constituents were the same as noted above, but the reaction volume in each well was 150 µL. Plates were kept at 37°C and shaken throughout the assay to keep the cells suspended. Changes in optical density were recorded at two wavelengths (450 and 550 nm) every 20 seconds for 5 minutes, as previously described. The difference in absorbance between identical wells with and without SOD was determined and cytochrome c reduction was calculated with the extinction coefficient 8.4 µmol/L·cm^-1, a value determined experimentally for the 550-nm filter used with the kinetic reader and for the path length of the 150 µL volume.

Oxygen consumption of intact neutrophils was measured using a Clark polarograph oxygen electrode (Yellow Springs Instrument Co, Yellow Springs, OH). Neutrophil suspensions in KRP-D at a final volume of 350 µL (1.5 × 10^6 cells) were preincubated at 37°C for 5 minutes. Oxygen consumption was followed for 10 minutes after activation with 200 ng/mL of PMA and maximal rates were determined and expressed as nanomoles per minute.
was added. The total volume was 280 µL. Optimal concentrations of subcellular fractions used in the assay were determined initially as 5 × 10^6 cell equivalents of membrane and 5 × 10^6 cell equivalents of cytosol, in agreement with previous studies.18,20,23,25 After incubation at 27°C for 3 minutes, the reaction was initiated by adding 20 µL of NADPH that was freshly prepared to give a final concentration of 200 µmol/L. Absorbance was measured in a single-beam spectrophotometer and \( O_2 \) was calculated as described for intact PMNs. Results were expressed as nanomoles of \( O_2 \) per minute.

In separate experiments, the amount of RT and 47/67 mix required for optimal superoxide generation in the presence of plasma membrane as noted above was determined. All cell components and purified cytosol fractions were from healthy adults. For 50 µL of 47/67 mix, 100 µL of RT provided optimal \( O_2 \) generation. Varied amounts of 47/67 mix were then assayed with 150 µL of RT. Twenty-five microlitres of 47/67 mix provided optimal \( O_2 \) generation with 100 µL RT. These amounts of RT and 47/67 mix were used in subsequent assays of \( O_2 \) generation to reconstitute cytosol from patient samples.

Spectrophotometric determination of cytochrome b558 in subcellular fractions. The amount of cytochrome b558 was calculated by measurement of reduced-minus-oxidized spectra in the absence or presence of sodium dithionate.31,36 For these studies, a 300 µL suspension containing specific granules or plasma membrane was analyzed in a single-beam spectrophotometer, scanning at 600 to 400 nm wavelength and using an absorbance coefficient of 21.6 mmol/L^-1 cm^-1.31,36 The protein content of the sample was determined as previously described.31 Results were expressed as picomoles per milligram of protein.

Western blot for cytochrome b558, p47-phox, and p67-phox. For Western blotting, SDS-PAGE was performed on samples of subcellular fractions containing 10 to 20 µg of protein using 1.0% (wt/vol) slab gels containing 1.0% (wt/vol) SDS at ambient temperature and standard conditions, as previously described.29 After electrophoresis, the gels were transferred to nitrocellulose at 1 amp for 1 hour in buffer containing 20% (vol/vol) methanol, 0.025 mol/L glycine, and 0.0015% (vol/vol) ethanolamine. The blots were processed as previously described31 and developed with 1:1,000 dilution of a mixture of monoclonal antibodies to p91-phox and p22-phox or a 1:2,000 dilution of goat antiserum to p47-phox and p67-phox. The blots for the subunits of cytochrome b558 were detected with rabbit antimouse IgG conjugated to horseradish peroxidase (20 mL of 1:1,000 dilution in phosphate-buffered saline [PBS]-Tween) and peroxidase substrate solution (H_2O_2 and 4-chloro-1-naphthol).31

Quantitation of p47-phox and p67-phox proteins. The cytosolic components p47-phox and p67-phox were quantitated with an immunonaautoradiographic assay based on a technique previously described.29 Western blots of cytosol were competed as described above. After incubation with goat antiserum to p47-phox and p67-phox for 90 minutes at room temperature, the blot was washed with PBS + 0.1% Tween, as noted above, and then incubated with 10 mL [20%] protein G (1 µCi/mL) for 1 hour at room temperature. After washing four times with PBS and 0.1% Tween, the nitrocellulose was dried and exposed to X-Omat AR films at -70°C overnight with an intensifying screen. With the autoradiograph as a guide, the areas containing the p47-phox and p67-phox bands were removed from the blot. Pieces of the same size from adjacent areas of the paper but containing no protein bands served to define background counts. All samples were counted in a gamma counter (Cobra Autogamma; Packard Instrument, Canbara, Canada). After subtracting background counts, the quantity of p47-phox and p67-phox in each sample was expressed as counts per minute per 10 µg of protein applied to the gel. Because of variability in the absolute amount of radioactivity per blot, burn patient samples were run with their controls and radioactivity was expressed as the percentage of control.

Reconstitution assays of burn cytosol activity by add-back experiments with p47-phox and p67-phox. Amounts of p47-phox and p67-phox were determined in the cytosol of burn patients and controls and in cytosolic fractions containing purified p47-phox and p67-phox by immunoneautoradiography as described above. The quantitative difference from control was calculated for each burn specimen. Activity of burn cytosol in the absence or presence of that quantity of purified p47-phox or p67-phox that would restore control levels of these proteins was measured in the SDS cell-free system. The activity assays contained reactants in the same concentration as noted, but the measurements were completed in the kinetic microplate reader under the previously defined conditions and a total volume of 150 µL. \( O_2 \) production was calculated with the extinction coefficient 8.4 mmol/L^-1 cm^-1 and expressed as nanomoles per minute. In other experiments, the amount of recombinant p47-phox or p67-phox to produce 50% correction to cytosol (300 ng of either protein) was added to the assay system. In these assays, the plasma membrane source was from healthy adult controls.

RESULTS

The respiratory burst of intact neutrophils from patients with thermal injury. The status of the respiratory burst by PMNs from burn patients and healthy adult controls was evaluated with measurements of \( O_2 \) generation and \( O_2 \) consumption. \( O_2 \) production was measured in response to PAF/fMLP and/or PMA between days 3 and 7 after thermal trauma in samples from 8 patients and their controls. Oxidase activity of PMNs from burn patients was half of that exhibited by cells from healthy controls (Table 1). In 4 of these 8 patients, similar results were found 10 to 15 days after thermal injury. In addition, PMNs from patients with thermal injury exhibited a lower \( O_2 \) consumption compared with healthy adult controls in response to PMA (Table 1). The degree of decreased \( O_2 \) consumption by patient PMNs was similar to the level of diminished \( O_2 \) production. These data reconfirm earlier studies that documented a diminished respiratory burst activity in neutrophils from individuals sustaining burn injury.4,5,26,27

Superoxide production by subcellular fractions of neutrophils. The decreased respiratory burst in PMNs from burn patients might be explained as a deficiency of either cytosolic or membrane-associated oxidase components or both. To evaluate these possibilities, \( O_2 \) generation by PMN subcellular fractions was measured in the SDS cell-free system. Samples from 19 burn patients and their controls were studied. Cytosol and plasma membrane from burn patients produced less \( O_2 \) than cytosol and plasma membrane from controls (Fig 1.7.6 ± 0.9 nmol/min [mean ± SEM] vs 12.9 ± 1.1, P < .01 by paired t-test). To further define the defect in the oxidase, various combinations of burn cytosol or plasma membrane were assayed with complementary fractions from healthy controls. The activity of the combination of control cytosol and burn membrane was not statistically different from the control cytosol, control membrane combination. In contrast, results from the burn cytosol and control membrane showed a significantly diminished activity (P < .01, by paired t-test) compared with any other combination that used control cytosol. Thus, the deficient oxidase activity in the PMNs from burn patients was associated with a defect in...
the contribution of cytosolic component(s) and not plasma membrane in the SDS cell-free system.

Evaluation of cytochrome b$_{558}$. Spectrophotometric techniques were used to quantitate cytochrome b$_{558}$ in specific granules (SG) and plasma membrane fractions of neutrophils. The content of cytochrome b$_{558}$ was 1.5 ± 0.3 pmol/µg protein and 1.3 ± 0.1 in burn and control SG, respectively (mean ± SEM, n = 9, P > .10 by paired t-test). The concentration of cytochrome b$_{558}$ in plasma membrane from patient PMNs was 10% to 15% of that found in SG and not different from control values (data not shown). In addition, analysis of the α (p22-phox) and β (gp91-phox) subunits by Western blot with monoclonal antibodies showed no qualitative changes in these proteins in SG (Fig 2) and plasma membrane (data not shown) from burn patients compared with healthy adults. Thus, no quantitative or qualitative defects in cytochrome b$_{558}$ could be identified in subcellular fractions from burn patients compared with healthy controls.

Superoxide anion production by membrane fraction, RT, and 47/67 mix. Previous studies showed that the PMN cytosol can be separated by ion exchange chromatography with CM-Sepharose into RT, which contains the Rac 2, and the eluate (47/67 mix), which has the p47-phox and p67-phox, both of which are required to achieve the full activity of cytosol in the SDS cell-free system. To explore the possibility that deficient cytosol activity could be related to a decrease in one or more oxidase components, cytosol from patient and control PMNs was separated by this technique. Fractionation of cytosol from burn patients was identical to that from controls (data not shown). When patient fractions or control fractions were assayed with their respective plasma membranes, ~80% of the respective whole cytosol activity was recovered.

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**Table 1. Oxidase Activity of Neutrophils From Burn Patients**

<table>
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<th>Stimulus</th>
<th>O$_2$ Generation*</th>
<th>O$_2$ Consumption*</th>
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<tr>
<td></td>
<td>Days 3-7 Postburn</td>
<td>Days 10-15 Postburn</td>
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<tr>
<td>PMA (200 ng/mL)</td>
<td>55 ± 5† (n = 8)</td>
<td>52 ± 6† (n = 4)</td>
</tr>
<tr>
<td>PAF (2 µmol/L) + fMLP (1 µmol/L)</td>
<td>56 ± 9† (n = 8)</td>
<td>49 ± 5† (n = 4)</td>
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Absolute values for O$_2$ generation from neutrophils from healthy adults: PMA stimulus, 7.0 ± 0.9 nmol/min/1.5 × 10$^6$ cells (mean ± SEM); PAF + fMLP, 7.4 ± 1.7. Neutrophils from burn patients: PMA: 3.9 ± 0.8, post burn days 3 through 7, and 3.9 ± 0.9, days 10 through 15; PAF + fMLP: 3.8 ± 0.8, days 3 through 7 post burn, and 4.7 ± 1.2, days 10-15. Absolute values for O$_2$ consumption: PMA, control neutrophils, 16.7 ± 0.2 nmol/min/1.5 × 10$^6$ cells; burn, 7.8 ± 0.4.

* Generation of O$_2$ and O$_2$ consumption by neutrophils from burn patients was expressed as the percentage of simultaneously tested neutrophils from healthy controls.
† Numbers represent the mean ± SEM, with the number of different patient-control pairs studied in parentheses.
‡ Results for burn neutrophils were statistically different from control neutrophils (paired t-test, P < .001).

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**Fig 1.** Superoxide anion generation by subcellular fractions of neutrophils from burn patients and healthy controls in the SDS cell-free system. Plasma membrane (5 × 10$^6$ cell equivalents) from burn or control neutrophils was assayed in the presence of cytosol (5 × 10$^6$ cell equivalents) from burn or control PMNs as described in the Materials and Methods. O$_2$ generation is expressed as nanomoles per minute. Rectangles and bars represent the mean ± SEM, with the number of experiments in parenthesis. *Results significantly different from control cytosol/control membrane (P < .01 by paired t-test).

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**Fig 2.** Western blot analysis of PMN specific granules from burn patients and healthy controls for the α (p22-phox) and β (gp91-phox) subunits of cytochrome b$_{558}$.
DEFICIENT OXIDASE COMPONENTS IN NEUTROPHILS FROM BURN PATIENTS

In subsequent experiments, the possibility that a deficiency in a cytosolic fraction was responsible for the decreased oxidase activity was evaluated using combinations of components (plasma membranes, RT, and 47/67 mix) from patients with thermal injury and healthy adult controls in the SDS cell-free system. Because no significant differences in chromatographic separation of control and burn samples were detected, identical volumes of burn or control cytosol fractions were used in these experiments. Ten evaluations were completed, 7 from individual patients 3 to 7 days after burn and 3 from days 10 to 15 postburn. The results showed that 47/67 mix obtained from burn patients had a decreased oxidase activity in the presence of RT and plasma membrane from either the control or patient PMNs (Fig 3). In contrast, both RT and membrane from burn patients in the presence of control 47/67 mix were able to generate O$_2^-$ at rates similar to the combination of control RT and 47/67 mix and control plasma membrane. Thus, the diminished cytosol activity of burn PMNs was explained by the deficient contribution of 47/67 mix to the oxidase activity in the SDS cell-free system.

Quantitation of p47-phox and p67-phox in cytosol. Quantitation of the p47-phox and p67-phox components in cytosol from burn patients was completed with an immunonuclear autoradiography assay of control cytosol. Varying amounts of cytosol, ranging from 5 to 75 μg of protein, were processed as described above. A linear relationship existed between radioactivity and the amount of cytosol assayed in the range of 10 to 50 μg protein. In subsequent studies, 10, 20, and 40 μg of cytosol from each burn patient and control were assayed, and the results were used to determine the content of p47-phox and p67-phox. Because of differences in the absolute amount of radioactivity between blotting runs, results for burn patients were expressed as the percentage of simultaneously run samples from controls. Samples from 16 different burn patients during the first week after injury and their controls were assayed. A representative example of Western blot analysis of a sample from a burn patient is shown in Fig 5. For the burn group, the amount of p47-phox was half of the control (Fig 5) and this was statistically significant (P < .01, by paired t-test). As a group, burn patients also exhibited a milder but statistically significant decrease in p67-phox.

Reconstitution of burn cytosol activity with p47-phox and p67-phox. If the deficiency in patient cytosol was related to a decrease in one or both cytosolic components, the addition of purified p47-phox and p67-phox to control cytosol should reconstitute the oxidase system in burn patients. This hypothesis was tested in the following experiments. Amounts of p47-phox and p67-phox in cytosol from 7 burn patients, their paired controls, and fractions containing p47-phox or p67-phox purified by chromatography with CM-Sepharose were determined by immunonuclear autoradiography. The amount of p47-phox and p67-phox missing in the burn cytosol compared with control was calculated, and those amounts of purified p47-phox or p67-phox required to replenish burn cytosol were added to the assay for oxidase activity. O$_2^-$ production by cytosol from controls and burn patients was 14.2 ± 1.3 nmol/min (mean ± SEM, n = 7) and 9.3 ± 0.9, respectively (the difference was significant; P < .01 by paired t-test). The addition of the missing amount of purified p47-phox to burn cytosol resulted in O$_2^-$ generation of 13.6 ± 1.2 (not different from control by paired t-test), whereas the addition of p67-phox did not enhance the activity of burn cytosol (8.9 ± 2.0, difference from control by paired t-test, P < .01). These data support the concept that the deficient p47-phox in cytosol from burn patients was the more important of the two cytosolic components in accounting for diminished oxidase activity.

Additional cytosol reconstitution studies were completed in samples from 5 additional patients using recombinant p47-phox and p67-phox. In these studies, content of p47-phox and p67-phox were quantitated in recombinant preparations and patient cytosol as described above, and patient cytosol was reconstituted with 50% recombinant p47-phox or p67-phox. Results for these experiments were similar to those completed with oxidase components purified from normal cytosol. Patient cytosol activity was again decreased compared with control (P < .005), and the addition of p47-phox...
Fig 4. A representative example of Western blot for quantitation of p47-phox and p67-phox in control cytosol. After SDS-PAGE electrophoresis, proteins were electroblotted onto nitrocellulose paper and subsequently incubated with [125I]protein G. The nitrocellulose paper was then exposed to film and areas of high radioactivity representing p47-phox and p67-phox were removed and counted. The relationship between radioactivity (in counts per minute) and amount of cytosol is plotted in the insert. A linear relationship exists for both p47-phox and p67-phox in the range of 5 to 50 μg of cytosolic protein.

Fig 5. Quantitation of p47-phox and p67-phox in cytosol from patients with thermal injury. Samples were analyzed by Western blot and a representative example is presented (top panel). Quantitation was completed by the immunoradiographic technique described in the Materials and Methods. The amount of p47-phox or p67-phox was calculated as a percentage of a simultaneously assayed sample from a healthy adult control. The graph (lower panel) shows results from 16 patients studied on days 3 through 7 after injury. Results are expressed as the mean (columns) ± SEM (error bars). Amounts of p47-phox and p67-phox were both different from control (P < .01).

DISCUSSION

Intact neutrophils play a critical role in the host’s defense against bacterial invasion. It is well documented that infection constitutes the most important threat to survival in patients successfully resuscitated after burn injury; and this immunodeficient state is, in part, related to abnormal PMN function.1-3 Defects in chemotaxis, adhesion, phagocytosis, and microbial killing have been described in PMNs harvested from burn patients.2-12 Recent reports26,27 documented a diminished oxidase activity in PMNs from burn patients, and this may contribute to the increased risk of infection by affecting the oxygen-dependent arm of PMN bactericidal activity. The biochemical basis for the defective oxidase activity in burn PMNs has not been well defined.

In the studies presented here, we investigated the nature of the decreased respiratory burst activity in PMNs from burn patients by analyzing the status of the oxidase and its enzymes.

Table 2. Reconstitution of Burn Cytosol With Recombinant p47-phox or p67-phox

<table>
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<th>Addition</th>
<th>O2 Production (nmol/min)</th>
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<tr>
<td>Control cytosol</td>
<td>19.1 ± 2.7*</td>
</tr>
<tr>
<td>Burn cytosol</td>
<td>5.9 ± 1.6†</td>
</tr>
<tr>
<td>Burn cytosol + p47-phox</td>
<td>14.0 ± 2.4</td>
</tr>
<tr>
<td>Burn cytosol + p67-phox</td>
<td>8.5 ± 2.5†</td>
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Activity measurements were completed with plasma membrane from control neutrophils and control cytosol or burn cytosol alone or in the presence of recombinant p47-phox or p67-phox. Results are expressed as nanomoles per minute.

* Numbers represent mean ± SEM for 5 burn patients and their controls.
† Results different from control (P < .01).
components. In the resting state, the constituents of the oxidase are confined within different compartments of the PMN. Components located in the plasma membrane and specific granules include the constituents of a flavohemoprotein, cytochrome b$_{558}$.$^{40-42}$ Over the past few years, a number of studies have documented that at least three and as many as five additional oxidase components are located in the cytosol.$^{16,17,43-45}$ When PMNs are stimulated, cytochrome b$_{558}$ and cytosolic components are translocated into the plasma membrane and the activity of the oxidase is expressed.$^{24,25,31}$ That abnormalities in one or more of these components was the cause of the defective respiratory burst activity of burn PMNs served as the hypothesis for this study.

Intact neutrophils from burn patients exhibited a diminished respiratory burst, confirming the results of earlier studies,$^{26,27}$ and the activity of subcellular fractions from burn patients in the SDS cell-free system was also blunted compared with control samples. Further studies documented a consistent, specific defect in the activity of cytosol. The contribution of plasma membranes from burn patients to O$_2^-$ production appeared comparable to that of membrane from healthy adults. In addition, quantitative and qualitative evaluation of cytochrome b$_{558}$ in specific granules and plasma membrane by spectrophotometric and Western blot techniques documented no difference between burn and control samples.

Fractionation studies using ion-exchange chromatography suggested that the deficient cytosol activity found in burn samples was likely related to changes in p47-phox and/or p67-phox. Using an immunoaautoradiography technique, a consistent decrease in the amount of p47-phox was found in cytosol from burn patients compared with that from normal controls. Some patients showed an additional decrease in p67-phox. The significance of this finding is uncertain, because the p67-phox appears to be the more unstable of the two proteins in cytosolic fractions and susceptible to degradation resulting in artifactualy low levels. In a subsequent series of studies, adding the deficient amount of purified p47-phox to the assay resulted in restoration of patient cytosol activity to control levels, confirming the significance of the quantitative deficiency of p47-phox. The purified p67-phox did not restore activity of the burn cytosol, suggesting that the modest decreases in this protein may not play a role in the diminished oxidase activity in these patients.

The current investigations focused on the contributions of cytosol and plasma membrane to oxidase activity and defined a specific deficiency in a cytosol component in PMNs from burn patients. Although the precise role of specific granules in the respiratory burst of the PMN is controversial, most available evidence suggests that these granules serve as a reservoir of cytochrome b$_{558}$ when the oxidase is assembled in the plasma membrane.$^{46}$ Furthermore, congenital absence of specific granules has been associated with abnormal respiratory burst and oxidase activity and diminished bactericidal activity.$^{27,48}$ In the data presented here, the concentration of cytochrome b$_{558}$ in subcellular fractions from patient PMNs was comparable to control. However, previous investigations in our laboratory and others have documented a specific granule deficiency.$^{3,49}$ By limiting the reserve of catalytic components, specific granule deficiency could also attenuate oxidase and the microbicidal activity of the cell, especially under conditions of excessive bacterial growth found in the burn wound. Thus, the deficiency in p47-phox may combine with other abnormalities to produce the defective cell function found in burn patients.

Hematologic changes documented in patients with burns may provide clues to the etiology of the oxidase defect. A decrease in absolute neutrophil counts after thermal injury has been previously reported.$^{50-52}$ Associated with the decrease in PMNs is a diminished myelopoietic activity defined by a decrease in myelopoietic growth factors and stem cells. Abnormalities in hematopoietic growth factors and other cytokines may result in an alteration of both proliferation and maturation of PMNs.$^{53-56}$ For example, interferon-γ (INF-γ) has been shown to decrease levels of p47-phox and its mRNA in phagocytic cells during in vitro incubation,$^{27}$ suggesting a possible cause for the cytosolic component deficiency documented in our studies. Alternatively, the presence of toxins in the serum of burn patients has been documented.$^{27}$ Although such inhibitors may play a protective role in postburn homeostasis by inhibiting excessive inflammatory response, they may also result in deficient phagocyte function by blunting opsonization and the respiratory burst.$^{27}$ Recent studies from this laboratory have shown the presence of a PAF-like substance in serum from burn patients that is capable of priming the PMN oxidase.$^{34}$ Excessive priming and/or activation of the PMN might result in an increased turnover, degradation, and depletion of p67-phox and/or p47-phox and their message. In addition, premature release of PMNs from the bone marrow during periods of stress (eg, burn injury) might adversely affect synthesis of cytosolic oxidase components. Many of these factors are found in other disease states such as trauma, overwhelming sepsis, massive transfusions, and administration of cytokines. Common abnormalities in the milieu in which the PMN must develop and work may be responsible for defective cell function.$^{3}$ Further studies will be required to define the etiology for the deficiency in p47-phox and p67-phox in burn patients.

Careful definition of the biochemical defects associated with altered oxidase activity has important clinical implications for patient care. In the past few years, there has been a great deal of interest in the use of recombinant cytokines to treat a variety of hematologic and immunologic disorders. A recent study by The International Chronic Granulomatous Disease Cooperative Study Group has documented the effectiveness of INF-γ therapy in reducing severe infections in patients with CGD and may hold promise for other groups of patients such as those with burns.$^{58}$ The use of other cytokines, such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF), may be especially helpful because of the documented myelopoietic defects in burn patients.$^{52}$ Both GM-CSF and G-CSF stimulate the growth of myeloid progenitors in the bone marrow and enhance neutrophil function, including cell-mediated toxicity, induction of the expression of chemotactic receptors mature PMNs, and priming of the respiratory burst.$^{53-56}$ As such, they provide
a useful strategy to restore myelopoiesis and correct defects in PMN function after burn injury. A recent, nonrandomized clinical trial using recombinant GM-CSF showed that treatment with this growth factor increased peripheral blood leukocyte counts and enhanced the respiratory burst activity of PMNs from burn patients. A phase III clinical trial with GM-CSF in patients with thermal injury is currently underway at this institution, and studies to determine if cytokine treatment will restore O₂ production and levels of p47-phox are being performed. Continued investigations in this area will help define strategies to reverse PMN defects in burn patients and reduce the incidence and/or severity of opportunistic infections.

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