Inhibition of Zymosan Activation of Human Neutrophil Oxidative Metabolism by a Mouse Monoclonal Antibody


We have studied a neutrophil-specific murine monoclonal antibody, PMN7C3 (IgG3), which specifically alters PMN oxidative metabolism stimulated by serum-opsonized zymosan (STZ) or Candida albicans (STC). Polymorphonuclear cell (PMNs) exposed to PMN7C3 show a significant depression in O$_2^-$ release (52.8% ± 2.5% of control), H$_2$O$_2$ release (44.4% ± 6.0% of control), and O$_2$ consumption (73.9% ± 2.6% of control) in response to STZ. O$_2^-$ release in response to phorbol myristate acetate (PMA) was modestly reduced (78.4% ± 3.7%) by PMN7C3 treatment, but not to the extent seen with STZ or STC. PMN7C3 did not affect O$_2^-$ release by PMNs stimulated by zymosan opsonized with IgG or by S. aureus, A 23187, or FMLP. PMN7C3 was not cytotoxic, did not trigger oxidative metabolism when used as a stimulus, did not alter STZ-induced degranulation, and did not interfere with binding or uptake of STZ by PMNs. Exposure of PMNs to PMN7C3 decreased PMN rosette formation with erythrocytes coated with C3b (64% of control) or C3bi (63% of control), but had no affect on rosette formation with IgG-coated erythrocytes. PMN7C3 does not bind to monocytes and had no affect on rosette formation by this cell type. Binding of antibody PMN7C3 to the neutrophil surface inhibits the oxidative response to opsonized STZ or STC, possibly in part by altering the function or expression of C3b and C3bi receptors. Monoclonal antibodies such as PMN7C3 provide highly specific probes that may be used to define the molecular features of the stimulus-coupled response of PMN activation.

HUMAN POLYMORPHONUCLEAR leukocytes (PMNs) require oxygen for optimum microbicidal activity, a fact supported by the observation that PMN function is suboptimum under anaerobic conditions or when PMNs lack the capacity to produce superoxide anion (O$_2^-$), as in chronic granulomatous disease of childhood (CGD). Activated PMNs reduce molecular oxygen to generate O$_2^-$ and H$_2$O$_2$ through a series of single electron transfers. These changes are generally believed to depend in part on a cyanide-insensitive O$_2^-$-forming oxidase, which is dormant in resting cells and becomes activated when PMNs are stimulated. Although the oxidative events that follow PMN stimulation have been well described, the precise mechanism of oxidative activation and the biochemical link coupling stimulus–membrane interaction and subsequent events in PMN oxidative metabolism are incompletely defined.

The recent application of hybridoma technology to the study of hemopoietic cells has facilitated detection, isolation, and identification of membrane components in various types of cells. We have characterized the biochemical and appearance during differentiation of three PMN-specific membrane antigens detected by three murine monoclonal antibodies. We report that one of the antibodies, PMN7C3, selectively alters the respiratory burst of PMNs stimulated with serum-opsonized zymosan or Candida albicans. We used PMN7C3 to probe events in PMN oxidative metabolism and have obtained evidence to suggest that the antigen to which PMN7C3 binds is involved in regulation of PMN oxidative responses to zymosan and Candida opsonized in whole serum. Our findings also demonstrate how monoclonal antibodies can be used to dissect the mechanism by which PMN oxidative metabolism is initiated.

MATERIALS AND METHODS

Cell Preparation

Venous blood from normal volunteers was collected in heparin (10 U/ml) and neutrophils separated by dextran sedimentation or Ficol-Hypaque-dextran sedimentation, followed by hypotonic lysis of erythrocytes, as previously described. For dextran sedimentation alone, the number of neutrophils in the preparation was dependent on the differential count in whole blood, but was usually about 65%–85%. The Ficol-Hypaque-dextran method yielded about 92–96% neutrophils. Mononuclear cells were obtained from the upper cell layer from the Ficol-Hypaque separation.

Reagents

Dimethyl sulfoxide (DMSO), ferricytochrome-C (horse heart cytochrome type IV), superoxide dismutase (SOD), A 23187, and scopoletin were obtained from Sigma Chemical Co., St. Louis, MO. Ferricytochrome-C and SOD were prepared in Hanks' balanced salt solution (HBSS) and stored at -20°C. Scopoletin was diluted to 1 mM in 50 mM phosphate buffer and stored at 4°C until use.

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Horseradish peroxidase (Worthington Biochemical Corporation, Freehold, NJ) was diluted in 50 mM phosphate buffer to 2.4 mg/ml and stored at -20°C.

**Stimuli**

Heat-killed Staphylococcus aureus 502A, heat-killed Candida albicans, or zymosan particles were opsonized with pooled human serum that had been stored undiluted at -70°C after collection.

In some experiments, zymosan was opsonized with purified human IgG. The IgG was prepared by precipitation of fresh human serum at 4°C in 50% ammonium sulfate, followed by multiple washes with 1.75 M ammonium sulfate until white. The precipitate was dissolved in 10 mM sodium phosphate buffer (pH 7.0) and dialyzed 18 hr against water to remove lipoproteins. The supernate was dialyzed against a 10 mM sodium phosphate buffer (pH 8.0) overnight and applied to a DEAE cellulose column equilibrated with the same phosphate buffer. Eluted IgG was concentrated by ammonium sulfate precipitation and adjusted to 5.0 mg/ml in a Tris-borate buffer (200 mM Tris, 20 mM EDTA, 30 mM borate, pH 9.1) for opsonization of zymosan. These particles will be referred to as IgG-treated zymosan (ITZ).

Opsonized particles were prepared fresh daily and washed in modified Hanks' salt solution (MHS) and resuspended in HBSS for use in assays of PMN function. *S. aureus* was used at a 500:1 particle-to-cell ratio, whereas zymosan and *C. albicans* were used at 100:1. These ratios were selected because they gave reproducible results and maximum stimulation of PMN respiratory activity. Phorbol myristate acetate (PMA), a soluble stimulus, was purchased from Consolidated Midland Inc., Brewster, NY. PMA was dissolved in DMSO at a concentration of 100 μg/ml and stored at -20°C until use. The final concentration of PMN in the assays was 100 ng/ml. The synthetic chemotactic peptide, formyl-methionyl-leucyl-phenylalanine (FMLP), was obtained from Peninsula Laboratories, San Carlos, CA, and used at a final concentration of 10 nM. The calcium ionophore, A 23187, was used at concentrations of 0.5-5.0 μM.

**Production of Monoclonal Antibodies**

Recently, we have developed in our laboratory eight monoclonal antibodies that bind to PMN, of which three have been described in detail.1 In brief, using the protocol of Lerner,4 we fused the spleen cells of BALB/c mice that had been immunized with human PMNs with the non-immunoglobulin-secreting 8-azaquainine-resistant myeloma line SP2/0 Ag14 using polyethylene glycol 1500. Fusion products were grown in selective media and supernates screened for binding to paraformaldehyde-fixed human PMNs. Cells from positive wells were subcloned by limiting dilution. With this technique, eight clones that secrete monoclonal antibodies that specifically bind to PMNs were isolated. All eight of these antibodies were screened for effects on PMN oxidative metabolism. Antibodies were obtained from culture media of growing hybridomas or from malignant ascites of mice that had been treated with trisance and injected intraperitoneally with growing hybridoma cells. Antibodies from the ascites were partially purified by 45% ammonium sulfate precipitation as previously described, yielding material that was 90% immunoglobulin and about 60% specific monoclonal antibody.

**Immunoglobulin Subclass Determination and Purification**

Immunoglobulin subclass of monoclonal antibody precipitated from culture media of growing hybridomas was determined by Ouchterlony analysis using subclass-specific rabbit anti-mouse immunoglobulin. IgG monoclonal antibody was further purified using DEAE cellulose chromatography. F(ab')2 fragments from the monoclonal IgG were produced by pepsin digestion and separation on a Sephadex G-200 column. Fab' fragments were obtained from reduction of F(ab')2 with dithiotreitol.16

The 45% ammonium-sulfate-precipitated immunoglobulin fraction of the monoclonal antibodies generated from malignant ascites was stored at a concentration of 3 mg/ml in 200 mM sodium bicarbonate buffer (pH 8.4). For each antibody, the dilution used for treatment of PMNs prior to stimulation was that at which there was maximal binding to PMNs as determined in a microtiter assay employing a β-galactosidase-linked sheep F(ab')2 anti-mouse Ig (Bethesda Research Lab., Bethesda, MD) to detect binding of monoclonal antibody (ELISA).3

**Exposure of PMNs to Antibody**

PMNs were exposed to a 1:100 dilution of antibody (approximately 30 μg/ml) in MHS for 30 min. Exposure was performed at 4°C to minimize internalization of antibodies by the PMNs. Cells were washed free of unbound antibody with cold MHS and resuspended in cold HBSS until use.

**Measurement of PMN Respiratory Burst**

Oxygen consumption was measured polarigraphically with a Clark electrode (Oxygraph Monitor, Yellow Springs Instruments Co., OH) and employing a technique previously described.11 Cell suspensions of 3 ml (2.5 × 10⁶ PMNs/ml) in HBSS were agitated continuously at 37°C. Data are expressed as nanomoles of O₂ consumed per 2.5 × 10⁶ PMNs per unit time. Extracellular release of H₂O₂ was measured by the extinction of scopoletin fluorescence, as previously described.12 Superoxide was measured by the SOD-inhibitable reduction of ferricytochrome-C, as determined by the absorbance at 551 nm using a double beam spectrophotometer. Determinations were made utilizing the interrupted technique of Babor et al.11 or a modification of the continuous assay of Newburger et al.14,15 Final concentrations of ferricytochrome-C in the cuvette were 60 μM in the interrupted assay and 72 μM in the continuous assay. In the continuous assay, cells, stimulus, and ferricytochrome-C were in the sample cuvette, while the same reagents plus SOD were in the reference cuvette.

**Preparation of EC3b, EC3bi, and EIgG**

EC3b were prepared as described previously using alternative pathway complement proteins purified from human serum.16 Sheep erythrocytes (E, 10⁶) first were incubated with 2 mg purified C3 and 25 μg trypsin for 45 sec at 37°C to form EC3b. A nickel-stabilized C3 convertase was formed on these cells by the addition of factors B and D in GVB-Ni (gelatin veronal buffer containing 0.15 mM nickelous chloride), and then 0.5 mg of purified C3 was added and the cells incubated 20 min at 37°C. This procedure resulted in the deposition of between 2 and 3 × 10⁵ C3b molecules per E. EC3bi were prepared by incubating EC3b for 30 min at 37°C in a heat-inactivated serum reagent as a source of factors H and I. This reagent was prepared by heating normal serum at 56°C for 60 min and absorbing the resultant serum 3 times at 37°C with a 10% volume of sheep E. The different bound C3 fragments on the EC3b and EC3bi were quantitated by two parallel radioimmunoassays using ¹²⁵I-monoclonal anti-C3c (Bethesda Research Laboratories, Gaithersburg, MD) or ¹²⁵I-monoclonal anti-C3g (kindly provided by Dr. Peter Lachmann, Medical Research Council, Cambridge, England), as described previously.16

EIgG were prepared by incubating E with a subagglutinating concentration of IgG anti-E (Cordis Laboratories, Miami, FL) for 30 min at 37°C. EIgG, EC3b, and EC3bi were washed and standard-
ized to $2 \times 10^6$ cells/ml in HBSS containing 20 mM HEPES and 0.25% bovine serum albumin (BSA) (Hanks-BSA) for use in rosette assays.

**Rosette Assays**

Rosette formation with EC3b and EC3bi was performed as described previously. PMNs ($10^6$/ml) and mononuclear cells ($3 \times 10^8$) were allowed to adhere to 12-mm diameter glass coverslips for 60 min at 37°C in 5% CO2-95% air. After removing nonadherent cells, cell monolayers were incubated with a 1/6 dilution of hybridoma culture medium, in a total volume of 0.2 ml of Hanks-BSA, for 30 min at 37°C. After preincubation with antibody, 0.1 ml of EC3b or EC3bi was added to the cell monolayers, and the total volume of the assay mixture brought to 1 ml with Hanks-BSA. After rosette formation had proceeded for 60 min at 37°C, nonadherent E were removed by washing the cell monolayers with buffer. Cell monolayers were then fixed in 1.5% glutaraldehyde-1% sucrose in 0.1 M phosphate buffer, pH 7.4. Rosettes were enumerated by phase-contrast microscopy, and 200 cells were counted on each coverslip. A rosette was defined as a monocyte or PMN containing 3 or more E bound to its surface.

PMN rosette formation with ElgG was performed using a centrifuge technique. PMNs (0.1 ml of $4 \times 10^6$/ml) were aliquoted into 10 x 75 mm plastic tubes; 0.02 ml of Hanks-BSA or monoclonal antibody supernates were added, and the tubes were incubated on a rotator for 15 min at 37°C. ElgG (0.1 ml) was added to each tube, and the cell mixture was then centrifuged for 5 min at 200 rpm in a Beckman TJ-6 centrifuge (Beckman Instruments Inc, Palo Alto, CA). The pelleted cells were then incubated at 22°C for 10 min. Cell pellets were gently resuspended with a Pasteur pipette, and 0.02 ml of cells were placed onto a slide and sealed under a coverslip. Rosettes were enumerated by phase-contrast microscopy.

**Other PMN Function Assays**

Release of lysozyme from stimulated PMNs was measured as the decrease in absorbance at 450 nm of a suspension of Micrococcus luteus as described. Phagocytosis of opsonized zymosan was assessed by light microscopy, employing a technique described.

**RESULTS**

**Superoxide Release**

The effect of each of the monoclonal antibodies on release of $O_2^-$ was initially examined. The stimulus used was serum-opsonized zymosan (STZ) at a particle-to-cell ratio of 100:1. Only PMN7C3 (30 μg/ml) reduced the amount of $O_2^-$ recovered (52.8% ± 2.5% of control), whereas the other 7 monoclonal antibodies that bind to PMNs had no significant effect on superoxide release (Fig. 1).

To determine if this effect reflected a generalized depression of the ability of PMNs to undergo the respiratory burst, $O_2^-$ release by cells pretreated with PMN7C3 and stimulated with a variety of other agents known to initiate oxidative metabolism was examined (Fig. 2). There was significant reduction in the amount of $O_2^-$ recovered after treatment of PMNs with PMN7C3 only when STZ or C. albicans was used as the stimulus. This effect was not seen with other stimuli of $O_2^-$ release, except for a very modest reduction seen when PMA was used as the stimulus (78.4% ± 3.7% of control).

Exposure of PMNs to PMN7C3 at concentrations as high as 300 μg/ml, tenfold that which was used in studies of oxidative metabolism, did not induce cytotoxicity as judged by the ability of PMNs to exclude trypan blue. Furthermore, the normal oxidative activity of PMNs after exposure to PMN7C3 when other stimuli are used (e.g., S. aureus, FMLP, A 23187, ITZ) excludes PMN7C3-mediated cytotoxicity as the mechanism of its effect on PMN function. In addition, this concentration of PMN7C3 did not itself trigger any $O_2^-$ release by PMNs.

**EFFECT OF NEUTROPHIL SPECIFIC MONOCLONAL ANTIBODIES ON SUPEROXIDE RELEASE**

![Graph showing the effect of monoclonal antibodies on superoxide release.](image-url)
We purified an IgG fraction of PMN7C3 (IgG3) ascites and prepared F(ab')2 and Fab' fragments. As noted above, intact PMN7C3 antibody did not stimulate PMN oxidative metabolism, nor did the fragments of PMN7C3, devoid of the Fc moiety, stimulate PMN respiratory activity. However, in the ELISA, both F(ab')2 and Fab' fragments competed with whole PMN7C3 for binding on the PMN surface, verifying functional integrity of the fragments after preparation.

A dose–response study of STZ-triggered $O_2^-$ release versus concentration (Fig. 3) demonstrated that the intact antibody and F(ab')2 fragment were equipotent inhibitors of $O_2^-$ release. In contrast, exposure of PMNs to Fab' fragments at 20 µg/ml failed to inhibit STZ-triggered $O_2^-$ release, although binding to PMNs at this concentration was clearly demonstrated in the ELISA.

Hydrogen Peroxide Release and Oxygen Consumption

To verify these findings in other assays of PMN oxidative metabolism, we studied STZ-triggered extracellular release of $H_2O_2$ and $O_2$ consumption. PMN7C3 depressed STZ-triggered $H_2O_2$ release in comparison to that released by PMNs not exposed to PMN7C3 (Table 1). In contrast, the release of $H_2O_2$ in response to stimulation with PMA was not significantly altered by pretreatment of PMNs with PMN7C3. The latency, that is, the interval between the exposure of PMNs to STZ and the onset of a measurable response, was markedly prolonged in PMN7C3-treated cells. The maximum rate and total amount of $H_2O_2$ release were also significantly depressed when PMNs were pretreated with PMN7C3 prior to stimulation with STZ.

The effect of PMN7C3 on a third measure of oxidative metabolism, $O_2$ consumption, was also examined. $O_2$ consumption by PMNs exposed to PMN7C3 was less than that of controls (Fig. 4). Especially striking was the prolongation of latency seen in the PMN7C3-treated cells. PMN7C3-treated PMNs had a latency of 115.0 ± 8.7 sec in comparison to a latency of 78.8 ± 8.0 sec for control PMNs ($p = 0.031$). Taken together, these data are consistent with the conclusion that PMN7C3 both prolongs the initiation and inhibits the continuation of the PMN respiratory burst.

Particle Binding

Because an alteration in particle–cell contact could easily explain these results, it was critical to assess the effect of PMN7C3 on binding of zymosan to PMNs. PMN7C3 did not alter the number of cell-associated STZ after 5 min of stimulation at a 10:1 particle-to-cell ratio. The percent of cells with one or more particles of zymosan associated with or ingested by PMNs was 35.3% ± 1.5% for control PMNs and 39.3% ± 1.8% for PMN7C3-treated PMNs ($n = 6$ experiments). Thus, these data suggest that PMN7C3 alters an event that follows the binding of STZ to the surface of the PMN.

In other experiments (data not shown), degranulation of PMNs in response to STZ was not altered by
Fig. 3. The effects of PMN7C3 as intact IgG3 and as F(ab')2 fragment on O2 release are compared at various concentrations of antibody. On a molar basis, both whole antibody and the F(ab')2 fragment are equipotent inhibitors of STZ-triggered O2 release.

preexposure of cells to PMN7C3, as determined by measurement of lysozyme release. PMN7C3 does not alter STZ-triggered release of O2 from monocytes (data not shown) and does not bind to monocytes as determined by ELISA.7

Erythrocyte Rosette Formation

The failure of PMN7C3 to block zymosan binding and uptake or to bind to monocytes suggested that it was not directed specifically at PMN receptors for C3b (CR1) or C3bi (CR3). To examine this possibility, we studied the effect of PMN7C3 on these receptors and on the PMN Fc receptor using specific rosette assays. The results are shown in Table 2. PMN7C3, but not a different PMN-specific antibody (PMN8C7), was found to inhibit both EC3b and EC3bi rosette formation with human PMNs. However inhibition did not occur in a concentration-dependent fashion, and complete inhibition was not seen even at concentrations of PMN7C3 where maximum binding occurs. This suggested that PMN7C3 binds to a site on the PMN membrane such that subsequent binding at both the CR1 and CR3 receptors is inhibited.

PMN7C3 did not inhibit EC3b or EC3bi rosette formation with human monocytes (data not shown). In a single experiment (data not shown), it did not inhibit PMN rosette formation with ElgG (Fc receptor).

DISCUSSION

Oxygen-dependent PMN microbicidal activity is an integral part of host defenses. The metabolic changes that follow stimulation of PMNs, known collectively as the respiratory burst, have been the subject of considerable study since Gerard and Baldridge first noted that stimulated PMNs consume O2.21

Interaction of a ligand with a surface receptor and resultant membrane perturbation initiates the stimulus-coupled response of PMN activation. Specific receptors for the Fc portion of IgG,18,22–27 the C3b component of complement,28–32 phorbol esters,33,34 and certain chemotactic factors, such as C5a35 and FMLP,36,37 have been identified on the PMN surface, although the role of each in activation and their interrelationship have not been clearly established.38

Chemical treatment of PMNs with impermeant sulfonic acids19,40 or neuraminidase41–43 alters the PMN surface in a predictable way, although exposure of PMNs to these agents affects subsequent cell activa-
Table 1. Effect of PMN7C3 on Release of H2O2

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>STZ</th>
<th>PMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>49.5 ± 4.3</td>
<td>22.5 ± 5.0</td>
</tr>
<tr>
<td>PMN7C3</td>
<td>71.3 ± 2.3*</td>
<td>27.5 ± 2.5</td>
</tr>
<tr>
<td>Maximum rate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.33 ± 0.03</td>
<td>1.73 ± 0.07</td>
</tr>
<tr>
<td>PMN7C3</td>
<td>0.17 ± 0.04*</td>
<td>1.40 ± 0.07</td>
</tr>
<tr>
<td>Total released</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.17 ± 0.10</td>
<td>6.4 ± 0.55</td>
</tr>
<tr>
<td>PMN7C3</td>
<td>0.52 ± 0.07*</td>
<td>4.1 ± 0.17</td>
</tr>
</tbody>
</table>

Extracellular release of H2O2 in response to STZ (100:1 particle-to-cell ratio) or PMA (100 ng/ml) was measured in 3 ways: measuring the time between onset of stimulation and detectable H2O2 release (latency), the maximum rate of H2O2 release (nmole/2.5 × 10⁶ PMNs/min), and the total amount of H2O2 released in 5 min (nmole/2.5 × 10⁶ PMNs). Data are the mean ± SEM for 6 experiments.

*Significant at $p < 0.031$ level, Pittman-Welch test, in comparison with similarly stimulated PMNs not exposed to PMN7C3.

Table 2. Effect of PMN7C3 on Neutrophil Receptors for C3b and C3bi*

<table>
<thead>
<tr>
<th>Target</th>
<th>Antibody</th>
<th>Percent Rosettes</th>
<th>Percent of Daily Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC3b</td>
<td>None</td>
<td>63</td>
<td>100</td>
</tr>
<tr>
<td>EC3b</td>
<td>PMN7C3</td>
<td>41†</td>
<td>54</td>
</tr>
<tr>
<td>EC3b</td>
<td>PMN8C7</td>
<td>67</td>
<td>109</td>
</tr>
<tr>
<td>EC3bi</td>
<td>None</td>
<td>63</td>
<td>100</td>
</tr>
<tr>
<td>EC3bi</td>
<td>PMN7C3</td>
<td>38†</td>
<td>63</td>
</tr>
<tr>
<td>EC3bi</td>
<td>PMN8C7</td>
<td>61</td>
<td>108</td>
</tr>
</tbody>
</table>

*Data are the mean for 6 experiments and are expressed as the percent rosettes formed and as the percent of daily control, where the daily control is that percent of rosettes formed in the absence of antibody pretreatment of PMNs.

†$p < 0.03$ in comparison to percent rosette formation with PMNs not preexposed to PMN7C3.

Monoclonal antibodies can be used as specific probes to study the function and biochemistry of membrane determinants in various cells. We have screened a group of anti-PMN monoclonal antibodies developed in our laboratory for their effect on PMN oxidative metabolism. We have found one (PMN7C3, a murine antibody of the IgG3 subclass) that is PMN-

membrane constituents and membrane activation and the nature of the oxidase system remain incompletely characterized.

Monoclonal antibodies can be used as specific probes to study the function and biochemistry of membrane determinants in various cells. We have screened a group of anti-PMN monoclonal antibodies developed in our laboratory for their effect on PMN oxidative metabolism. We have found one (PMN7C3, a murine antibody of the IgG3 subclass) that is PMN-

...
specific and profoundly alters the respiratory activity of PMNs stimulated by opsonized zymosan or C. albicans.

There are a number of potential mechanisms by which PMN7C3 could alter O$_2^-$ release. First, PMN7C3 could in some way interfere with the assay employed, thereby artifactually resulting in a decrement in the amount of O$_2^-$ recovered. The effect of PMN7C3 on O$_2^-$ release is paralleled in assays of O$_2$ consumption and H$_2$O$_2$ release in response to STZ, thereby establishing that the antibody clearly alters PMN respiratory activity rather than artifactually decreasing recovery of the products of oxygen metabolism. Differences in the magnitude of inhibition by PMN7C3 on O$_2$ consumption, O$_2^-$ release, and H$_2$O$_2$ release reflect differences in the time at which measurements were made (e.g., O$_2$ consumption continually during 10 min, O$_2^-$ release after 10 min, and H$_2$O$_2$ release in the first minute after stimulation), as well as the conditions under which cells were stimulated (in the O$_2$ consumption and O$_2^-$ release assays, cells are continually mixed, while in the H$_2$O$_2$ assay cells are stationary).

Second, PMN7C3 could exert its effect on PMN function by inducing a generalized depression of PMN metabolic activity. PMN7C3-treated PMNs undergo a normal oxidative burst when stimulated with opsonized S. aureus, IgG-coated zymosan, A 23187, and FMLP, suggesting that the potential for full respiratory activity is preserved after exposure to PMN7C3. Furthermore, the inhibitory effect of PMN7C3 is specific for oxidative metabolism, since zymosan-elicited degranulation is normal in PMN7C3-treated PMNs. Dissociation of degranulation from events integral to oxidative metabolism has been reported previously, and Cotter et al. have reported equally specific effects with their monoclonal antibodies NCD 1 and NCD 3.

Third, PMN7C3 could inhibit STZ-triggered O$_2^-$ release by reducing the binding or uptake of STZ particles by the PMNs, thereby reducing respiratory activity simply by inhibiting particle–cell contact. PMN7C3 does not alter the number of PMN-associated STZ assessed visually after 5 min of exposure of PMNs to STZ. Since particle–cell contact is unaltered after PMN7C3 treatment of PMNs, the antibody must modify some event occurring after binding of STZ to the cell surface but which is restricted to metabolic pathways important in the oxidative burst of PMNs and which is unique to the PMN response to serum-opsonized zymosan or C. albicans.

There is no inhibition of the oxidative response when S. aureus, IgG-coated zymosan, FMLP, or A 23187 are used to stimulate PMNs, although there is slight inhibition of PMA stimulation. These observations support suggestions of other investigators that there are multiple mechanisms for PMN stimulation. McPhail et al., using impermeant surface protein inhibitors, examined O$_2^-$ release by PMNs activated with various stimuli. Respiratory activity after stimulation by cononavalin-A or FMLP was inhibited by pretreatment of PMNs with p-diazobenzene sulfonic acid (DASA), whereas O$_2^-$ release in response to PMA, sodium fluoride, or A 23187 was unaltered by DASA treatment. Although the membrane alteration induced by DASA treatment may not be specific, that study strongly suggests that there are multiple mechanisms for PMN activation.

Recent reports of patients with frequent infection and selective defects in PMN activation give clinical import to the concept of multiple mechanisms of activation of PMN oxidative metabolism. In these cases, PMNs from subjects with recurrent severe infections showed defects in oxidative metabolism that were specific for the type of stimulus used: in one case, soluble versus particulate stimuli, and in the other case, opsonization of latex particles with serum or IgG. Our study supports the hypothesis of multiple mechanisms of PMN activation. The inhibitory effect of PMN7C3 is stimulus-specific, confined to PMN responses to yeast (either zymosan or C. albicans) opsonized in whole human serum. Zymosan requires opsonization to interact with PMNs and activate the respiratory burst. Although zymosan activates the alternate pathway of complement, opsonization of zymosan is dependent on both pathways of complement as well as IgG. Thus, the opsonic requirements for optimum PMN activation in response to zymosan are complex and not simply mediated through coating the particle with IgG or C3b.

Human PMNs have surface receptors for the Fc portion of IgG and for fragments of complement, notably C3b and C3bi. Prior studies have suggested that attachment of sheep erythrocytes opsonized with C3b is mediated through the C3b receptor but that such opsonization is not sufficient for phagocytosis of the particle, nor is such attachment sufficient stimulus for activation of the respiratory burst or degranulation. Opsonization of particles with IgG is sufficient for activation of the cell even in the absence of particle ingestion. These studies suggested the Fc and C3b receptors act synergistically to augment the particle–PMN interaction and thereby facilitate particle ingestion and PMN activation.

When zymosan was opsonized in whole serum, containing IgG and complement, the respiratory burst of PMN7C3-treated PMNs was significantly depressed.
in comparison to that of untreated PMNs. However, control and PMN7C3-treated PMNs released the same amount of $O_2^-\text{ when stimulated by zymosan opsonized with human IgG (ITZ, Fig. 2). This suggests PMN7C3 does not inhibit respiratory activity by modifying Fc-dependent cell activation. Although PMN7C3 could exert its effects by directly binding to the C3b receptor, there are a number of arguments against this interpretation.}

It is unlikely that PMN7C3 binds directly to the C3b receptor on PMNs for three reasons. First, PMN7C3 inhibits rosette formation by sheep erythrocytes coated with either C3b or C3bi. Myeloid cells have two distinct receptors for complement fragments, one binding C3b and the other C3bi.29 Binding of a monoclonal antibody such as PMN7C3 to both receptors would require the presence of an identical epitopic site on both receptors, a possible but somewhat improbable event. Second, these two receptors for C3 fragments have been described in mononuclear phagocytes58 and erythrocytes.30 However, the antigen to which PMN7C3 binds is PMN-specific, since this antibody fails to bind to monocytes or erythrocytes.7 Third, the receptor for C3b has been established to be a single polypeptide chain of 205,000 molecular weight30,39 and that for C3bi is postulated to be the same as Mac-1, an antigen containing two polypeptides of 170,000 and 95,000 molecular weight.40 Employing two-dimensional polyacrylamide gel electrophoresis of PMN membrane-enriched samples followed by immunoautoradiography, Malech et al. found the antigen to which PMN7C3 binds to migrate as two broad high molecular weight bands, at 155–280,000 and 75–125,000 molecular weight, respectively.7

To explain interference with C3b receptor-mediated functions without evidence for direct binding of PMN7C3 to the C3b receptor, one hypothesis is that PMN7C3 binding could result in limited access of C3b- and C3bi-coated particles to the site and decrease C3b-mediated metabolic activity by virtue of steric hindrance due to binding to an associated protein rather than by occupancy of the actual CR1 and CR3 receptors. In this respect, it is of interest that the divalent F(ab')2, but not the monovalent Fab' fragments of PMN7C3 are inhibitory. Fab' fragments may be too small to produce sufficient steric hindrance. Another hypothesis is that there may be a requirement for cross-linking of PMN7C3 antigenic determinants in order for antibody binding to inhibit STZ-stimulated cell activation. Such cross-linking may restrict mobility of zymosan-binding sites in the plane of the PMN membrane or may initiate the ingestion of both the PMN7C3 antigenic molecule and a specific subset of other surface components through such mechanisms as patching and endocytosis. Of note, Cotter et al.50,51 also noted that their monoclonal antibodies, NCD 1 and NCD 3, required divalent binding of antibody to the PMN surface for inhibition of degranulation and chemotaxis, respectively.

It is clear that PMN7C3 does not alter the function of the oxidase per se, since cell activation by most stimuli proceeds normally in cells exposed to the antibody (Fig. 1). Studies are currently underway to define the sites of binding of PMN7C3 to subcellular fractions of resting and activated PMNs. Such work may help elucidate the details of the stimulus-coupled activation of the multicomponent electron transport chain proposed as a model for the PMN oxidase system.81

REFERENCES

37. Goetzl EJ, Foster DW, Goldman DW: Specific effects on human neutrophils of antibodies to a membrane protein constituent of neutrophil receptors for chemotactic formyl-methionyl peptides. Immunology 45:249, 1982
54. Hakansson L, Venge P: Kinetic studies of neutrophil phagocytosis: V. Studies on the cooperation between the Fc and C3b receptors. Immunology 47:687, 1982
56. Hed J, Stendahl O: Differences in the ingestion mechanisms
of IgG and C3b particles in phagocytosis by neutrophils. Immunology 45:727, 1982

57. Wilton JWA, Renggli HH, Lehner T: The role of Fc and C3b receptors in phagocytosis by inflammatory polymorphonuclear leukocytes in man. Immunology 32:955, 1977


59. Dobson NJ, Lambris JD, Ross GD: Characteristics of isolated erythrocyte complement receptor type one (CR 1, C4b-C3b receptor) and CR 1-specific antibodies. J Immunol 126:693, 1981


Inhibition of zymosan activation of human neutrophil oxidative metabolism by a mouse monoclonal antibody

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