Polymorphonuclear Leukocyte Dysregulation During the Systemic Inflammatory Response Syndrome

By H. Hank Simms and R. D’Amico

Altered polymorphonuclear leukocyte (PMN) function is thought to contribute to organ dysfunction during the systemic inflammatory response syndrome (SIRS). To test this hypothesis, we evaluated whole blood PMN function adherent to fibronectin or laminin in patients with mild or severe acute pancreatitis as a paradigm for SIRS. Whole-blood PMN intracellular H₂O₂ production, expression of CD32w (FcγRII), CD16 (FcγRIII), and phagocytosis were performed using dichlorofluorescin diacetate, fluorescein isothiocyanate-labeled anti-CD32w, CD16, and serum-opsonized fluorescent microspheres. Group I (n = 7) represents normal control individuals; group II (n = 11) represents patients with mild acute pancreatitis. Group III (n = 15) represents critically ill patients with severe acute pancreatitis. Adherence of PMN from groups I and II to matrix proteins resulted in a 5% to 20% increase in each PMN function assayed whereas adherence of PMN from group III to matrix proteins resulted in 50% to 75% increases in each PMN function assayed. Pertussis toxin, pentoxifylline, and dibutyryl cyclic adenosine monophosphate (cAMP) each reduced group I-II H₂O₂ production and phagocytosis. Pentoxifylline and dibutyryl cAMP but not pertussis toxin reduced group III H₂O₂ production. Both intracellular H₂O₂ and phagocytosis assays from group III but not groups I-II showed exaggerated upregulation when exposed to NaF (4 mmol/L). Anti-interleukin-6 reduced the increase in intracellular H₂O₂ production in group III patients and significantly altered the exaggerated oxidative response to NaF. Longitudinal studies of group III whole-blood PMN showed persistent upregulation of intracellular H₂O₂ production in those patients whose hospital courses were complicated by multiple system organ failure. These results demonstrate abnormal whole blood PMN function during the systemic inflammatory response syndrome in the presence of fibronectin, or laminin and that this is mediated in part via a pertussis toxin insensitive altered guanosine triphosphate-binding protein.

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THE SYSTEMIC inflammatory response syndrome has been recently recognized as appropriate terminology to define the host response to an inflammatory process independent of its cause. This syndrome has both infectious and noninfectious etiologies including pancreatitis, trauma, and the exogenous administration of inflammatory mediators. While the development of organ system dysfunction may complicate the systemic inflammatory response syndrome, it is not yet clear if the underlying pathophysiologic mechanisms are the same in both clinical entities.

Polymorphonuclear leukocytes (PMN) are believed to play an important role in the pathogenesis of the systemic inflammatory response syndrome. PMN superoxide anion, hydrogen peroxide, and hydroxyl radicals injure endothelial cells. Myeloperoxidase plus superoxide derivatives may form hypochlorous acid and acid-free chlorine. These products are toxic to endothelial cells, mitochondria, and collagen. PMN microemboli may occlude arterioles, increasing the risk to tissues from ischemia/reperfusion injury. Lastly, various inhibitors of PMN activation have proved beneficial in increasing survival rates in several models of sepsis and inflammation.

However, at sites of acute inflammation PMN chemotaxis brings the PMN into contact with interstitial matrix proteins. The role of matrix proteins vis-a-vis PMN during the systemic inflammatory response syndrome remains unclear. Furthermore, most previously published reports investigating PMN function have used purified PMN preparations; however, the process of purifying PMN greatly alters both receptor expression and oxidative metabolism capabilities.

For these reasons, we investigated the relationship between whole-blood PMN and the interstitial matrix proteins, fibronectin and laminin. We hypothesized that baseline whole blood PMN function would be altered in patients with the systemic inflammatory response syndrome. Furthermore, we hypothesized that the normal regulatory effect of matrix proteins on PMN function (eg, to support phagocytosis) would be modulated during the systemic inflammatory response syndrome. To investigate these hypotheses, we evaluated whole-blood PMN from patients with severe, acute pancreatitis as a paradigm for the systemic inflammatory response syndrome. We report here that whole-blood PMN function from patients with the systemic inflammatory response syndrome is markedly upregulated in the presence or absence of matrix proteins. Furthermore, longitudinal studies of PMN function in patients with the systemic inflammatory response syndrome showed ongoing dysregulation of PMN function in those patients whose systemic inflammatory response syndrome was complicated by multiple system organ failure and death.

MATERIALS AND METHODS

Buffers and reagents. Phosphate-buffered saline (PBS), HBSS Hanks’ buffered saline solution (HBSS) with and without Ca²⁺ and Mg²⁺ (HBSS⁺, HBSS⁻), and RPMI-1640 medium containing L-glutamine were obtained from GIBCO Laboratories (Grand Island, NY). Reagents were obtained from the following sources: lymphocyte separation medium (Litton Bionetics, Kensington, MD); fibronectin, laminin, albumin, sodium fluoride (NaF), dibutyryl cyclic adenosine monophosphate (cAMP), pentoxifylline, ethidium bromide, anti-PMN, and anti-PMN antibodies; dihydrochlorofluorescein diacetate, dichlorofluorescein, dihydrofluorescein, and fluorescein isothiocyanate-labeled anti-CD32w, anti-CD16, and anti-PMN antibodies; anti-interleukin-6, anti-interleukin-11, and anti-interleukin-6 antibodies; and anti-interleukin-11 and anti-interleukin-6 antibodies.

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PMN FUNCTION DURING INFLAMMATION

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Table 1. Patient Profile

<table>
<thead>
<tr>
<th></th>
<th>Group II (n = 11)</th>
<th>Group III (n = 15)</th>
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</thead>
<tbody>
<tr>
<td>Age (yrs)</td>
<td>50.6 ± 14.8</td>
<td>55.6 ± 18.2</td>
</tr>
<tr>
<td>Mean no. of Ranson’s Criteria</td>
<td>1.4 ± 0.9</td>
<td>5.1 ± 1.3*</td>
</tr>
<tr>
<td>WBC (&gt;10^9/mL)</td>
<td>8.6 ± 2.5</td>
<td>14.2 ± 5.3*</td>
</tr>
<tr>
<td>PMN (total)</td>
<td>5.1 ± 1.6</td>
<td>8.9 ± 4.8</td>
</tr>
<tr>
<td>PMN (bands)</td>
<td>0.42 ± 0.08</td>
<td>1.9 ± 0.9</td>
</tr>
<tr>
<td>Monocytes</td>
<td>1.86 ± 0.9</td>
<td>2.7 ± 1.1</td>
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<tr>
<td>Lymphocytes</td>
<td>0.26 ± 0.04</td>
<td>0.5 ± 0.5</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>37.4 ± 2.3</td>
<td>39.8 ± 2.4</td>
</tr>
<tr>
<td>Male/female patients</td>
<td>7/4</td>
<td>8/7</td>
</tr>
<tr>
<td>Serum Ca2+ (mg/dL)</td>
<td>10.1 ± 1.1</td>
<td>8.3 ± 2.6*</td>
</tr>
<tr>
<td>Serum amylase</td>
<td>263 ± 38.9</td>
<td>922 ± 158*</td>
</tr>
</tbody>
</table>

Abbreviation: WBC, white blood cells.
* P < .05 comparing group II v. group III.

bromide, and pertussis toxin (Sigma Chemical Co, St Louis, MO); dichlorofluorescein diacetate (DCF-DA) was purchased from Eastman Kodak ( Rochester, NY); and dextran T300 (Pharmacia Fine Chemicals, Uppsala, Sweden). Fluorescent microspheres (Cova-
spheres CX reagent, 0.9 µm) were purchased from Duke Scientific Corp (Palto Alto, CA). Monoclonal anti-tumor necrosis factor-α (anti–TNF-α), anti–interleukin-1α (anti–IL-1α), anti–IL-6, and anti–IL-8 were purchased from UBI Technology (Lake Placid, NY).

Patient profile Three groups of patients were evaluated: group I (n = 7) represents normal control volunteers; group II (n = 11) represents patients with acute gallstone pancreatitis. These patients all had abdominal pain, cholecystitis by ultrasound, and elevated serum amylase levels (>150 IU/mL). Group III (n = 15) represents critically ill patients with acute gallstone pancreatitis. All patients in group III were in the Surgical Intensive Care Unit (SICU) and most (80%) showed respiratory failure requiring ventilating (FiO2 > 0.5 to maintain PaO2 ≥ 90% O2 saturation). All patients had fever (temperatures > 101°F), abdominal pain, cholecystitis, elevated serum amylase levels (>300 IU/mL), and no other cause for their pancreatitis. Patients with acute alcohol-induced pancreatitis were excluded from the study. A patient profile on groups II and III is provided in Table 1. All patients were studied within 24 hours of admission to the hospital or SICU. At the time of whole-blood PMN sampling, patients were receiving antibiotics (Tagamet, Smith, Kline, Beecham, Malvern, PA) and anxiolytics as needed. No patients were receiving antibiotic therapy at the time of PMN whole-blood sampling. To clarify that bacterial infection was not present in any patient at the time of whole-blood sampling, CATscan guided aspirations were performed on any significant peripancreatic fluid collection found on admission CATscans. All aspirations were culture negative at the time of whole-blood sampling. This study was approved by the Rhode Island Hospital Committee on Human Welfare.

Preparation of whole-blood samples. Ten milliliters of whole blood was withdrawn from either peripheral or central venous catheters and immediately added to heparinized, endotoxin-free glass tubes. One hundred microliters of whole blood was then transferred to separate glass tubes (11 × 75 mm) Vacutainer Tubes (Becton Dickinson, Franklin Lakes, NJ) precoated with buffer or 1 µg of fibronectin or laminin before flow cytometry experiments. This concentration of matrix proteins was chosen based on previous experiments investigating purified PMN respiratory burst and cidal activity.18 Coating of the glass tubes with matrix proteins was confirmed by the Bio-Rad Protein Assay (Bio-Rad, Richmond, CA) with average OD275 of 0.1 to 0.2 per tube. Control assays using 100 µL of 35Cr-labeled purified PMN showed adherence of 2.5 ± 0.3 x 10^9 PMN in the presence of matrix proteins. Approximately 5.0 × 10^9 PMN were added to each glass tube in these experiments. This was 10% to 15% greater than the number of PMN adhered in the presence of buffer and represented approximately 90% to 95% of the number of adherent PMN per glass tube studied in whole-blood assays.

Monoclonal antibodies (MoAbs). Fluorescein isothiocyanate (FITC)-labeled anti-CD32w and CD16 were purchased from Med-arex Corp (West Lebanon, NH). FITC-labeled mouse monoclonal control antibodies anti-human IgG, IgG1, IgG2a, IgG2b, anti-CD35, and anti-CD11b/CD18 were purchased from Becton Dickinson Immunosystems (Mountain View, CA). FITC-labeled anti-LFA-1 was purchased from Tago Pharmaceuticals (Burlingame, CA).

Preparation of fluorescent microspheres for phagocytosis. Fluorescent microspheres were opsonized with pooled normal human serum (vol/vol) for 30 minutes at 37°C. The microspheres were then washed three times with 4°C PBS before their addition to the whole-blood aliquots. To confirm that the fluorescent microspheres were, in fact, opsonized, four separation preparations of microspheres were incubated with FITC-labeled murine antihuman IgG, murine anti-CD35, murine anti-CD11b/CD18, or murine isotypic controls. The results of these preparations are shown in Table 2 and demonstrate that most fluorescent microspheres were opsonized with IgG and C3b.

Flow cytometry. Three separate components of whole-blood PMN function were assayed via flow cytometry. First, the production of intracellular H2O2 production was assessed by the formation of intracellular 2',7'-dichlorofluorescein.19 Aliquots of 100 µL of whole blood were added to buffer, fibronectin, or laminin for 60 minutes at 37°C. The aliquots of whole blood were then loaded by incubation with 100 µmol/L 2',7'-dichlorofluorescein diacetate (DCF-DA; Eastman Kodak, Rochester, NY) for 30 minutes at 37°C. Intracellular H2O2 production was stopped by addition of 200 µL ice-cold PBS and intracellular fluorescence was determined by flow cytometry. Whole-blood samples were then processed using the Immunology Workstation (Coulter Corp, Hialeah, FL) as described below. Secondly, the expression of CD32w and CD16 were assayed using FITC-labeled MoAbs. Aliquots of 100 µL of whole blood were added to buffer, fibronectin, or laminin for 60 minutes at 37°C. The aliquots of whole blood plus 10 µg of FITC-labeled MoAbs were then incubated for 30 minutes at 37°C in the presence or absence of matrix proteins. Whole-blood samples were then processed using the Immunology Workstation as described below. Thirdly, phagocytosis was assayed using serum-opsonized fluorescent microspheres. Aliquots of 100 µL of whole blood were added to buffer, fibronectin, or laminin for 60 minutes at 37°C. The aliquots of whole blood plus 10 µL of serum-opsonized fluorescent microspheres were incubated for 30 minutes at 37°C. Phagocytosis was stopped by bringing the whole blood to 4°C. In experiments involving matrix proteins, phenylmethylsulfonyl fluoride (1 mM) and aprotinin (100 µg/mL) were added to whole-blood aliquots before placing the whole blood on matrix proteins to prevent PMN-derived protease degradation of the matrix proteins. To insure measurement of phagocytosis and not membrane-bound unengaged microspheres, control experiments using ethidium bromide and cytotoxicin D were performed to quench fluorescence of free or membrane-bound microspheres.20 Fluorescence microspheres were stained with ethidium bromide (final concentration 50 µg/mL) for 5 minutes at 4°C after the phagocytosis assay had been performed. The process of resonance energy transfer, FITC fluorescence was quenched. Because it is well known that ethidium bromide does not penetrate intact cell membranes, internal ingested microspheres cannot be affected by ethidium bromide, and thus the microspheres

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retain their green fluorescence. Therefore, green fluorescence of any PMN after ethidium bromide quenching can only be explained by the presence of internal ingested microspheres. As an additional control to insure measurement of phagocytosis, aliquots of whole blood were pretreated with cytochalasin D (final concentration, 5 

\text{pmol/L}) to inhibit phagocytosis. Fluorescent microspheres were added and phagocytosis performed as described above. In this protocol, phagocytosis but not adherence is inhibited. For all assays after the end of the experimental period, aliquots were placed in the Coulter Immunology Workstation. Subsequently, erythrocytes were lysed with formic acid (1.2 mL/L), PMN membranes were then stabilized by treating the PMN with a solution of sodium carbonate (6.0 g/L), sodium chloride (14.5 g/L), and sodium sulfate (31.3 g/L) followed by fixation of the PMN with 1% parafomaldehyde using the commercially available reagent Reagent Immuno-lyse (Coulter Corp.). This process essentially removes all adhered cells from the surface of each tube for fluorescence-activated cell sorter (FACS) analysis. Fading of immunofluorescence for all assays was retarded by using p-phenylenediamine.21 FACS analyses were then performed using a Becton Dickinson FACS 440 flow cytometer, equipped with a Coherent Innova 90 5 W argon laser. The laser was operated at 0.5 W, with an excitation length of 488 nm. Statistical analysis was performed on an IBM AT computer, using Cellsoft flow cytometry software. After defining the population of interest in each sample by gating to exclude particles of subcellular size and possible cell aggregates, analysis was performed on a minimum of 6,500 (estimate) cells per sample using computer-generated histograms.

The effect of nonspecific binding by FITC was neutralized by running a control sample for each test batch. Control samples were identical cell suspensions, subjected to FITC, but not to the specific antibodies being tested. Positivity in test samples was defined as a level of fluorescence exceeding the maximum fluorescence level seen in the control sample.

Statistical analysis. Differences among groups of patients were analyzed using one-way ANOVA. If a significant f-value was obtained, then post-hoc analysis was performed using the Scheffe f-test. A \( P < .05 \) was considered statistically significant.

RESULTS

PMN function is upregulated during the systemic inflammatory response syndrome (SIRS). Whole-blood PMN from normal controls and patients with the systemic inflammatory response syndrome were studied in the presence or absence of matrix proteins to determine the levels of activation of PMN in patients during SIRS. The results of these experiments for intracellular H\(_2\)O\(_2\) production, CD32w, CD16 expression, and phagocytosis are shown in Tables 2 through 5, respectively. The rationale for studying these three aspects of PMN function is that deficient oxidative metabolism, Fc receptor expression, or phagocytosis would each impair PMN cidal capability. Control experiments with purified PMN showed that matrix proteins by themselves only increased PMN adherence by approximately 10% (data not shown). Adherence of whole-blood group I PMN on matrix proteins resulted in minimal increases in each PMN function assayed and showed an approximately 0% to 10% increase for each PMN function assayed. Group II PMN function demonstrated an approximately 20% to 30% increase over that seen in normal controls. Adherence of group III PMN to fibronectin or laminin resulted in 50% to 75% increases in each PMN function assayed. Furthermore, group III PMN function was increased over group I or II for each PMN function assayed in the presence of buffer, fibronectin, or laminin. DCF-DA has recently been shown to also measure nitric oxide production and therefore control assays were performed using catalase. The addition of catalase (240 U/mL) to the whole-blood assays involving DCF-DA reduced the maximal mean channel fluorescence by approximately 90%. For example, the mean channel fluorescence for group III patients in the presence of laminin was reduced from 199 ± 17.4 to 18.5 ± 1.8 (\( n = 7; \ P < .001 \)). These results show that the major intracellular product being measured in the whole-blood assays using DCF-DA was H\(_2\)O\(_2\) and not nitric oxide.

To determine if the increase in Fc receptors represented a specific increase in receptor expression or increased surface area on activated PMN, control experiments using FITC anti-LFA-1 (\( \alpha\)\( \beta\) integrin) were performed with group III PMN. Staining of group III PMN with FITC anti-LFA-1 was not statistically different from groups I or II and failed to show significant increases in the presence of matrix proteins. The percent positive PMN were 51.8% ± 2.7%, 53.1% ± 2.9%, and 55.6% ± 2.0% for buffer, fibronectin, and laminin, respectively. The mean channel fluorescence was 61.8 ± 3.0, 64.2 ± 3.1, and 60.1 ± 2.8 for buffer, fibronectin, and laminin, respectively (\( P = \) not significant [NS] from groups I, II for CD32w or CD16 expression [see Table 4]). To confirm that phagocytosis and not adherence of fluorescent microspheres was being measured, control experiments using cytochalasin D were performed and never showed greater than 5% positive PMN for fluorescence. In addition, ethidium bromide treatment of whole-blood PMN did not sig-

<table>
<thead>
<tr>
<th>Table 2. Opsonization of Fluorescent Microspheres</th>
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<tr>
<td>Antibody</td>
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</tr>
<tr>
<td>FlTC anti-human IgG</td>
</tr>
<tr>
<td>FlTC anti-CD3b</td>
</tr>
<tr>
<td>FlTC anti-CD11b/CD18</td>
</tr>
<tr>
<td>FlTC anti-MsgG1</td>
</tr>
<tr>
<td>FlTC anti-MsgG2a</td>
</tr>
<tr>
<td>FlTC anti-MsgG2b</td>
</tr>
</tbody>
</table>

Control: represents fluorescent microspheres without serum, \( n = 4 \) separate preparations of microspheres; Experiments 1-4: represent fluorescent microspheres with serum; % Pos, % positive PMN for a particular antibody; MCF, mean channel fluorescence.
significantly reduce the percent positive PMN or mean channel fluorescence for groups I-III in the presence or absence of matrix proteins (data not shown). These results show that phagocytosis rather than adherence was being measured.

The addition of serum from group III patients to group I whole-blood PMN increased intracellular H2O2 production, Fc receptor expression, and phagocytosis by 10% to 20%. However, these increases were still significantly below that seen with group III whole-blood PMN. (Mean channel fluorescence for group I PMN added to buffer in the presence of serum were 45 ± 4.4, 57 ± 5.1, and 46 ± 4.2 for H2O2 production, CD32w expression, and phagocytosis, respectively. n = 5; P = NS comparing values to buffer without serum for group III patients [see Tables 3 through 5].)

Guanosine triphosphate (GTP)-binding protein mediates PMN upregulation during SIRS. Inhibitors of various intracellular signal transduction pathways contributing to the PMN oxidative burst and phagocytosis were used to determine possible mechanisms contributing to the upregulation of PMN function seen during SIRS. The results of these experiments are shown in Figs 1 and 2. Dibutyryl cAMP, pentoxifylline, and pertussis toxin reduced PMN oxidative metabolism and phagocytosis in groups I-II but pertussis toxin had no effect on group III patients. No effect of dibutyryl cAMP, pentoxifylline, or pertussis toxin was seen on CD32w or CD16 expression in any group of patients.

G-protein activation exacerbates PMN dysfunction during SIRS. The previous results suggested disordered G-protein function during SIRS as a contributory factor in the PMN dysfunction observed. To further test this hypothesis, PMN were incubated with sodium fluoride at a dose that is stimulatory for the Gi component of the G-protein complex followed by oxidative metabolism and phagocytosis assays. The results of these experiments are shown in Fig 3. Activation of G-proteins with sodium fluoride in groups I-II resulted in a 10% to 20% increase over baseline of both intracellular H2O2 and phagocytosis. PMN from group III patients showed an exaggerated upregulation of both functions with 35% to 55% increases over baseline being observed. In contrast, incubation of PMN from groups I-III with phorbol myristate acetate (10 ng/mL) resulted in maximal and similar levels of H2O2 production for all groups of patients (data not shown).

Increased PMN oxidative metabolism during SIRS is endotoxin independent. Whole-blood PMN from group III PMN were incubated with various inhibitors of proximal inflammatory mediators to determine their relative importance in the exaggerated oxidative metabolism seen in these patients. The results of these experiments are shown in Fig 4. Incubation of group III PMN with anti-IL-6 significantly reduced the increase in PMN oxidative metabolism seen during the systemic inflammatory response syndrome. In contrast, polymyxin B, used to bind endotoxin, did not significantly reduce the increase in PMN oxidative metabolism seen during SIRS. Incubation of group III PMN with anti-IL-18 did not affect the group III PMN oxidative burst (data not shown); incubation of group III with anti-IL-8 further increased the group III PMN oxidative burst. (Mean channel fluorescence for control vs anti-IL-8 was 115 ± 12 vs 158 ± 19, 173 ± 22 vs 248 ± 19, and 169 ± 18 vs 241 ± 23 for

Table 3. Intracellular H2O2 Production

<table>
<thead>
<tr>
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<th>Buffer</th>
<th>Fibronectin</th>
<th>Laminin</th>
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<tr>
<td>% Pos PMN</td>
<td>MCF</td>
<td>% Pos PMN</td>
<td>MCF</td>
</tr>
<tr>
<td>Group I (n = 6)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD32w</td>
<td>40 ± 4.2</td>
<td>44 ± 4.2</td>
<td>43 ± 4.1</td>
</tr>
<tr>
<td>CD16</td>
<td>43 ± 4.3</td>
<td>51 ± 4.6</td>
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<tr>
<td>Group II (n = 10)</td>
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<td></td>
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</tr>
<tr>
<td>CD32w</td>
<td>51 ± 2.8</td>
<td>61 ± 4.9</td>
<td>59 ± 3.8</td>
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<tr>
<td>CD16</td>
<td>54 ± 3.1</td>
<td>83 ± 7.2</td>
<td>62 ± 4.3</td>
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<tr>
<td>Group III (n = 13)</td>
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</tr>
<tr>
<td>CD32w</td>
<td>77 ± 4.1</td>
<td>142 ± 13.3*</td>
<td>93 ± 3.8t</td>
</tr>
<tr>
<td>CD16</td>
<td>75 ± 6.1</td>
<td>201 ± 10.8*</td>
<td>96 ± 4.11</td>
</tr>
</tbody>
</table>

Abbreviations: MCF, mean channel fluorescence; % Pos PMN, percent positive PMN.
* P < .01 comparing CD32w or CD16 expression for group I or II vs group III.
† P < .05 comparing CD32w or CD16 expression for group I or II vs group III.

Table 4. Fc Receptor Expression

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<thead>
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<th>Buffer</th>
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<th>Laminin</th>
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<tr>
<td>% Pos PMN</td>
<td>MCF</td>
<td>% Pos PMN</td>
<td>MCF</td>
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<tr>
<td>Group I (n = 6)</td>
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<tr>
<td>CD32w</td>
<td>40 ± 4.2</td>
<td>44 ± 4.2</td>
<td>43 ± 4.1</td>
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<td>CD16</td>
<td>43 ± 4.3</td>
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<td>45 ± 2.9</td>
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<td>Group II (n = 10)</td>
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<td>CD32w</td>
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<tr>
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<td>83 ± 7.2</td>
<td>62 ± 4.3</td>
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<tr>
<td>Group III (n = 13)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>CD32w</td>
<td>77 ± 4.1</td>
<td>142 ± 13.3*</td>
<td>93 ± 3.8t</td>
</tr>
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<td>CD16</td>
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<td>201 ± 10.8*</td>
<td>96 ± 4.11</td>
</tr>
</tbody>
</table>

Abbreviations: MCF, mean channel fluorescence; % Pos PMN, percent positive PMN.
* P < .01 comparing CD32w or CD16 expression for group I or II vs group III.
† P < .05 comparing CD32w or CD16 expression for group I or II vs group III.
buffer, fibronectin, and laminin, respectively \( [n = 15; P < 0.04 \text{ comparing control } v \text{ anti-IL-8 in the presence of fibro-}

To determine if IL-6 was in part responsible for the altered G-protein activation previously observed, group III PMN were preincubated with anti-IL-6 followed by the addition of sodium fluoride. The results of these experiments are shown in Fig 5. Pretreatment of group III PMN with anti-IL-6 significantly reduced both the percent increase over baseline and absolute level of mean channel fluorescence for intracellular \( \text{H}_2\text{O}_2 \) production to levels seen with groups I and II.

**Increased PMN oxidative metabolism coincide with MSOF in SIRS.** Group III patients were studied longitudinally to determine if exaggerated PMN oxidative metabolism corresponded to complications following SIRS. Those patients whose systemic inflammatory response syndrome was complicated by multiple system organ failure and subsequent death \( (n = 9) \) had persistent upregulation of PMN oxidative metabolism. Those patients \( (n = 6) \) whose SIRS either resolved or did not develop multiple systems organ failure showed a return of PMN oxidative metabolism to near-normal levels (Fig 6).

**Increased phagocytosis during SIRS is CD32w dependent.** Because phagocytosis represents a critical aspect of PMN function, further studies were performed to investigate the mechanisms behind the increased phagocytosis observed during SIRS. The results of the first series of experiments in this regard are shown in Fig 7 and demonstrate that an anti-CD32w but not an anti-CD16 MoAb significantly blocked the increase in phagocytosis seen in group III patients during SIRS. Further, when surface CD16 was removed using phosphatidyl-inositol-linked phospholipase C (PIPLC), no decrease in phagocytosis was seen (data not shown).

**Increased phagocytosis during SIRS relies on receptor transport.** To confirm that group III PMN relied on recruitment of intracellular stores of CD32w receptors to increase phagocytosis, PMN cytoplasts were prepared as described. These cytoplasts are essentially devoid of intracellular granules and flow cytometry demonstrated almost no staining when using FITC-labeled anti-CD32w (data not shown). The results of these experiments are shown in Fig 8 and demonstrate that PMN cytoplasts isolated from group III patients did not exhibit increased phagocytosis in contrast to whole PMN isolated from these patients.

**DISCUSSION**

SIRS has been recently recommended as the terminology most appropriate to standardize patient care relating to multiple previous definitions involving certain infectious and inflammatory conditions. While the physiologic alterations in the syndrome are well described, underlying pathophysiologic mechanisms remain less clear. Several lines of evidence suggest that the PMN may play a role in the underlying tissue change and organ system dysfunction that occur during SIRS. Weiss detailed the central role that PMN play in tissue destruction during inflammation. Oxidants and hydroxyls induced primarily by NADP oxidase-activated PMN may damage adjacent endothelium and promote capillary leak. Neutrophil elastase may produce lung injury and levels of PMN elastase correlate with the severity of SIRS. Furthermore, elevated levels of the IL-1 \( \beta \) receptor appear on the PMN during the sepsis syndrome. Finally, scavengers of PMN oxidative products have been shown to be protective against pulmonary capillary leaks and gastrointestinal injury after severe burns.

Our results show that SIRS is associated with a multifaceted upregulation of neutrophil function (Tables 3 through 5). In particular, intracellular \( \text{H}_2\text{O}_2 \) production, phagocytosis, and Fc receptor expression were all markedly upregulated in patients with SIRS. While phagocytosis of serumopsonized fluorescent covaspheres would depend on both complement and Fc receptor expression, increased Fc receptor expression at least partially explains the observed increase in phagocytosis and oxidative metabolism during SIRS. The critical importance of CD32w (FcγR II) in particular was demonstrated by the ability of an anti-CD32w MoAb to block the increase in phagocytosis seen during SIRS (Fig 7). Further, SIRS appears to be recruiting intracellular CD32w receptors to participate in phagocytosis as evidenced by the fact that PMN cytoplasts (devoid of intracellular granules containing CD32w receptor) do not show increased phagocytosis during SIRS. In addition, the degree of upregulation of each PMN function was clearly greatest in the patients whose inflammatory insult was the largest (group III) at the time of whole-blood PMN sampling. Furthermore, as these studies were performed on whole-blood PMN, natural upregulation secondary to PMN preparation was largely eliminated. The fact that exposure of serum from group III patients to control PMN did not markedly affect their function suggests that other cell lines (eg, monocytes) act in a paracrine fashion to upregulation PMN function during severe SIRS. The upregulation of PMN phago-
and Fc receptor expression suggests multiple integrin receptor activation during SIRS. Specifically, the ability of fibronectin to augment PMN function suggests that the α5β1 integrin receptor is being used while the ability of laminin to upregulate complement receptors suggests utilization of α6β3 integrin receptor.\(^{35}\)

Bone\(^{36}\) has recently called attention to the fact that pa-

The cytosis observed during SIRS is particularly relevant in light of the recent finding that phagocytosis is associated with leakage of reactive oxygen species.\(^{34}\) The significant differences exerted by fibronectin and laminin compared to buffer with regard to oxidative metabolism, phagocytosis,
Fig 3. One hundred microliters of whole blood was adhered in the presence of buffer, fibronectin (1 μg), or laminin (1 μg) for 60 minutes at 37°C. Aliquots (100 μL) of whole-blood PMN were incubated with 100 μmol/L of DCF-DA or 10 μL of serum-opsonized fluorescent microspheres plus sodium fluoride (4 mmol/L) for 30 minutes at 37°C. Results are the percent increase of mean channel fluorescence over baseline (without sodium fluoride) for intracellular H₂O₂ production and phagocytosis. (A) group I; (B) group II; (C) group III. Sodium fluoride increased intracellular H₂O₂ production and phagocytosis for group III, group II, group I. P < .01 comparing H₂O₂ production or phagocytosis for group I or II versus group III in the presence of buffer, fibronectin, or laminin. (■) H₂O₂; (□) phagocytosis.

Fig 4. Aliquots (100 μL) of whole-blood PMN were adhered in the presence of buffer, fibronectin (1 μg), or laminin (1 μg) for 60 minutes at 37°C. After adherence, the aliquots of whole-blood PMN were incubated with 100 μmol/L of DCF-DA as described above ± anti-IL-6 (1 μg), anti-TNF-α (1 mg), anti-TNF-α + anti-IL-6, or polymyxin B (1 mg) for 30 minutes at 37°C. Mean channel fluorescence for group III intracellular H₂O₂ production is shown. Anti-IL-6 reduced group III intracellular H₂O₂ production. n = 13; P < .05 comparing mean channel fluorescence for control versus anti-IL-6 or anti-TNF-α + anti-IL-6. (■) Control; (□) anti-IL-6; (☑) anti-TNF; (☑) anti-TNF + anti-IL-6; (☑) polymyxin B.

Patients with SIRS often have no evidence of gram-negative infection. Our results with polymyxin B corroborate this finding in that the increase in PMN oxidative metabolism seen during SIRS could not be reduced with polymyxin B but only with MoAbs directed against inflammatory mediators (Fig 4). Furthermore, in the small group of patients in which endotoxin levels were assayed (n = 3 of group III) by the limulus lysate assay, detectable levels of endotoxin in the serum of these patients were not observed (data not shown).

Normal PMN oxidative metabolism is dependent on a variety of intracellular signaling transduction mechanisms. These include levels of both intracellular cAMP and polyphosphoinositide levels, surface receptors coupled to the G-protein stimulatory for adenylate cyclase, and protein kinase c activation. Experiments were performed with dibutyryl cAMP (a membrane permeable analogue of cAMP), pentoxifylline, pertussis toxin, and PMA to investigate the relative importance of each pathway in the upregulation of PMN function seen during SIRS. G-protein activation and/or abnormalities of function have previously been observed in cytokine-stimulated human PMN. IL-8, NAP-2, and gro-MGSA have all been shown to activate G-protein. Our findings extend this observation by pointing out that abnormalities of G-protein downregulation (lack of ability of pertussis toxin to reduce PMN oxidative metabolism) contribute to the PMN dysfunction seen during SIRS (Figs 1 and 2). Furthermore, G-protein activation with NaF resulted in an exaggerated PMN response in group III patients (Fig 3). In contrast, activation of protein kinase c with PMA resulted in maximal and similar levels of H₂O₂ production by all groups of patients. Taken together, our results with pertussis toxin and NaF suggest that on whole-blood PMN from patients with severe SIRS, in vivo priming induces the addition of a pertussis-toxin-insensitive G protein to the cell surface.
IL-6 has been previously shown to be an important indicator of the extent of an inflammatory stimulus. The finding that anti-IL-6 reduced PMN oxidative metabolism to more normal levels strongly suggests a role for IL-6 as an important inflammatory mediator during SIRS. Furthermore, incubation of group III PMN with anti-IL-6 reduced subsequent G-protein activation with NaF to levels no different from that seen in groups I and II. While the combination of anti-TNF-α plus IL-6 further reduced PMN oxidative metabolism, this reduction was not statistically different from that seen with anti-IL-6 alone (Fig 5). Multiple organ system failure may be a consequence of both bacterial/nonbacterial inflammation. Several studies have shown a positive correlation between PMN-mediated endothelial injury and release of a variety of active substances from PMN. These substances have included elastase, reactive oxygen species, and platelet-activating factors. Our results show persistent upregulation of PMN oxidative metabolism in the subset of patients who develop multiple organ system failure after SIRS. While these results do not prove that the activated PMN is the sole source of organ dysfunction during SIRS, they strongly suggest that following in vitro PMN function may be a useful clinical marker.
for the subset of patients with SIRS who are at risk for multiple system organ failure.

Taken together, our results show a marked upregulation of many aspects of PMN function during SIRS. This upregulation was also observed on biologically relevant surfaces (fibronectin, laminin). Furthermore, the important inflammatory mediator IL-6 is involved in PMN upregulation that is mediated in part via disordered intracellular G-protein function.

**REFERENCES**


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