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Lysosomal Enzyme Release from Guinea Pig Polymorphonuclear Leukocytes by Influenza Virus

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ABSTRACT

Extracellular release and subsequent decrease of intracellular lysosomal enzymes of guinea pig polymorphonuclear leukocytes (PMNLs) by influenza virus were observed. Total enzyme activity was also decreased in all the enzymes assayed. After incubation of the PMNLs with influenza virus at 37°C for 20 min, the degree in decrease of total enzyme activity varied from enzyme to enzyme: 50% in myeloperoxidase (MPO), 20% in acid phosphatase, 10% in N-Ac-β-glucosaminidase and β-glucuronidase and 5% in lysozyme, respectively.

Since MPO assumed to play a critical role in chemiluminescent response of luminol-enhanced system, the dysfunction in oxidative metabolism of PMNLs induced by influenza virus seems to be attributed to intraphagosomal and/or extracellular inactivation of MPO of PMNLs during the process of direct stimulation of respiratory burst.

A variety of viruses has been known to affect neutrophil function in vitro, especially influenza virus has been studied most widely in this respect. Abramson et al[1,2,4] demonstrated in a series of studies using luminol-enhanced chemiluminescence (CL) reaction that influenza virus initiated rapid respiratory burst of resting polymorphonuclear leukocytes (PMNLs), while it caused strong depression of subsequent CL response induced by phorbol myristate acetate (PMA).

At least two mechanisms are involved in the production of CL during the process of phagocytosis of PMNLs or direct stimulation of PMNLs membrane without phagocytosis: one is the generation of singlet oxygens by dismutation of superoxide anions during the process of activation of NADPH-oxidase in PMNLs membrane and another is that by activation of myeloperoxidase(MPO)-hydrogen peroxide(H_2O_2)-halide system[5]. Although it is difficult to demonstrate exact role of reactive oxygen metabolites during a series of reaction generally called "respiratory burst", the former is assurmed to play a critical role in luminol-nonenhanced CL and the latter in luminol-enhanced one of PMNLs[10,11]. Recently, the generation of hydroxy radical was proposed by Repine et al[20] as another mechanism in the production of CL.

Previously, we reported the effect of influenza virus on the luminol-enhanced CL response of guinea pig PMNLs[16], and demonstrated that viral neuraminidase was not a critical component for initiation of respiratory burst of resting PMNLs and subsequent depressed function of CL response by PMA. These findings were also confirmed with human PMNLs by Abramson et al[4].

On the other hand, PMNLs release reactive oxygen metabolites and lysosomal enzymes not

only into phagosomes[14,17,23,24] but also into the extracellular circumstances during phagocytosis or direct stimulation of PMNLs membrane. Some of them exhibit bactericidal activity at environment close to the external surface of PMNLs[18]. Moreover, the lysosomal enzyme activity might be oxidatively inactivated with reactive oxygen metabolites in phagolysosome[17,23].

These facts mean that the intracellular level of lysosomal enzymes is reduced during the activation of PMNLs. In order to elucidate a possible mechanism in modification of the oxidative metabolism of guinea pig PMNLs by influenza virus, we investigated the effect of the virus on the extracellular release and/or intracellular aspect of lysosomal enzymes.

MATERIALS AND METHODS

1. Preparation of guinea pig PMNLs

Guinea pig PMNLs were collected according to the method previously reported[16] using a female Hartley guinea pig, weighting approximately 400 g. The cells were washed with and suspended in Eagle's MEM(3) (Nissui Seiyaku Kogyo, Japan) buffered at pH 7.4 with 10 mM N-2-hydroxyethylpiperadine-N-2'-ethansulfonic acid (HEPES: Dojindo Laboratories, Japan) (MEM-HEPES). Cell suspensions were adjusted at 5×10^6 cells per 0.9 ml for determination of lysosomal enzyme release and per 0.7 ml for determination of luminol-enhanced CL activity, respectively.

2. Influenza virus preparation

Influenza A/USSR/92/77(HlNl) and B/Kanagawa/3/76(B) viruses were propagated in MDCK cell cultures according to the method described by Tobita et al[22] and the partially purified viruses were prepared by the method previously described[16]. The hemagglutination titers of A/USSR/92/77 and B/Kanagawa/3/76 viruses were 1:5,120 and 1:2,560, respectively. Unopsonized virus preparations were used in all the assays.

3. Lysosomal enzyme release from guinea pig PMNLs

Extracellular release of lysosomal enzymes from guinea pig PMNLs was examined by incubation of the PMNLs with influenza virus at 37°C in water bath (shaken at 60 cycles/min). After incubation for various times the reactions were terminated by standing in ice bath and centrifuged immediately at $800 \times g$ for 10 min. The supernatant was harvested and enzyme activities released were determined. Sedimented PMNLs were solubilized with 1 ml of 0.1% Triton X-100 in MEM-HEPES and the supernatant fluid after centrifugation was used for determination of intracellular enzyme activity. The total enzyme activity of PMNLs was determined at 0-time after solubilizing non-incubated cells with 0.1% Triton X-100.

Stability of enzyme activity released from PMNLs into the medium was confirmed with the supernatant fluid of solubilized cells at 0-time by incubating the fluid at 37°C for various times.

One μg/ml phorbol 12-myristate 13-acetate (PMA: LC Service Corporation, USA), soluble stimulant and 1 mg/ml opsonized zymosan, particulate stimulant, were also employed as control stmuli. Opsonization of zymosan was performed according to the method described by Kobayashi et al[17] using Zymosan A (Sigma Chemicals, USA) and fresh autologous guinea pig serum.

4. Measurement of lysosomal enzyme activity

The activities of acid phosphatase, N-Ac-β-glucosaminidase and β-glucuronidase were determined fluorometrically using 4-methylumbelliferone (4-MU) derivatives (Koch Light Laboratories, England) as substrates according to the method described previously[15]. The concentration of the substrates was 0.2 mM 4-MU-phosphate in 0.1 M acetate buffer (pH 5.5) for acid phosphatase activity, 0.2 mM 4-MU-2-acetamide-2-deoxy-β-D-glucopyronoside in 0.1 M citrate buffer (pH 5.5) for N-Ac-β-glucosaminidase activity and 0.2 mM 4-MU-β-D-glucuronide in 0.1 M acetate buffer (pH 3.5) for β-glucuronidase activity, respectively. All the assay buffers contained 0.1% Triton X-100.

After incubation of 100 μl of each buffered substrate with an equal volume of enzyme preparation at 37°C for 20 min in water bath, reaction was terminated by adding 3.3 ml of 50 mM glycine buffer (pH 10.4) containing 5 mM ethylenediaminetetraacetic acid (EDTA), and the fluorescent intensity of 4-MU liberated enzymologically was measured with a fluorescent spectrophotometer (Hitachi 204: Hitachi Seisakusho, Japan) with an exitation wavelength of 365 nm and an emission wavelength of 450 nm. Enzyme activity was expressed as pmoles of substrate

hydrolyzed per min per 100 μl of sample.

Lysozyme activity was calculated from the reduction rate in turbidity of the suspension of *Micrococcus luteus* ATCC 4698 (Boehringer Mannheim GmbH, West-Germany). *M.luteus* was suspended at 0.1 mg/ml in 0.1 M sodium phosphate buffer (pH 5.5), 2.5 ml of which prewarmed at 25°C added with 0.1 ml of enzyme sample. The mixture was incubated at 25°C and the decrease of optical density was monitored at 450 nm with a spectrophotometer (Hitachi 100-50: Hitachi Seisakusho, Japan) equipped with a temperature constant cell holder. Enzyme activity was expressed as absorbance rate per min per 100 μl of sample by using hen egg white lysozyme (Sigma Chemicals, USA) as a standard.

Determination of MPO activity was performed according to the method described by Bretz et al[7] with some modifications. The substrate employed was 50 μM H_2O_2 (Wako Jyunyaku Kogyo, Japan) and 320 μM o-dianisidine (Sigma Chemicals, USA) in 0.05 M sodium phosphate buffer (pH 5.0). The assay was started by adding 0.1 ml of sample to 0.9 ml of the substrate solution. Incubation was carried out at 20°C and the increase of optical density associated with oxidation of o-dianisidine was monitored at 460 nm with a spectrophotometer equipped with temperature constant micro cell holder. Enzyme activity was expressed as absorbance rate per min per 100 μl of sample by using horse radish peroxidase (Sigma Chemicals, USA) as a substrate.

5. Luminol-enhanced CL assay

CL was measured with Lumicountor ATP-237 (Toyo Kagaku Sangyo, Japan) at ambient temperature. After pre-incubation of 5 × 10^6 cells of PMNLs/0.7 ml with 1 μmol/0.1 ml of luminol (3-aminophthaloylhydrazine: Tokyo Kasei Kogyo, Jăpan) in a siliconized glass cuvette (55 × 16.5 mm: Pyrex, Iwaki Glass, Japan) at 37°C in water bath for 5 min, a 0.1 ml volume of influenza virus preparation was added to the mixture. The CL intensity was measured every 30 sec and the effect of virus on the oxidative metabolism of resting PMNLs was monitored. Then, the mixture was stimulated with 1 μg/0.1 ml of PMA for monitoring PMA-induced CL[16].

RESULTS

1. Effect of influenza virus on luminol-enhanced CL response of guinea pig PMNLs

Both influenza A/USSR/92/77 and B/Kanagawa/3/76 viruses rapidly stimulated oxidative burst of resting PMNLs and the peak CL initiated by them was 2 and 2.5 times as high as that induced by PBS(−), respectively (Fig. 1). However, the peak CL response induced by PMA was significantly depressed and the value with each virus was counted to be 30% and 45% of that of PBS(−)-incubated cells, respectively.

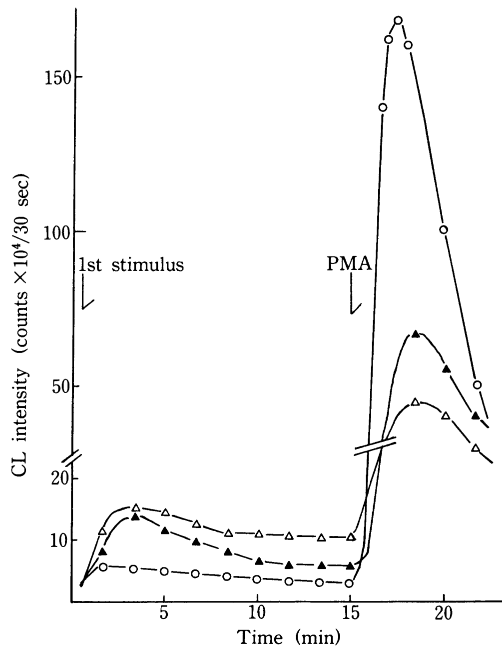

Fig. 1. Luminol-enhanced CL response of guinea pig PMNLs. PMNLs were initially stimulated with influenza A/USSR/92/77 (—△—), B/Kanagawa/3/76 (—▲—) virus or PBS(−)(—○—) at 0-time, and subsequently stimulated with PMA 15 min after incubation.

Although depression of PMA-induced CL response by influenza virus was dose-dependent upon the virus employed, the initiation of oxidative burst of resting PMNLs was not (Table 1). No direct correlation was observed between the direct stimulation of oxidative burst and subsequent depression of PMA-induced CL response.

2. Effect of influenza virus on lysosomal enzyme release from guinea pig PMNLs

Extracellular activity of MPO from PMNLs was unexpectedly low. However, significant

Table 1. Effect of influenza virus on luminol-enhanced CL response of guinea pig PMNLs

Influenza virus[2]	CL intensity[1] Resting peak Cl	Resting %	PMA-induced peak CL	PMA-induced %
A/USSR/92/77				
×1	14.6	261	45.2	30
2	8.1	144	95.4	63
4	7.1	126	111.6	74
8	6.1	108	164.9	109
B/Kanagawa/3/76				
×1	12.3	218	67.5	45
2	12.2	217	114.5	75
4	11.8	210	173.0	114
PBS(−)-control	5.6	100	151.7	100

1) CL intensity was expressed as counts × 10^4/sec.
2) The partially purified and unoponized viruses were used, and ×1 corresponded to the HA titers of 1:5, 120 in A/USSR/92/77 and 1:2, 560 in B/Kanagawa/3/76, respectively.

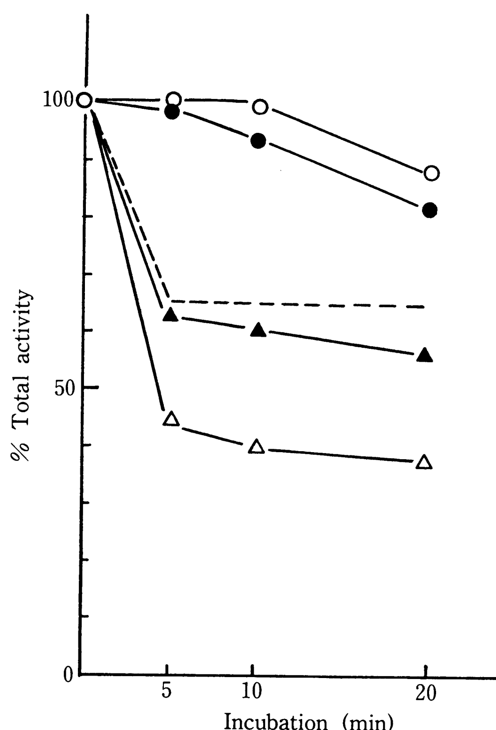

Fig. 2. Time course of intracellular decrease of MPO activity of guinea pig PMNLs associated with incubation with MEM-HEPES(—○—), PBS(−)(—●—) and influenza A/USSR/92/77(—△—) and B/Kanagawa/3/76(—▲—) viruses at 37°C. The dotted line shows the stability of MPO in the supernatant fluid of solubilized cells after released with 0.1% Triton X-100 at 0-times.

decrease of intracellular MPO activity was observed five min after incubation of guinea pig PMNLs with influenza virus at 37°C and the activity incubated with each virus was reduced to 43% and 52% of the activity at 0-time, respectively (Fig. 2). Spontaneous decrease of the enzyme activity was observed in MEM-HEPES- or PBS(−)-control cells and the decrease seemed to reach the plateau at 10 or 20 min after incubation.

The extra- and intracellular distribution of other lysosomal enzymes were examined 20 min after incubation at 37°C with various stimuli. The results are summarized in Fig. 3. Increase of extracellular release and subsequent decrease of intracellar levels of lysosomal enzyme activity by influenza virus were observed in all the enzymes employed. In all the cases, decrease of total enzyme activity was also observed. The degree in total enzyme activity varied from enzyme to enzyme, that is, 50% in MPO, 20% in acid phosphatase, 10% in N-Ac-β-glucosaminidase and β-glucuronidase and 5% in lysozyme. An investigation was made on each enzyme release from non-stimulated PMNLs treated with Triton X-100 as to whether the decrease of total activity was due to the instability of the enzyme at the extracellular circumstance or not. All the enzymes except MPO were almost stable in the medium as well as in PMNLs (Fig. 2 and 3).

DISCUSSION

It has been reported that a rapid respiratory

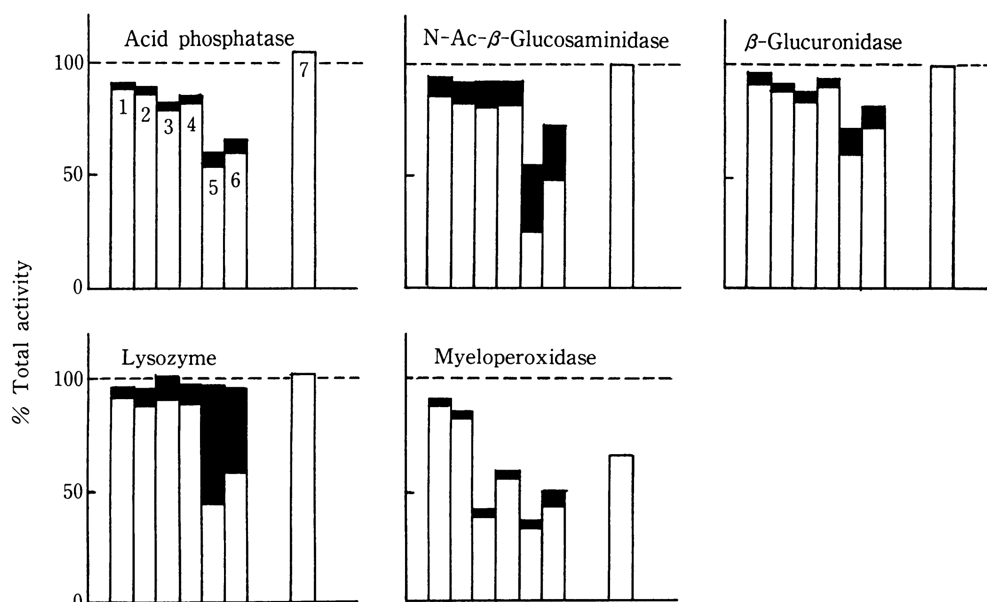

Fig. 3. Intra- and extracellular distribution of lysosomal enzyme activity of guinea pig PMNLs during stimulation with various stimuli at 37°C for 20 min. Open bars show intracellular level on each enzyme activity and shaded bars show extracellular one, and numbers in the bar show the stimuli employed: MEM-HEPES(1), PBS(–) (2), A/USSR/92/77(3), B/Kanagawa/3/76(4), PMA(5) and opsonized zymosan(6). Bar designated as 7 shows the stability of each enzyme activity in the supernatant fluid of solbilized cells after released with Triton X-100 at 0-time.

burst of PMNLs occurs when the PMNLs are incubated with influenza virus in vitro but the luminol-enhanced CL response of the PMNLs induced by subsequent PMA or opsonized zymosan stimulation is strongly depressed[1,2,4,16]. No direct correlation is observed between the direct initiation of oxidative metabolism and subsequent depression of PMA-induced CL response of PMNLs by influenza virus[1,16]. However, a possibility does not seem to be denied that the direct stimulation of respiratory burst of PMNLs by influenza virus modifies the reactivity of the cells to PMA or opsonized zymosan, although the mechanism remains unknown.

It is well known that generation of CL is mainly dependent on activation of MPO-H_2O_2-halide system in luminol-enhanced system[10,11], and that depletion of any one of these components results in a complete ablation of the response[9,11].

On the other hand, lysosomal enzyme release from PMNLs into extracellular circumstances as well as into phagolysosomes during the activation process are widely recognized[14,17,18,23,24]. Recently, inactivation of lysosomal enzymes in phagolysosomes by oxidative metabolites has been suggested[17,23]. In the present study, extracellular release and subsequent intracellular decrease of lysosomal enzymes as well as decrease of total enzyme activity by influenza virus were observed with each enzyme. The degree of these changes varied from enzyme to enzyme. particularly, the intracellular decrease of MPO, which is considered to play a critical role in luminol-enhanced CL response of PMNLs[10,11], was very remarkable.

Although a lot of reports have been presented about lysosomal enzyme release from activated PMNLs, only a few appeared about MPO[3,6]. Abramson et al[3] demonstrated MPO release from human PMNLs by influenza virus whereas significant depression of the release subsequently induced by opsonized zymosan. However, as is obvious from our present study and the results described by Kobayashi et al[17], measurement of the enzyme activity released only in the medium from PMNLs is not sufficient enough for the estimation of the effect of influenza virus on lysosomal enzymes.

The decrease of total lysosomal enzyme activity associated with activation of PMNLs was investigated in detail by Voetman et al[23] and

Kobayashi et al[17] independently. They suggested inactivation of lysosomal enzymes with oxidative metabolites in phagolysosomes, because the decrease was not observed in PMNLs from patients with chronic granulomatous disease which failed to generate the normal respiratory burst and decrease of total enzyme activity by unopsonized influenza virus were confirmed. Since all the enzymes except MPO were fairly stable in extracellular circumstances as well as within PMNLs, the decrease of total enzyme activity was likely due to oxidative inactivation in phagolysosomes[17,23]. The activity of MPO decrease immediately after releasing from PMNLs by the virus so that extracellular inactivation might be involved in total MPO activity to some extent. However, the degree of extracellar inactivation of this enzyme was not so significant and at least 40 to 50% of MPO activity was inactivated intracellularly as in other enzymes based on calculation. Remarkable lysosomal enzyme release and decrease of total enzyme activity were also observed by treatment of PMNLs with both PMA and opsonized zymosan, but were essentially different from those by influenza virus.

It is considered that extracellular enzyme release and subsequent decrease of intracellular enzyme activity are attributed to the subcellular location of each enzyme and selective activity of stimulant employed. Generally, four enzymes employed except lysozyme are known to be mainly present in azurophilic granules, while lysozyme is in specific granules[8,21]. Moreover, PMA causes degranulation of specific granules selectively[12,19]. In our present study, it was difficult to find any essential difference between the effects of the two stimuli, PMA and opsonized zymosan, although Bentwood and Henson[6] reported MPO released from PMNLs by PMA.

The exact mechanism involved in stimulation of guinea pig PMNLs by nonopsonized influenza virus is still unclear, but it seems interesting that influenza virus causes a selective decrease of intracellular MPO activity.

Recently, Haug et al[13] reported that lipid moiety in normal rat tissue homogenates inhibited luminol-enhanced CL of human PMNLs induced by PMA or *Escherichia coil*. The fact should be kept in mind in estimating the results obtained from experiments using obligate intracellular parasites, especially enveloped virus.

In the present study, we demonstrated a marked loss of intracellular MPO activity of guinea pig PMNLs when incubated with influenza virus in vitro. Since the MPO-H_2O_2-halide system is one of the most potential bactericidal systems, the findings may provide a new idea for explaining the depressed resistance against secondary infections of other microorganisms in influenza virus infection.

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