Effects of Neuraminidase on \( \text{O}_2 \) Consumption and Release of \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) From Phagocytosing Human Polymorphonuclear Leukocytes

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A SERIES OF investigations have shown that polymorphonuclear leukocytes (PMN) generate oxygen-dependent bactericidal intermediates such as \( \text{O}_2^- \), \( \text{H}_2\text{O}_2 \), and \( \text{OH}^- \) during phagocytosis. The mechanism for this metabolic activation is still unclear, but investigators have recently demonstrated that perturbations of the PMN plasma membrane may play important roles in the induction of this metabolic change.

It is well known that sialic acids are common components of the plasma membrane of mammalian cells. Tsan et al. have reported that treatment of PMN with bacterial neuraminidase resulted in complete inhibition of both \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) release during phagocytosis. Therefore, they concluded that sialic acid of the plasma membrane was essential for the generation of \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) by phagocytosing PMN. However, evidence against Tsan’s reports has been presented by some laboratories as follows. Zakhireh and Root reported that immature bone marrow myelocytes, which have membranes rich in sialic acid, did not generate \( \text{O}_2^- \). In our previous report, human PMN collected through the procedure of filtration released a larger amount of \( \text{H}_2\text{O}_2 \) than that from PMN collected by dextran sedimentation. In addition, we observed that the former cells contained less membrane sialic acid than the latter cells. On the other hand, Kraemer has pointed out that commercially available neuraminidase from \( \text{Clostridium perfringens} \) may not be satisfactory for investigations of membrane-bound sialic acid unless it can be further purified. Therefore, in the present study, the role of sialic acid in PMN oxidative metabolism was studied using purified neuraminidase to clarify the relationship between membrane sialic acid and the generation of \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \).

Neuraminidase type X (NMD-type-X, Sigma Chemical Co., St. Louis, Mo.), which is obtained from a further purification of neuraminidase type V (NMD-type-V, Sigma), markedly enhanced the release of \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) from phagocytosing human polymorphonuclear leukocytes (PMN). In contrast, \( \text{O}_2^- \) consumption by NMD-type-X-treated PMN was identical to that of untreated PMN. Morphological observations suggested that the enhancement of \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) release was caused by excessive release of the oxygen metabolites into the extracellular medium from incompletely formed phagocytic vacuoles as was observed with cytochalasin-B-treated cells. Our observations are in contrast to the previous reports of Tsan et al. that showed complete inhibition of both \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) release from phagocytosing PMN by the treatment with NMD-type-V.

MATERIALS AND METHODS

Chemicals

Neuraminidase type V (designated as NMD-type-V, specific activity 0.31 U/mg protein) from \( \text{Clostridium perfringens} \), neuraminidase type X (designated as NMD-type-X, specific activity 150 U/mg protein) from \( \text{Clostridium perfringens} \), superoxide dismutase (SOD), and scopoletin were purchased from Sigma Chemical Co., St. Louis, Mo. Cytochrome-c (cyt-c) and horseradish peroxidase grade I (HRP) were purchased from Boehringer Co., Mannheim. Acetylated cyt-c was prepared by the previously reported procedure. Dowex 1-X8, 100-200 mesh was purchased from Dow Chemical Co., Detroit, Mich. Resorcinol was obtained from Merck Co., Darmstadt. Other reagents were of analytical grade.

Measurement of \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) in the Cell-Free System

The reduction of cyt-c was measured at 37°C by recording the absorption increase at 550-540 nm for the \( \text{O}_2^- \) assay. The reaction mixture contained 50 \( \mu \text{M} \) xanthine, 50 \( \mu \text{M} \) cyt-c, 0.1 mM EDTA, and 5 \( \mu \text{g} \) catalase in 1 ml of 50 mM sodium phosphate buffer, pH 7.4, with or without 0.1 U NMD-type-V or NMD-type-X. The reaction was initiated by the addition of 1.2 \( \mu \)g xanthine oxidase. \( \text{H}_2\text{O}_2 \) was measured by the scopoletin fluorescent assay as previously described. The reaction mixture contained 2 or 10 \( \mu \text{M} \) scopoletin and 20 \( \text{nM} \) or 1.25 \( \mu \text{M} \) HRP in 2 ml of 50 mM sodium phosphate buffer, pH 7.4, with or without 0.1 U/ml NMD-type-V or NMD-type-X. When indicated, 2 \( \mu \text{M} \) azide was added to the reaction mixture. The \( \text{H}_2\text{O}_2 \) measurement was performed at 37°C by tracing the emission decrease at 470 nm with an excitation wavelength of 380 nm after addition of various amounts of \( \text{H}_2\text{O}_2 \) in a Hitachi MPF-4 spectrofluorometer with a Hitachi data processor.

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Cell Preparation

Heparinized venous blood drawn from healthy adult donors was used to obtain PMN by dextran sedimentation and hypotonic treatment as reported previously. Collected cells, which contained 80%-83% PMN, were suspended in Ca²⁺-free Krebs-Ringer phosphate buffer (KRP), pH 7.4, at a final concentration of 1 × 10⁶ PMN/ml.

Treatment of Cells With Neuraminidases

Using N-acetylneuraminylactose as substrate, 1 U of neuraminidase liberates 1.0 amole of N-acetylated neuraminic acid/min at pH 5.0, 37°C. Cells were incubated in KRP at 37°C for 30 min with or without various amounts of NMD-type-V or NMD-type-X, and then the reaction was stopped by chilling in ice water. Each cell suspension was washed once with chilled KRP. There was no difference in the oxidative metabolism between washed and unwashed NMD-type-X-treated PMN. Therefore, the effects of neuraminidase treatment on the oxidative metabolism of PMN were studied by using the NMD-type-X-treated and unwashed PMN. These procedures did not affect the viability of cells (trypan blue dye exclusion). Cells incubated without neuraminidase were used as control.

Measurement of Sialic Acid

Sialic acid content was assayed basically by the ion-exchange method. After PMN were incubated at 37°C for 30 min with or without 0.1 U/ml NMD-type-X as described above. Each reaction mixture was centrifuged at 400 × g for 5 min. Both the pellets and supernatants were sonicated at 0°C for 1 sec and hydrolyzed with 0.1 N H₂SO₄ at 80°C for 1 hr. The hydrolysates were filtered over Dowex 1-X8 columns. After sialic acid was eluted from the columns with 0.5 N formic acid, the effluents were lyophylized. The content of sialic acid was determined by the method of Jourdan et al. Resorcinol was twice recrystalized before the measurement.

Measurement of O₂ Consumption by Cells

The rate of O₂ consumption was measured with a Clark type oxygen electrode at 37°C. Each cell suspension contained 0.7–1.4 × 10⁷ PMN, 0.49 mg protein of anti-E. coli antiserum, and 5 mM glucose in 1.4 ml KRP. After measurement of O₂ consumption in the resting state, heat-killed E. coli were added to the cell suspension at a bacteria to cell ratio of 100:1 to measure the O₂ uptake by phagocytosis.

Measurement of H₂O₂ Release From Cells

H₂O₂ was measured by the modified scopoletin fluorescent assay as described previously. Each cell suspension contained 1.0–2.0 × 10⁷ PMN, 0.1 M glucose, 0.2 μM scopoletin, 20 nM HRP, and 2.0 mM azide in 2.0 ml of KRP. The H₂O₂ released was determined for 4 min at 37°C after the addition of opsonized E. coli at a cell to bacteria ratio of approximately 1000:1 or after addition of 50 μM myristic acid.

Electron Microscopic Study

For morphological studies, phagocytosis was carried out in the same way as described in the section on O₂ consumption. Thirty or 120 sec after the incubation of cells with bacteria, an aliquot of the cell suspension was dropped into chilled 0.1 M Millonig buffer, pH 7.2, and centrifuged at 80 × g for 5 min at 4°C. The pellets were then used for electron microscopic studies according to standard techniques. The pellets were fixed in 2.5% glutaraldehyde, and they were subsequently fixed in 1.5% osmium in 0.1 M Millonig buffer, pH 7.2, for 1 hr. After osmium fixation, the pellets were dehydrated with a series of increasing ethanol concentrations and embedded in Poly/Bed 812. After polymerization, ultrathin sections were cut and stained with uranyl acetate and lead citrate. Observations were performed with a JEOL-1000 electron microscope.
RESULTS

Effects of NMD Types V and X on the Scopoletin Fluorescent Assay and the cyt-c Reduction Assay in the Cell-Free System

NMD-type-V that was dissolved at 0.1 U/ml as described by Tsan et al. exhibited a much greater light absorption than did NMD-type-X. Furthermore, NMD-type-V highly quenched the fluorescence intensity of scopoletin as compared to NMD-type-X. This fact suggested that NMD-type-V may interfere with both the fluorometric and spectrophotometric assays for \( \text{H}_2\text{O}_2 \) and \( \text{O}_2 \), so the effects of these neuraminidases on both assays were examined. When NMD-type-V was added to the assay mixture for the scopoletin fluorescent assay of Root et al. (Fig. 1a), the intensity decrease that accompanies scopoletin oxidation by \( \text{H}_2\text{O}_2 \) and HRP was completely inhibited (open circles), whereas NMD-type-X did not interfere with the scopoletin fluorescent assay (closed circles). As shown in Fig. 1b, in the case of \( \text{H}_2\text{O}_2 \) measurement by the assay in Tsan’s report, NMD-type-V inhibited the decrease of emission intensity by approximately 50% (open circles), whereas NMD-type-X did not interfere with the assay (closed circles). In subsequent experiments, effects of these neuraminidases on the \( \text{O}_2 \) assay was examined by following the reduction of cyt-c. As shown in Fig. 2b, NMD-type-V caused a reduction of cyt-c even in the presence of SOD. In contrast, NMD-type-X did not have such an effect on the cyt-c reduction assay (Fig. 2c).

Effects of NMD Types V and X on the \( \text{O}_2 \) Consumption by PMN

Since NMD-type-V interfered with both the scopoletin fluorescent and cyt-c reduction assays as described above, the rates of \( \text{O}_2 \) consumption in the presence of these two neuraminidases were examined to determine the actual effects of these enzymes on the oxygen metabolism in PMN. Figure 3 (a–c) shows a comparison of the effects of the two types of neuraminidases on the rates of \( \text{O}_2 \) consumption in both resting
and phagocytosing states. NMD-type-V (Fig. 3b) inhibited the rate of O$_2$ consumption by about 50% in the resting state and by about 70% during the uptake of *E. coli* as compared to those of the control PMN (Fig. 3a). In contrast, the treatment of cells with NMD-type-X did not affect the rate of O$_2$ consumption by resting PMN as shown in Fig. 3c. This trace seemed to enhance the respiratory burst by about 20% during phagocytosis, but no statistically significant difference ($p < 0.2$) in the O$_2$ uptake was observed between PMN treated with various amounts of NMD-type-X (2–200 mU/ml) and control PMN (data not shown). Based on the above data, neuraminidase treatment was conducted with NMD-type-X at a concentration of 0.1 U/ml in the following experiments. The treatment of cells with 0.1 U/ml NMD-type-X removed 29%–45% of the cellular sialic acid.

**Effects of NMD-Type-X on O$_2$ and H$_2$O$_2$ Release During Phagocytosis**

Figure 4 (a and b) shows the effect of NMD-type-X on O$_2$ release from phagocytosing PMN. The rate of O$_2$ release was determined by the acetylated cyt-c reduction assay, since acetylated cyt-c is more specific for O$_2$ than is unmodified cyt-c.$^{14}$ The rate of O$_2$ release from NMD-type-X-treated PMN was enhanced by about 80% (Fig. 4b) as compared to that from control PMN (Fig. 4a). Figure 5 (a–d) shows the effects of NMD-type-X on H$_2$O$_2$ release from phagocytosing and myristate added PMN. The rate of H$_2$O$_2$ release from NMD-type-X-treated phagocytosing PMN was enhanced by about 100% (Fig. 5b) as compared to that from the control PMN (Fig. 5a). However, when cells were stimulated by the nonphago-
cytable agent, myristic acid, the rate of \( \text{H}_2\text{O}_2 \) release from NMD-type-X-treated PMN was slightly decreased (Fig. 5d) as compared to that from the control PMN (Fig. 5c).

**Morphological Study of NMD-Type-X-Treated PMN During the Phagocytic Process**

Figure 6 (a–c) shows the characteristic electron microscopic findings for the control (a and b) and NMD-type-X-treated PMN (c and d) that were incubated with *E. coli*. The control PMN (a and b) presented the ingestion of the bacteria and the formation of phagocytic vacuoles within 30 sec after addition of bacteria (a). In contrast, the NMD-type-X-treated PMN (c and d) have scarcely ingested bacteria at times up to 120 sec. The added bacteria merely attached to the membranes of the NMD-type-X-treated PMN. Such findings were observed with all the other specimens of NMD-type-X-treated PMN. Figure 6e is a high magnification micrograph of a portion of the NMD-type-X-treated PMN (c) at the site of attachment of the bacteria. Moore et al.\(^{21}\) reported that there were constant spacings between the PMN membrane and bacteria at the attachment sites of bacteria. In fact, there were spacings between the phagocytosed bacteria and the membranes of the phagosomes (Fig. 6, a and b). However, no spacing between the NMD-type-X-treated PMN membrane and bacteria was observed (Fig. 6e).

**DISCUSSION**

Noseworthy et al.\(^{22}\) demonstrated that the use of impure neuraminidase could bring about erroneous results, such as the inhibition of glycolysis and phagocytosis in PMN. The high absorptivity and fluorescence-quenching ability of NMD-type-V showed that it contained significant amounts of contaminants. Therefore, the present experiments were carried out to reexamine the reports of Tsan et al.\(^{1,2}\) regarding the effects of neuraminidase on \( \text{O}_2 \) and \( \text{H}_2\text{O}_2 \) generation by phagocytosing PMN.

Our data indicated that the results reported by Tsan et al.\(^{1,2}\) were artifacts as demonstrated by the following observations.

1. NMD-type-V interfered with the fluorescent assay for \( \text{H}_2\text{O}_2 \). The reasons for this inhibition have not been resolved in the present experiment but several possible mechanisms can be proposed. NMD-type-V may contain some hydrogen donors (A'H\(_2\)) which may competitively inhibit the oxidation of scopoletin (ScH\(_3\)) by the HRP-H\(_2\text{O}_2\) complex as shown by following equations:\(^{18}\)

\[
\text{HRP} + \text{H}_2\text{O}_2 \rightarrow \text{HRP-H}_2\text{O}_2 \text{ complex} \quad (1)
\]
HPR-H$_2$O$_2$ complex + ScH$_2$ → HPR
+ Sc + 2H$_2$O (2)
HPR-H$_2$O$_2$ complex + $\Lambda'H_2$ → HPR
+ $\Lambda'$ + 2H$_2$O (3)

where HPR is free horseradish peroxidase and HPR-H$_2$O$_2$ is the enzyme H$_2$O$_2$ complex. NMD-type-V quenched the emission of scopoletin, suggesting that it contains unidentified contaminants, some of which may react with HPR-H$_2$O$_2$ complex as hydrogen donor ($\Lambda'H_2$).

(2) NMD-type-V interfered with the spectrophotometric assay for O$_2$. It seems that NMD-type-V contains some reducing agent that can cause O$_2$-independent cyt-c reduction.

(3) It is clear that the inhibition of O$_2$ consumption
by NMD-type-V was independent of the neuraminidase activity of the enzyme. The enzymatic activities of both NMD types V and X used in the present experiments were equal (0.1 U/ml), so that the inhibition by NMD-type-V was caused by its contaminants. In order to avoid the interferences as described above, PMN should be treated with more purified neuraminidase, i.e., NMD-type-X.

Contrary to the reports of Tsan et al., the present study clearly showed that the rate of $\text{O}_2$ and $\text{H}_2\text{O}_2$ release from phagocytosing PMN was enhanced by the pretreatment of cells with NMD-type-X. The enhancement of the rate of $\text{O}_2$ and $\text{H}_2\text{O}_2$ release may be caused by accelerated phagocytosis because the magnitude of the respiratory burst seems to closely parallel that of phagocytosis. However, this speculation is not consistent with the following observations. Since $\text{O}_2$ is formed by a one-electron reduction of $\text{O}_2$ by NADPH oxidase of PMN, the rate of $\text{O}_2$ consumption by NMD-type-X-treated PMN should be
increased during phagocytosis in proportion to the rate of 
O$_2$ and H$_2$O$_2$ release. However, such an enhance-
ment of O$_2$ consumption was not observed in the 
NMD-type-X-treated PMN. Noseworthy et al.\textsuperscript{22}
reported that neuraminidase treatment of cells did not 
cause an enhancement of the phagocytic rate in the 
absence of antiserum, and Constantpoulos and Naj-
jar\textsuperscript{24} showed the necessity of sialic acid for enhance-
ment of phagocytic activity in the presence of anti-
serum. From these observations, the enhancement of O$_2$
and H$_2$O$_2$ release from phagocytosing NMD-type-
X-treated PMN cannot be explained by its phagocytic 
activity. Both the O$_2$ and H$_2$O$_2$ measured correspond 
only to the oxygen intermediates released from the 
cells into the external medium because neither cyt-c 
or HRP can penetrate into a cell through the plasma 
membrane. Thus, the enhancement of O$_2$ and H$_2$O$_2$
release from NMD-type-X-treated PMN during pha-
gocytosis may be caused by excessive extracellular 
release of oxygen intermediates through the incom-
pletely formed phagocytic vacuoles as observed in 
cytochalasin-B-treated cells.\textsuperscript{23} This speculation seems 
to be supported by the fact that no difference in H$_2$O$_2$
release between the control and the NMD-type-X-
treated PMN was observed by the addition of myrist-
tate, which stimulates the oxidative metabolism of 
PMN without formation of phagocytic vacuoles.\textsuperscript{24} In 
addition, morphological observations of NMD-type-
X-treated PMN are in accord with this speculation. 
NMD-type-X-treated PMN were able to attach to the 
bacteria, but they seemed to be unable to ingest 
the bacteria and failed to form phagocytic vacuoles. Since 
anchorage of bacteria to the plasma membrane per se 
caused the respiratory burst, NMD-type-X-treated 
PMN seemed to freely release O$_2$ and H$_2$O$_2$ into the 
extracellular medium.

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H Suzuki, T Kurita and K Kakinuma