Stimulation of the Hexose Monophosphate Shunt Independent of Hydrogen Peroxide and Superoxide Production in Rabbit Alveolar Macrophages During Phagocytosis

By Min-Fu Tsan

Phagocytosis and oxidative metabolism of human polymorphonuclear leukocytes (PMN) and rabbit alveolar macrophages (AM) were studied. Human PMN ingested a mean of 12 polyvinyl toluene latex particles (2 μm in diameter) per cell. There was stimulation of O$_2^-$ and H$_2$O$_2$ production, light emission, and activation of the hexose monophosphate shunt during phagocytosis by human PMN. Rabbit AM ingested 51 latex particles (2 μm in diameter) per cell. There was no stimulation of the production of O$_2^-$ and H$_2$O$_2$ or light emission associated with phagocytosis by rabbit AM, while the hexose monophosphate shunt was activated. Similar metabolic changes were obtained in both cell types when opsonized zymosan was used as phagocytic particles. 14C-Glucose-oxidation was stimulated by H$_2$O$_2$ and methylene blue in both resting human PMN and rabbit AM. It is concluded that activation of the hexose monophosphate shunt in rabbit AM during phagocytosis is independent of O$_2^-$ and H$_2$O$_2$ production.

Phagocytosis by polymorphonuclear leukocytes (PMN) is accompanied by a burst of oxidative metabolic changes, including increased oxygen consumption, hexose monophosphate shunt (HMS) activity, and hydrogen peroxide (H$_2$O$_2$), and superoxide (O$_2^-$) production. Despite intensive investigations, the mechanism by which this oxidative metabolism is stimulated during phagocytosis is still unclear. O$_2^-$ production is probably the initial step. Most, if not all H$_2$O$_2$ is generated via O$_2^-$ as an unstable intermediate. Although HMS activation is thought to be dependent on H$_2$O$_2$, the connection between O$_2^-$ and H$_2$O$_2$ production and the activation of the HMS remains unclear.

Two enzymes have been implicated for the production of O$_2^-$ and H$_2$O$_2$. Briggs and co-workers have provided evidence that a membrane-bound NADH oxidase is responsible for the production of H$_2$O$_2$. More recent evidence, however, has suggested that a granular enzyme, NADPH oxidase, is activated during phagocytosis and is responsible for the O$_2^-$ production. In the presence of adequate substrate (glucose-6-phosphate), oxidation of glucose via the HMS in leukocytes is controlled by the availability of the coenzyme, NADP$^+$. If an NADH oxidase is responsible for the H$_2$O$_2$ production, then the link between NADH oxidation and the resultant supply of NAD$^+$ for the stimulation of HMS could be achieved by the utilization of H$_2$O$_2$ via the...
glutathione cycle, a NADPH-linked lactate dehydrogenase, or a NADPH/NAD⁺ transhydrogenase. On the other hand, if a NADPH oxidase is responsible for the generation of O₂⁻, then the resultant NADP⁺ would account for the stimulation of the HMS.

We have previously demonstrated that the activity of HMS can be stimulated during phagocytosis in human PMN without concomitant stimulation of O₂⁻ and H₂O₂ production. Removal of membrane sialic acid from human PMN by bacterial neuraminidase eliminates the stimulation of O₂⁻ and H₂O₂ production associated with phagocytosis, while the desialylated PMN retain normal capacity for phagocytosis and activation of the HMS. In this study, we have demonstrated that in rabbit alveolar macrophages (AM), there is no stimulation of the production of O₂⁻ and H₂O₂ and chemiluminescence during phagocytosis, while HMS activity is stimulated.

**MATERIALS AND METHODS**

**Chemicals**

1-14C-glucose and 6-14C-glucose were obtained from Amersham/Searle Corp., Arlington Heights, Ill. Ferricytochrome C (horse heart, type VI), nitroblue tetrazolium (NBT), methylene blue, scopoletin (6-methyl-7-hydroxy-1:2-benzopyrone), horseradish peroxidase (HRP, type II, E.C. 1.11.1.7), and superoxide dismutase (SOD, bovine blood, E.C. 1.15.1.1) were obtained from Sigma Chemical Co., St. Louis, Mo. H₂O₂ (as a 30.2% solution) was obtained from Fisher Scientific Co., Pittsburgh, Pa. Ficoll was obtained from Pharmacia, Uppsala, Sweden, and sodium diatrizoate (Hypaque) from Winthrop Laboratories, New York, N.Y. Polyvinyl toluene latex (2.02 μm in diameter) and polystyrene latex (0.79 μm in diameter) were obtained from the Dow Chemical Co., Indianapolis, Ind.

**Isolation of Cells**

Rabbit AM were obtained by the method of Myrvik et al. with some modification as described previously. Human PMN were obtained from normal individuals as previously described. The cells were harvested with modified Hanks’ balanced salt solution.

**Quantification of Phagocytosis**

Quantitative measurement of phagocytosis of latex particles was performed as described previously using a cell monolayer technique. Two million PMN per monolayer and 0.3 million AM per monolayer were used.

**Measurement of Ferricytochrome C Reduction**

Superoxide production by human PMN or rabbit AM was measured by SOD-inhibitable reduction of ferricytochrome C according to Babior et al. The reaction mixtures contained 3 ml of cells (1 × 10⁷ PMN/ml or 2 × 10⁶ AM/ml) and 50 nmoles ferricytochrome C in a final volume.

Abbreviations used in this paper: PMN, polymorphonuclear leukocytes; HMS, hexose monophosphate shunt; AM, alveolar macrophages; NADH and NAD⁺, reduced and oxidized nicotinamide adenine dinucleotide; NADPH and NADP⁺, reduced and oxidized nicotinamide adenine dinucleotide phosphate; NBT, nitroblue tetrazolium; HRP, horseradish peroxidase; SOD, superoxide dismutase.

*Horseradish peroxidase, approximately 160 purpurogallin U/mg solid. One unit will form 1 mg purpurogallin in 20 sec at pH 6.0, 20°C.

of 4 ml of modified Hanks' solution. Experimental additions yielded the following concentrations: 0.79 μm polystyrene latex particles, 0.17% (by weight), providing more than 200 particles per PMN,21 and SOD, 200 U/ml. The reaction mixtures were stored on ice. Two ml of each reaction mixture was kept at 0°C for use as a blank; the remainder was incubated for 30 min at 37°C. The reaction was terminated by placing the flasks in ice. After centrifugation of blanks and incubated mixtures at 20,000 g for 10 min at 4°C, the supernatant was passed through a 0.22-μm Millipore filter to remove all the remaining particles and cells. Ferricytochrome C reduction was determined by measuring the absorbance of the incubated supernatant at 550 nm with a Beckman spectrophotometer using the nonincubated blank as reference. The difference of absorbance at 550 nm in the absence and presence of SOD was taken as a measurement of O2−.

**Measurement of NBT Reduction**

Quantitative measurement of NBT reduction was performed as described by Baehner and Nathan.22 To 15-ml conical plastic centrifuge tubes the following were added: 0.4 ml (0.35 ml in tubes containing latex particles) modified Hanks' solution; 0.1 ml 10 mM KCN; 0.4 ml 1% NBT in normal saline, and 0.05 ml 3.3% polystyrene latex particles (0.79 μm) in tubes designated for phagocytosis. The mixture was preincubated in a shaking water bath at 37°C for 15 min. Then 0.1 ml of human PMN (2.5 x 107/ml) or rabbit AM (5 x 106/ml) was added to each tube. The reaction was allowed to proceed for 15 min, when it was stopped by the addition of 10 ml of 0.5 N hydrochloric acid. The tubes then were centrifuged at 1000 g for 15 min. The supernatant was aspirated, and the visible purple granule button was extracted for 10 min with 2 ml of pyridine in a boiling water bath under an exhaust hood. The tubes were centrifuged at 1000 g for 10 min and a second extraction with 2 ml of pyridine was repeated. The extracts were combined and the optical density (OD) at the purple color of the reduced NBT was determined at 515 nm against a pyridine blank. The optical density of the pyridine extract of a mixture of cells and NBT which had been incubated for only 10 sec was determined and was used as a reagent blank. The results were expressed as the increase in OD/15 min/2.5 x 106 PMN or 0.5 x 106 AM.

**Measurement of Chemiluminescence**

Light emission was measured in a Packard Tri-carb model 3003 liquid scintillation counter (Packard Instrument Co., Downers Grove, Ill.) in the off coincidence mode, with the window setting of 70 to maximum and 9% gain. The temperature inside the counter was maintained at 4°C. The reaction mixtures were placed in 20-ml glass scintillation vials which had been kept in the dark for at least 24 hr. The experiments were performed at room temperature in dim light to minimize spurious light emission from the vials. Under these conditions the light emission from empty vials was about 400-500 cpm. The results were expressed as counts per minute above background.

For the study of light emission by resting cells, 1 x 107 human PMN or 2 x 106 rabbit AM were used. When the light emission by phagocytosing cells was studied, polystyrene latex particles (0.79 μm) were added to a final concentration of 0.17%. Control experiments consisted of Hanks' solution in the presence and absence of particles. Each sample was counted for 30 sec and then immediately removed from the counter. The samples then were placed in an incubator with constant agitation at room temperature and counted at desired intervals.

**Measurement of H2O2 Production**

Measurement of H2O2 production by human PMN or rabbit AM was performed as described previously.16 Briefly, human PMN (1 x 107) or rabbit AM (2 x 106) were incubated with sodium azide (2 mM) in the presence and absence of latex particles (0.17%) in 1 ml of modified Hanks' solution at 37°C for 30 min. Sodium azide was used to inhibit the breakdown of H2O2 by cellular enzymes. At the end of incubation, cells were removed by centrifugation at 4°C at 200 g for 10 min. The supernatant then was passed through a 0.22-μm millipore filter to remove the remaining latex particles. The concentration of H2O2 in the clear solution was then determined by the disappearance of scopoletin fluorescence in the presence of HRP as described previously.16
Measurement of $^{14}$C-Glucose Oxidation

Measurement of $^{14}$CO$_2$ production by human PMN or rabbit AM was done as described previously; $^{19}$ 1 x 10$^7$ human PMN or 2 x 10$^6$ rabbit AM were used. Radioactive substrates and particles were then added to stimulate phagocytosis in a final volume of 2 ml of modified Hanks' solution. The final concentration of particles was 0.17% by weight. For the study of 1-$^{14}$C-glucose oxidation, 0.1 μCi was added to each flask, with a final glucose concentration of 5 mM. For the study of 6-$^{14}$C-glucose oxidation, 0.2 μCi and a final concentration of glucose of 0.5 mM were used. When cells were not present in the incubation medium, the radioactivity recovered from glucose was negligible. Nevertheless, this control was run each time to ensure that the $^{14}$CO$_2$ measured had been produced by cells. In each experiment, duplicate samples were measured and the results averaged.

Opsonization of Zymosan

Zymosan (Immunological Reagent, ICN Pharmaceuticals, Cleveland, Ohio) was opsonized according to Hohn and Lehrer $^{24}$ by adding pooled serum to achieve a concentration of 5 mg/ml. The mixture was incubated for 30 min at 37°C. Opsonized zymosan was then washed twice and suspended in modified Hanks' solution at a concentration of 50 mg/ml.

Statistical Significance

The statistical significance of each experiment was determined by paired differences. $^{23}$

RESULTS

Phagocytosis by Human PMN and Rabbit AM

Table 1 shows the amount of latex particles ingested by human PMN and rabbit AM after 30 min of incubation at 37°C. The results have been corrected for the attachment of particles to the cell surface by subtracting the amount of latex particles taken up by monolayers at 0°C. $^{20}$ The average number of particles ingested by each human PMN was 12 ± 0.5 (mean ± SE) and 51 ± 4 for each rabbit AM, as calculated from the extinction coefficient (2.4 x 10$^{-3}$ OD U/μg/ml at 274 nm) and the density (1.03 g/ml at 20°C) of polyvinyl toluene.

Production of O$_2^-$ by Human PMN and Rabbit AM

O$_2^-$ production was measured by SOD-inhibitable ferricytochrome C reduction and NBT reduction. In the experiments with NBT reduction, no SOD was used. Recent evidence, however, has indicated that O$_2^-$ production during phagocytosis is responsible for the reduction of NBT by human PMN. $^4$ As shown in Tables 2 and 3, there was a marked stimulation of O$_2^-$ production during phagocytosis by human PMN. In contrast, rabbit AM had no significant stimulation of either ferricytochrome C reduction or NBT reduction during phagocytosis.

Table 1. Phagocytosis of Latex Particles by Human PMN and Rabbit AM

<table>
<thead>
<tr>
<th></th>
<th>Human PMN (2 x 10$^6$/monolayer)</th>
<th>Rabbit AM (0.3 x 10$^6$/monolayer)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OD absorbance at 274 nm</td>
<td>0.102 ± 0.008 (8)</td>
<td>0.082 ± 0.006 (16)</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SEM of the dioxane-extracted polyvinyl toluene latex particles per monolayer. Number in parentheses indicates the number of experiments.
Table 2. Reduction of Ferricytochrome C by Human PMN and Rabbit AM

<table>
<thead>
<tr>
<th></th>
<th>Human PMN</th>
<th>Rabbit AM</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Resting</td>
<td>Phagocytosing</td>
</tr>
<tr>
<td>OD absorbance at 550 μm</td>
<td>0.030 ± 0.009 (7)</td>
<td>0.093 ± 0.010 (7)</td>
</tr>
<tr>
<td>p value</td>
<td>&lt;0.005</td>
<td>&gt;0.4</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SEM (OD absorbance at 550 nm/30 min/1.5 x 10⁷ PMN or 3 x 10⁶ AM). Number in parentheses indicates the number of experiments.

Production of H₂O₂ by Human PMN and Rabbit AM

Rister and Baehner²⁵ have demonstrated that guinea pig AM have three to five times higher SOD activity than do PMN or monocytes. It is possible that our failure to detect significant O₂⁻ production by rabbit AM during phagocytosis was due to the presence of high SOD activity in this cell type. If this were the case, we would be able to detect stimulation of H₂O₂ production by rabbit AM during phagocytosis, since the enzymatic product of SOD would be H₂O₂. There was a marked stimulation of H₂O₂ production by human PMN during phagocytosis (Table 4). However, no such stimulation of H₂O₂ production by rabbit AM was noted. In this experiment, sodium azide was present in the incubation medium to inhibit the breakdown of H₂O₂ by cellular enzymes, such as catalase.

Production of Chemiluminescence by Human PMN and Rabbit AM

The emission of light associated with the ingestion of particles by PMN was initially thought to be due to the production of singlet oxygen by PMN during phagocytosis.²⁶ Recent evidence, however, has indicated that this light emission is the result of reactions between certain unspecified constituents of the ingested particles and some or all of the oxidizing agents, such as H₂O₂, O₂⁻, etc.²⁷,²⁸ Therefore, light emission by human PMN and rabbit AM was also studied. Human PMN emitted light immediately after the addition of particles (Fig. 1). It reached a maximum after 10 min. By 90 min, light emission had returned to the baseline. In contrast, rabbit AM had essentially no light emission during phagocytosis. The pattern of light emission by human PMN during phagocytosis was similar to those reported by Cheson et al.²⁷ and Rosen and Klebanoff.²⁸ though the actual counts were lower than those previously reported.²⁷,²⁸ This difference is probably due to the difference in the setting of the scintillation

Table 3. Reduction of Nitroblue Tetrazolium by Human PMN and Rabbit AM

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<thead>
<tr>
<th></th>
<th>Human PMN</th>
<th>Rabbit AM</th>
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<tbody>
<tr>
<td></td>
<td>Resting</td>
<td>Phagocytosing</td>
</tr>
<tr>
<td>OD absorbance at 515 μm</td>
<td>0.046 ± 0.005 (4)</td>
<td>0.280 ± 0.033 (4)</td>
</tr>
<tr>
<td>p value</td>
<td>&lt;0.01</td>
<td>&gt;0.6</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SEM (OD absorbance of 515 nm/15 min/2.5 x 10⁸ PMN or 0.5 x 10⁶ AM). Number in parentheses indicates the number of experiments.
Table 4. \( \text{H}_2\text{O}_2 \) Production by Human PMN and Rabbit AM

<table>
<thead>
<tr>
<th></th>
<th>Human PMN</th>
<th>Rabbit AM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting</td>
<td>2.13 ± 0.93(7)</td>
<td>0.161 ± 0.097(3)</td>
</tr>
<tr>
<td>Phagocytosing</td>
<td>297 ± 101(7)</td>
<td>0.075 ± 0.006(3)</td>
</tr>
<tr>
<td>( p ) value</td>
<td>&lt;0.001</td>
<td>&gt;0.8</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SEM \((10^{-10} \text{ moles H}_2\text{O}_2/\text{hr}/10^6 \text{ cells})\). \( \text{H}_2\text{O}_2 \) was determined by the disappearance of scopoletin fluorescence in the presence of horseradish peroxidase. Number in parentheses indicates the number of experiments.

counter. Under our setting, the light emission from empty vials was 400–500 cpm (see Materials and Methods), whereas those reported in the literature\(^2^7\)\(^2^8\) had background noise in the thousands. If we increased the gain from 9\% to 20\%, our background increased to 3000–4000 cpm, and the peak light emission was up to 20,000 cpm by phagocytosing PMN. Under this setting, there was still no chemiluminescence by rabbit AM, either at rest or during phagocytosis.

**Oxidation of Glucose by Human PMN and Rabbit AM**

As shown in Table 5, both \( 1\text{-}^{14}\text{C}-\text{glucose} \) and \( 6\text{-}^{14}\text{C}-\text{glucose} \) oxidation were stimulated in human PMN during phagocytosis. In rabbit AM, \( 1\text{-}^{14}\text{C}-\text{glucose} \) oxidation was significantly stimulated, while \( 6\text{-}^{14}\text{C}-\text{glucose} \) oxidation remained relatively unchanged. It was also evident from the data shown that in the resting stage, AM oxidized more \( 6\text{-}^{14}\text{C}-\text{glucose} \) than did PMN. These observations are consistent with those of Oren et al.\(^2^9\)

![Fig. 1](image-url)  

*Fig. 1. Light emission by human PMN and rabbit AM. The results are the means of three experiments. Vertical bars indicate 1 SEM. PMN or AM, resting cells in Hanks' solution; PMN + LP or AM + LP, cells plus latex particles; Hanks', Hanks' solution alone; Hanks' + LP, Hanks' solution plus latex particles.*
Table 5. Glucose Oxidation by Human PMN and Rabbit AM

<table>
<thead>
<tr>
<th></th>
<th>Human PMN</th>
<th>Rabbit AM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Resting</td>
<td>Phagocytosing</td>
</tr>
<tr>
<td>1-14C-glucose oxidation (5 mM)</td>
<td>13.2 ± 3.3 (6)</td>
<td>36.9 ± 7.3 (6)</td>
</tr>
<tr>
<td>p value</td>
<td>&lt;0.005</td>
<td></td>
</tr>
<tr>
<td>6-14C-glucose oxidation (0.5 mM)</td>
<td>0.37 ± 0.17 (4)</td>
<td>1.01 ± 0.28 (4)</td>
</tr>
<tr>
<td>p value</td>
<td>&lt;0.05</td>
<td></td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SEM (nmole) of the 14CO2 released after 1 hr of incubation (1 × 107 PMN or 2 × 106 AM). The radioactivity added to each flask was 0.1 μCi for 1-14C-glucose and 0.2 μCi for 6-14C-glucose. Number in parentheses indicates the number of experiments.

Effect of H2O2 and Methylene Blue on 1-14C-Glucose Oxidation by Human PMN and Rabbit AM

Supportive evidence that stimulation of the HMS in PMN during phagocytosis is dependent on H2O2 is that HMS could be stimulated by exogenous H2O2, probably through the glutathione cycle, and PMN produced H2O2 during phagocytosis. In rabbit AM, no stimulation of H2O2 production was noted during phagocytosis, while HMS was stimulated. The effect of H2O2 was studied to determine whether it also stimulates the HMS activity of rabbit AM. Methylene blue, an electron acceptor, has been shown to stimulate 1-14C-glucose oxidation by oxidizing NADPH in red cells and PMN. As shown in Table 6, both exogenous H2O2 and methylene blue stimulated 1-14C-glucose oxidation in both human PMN and rabbit AM.

Oxidative Metabolic Changes of Human PMN and Rabbit AM During Phagocytosis of Opsonized Zymosan

The above metabolic studies were performed with polystyrene latex as phagocytic particles. The concentration of PMN used was five times that of AM because rabbit AM ingested about 5 times as much latex particles as human PMN. It could be argued that our failure to detect stimulation of O2⁻ and H2O2 production and light emission by rabbit AM was due to the smaller number of AM used and the type of particles used for phagocytosis. In order to test this possibility, the following experiments were performed with the same

Table 6. Effect of H2O2 and Methylene Blue on 1-14C-Glucose Oxidation in Resting Human PMN and Rabbit AM

<table>
<thead>
<tr>
<th>1-14C-Glucose Oxidation</th>
<th>Control</th>
<th>+H2O2 (4 mM)</th>
<th>+Methylene Blue (0.15 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human PMN</td>
<td>11.7 ± 2.5 (6)</td>
<td>29.0 ± 4.2 (6)</td>
<td>31.8 ± 2.5 (6)</td>
</tr>
<tr>
<td>p value (vs. control)</td>
<td>&lt;0.005</td>
<td>&lt;0.005</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>Rabbit AM</td>
<td>4.8 ± 0.5 (4)</td>
<td>8.1 ± 0.7 (4)</td>
<td>7.1 ± 0.7 (4)</td>
</tr>
<tr>
<td>p value (vs. control)</td>
<td>&lt;0.02</td>
<td>&lt;0.02</td>
<td>&lt;0.02</td>
</tr>
</tbody>
</table>

Results are expressed on the same basis as those in Table 5.
Table 7. Oxidative Metabolic Changes of Human PMN and Rabbit AM During Phagocytosis of Opsonized Zymosan

<table>
<thead>
<tr>
<th></th>
<th>Human PMN</th>
<th>Rabbit AM</th>
<th>Human PMN</th>
<th>Rabbit AM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Resting</td>
<td>Phagocytosing</td>
<td>Resting</td>
<td>Phagocytosing</td>
</tr>
<tr>
<td><strong>O$_2$</strong> production</td>
<td>0.020 ± 0.012 (3)</td>
<td>0.107 ± 0.008 (3)</td>
<td>0.003 ± 0.002 (3)</td>
<td>0.006 ± 0.005 (3)</td>
</tr>
<tr>
<td><strong>H$_2$O$_2$</strong> production</td>
<td>1.49 ± 0.01 (3)</td>
<td>216.3 ± 53.1 (3)</td>
<td>0.46 ± 0.21 (3)</td>
<td>0.37 ± 0.26 (3)</td>
</tr>
<tr>
<td>1-C-Glucose-oxidation</td>
<td>7.6 ± 0.9 (4)</td>
<td>34.2 ± 4.5 (4)</td>
<td>6.4 ± 0.7 (4)</td>
<td>18.8 ± 2.6 (4)</td>
</tr>
<tr>
<td>6-C-Glucose-oxidation</td>
<td>0.18 ± 0.02 (4)</td>
<td>0.63 ± 0.08 (4)</td>
<td>2.21 ± 0.38 (4)</td>
<td>2.06 ± 0.10 (4)</td>
</tr>
</tbody>
</table>

Experimental conditions were exactly the same as those in Tables 2, 4, and 5, except that 5 x 10$^6$/ml PMN or AM, and 5 mg/ml opsonized zymosan were used. The results are expressed as mean ± 1 SEM (O$_2$, OD absorbance at 550 nm/30 min/7.5 x 10$^6$ cells; H$_2$O$_2$, 10$^{-10}$ moles H$_2$O$_2$/hr/10$^6$ cells; glucose oxidation, nmols of $^{14}$CO$_2$ release/hr/5 x 10$^6$ cells). Number in parentheses indicates the number of experiments.

In this study, we have demonstrated that in rabbit AM, O$_2$ or H$_2$O$_2$ production is not stimulated and no light is emitted during phagocytosis. The HMS, however, is activated. This finding is in contrast to that in PMN, in which phagocytosis is associated with a burst of oxidative metabolism, including increased oxygen consumption, stimulation of O$_2$ and H$_2$O$_2$ production, light emission, and HMS activation. Our results are consistent with those of De Chatelet et al., who also found that rabbit AM do not produce O$_2$ during phagocytosis, while 1-C-glucose oxidation is markedly stimulated. In their study, however, H$_2$O$_2$ was not measured.

DISCUSSION

In this study, we have demonstrated that in rabbit AM, O$_2$ or H$_2$O$_2$ production is not stimulated and no light is emitted during phagocytosis. The HMS, however, is activated. This finding is in contrast to that in PMN, in which phagocytosis is associated with a burst of oxidative metabolism, including increased oxygen consumption, stimulation of O$_2$ and H$_2$O$_2$ production, light emission, and HMS activation. Our results are consistent with those of De Chatelet et al., who also found that rabbit AM do not produce O$_2$ during phagocytosis, while 1-C-glucose oxidation is markedly stimulated. In their study, however, H$_2$O$_2$ was not measured.

Rister and Baehner have shown that guinea pig AM have 3–5 times higher SOD activity than PMN. Thus, the failure to detect significant O$_2$ production by rabbit AM may be due to the presence of extremely high SOD activity. One way to answer this question is to measure H$_2$O$_2$, since the enzymatic product of SOD is H$_2$O$_2$. We used a highly sensitive method, but no stimulation of H$_2$O$_2$ production by rabbit AM was detected. This observation is in variance with that of Gee et al., who found stimulation of H$_2$O$_2$ production by rabbit AM during phagocytosis. The reason for this discrepancy is not clear. The measurement of small amounts of H$_2$O$_2$ is difficult. Most techniques used to measure H$_2$O$_2$ production by PMN have been indirect and lack specificity and precision. The determination of H$_2$O$_2$ by the oxidation of 14C-formate is widely
used for this purpose, but it has several disadvantages. This method leads to a high background value even in the absence of cells, probably due to the volatility of formate.\textsuperscript{2}\textsuperscript{1}\textsuperscript{3}\textsuperscript{4} As a consequence, the variation of the results is large and results are difficult to interpret.\textsuperscript{3}\textsuperscript{4}

There is good evidence that the stimulation of H$_2$O$_2$ production during phagocytosis is generated via superoxide as an intermediate.\textsuperscript{3}\textsuperscript{5} Recently Gee and Khandwala\textsuperscript{3}\textsuperscript{6} reported that during phagocytosis they found no stimulation of O$_2^-$ production by rabbit AM (actually there was a 50\% inhibition). This finding seems difficult to explain in view of their previous observation that H$_2$O$_2$ production was stimulated in rabbit AM during phagocytosis. Our measurement of H$_2$O$_2$ is based on the oxidation of scopoletin by H$_2$O$_2$ in the presence of HRP. The technique is highly sensitive (to 0.01 $\mu$M)\textsuperscript{3}\textsuperscript{5} and specific. In addition, in rabbit AM there was no stimulation of O$_2^-$ production or light emission during phagocytosis. These findings tend to support our conclusion that there is no stimulation of H$_2$O$_2$ production by rabbit AM during phagocytosis.

There appears to be some species variation. In all species studied, including rabbit,\textsuperscript{3}\textsuperscript{1} PMN produce O$_2^-$ and H$_2$O$_2$ during phagocytosis. In most species, such as the guinea pig,\textsuperscript{3}\textsuperscript{7} mouse,\textsuperscript{3}\textsuperscript{7} and human,\textsuperscript{3}\textsuperscript{8} but not in the rabbit, macrophages or monocytes also generate O$_2^-$ and H$_2$O$_2$ during phagocytosis. Thus, rabbit AM appear unique in this respect.

The assumption that stimulation of the HMS in PMN during phagocytosis is dependent on the production of H$_2$O$_2$ is based on the following observations: (1) PMN produce H$_2$O$_2$ during phagocytosis, and its production precedes the stimulation of HMS.\textsuperscript{3}\textsuperscript{5} (2) Exogenous H$_2$O$_2$ stimulates the activity of the HMS, probably via the glutathione cycle.\textsuperscript{3}\textsuperscript{9} (3) PMN from patients with chronic granulomatous disease do not generate H$_2$O$_2$ during phagocytosis. They also do not demonstrate stimulation of the HMS.\textsuperscript{3}\textsuperscript{1}\textsuperscript{3}\textsuperscript{3}\textsuperscript{4} (4) Phagocytosis of SOD-coated latex particles markedly enhances the stimulation of HMS.\textsuperscript{4} This enhancement is partially prevented by catalase. Therefore, it appears that H$_2$O$_2$ contributes to the stimulation of HMS activity during phagocytosis. However, whether stimulation of HMS during phagocytosis is solely due to H$_2$O$_2$ is an unanswered question. We have previously demonstrated that the removal of surface sialic acid from human PMN prevents the stimulation of O$_2^-$ and H$_2$O$_2$ production during phagocytosis, while the HMS activation occurs normally.\textsuperscript{3}\textsuperscript{5} This finding strongly suggests that the HMS can be activated independently of H$_2$O$_2$ during phagocytosis. Recent evidence\textsuperscript{8}\textsuperscript{5} has suggested that a granular enzyme, NADPH oxidase, is responsible for the production of O$_2^-$ in PMN during phagocytosis. The resultant NADP$^+$ can stimulate HMS directly, independent of H$_2$O$_2$.\textsuperscript{1}\textsuperscript{5} In this study, we have further demonstrated that in rabbit AM, HMS activity is stimulated without concomitant stimulation of H$_2$O$_2$ production during phagocytosis.

The factors that regulate HMS activity have been a subject of intense investigation. Recently, Eggleston and Krebs\textsuperscript{4}\textsuperscript{0} confirmed the observation of Negelein and Haas\textsuperscript{4}\textsuperscript{1} that hepatic glucose-6-phosphate dehydrogenase (G6PD) is inhibited by NADPH. The inhibition by NADPH is competitively reversed by NADP$^+$ and depends on the ratio of free NADPH and NADP$^+$. Physio-
logic concentrations of free NADPH and NADP⁺ almost completely inhibit glucose-6-phosphate dehydrogenase. Oxidized glutathione (GSSG) at physiological concentrations counters the inhibition by NADPH, suggesting that it exerts a fine control over the HMS.⁴⁰ In PMN, in the presence of adequate glucose-6-phosphate, the concentration of NADP⁺ plays a regulatory role in HMS activity.⁴⁰ The factors that regulate the HMS activity in macrophages are less well understood. In this study, we have also shown that both H₂O₂ and methylene blue stimulate 1⁴C-glucose oxidation by rabbit AM, suggesting that in rabbit AM, as in human PMN, NADP⁺ may also play a regulatory role.

Oxidative metabolism associated with phagocytosis is essential for the killing of bacteria by PMN.⁶ O₂⁻, H₂O₂, and their derivatives, such as hydroxyl radicals, are directly or indirectly implicated in the killing of bacteria.⁶ PMN from patients with chronic granulomatous disease fail to produce O₂⁻ and H₂O₂ or to activate HMS during phagocytosis. They also demonstrate markedly impaired killing of catalase-positive bacteria.³⁰,⁴² We have shown that rabbit AM do not exhibit enhanced production of O₂⁻ and H₂O₂ during phagocytosis, but have normal activation of the HMS. Preliminary results have revealed that they are also less efficient in the killing of Staphylococcus aureus (Tsan and Newman, in preparation). This finding confirms the important role of phagocytosis-associated oxidative metabolism in bacterial killing.

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Stimulation of the hexose monophosphate shunt independent of hydrogen peroxide and superoxide production in rabbit alveolar macrophages during phagocytosis

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