Neutrophils Adherent to a Nonphagocytosable Surface (Glomerular Basement Membrane) Produce Oxidants Only at the Site of Attachment

By Margret C.M. Vissers, W. Antony Day, and Christine C. Winterbourn

Adherence of neutrophils to glomerular basement membrane containing immunoglobulin G aggregates was accompanied by a marked increase in oxygen uptake (eightfold). Very little of the O₂ consumed was recovered as superoxide, measured by cytochrome c reduction, or as H₂O₂, measured with horseradish peroxidase and scopoletin. When neutrophils were incubated with the basement membrane preparation in the presence of cerium chloride to detect H₂O₂, electron micrographs showed cerium perhydroxide deposits in the contact area between the cells and the basement membrane, but not on the remainder of the cell surface. The results imply that superoxide is produced only where the plasma membrane is in contact with the basement membrane matrix, and that it mostly breaks down to H₂O₂ or undergoes other reactions at this site. The longer lifetime of H₂O₂ compared with that of superoxide allows some of the H₂O₂ produced to be detected in the medium. The results also suggest that the area of contact between the neutrophil and surfaces such as basement membrane is inaccessible to proteins in the medium, eg, cytochrome c. Circulating scavengers such as superoxide dismutase or catalase, or proteolytic inhibitors, may therefore be unable to control events occurring at this site.

e 1985 by Grune & Stratton, Inc.

From the Molecular Pathology Laboratory, and the Department of Pathology, Christchurch Clinical School of Medicine, Christchurch, New Zealand.

Supported by the Medical Research Council of New Zealand.

Address reprint requests to Dr M.C.M. Vissers, Molecular Pathology Laboratory, Christchurch Clinical School of Medicine, Christchurch Hospital, Christchurch, New Zealand.

© 1985 by Grune & Stratton, Inc.

0006-4971/85/6601-0024$03.00/0

MATERIALS AND METHODS

Basement membrane was prepared from glomeruli isolated from histologically normal postmortem human kidneys and impregnated with IgG aggregates by heating a suspension of basement membrane in 10 mg/mL purified IgG to 63 °C for 30 minutes as previously described. Free IgG aggregates were removed by repeated low speed centrifugation (120 g for five minutes). Neutrophils were prepared from the peripheral blood of normal donors by Ficoll-Hypeaque centrifugation, dextran sedimentation, and hypotonic lysis of red cells. Cell preparations were at least 99% viable by trypsin blue exclusion and regularly contained 95% to 98% neutrophils and 2% to 5% eosinophils. All biochemicals used were obtained from Sigma Chemical Corp, St Louis.

Measurement of O₂ consumption, O₂ production, and H₂O₂ production. The oxygen consumed, and the O₂⁻ and H₂O₂ produced by neutrophils were measured in separate experiments carried out concurrently under identical conditions. The cells, suspended at 10⁷ per milliliter in phosphate-buffered saline, pH 7.4, supplemented with 1 mmol/L MgCl₂, 1 mmol/L CaCl₂, and 1 mg/mL glucose, were stimulated at 37 °C with either 1.5 mg/mL basement membrane containing IgG aggregates, 1 mg/mL heat aggregated IgG or 0.1 µg/mL phorbol myristate acetate (PMA). Oxygen consumption was measured continuously in a Clark-type oxygen electrode with a Biological Oxygen Meter (Yellow Springs Instrument Co, Yellow Springs, Ohio) before and following the addition of stimulant.

O₂⁻ production was determined by measuring the superoxide dismutase-inhibitable reduction of ferricytochrome c (100 µmol/L), in the presence of 1,720 units catalase. The absorbance at 550 nm was either monitored continuously (ΔΔ red-ox = 21.1 × 10⁻⁶), or the reaction was stopped at various times by cooling to 0 °C and the change in A₅₅₀ measured. Inhibition by superoxide dismutase was determined in the presence of 25 µg/mL enzyme.

H₂O₂ production was measured by incubating neutrophils in the presence of 50 µmol/L scopoletin and 40 U/mL horseradish peroxidase. The reaction was stopped by the addition of 0.1 mol/L borate buffer, pH 10.0, and the H₂O₂ concentration determined from the reduction in fluorescence of scopoletin read at 390 nm (ex) and 470 nm (em).

Electron microscopic localization of H₂O₂ production. The method used was basically that of Briggs et al. Neutrophils were stimulated with basement membrane containing IgG aggregates, or with PMA, and left at 37 °C for five minutes. They were then washed once by centrifugation in 0.1 mol/L Tris maleate buffer, pH 7.5, containing 7% sucrose and 1 mmol/L aminotriazole, and resuspended in pre-warmed 0.1 mol/L Tris maleate, pH 7.5, containing 7% sucrose, 10 mmol/L aminotriazole, 1 mmol/L CeCl₃, and 2% to 5% eosinophils. All biochemicals used were obtained from Sigma Chemical Corp, St Louis.

Polyformnuclear leukocytes (neutrophils) are a major component of the inflammatory response, and a number of inflammatory stimuli modulate cell adherence. When immune complexes or aggregates are fixed on a nonphagocytosable surface, neutrophils spread across the surface in an attempt to engulf it. They also undergo a respiratory burst under these conditions, but only low levels of O₂⁻ have been detected and the site of oxygen metabolite production has not been determined. Whether O₂⁻ and H₂O₂ are produced at the interface or over the whole cell surface of an adherent neutrophil is an important consideration for its directed action against a target cell or tissue.

We have applied the cytochemical method of Briggs et al, which uses CeCl₃ for localizing H₂O₂ production, to a model of inflammatory tissue damage to extracellular matrices. We have previously shown that neutrophils adhere to glomerular basement membrane impregnated with immunoglobulin G aggregates and degranulate, resulting in proteolytic degradation of the basement membrane matrix. In this paper we examine the respiratory burst of the cells and the site of H₂O₂ production under these conditions.
0.71 mmol/L NADH, 1 mmol/L MgCl₂, 1 mmol/L CaCl₂ and 1 mg/mL glucose. After five minutes and 15 minutes further incubation at 37 °C, the sample was fixed for one hour at room temperature in 2% formaldehyde and 2.5% glutaraldehyde in cacodylate buffer. The fixed sample was washed for one hour at pH 6.0 to remove any free cerium precipitate, postfixed in 1% osmium tetroxide, dehydrated through alcohol series, and embedded in Spurr’s resin.

Ultrathin (pale gold) sections were cut on a LKB Ultratome III (LKB Produkter AB, Bromma, Sweden) and viewed in a JEM 100B (Joel Ltd, Nakagami, Akishima, Tokyo) transmission microscope operated at 80 kV. Samples for scanning electron microscopy were fixed in buffered 2.5% glutaraldehyde, postfixed in osmium, dehydrated, and critical point dried in a Polaron E3000 unit (Watford, Hertfordshire, England). Specimens were sputter coated with gold in a Polaron E5100 and viewed in an ISI 40 scanning electron microscope operated at 30 kV.

RESULTS

Scanning electron micrographs showed that within five minutes of addition, neutrophils spread across the surface of basement membrane containing IgG, and were so tightly adherent that the junction between the cells and the basement membrane was not easily distinguishable (Fig 1A). In the absence of IgG aggregates the cells were not closely associated with the membrane (Fig 1B).

Figure 2 shows that there was a rapid increase in the rate of oxygen consumption when basement membrane containing IgG aggregates was added to neutrophils. The rate of uptake was almost constant for five minutes, then gradually decreased. In comparison, when the cells were stimulated with IgG aggregates in suspension, or with PMA, oxygen uptake continued linearly over the time period monitored until all the oxygen was consumed (Fig 2).

The amounts of oxygen consumed, and of O₂⁻ and H₂O₂ detected in the medium after five minutes are shown in Table 1. When neutrophils were stimulated with basement membrane containing IgG, the amount of O₂⁻ detected with cytochrome c represented only approximately 5% of the oxygen consumed over this period. The amount of H₂O₂ detected was 25% of the oxygen consumed and was always higher than the O₂⁻. The concentration of O₂⁻ and H₂O₂ measured did not increase when the incubation time was extended to one hour. By comparison, when the cells were stimulated with IgG aggregates or with PMA, 50% to 90% of the oxygen consumed was recovered as O₂⁻ and 20% to 25%
as H$_2$O$_2$ (Table 1). With these stimuli the amount of O$_2^-$ always exceeded the H$_2$O$_2$. Basement membrane alone did not stimulate oxygen uptake or O$_2^-$ production (Table 1). Neither did it interfere with cytochrome c reduction or H$_2$O$_2$ detection by PMA-stimulated neutrophils (results not shown).

In contrast to the findings with cytochrome c, when nitroblue tetrazolium (NBT) was used as a probe for O$_2^-$ production, it was readily reduced by neutrophils stimulated with basement membrane containing IgG aggregates to the blue formazan, which was detectable by eye. The insoluble formazan adhered to the basement membrane and neutrophils, and reliable quantification was not possible.

To localize the site of production of oxygen metabolites, neutrophils were incubated in the presence of CeCl$_3$, which reacts with H$_2$O$_2$ to form an electron dense precipitate of cerium perhydroxide. When neutrophils were stimulated with basement membrane containing IgG, this electron dense material was visible only in the area between the cells and the basement membrane and where there was cell-to-cell contact (Fig 3A through C). It was not seen where the plasma membrane was not in contact with the basement membrane (Fig 3A through C). When the basement membrane did not contain IgG aggregates, no cerium precipitate was visible (Fig 3D). This is in keeping with our findings that neutrophils are not stimulated by this material (Fig 1B, Table 1, and Vissers et al$^{16}$). In contrast, when a soluble stimulus was used, cerium precipitate was seen over the entire neutrophil plasma membrane, and inside vacuoles (Fig 4), in agreement with the results of Briggs et al.$^{15}$

**DISCUSSION**

Oxygen metabolites are easily measured in the medium of neutrophils stimulated with a soluble or phagocytosable stimulus.$^{1-4,7,9}$ In theory, each mole of O$_2$ consumed should produce one mole of O$_2^-$ and half as much H$_2$O$_2$. Some studies have found close to this stoichiometry,$^{2,7,8,15-19}$ but generally, less H$_2$O$_2$ than O$_2^-$ is detected, which probably reflects the technical difficulty of trapping all the H$_2$O$_2$ formed.$^{2,13,18}$ Our results with soluble stimuli agree well with those of others,$^{2,7,8,18}$ most of the O$_2$ consumed could be recovered as O$_2^-$ and much as H$_2$O$_2$.

The situation is very different, however, when basement membrane containing IgG aggregates is added to neutrophils. While there is a marked increase in O$_2$ consumption by the cells, O$_2^-$ production is virtually undetectable in the medium by cytochrome c reduction. However, NBT reduction, which with soluble or particulate stimuli represents O$_2^-$ production,$^{20}$ does occur. This implies that O$_2^-$ could be produced at a site accessible to NBT, a small, readily diffusible molecule, but not to cytochrome c. H$_2$O$_2$ measured with horseradish peroxidase and scopoletin, is also low compared with O$_2$ uptake, but is significantly higher than cytochrome c reduction. The electron microscopic studies, using CeCl$_3$ to localize the site of H$_2$O$_2$ production, show H$_2$O$_2$ production only in the space between the neutrophil and the basement membrane, and not over the rest of the cell surface. The absence of cerium deposit from the neutrophil membrane exposed to the medium is not due to its diffusing away since it is seen over the whole surface of PMA-stimulated neutrophils.

Taken together, our findings imply that when neutrophils encounter immune complexes on a nonphagocytosable surface, they produce O$_2^-$ only where the plasma membrane is in contact with the surface. Almost all this O$_2^-$ reacts at this site, either by spontaneous dismutation to H$_2$O$_2$ or in other ways. Much of the H$_2$O$_2$ also appears to react locally, probably with myeloperoxidase which is also released. How-

**Table 1.** O$_2$ Consumption, O$_2$ Production and H$_2$O$_2$ Production by Neutrophils

<table>
<thead>
<tr>
<th>Addition to Neutrophils</th>
<th>O$_2$ Consumed</th>
<th>O$_2$ Produced</th>
<th>H$_2$O$_2$ Produced</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>5.5 ± 3.0 (16)</td>
<td>0.9 ± 0.4 (7)</td>
<td>0.4 ± 0.2 (4)</td>
</tr>
<tr>
<td>Basement membrane</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>containing IgG</td>
<td>43.0 ± 4.0 (5)</td>
<td>2.8 ± 2.8 (8)</td>
<td>5.5 ± 1.4 (5)</td>
</tr>
<tr>
<td>aggregates</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basement membrane</td>
<td>6.3 ± 0 (2)</td>
<td>0.5 ± 0.3 (3)</td>
<td>ND</td>
</tr>
<tr>
<td>containing IgG</td>
<td>166.0 ± 15.0 (6)</td>
<td>67.0 ± 16.0 (6)</td>
<td>21.0 ± 2.0 (5)</td>
</tr>
<tr>
<td>aggregates</td>
<td>213.0 ± 55.0 (5)</td>
<td>185.0 ± 15.0 (6)</td>
<td>21.0 ± 7.0 (5)</td>
</tr>
</tbody>
</table>

Incubation conditions were as detailed in Materials and Methods. Results shown are the mean ± SD of (n) experiments. ND, not done.
Fig 3. Localization of H₂O₂ production by neutrophils (N) adherent to basement membrane (BM) containing IgG aggregates. (A, B, C) Electron-dense cerium deposits (arrows) are seen in the space between the basement membrane and the cells, and where there is cell-to-cell contact. No precipitate is present on the rest of the cell surface. (D) In the absence of IgG aggregates no cerium precipitate is seen between cells or on the basement membrane. Bars equal 1 μm.
ever, because $\text{H}_2\text{O}_2$ has a longer lifetime than $\text{O}_2^-$, some can diffuse into the medium and account for the $\text{H}_2\text{O}_2$ measured in our system.

Other studies carried out with neutrophils on nonphagocytosable surfaces are compatible with this proposal. The quantitative relationship between $\text{O}_2$ uptake and oxidant production has not been investigated, but studies with neutrophils adherent to micropore filters, through which limited access of cytochrome c would be expected, have shown low $\text{O}_2^-$ production. Other studies have measured chemiluminescence, which although basically not quantitative, should detect oxidant production even at inaccessible sites. Our findings can be considered as an extension of those of Ohno et al who, using the cerium chloride technique, showed that with particulate stimuli there is some localization of oxidant production within phagosomes. With nonphagocytosable surfaces such as basement membrane, the interface with the neutrophils' plasma membrane is the equivalent of the phagosome.

The inability to measure $\text{O}_2^-$ with cytochrome c in our system also suggests that proteins such as cytochrome c cannot gain access to the area of cell adherence. A similar situation is seen with macrophages, which exclude proteins from their sites of attachment to glass slides coated with IgG. The ability of phagocytes to prevent diffusion of proteins in or out of such a site may be important in vivo, since it would allow a cell to direct oxidants and granule enzymes at its target, with minimal risk to the surrounding tissue. However, the same mechanism could aggravate damage by neutrophils stimulated by immune complexes embedded in tissue, since it would prevent control of the degradative processes by oxidant scavenging enzymes such as superoxide dismutase or catalase, and by inhibitors of the proteases that are also released by the cells.

ACKNOWLEDGMENT

We are grateful to Helen Morrin and Sue Townsend for technical assistance.

REFERENCES

16. Vissers MCM, Winterbourn CC, Hunt JS: Degradation of
glomerular basement membrane by human neutrophils *in vitro*. Biochim Biophys Acta 804:154, 1984


Neutrophils adherent to a nonphagocytosable surface (glomerular basement membrane) produce oxidants only at the site of attachment

MC Vissers, WA Day and CC Winterbourn