Hiroshima Journal of Medical Sciences
Vol.34, No.1, 81~86, March, 1985
HIJM 34-13

Depression of Luminol-Enhanced Chemiluminescence of Guinea Pig Polymorphonuclear Leukocytes by Influenza Virus with Special Reference to Neuraminidase

Katsuhiro KIYOTANI[1], Naomi TAKEI[2] and Yoshiyasu MATSUO[1]

1) Department of Bacteriology, Hiroshima University School of Medicine, 1-2-3, Kasumi, Minami-ku, Hiroshima 734, Japan

2) Hiroshima Prefectural Institute of Public Health, 1-5-70 Ujina-kanda, Minami-ku, Hiroshima 734, Japan

(Received December 19, 1984)

Key words: Luminol-enhanced chemiluminescence, Influenza virus, Guinea pig PMNL, Neuraminidase

ABSTRACT

Effect of influenza virus on luminol-enhanced chemiluminescent response of guinea pig polymorphonuclear leukocytes (PMNL) was investigated in order to elucidate a possible mechanism by which influenza virus modified the oxidative metabolism of PMNL.

Influenza viruses, strains A/USSR/92/77(H1N1) and B/Kanagawa/3/76(B), rapidly stimulated oxidative burst of resting PMNL and the peak chemiluminescence initiated by them was 5 to 15 times as high as that induced by PBS(−), while the peak chemiluminescent response induced by phorbol myristate acetate (PMA) delayed and the values were counted to be approximately 40% of that of PBS(−)-incubated cells. No significant changes were observed in both activities to enhance chemiluminescent response of resting PMNL and to depress PMA-induced chemiluminescent response when influenza virus with heat-inactivated neuraminidase activity at 56°C for 20 min was used. Moreover, neither initiation of oxidative burst of PMNL nor depression of PMA-induced chemiluminescent response was observed with purified neuraminidase extracted from influenza virus.

It seems likely that viral neuraminidase is at least not a critical component to modify the oxidative metabolism of guinea pig PMNL.

Influenza virus infection is associated, as is well known, with secondary infections by bacteria and fungi[10,11,22], which are attributed to decreased defence mechanism of the host, mainly depression of polymorphonuclear leukocytes (PMNL) functions such as chemotaxis[18], phagocytosis or intracellular killing of microorganisms[1,19]. As the oxygen-dependent antimicrobial system is the most potent microbicidal mechanism of PMNL[14,15], it seems most probable that the decrease of defence mechanism associated with influenza virus infection is due to dysfunction in processes of oxidative metabolism of PMNL.

Faden et al[7] demonstrated that newcastle disease virus, herpes simplex virus, vaccinia virus and reovirus depressed luminol-enhanced chemiluminescence of human PMNL induced with opsonized zymosan. On the other hand, Mills et al[16] reported that influenza virus rapidly stimulated the respiratory burst and enhanced chemiluminescence of PMNL. Abramson et al[1,2] observed that influenza virus strongly depressed luminol-enhanced chemiluminescence of human PMNL induced with phorbol myristate acetate (PMA) or opsonized zymosan, in spite of a rapid enhancement of oxidative metabolism of resting PMNL[3].

However, no appreciable information is available as to the mechanisms by which influenza virus causes oxidative burst or depresses PMNL function.

We investigated the effect of viral neuraminidase on luminol-enhanced chemiluminescence of guinea pig PMNL.

MATERIALS AND METHODS

1. Preparation of guinea pig PMNL

The peritoneal exudate cells from female guinea pigs of the Hartley strain, weighing approximately 400g, were collected 18 hr after peritoneal injection of 12% purified casein (Difco Laboratories, USA) in PBS(−), pH 7.4 at a dose of 20 ml/kg. The cells were washed with and suspended in Eagle MEM (3)(Nissui Seiyaku Co, Japan) buffered at pH 7.4 with 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES: Dojindo Laboratories, Japan)(MEM-HEPES). Red blood cells contaminated were thoroughly treated with hypotonic lysis with distilled water.

2. Preparation of influenza virus suspensions

Influenza virus A/USSR/92/77 (H1N1) and B/Kanagawa/3/76(B) strains were propagated in MDCK cells according to the method described by Tobita et al[20] at 34°C for 3 to 4 days. After centrifugation of each culture fluid at 1,500 $\times g$ for 30 min, the supernatant was ultracentrifugated at 100,000 $\times g$ for 60 min. The precipitate was washed once with PBS(−) at 100,000 $\times g$ for 60 min. The resultant precipitate was resuspended in approximately 1 : 50 volume of PBS(−) and stored at 4°C overnight in a refrigerator. The cell debris was removed by centrifugation at 1,500 $\times g$ for 30 min and the supernatant fluid was used throughout the experiments.

3. Measurement of neuraminidase activity

Fluorometric determination of neuraminidase activity by the use of 4-methylumbelliferyl-N-Ac-α-D-neuraminide (Koch Light Laboratories, England) as a substrate was performed according to the method described previously[13]. Colorimetric determination of the activity was performed according to the method described by Aymard-Henry et al[4] using fetuin as a substrate. Enzyme activities were expressed as pmol of 4-methylumbelliferone or N-Ac-α-neuraminic acid liberated per 100 μl of sample per hr. As controls, purified neuraminidase from influenza virus (Calbiochem-Behring for Research Biochemicals and Immunochemicals, USA) and that from *Clostridium perfringens* (Boehringer Manheim GmbH, W-Germany) were used.

4. Chemiluminescence assay in luminol-enhanced system

Both luminol (3-aminophthaloylhydrazine: Tokyo Kasei Co., Japan) and phorbol 12-mrystate 13-acetate (PMA : LC Service Corporation, USA) were dissolved in dimethyl sulfoxide (DMSO : Wako Junyaku Co., Japan) and stock solutions of 10 mg/ml and 2 mg/ml, respectively, were stored at −80°C. When they were actually used, these stock solutions were diluted with MEM-HEPES to obtain appropriate concentrations.

Chemiluminescence was measured with Lumicountor ATP-237 (Toyo Kagaku Sangyo Co., Japan) at the ambient temperature.

After pre-incubation of 5×10^6 cells of PMNL/0.7 ml with 1 μmol/0.1 ml of luminol in siliconized cuvette (55×16.5 mm : Pyrex) at 37°C in a water bath for 5 min, 0.1 ml of influenza virus preparation was added. The chemiluminescence intensity was measured every 15 sec and the effect of the virus on the oxidative metabolism of resting PMNL was monitored. Then, the mixture was stimulated with 0.5 μg/0.1 ml of PMA and the PMA-induced chemilumunescence was monitored.

RESULTS

1. Effect of influenza virus on luminol-enhanced chemiluminescent response of guinea pig PMNL

Hemagglutination and neuraminidase activities of the influenza virus preparations or purified neuraminidases employed were summarized in Table 1.

Effects of influenza viruses on luminol-enhanced chemiluminescent responses of guinea pig PMNL were shown in Fig. 1. Both A/USSR/92/77 and B/Kanagawa/3/76 viruses rapidly stimulated oxidative burst of resting PMNL and the peak chemiluminescence initiated by them was 5 and 15 times as high as that induced by PBS(−), respectively. However, the peak chemiluminescent response induced by PMA delayed and the values were counted to

Table 1. Hemagglutination and neuraminidase activities of influenza viruses and pure neuraminidases employed

	HA titer	Neuraminidase activity*	
		(1)	(2)
Influenza virus			
A/USSR/92/77	2,048	189,000	414,750
B/Kanagawa/3/76	2,048	83,800	1,112,678
Pure neuraminidase			
Influenza virus	<4	176,300	135,200
Cl. perfringens	<4	308,000	4,080,000

*Neuraminidase activity was determined fluorometrically using 4-methylumbelliferyl-N-Ac-α-D-neuraminide(1) or colorimetrically using fetuin as substrate (2). The activities were shown as pmol/hr/100 μl of sample.

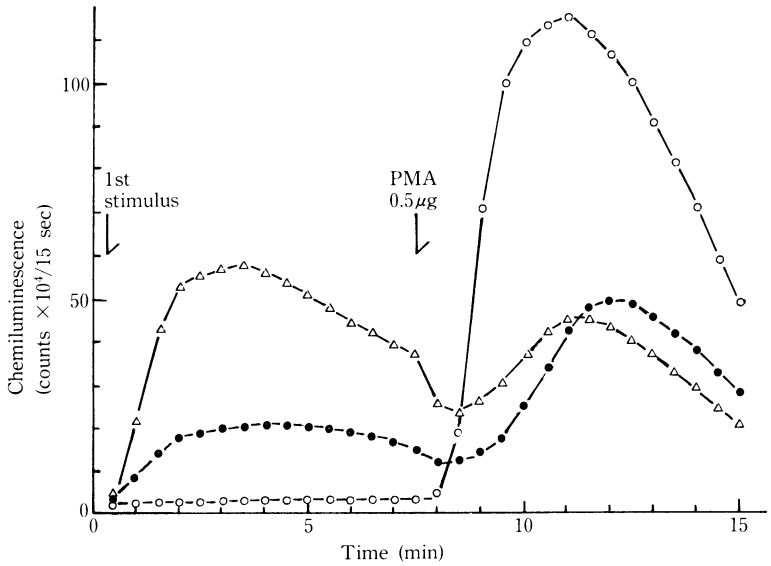

Fig. 1. Luminol-enhanced chemiluminescent response of guinea pig PMNL which were initially stimulated with influenza virus A/USSR/92/77 (—•—), B/Kanagawa/3/76 (—Δ—) strains and PBS (—) (— o —), and subsequently stimulated with PMA.

be approximately 40% of that of PBS(—)-incubated cells. Such responses were viral dose-dependent (Table 2). No direct correlation was observed between the enhance and/or depression of luminol-enhanced chemiluminescence and viral hemagglutination and/or neuraminidase activity.

2. Effect of the neuraminidase on luminol-enhanced chemiluminescent response of guinea pig PMNL

Effects of the commercially available purified neuraminidases on luminol-enhanced chemiluminescent response of guinea pig PMNL were shown in Fig. 2. No effect was observed on resting PMNL. However, PMA-induced chemiluminescent response was slightly inhibited by the neuraminidase from influenza virus, while it was somewhat enhanced by the bacterial neuraminidase. These phenomena are unlikely to depend upon enzymatic activity of the purified neuraminidases employed, because the chemiluminescent response was not significantly affected even after incubation of PMNL with the

Table 2. Effect of influenza virus on luminol-enhanced chemiluminescent responses of guinea pig PMNL in resting state and induced by PMA

Influenza virus	Chemiluminescent intensity*			
	Resting		PMA-induced	
	Peak CL	%	Peak CL	%
A/USSR/92/77				
×1	21.2	558	49.5	42.7
2	16.3	429	60.9	52.6
4	10.6	279	62.3	52.9
8	7.9	208	73.4	63.4
B/Kanagawa/3/76				
×1	58.2	1,532	45.8	39.6
2	36.3	955	62.6	54.1
4	23.3	613	70.9	61.2
PBS(—)-control	3.8	100	115.8	100

*Chemiluminescent intensity was expressed as counts $\times 10^4$ per 15 sec.

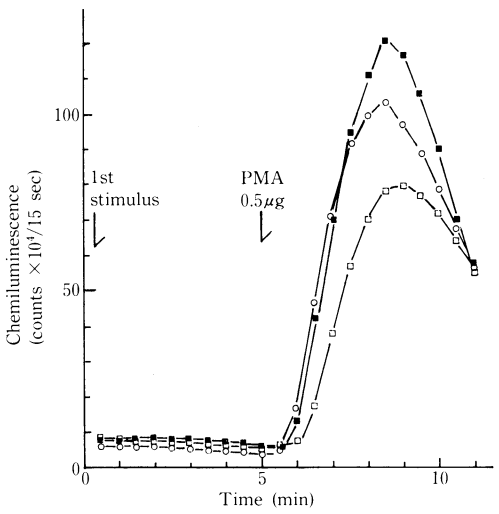

Fig. 2. Luminol-enhanced chemiluminescent response of guinea pig PMNL which were stimulated with purified neuraminidase extracted from influenza virus (— □ —) and *Clostridium perfringens* (— ■ —), and subsequently stimulated with PMA.

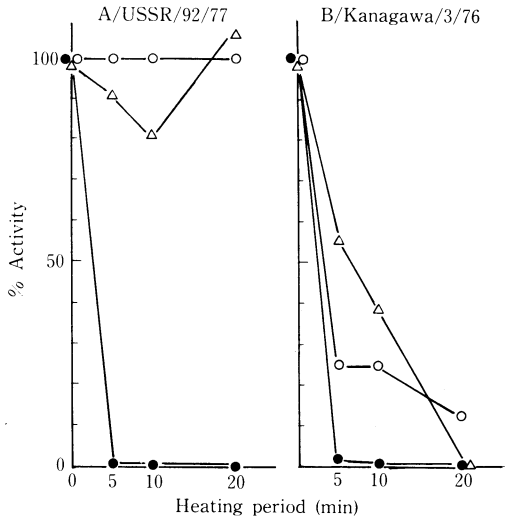

Fig. 3. Heat-inactivated pattern of influenza virus hemagglutination (— o —), neuraminidase (—•—) and chemiluminescence inhibition activities (—Δ—) at 56°C for the time indicated.

enzymes at 37°C for 60 min prior to stimulation with PMA (Data not shown).

The viruses were exposed to a temperature of 56°C for various lengths of time, and their activities of hemagglutination, neuraminidase and inhibition of PMA-induced chemiluminescence were investigated. As shown in Fig. 3, neuraminidase activities of both virus strains were almost completely inactivated within 5 min. Hemagglutinating activity of A/USSR/92/77 virus remained intact for over 20 min, while that of B/Kanagawa/3/76 virus was rapidly inactivated. The inhibitory activities of these viruses thus treated against PMA-induced chemiluminescence was correlated with hemagglutinating activities, although no effect on enhancement of

chemiluminescence of resting PMNL was observed.

DISCUSSION

The oxygen-dependent antimicrobial system, especially the myeloperoxidase-hydrogen peroxide-halide system, is the most potent microbicidal property of PMNL[14,15]. It seems most probable that the PMNL dysfunction associated with influenza virus infection is based on the depression of a part of processes of the oxidative metabolism of PMNL. Although it is generally known that influenza virus infection suppresses chemotaxic or phagocytic activity of host cells, reports are scanty regarding the relevance of influenza virus to the oxidative metabolism of PMNL[1,2,3,7].

Fisher and Ginsberg[8,9] first proposed the possibility that influenza virus affects the oxidative metabolism of guinea pig PMNL. They demonstrated reduction in phagocytosis and glycolysis of PMNL by influenza virus A/PR8 and B/Lee/40 viruses as well as receptor destroying enzyme (RDE). the reduction was not observed with heat-inactivated influenza viruses and RDE. They assumed that the reductive activity might be mediated by viral neuraminidase, since the activity was dependent on calcium ion.

These findings were contradicted by Noseworthy et al[17]. According to Tsan et al[21], removal of 20% of total sialic acid of human PMNL by bacterial neuraminidase showed no effect on phagocytosis of latex particles and activation of hexose monophosphate shunt, whereas such treatment prevented superoxide production associated with either phagocytosis or concanavalin A-stimulation. Recently, Mills et al[16] and Abramson et al[3] demonstrated by luminol-enhanced chemiluminescence assay method that influenza virus rapidly stimulated respiratory burst of resting human PMNL but strongly inhibited PMA- or opsonized zymosan-induced chemiluminescent response. Nonetheless, no clear explanation has been offered about mechanisms by which influenza virus causes oxidative burst on the one hand and disturbs PMNL function such as PMA- or opsonized zymosan-induced superoxide production on the other.

Initiation of respiratory burst of resting PMNL by influenza virus occurs rapidly in the absence of serum so that the response is likely to be attributed to direct stimulation of NADPH-oxidase in PMNL membrane. Mills et al[16] presumed that heat-labile factors on the virus envelope may be involved in oxidative stimulation of PMNL, because no response was observed when the virus treated at 80°C for 30 min was used in luminol-enhanced chemiluminescence assay, whereas it occurred when the live or ultraviolet irradiated virus was added. Abramson et al[3] reported that respiratory burst of PMNL was activated by stimulation of viral glycoproteins formed as a liposome (i.e., membrane structure) but not by glycoproteins as rosettes (i.e., glycoprotein aggregates), and suggested that membrane structure of influenza virus was necessary for the induction of respiratory burst of PMNL.

Since influenza virus bounds to the specific receptor on cell surface, it seems more likely that the virus would modify the oxidative metabolism of PMNL by hemagglutinin spike rather than by neuraminidase spike. In the present study, however, a significant difference was observed in the degree of inhibition of respiratory burst of resting PMNL between A/USSR/92/77 and B/Kanagawa/3/76 virus strains with almost the same hemagglutination activity. Furthermore, the activity of virus preparations exposed to heating of 56°C for 20 min for the initiation of respiratory burst of PMNL was not affected and the activity of purified neuraminidase extracted from influenza virus was not demonstrated. From the results, the participation of either viral hemagglutinin or neuraminidase in respiratory burst of PMNL was contradictory.

The inhibitory mechanism of influenza virus to chemiluminescence of PMNL induced by PMA remains unclear like that of initiation of respiratory burst. Mills et al[16] and Abramson et al[3] indicated that both virus infectivity and hemagglutination activity were not critical in PMNL dysfunction. Faden et al[7] suggested that viral neuraminidase might play an important role in PMNL dysfunction. However, our results revealed that no participation of viral neuraminidase was observed in the PMNL dysfunction irrespective of difference between the two virus strains in calcium ion-dependency and substrate specificity[12] (Table 1). A/USSR/92/77 virus with

lost neuraminidase activity after heating showed almost the same degree of depression of PMA-induced chemiluminescence as the non-heated control. PMA-induced chemiluminescence was not significantly affected by purified neuraminidase from influenza virus. These findings suggest that viral neuraminidase is not at least a critical component for induction of respiratory burst and subsequent depressed function of PMNL.

Another interesting problem in modification of PMNL oxidative metabolism by influenza virus is whether or not any interaction exists between initiation of respiratory burst of resting PMNL and depression of PMA-induced chemiluminescence. Although Abramson et al[3] stated a negative opinion, this is a matter to be solved in future research.

REFERENCES

1. **Abramson, J.S., Giebink, G.S., Mills, E.L. and Quie, P.G.** 1981. Polymorphonuclear leukocyte dysfunction during influenza virus infection in chinchillas. J. Infect. Dis. **143**: 836-845.
2. **Abramson, J.S., Mills, E.L., Giebink, G.S. and Quie, P.G.** 1982. Depression of monocyte and polymorphonuclear leukocyte oxydative metabolism and bactericidal capasity by influenza A virus. Infect. Immun. **35**: 350-355.
3. **Abramson, J.S., Lyles, D.S., Heller, K.A. and Bass, D.A.** 1982. Influenza A virus-induced polymorphonuclear leukocytes dysfunction. Infect. Immun. **37**: 794-799.
4. **Aymard-Henry, M., Coleman, M.T., Dower, R., Laver, W.G., Schild, G.C. and Webster, R.G.** 1973. Influenza virus neuraminidase and neuraminidase-inhibition test procedures. Bull. W.H.O. **48**: 199-202.
5. **Dahlgren, C. and Stendahl, O.** 1983. Role of myeloperoxidase in luminol-dependent chemiluminescence of polymorphonuclear leukocytes. Infect. Immun. **39**: 736-741.
6. **Dechatelet, L.R., Long, G.D., Shirley, P.S. and Cohen, M.S.** 1982. Mechanism of the luminol-dependent chemiluminescence of human neutrophils. J. Immunol. **129**: 1589-1593.
7. **Faden, H., Sutyla, P.S. and Ogra, P.L.** 1979. Effect of viruses on luminol-dependent chemiluminescence of human neurtophils. Infect. Immun. **24**: 673-678.
8. **Fisher, T.N. and Ginsberg, H.S.** 1956. The reaction of influenza viruses with guinea pig polymorphonuclear leukocytes. II. The reduction of white blood cell glycolysis by influenza viruses and receptor-destroying enzyme (RDE). Virology **2**: 637-655.
9. **Fisher, T.N. and Ginsberg, H.S.** 1956. The reaction of influenza viruses with guinea pig polymorphonuclear leukocytes. III. Studies of the mechanism by which influenza viruses inhibit phagocytosis. Virology **2**: 656-664.
10. **Fisher, J.J. and Walker, D.H.** 1979. Invasive pulmonary aspergillosis associated with influenza. J. Am. Med. Assoc. **241**: 1493-1494.
11. **Hers, J.F., Goslings, W.R., Masurel, N. and Mulder, J.** 1957. Death from asiatic influenza in the Netherlands. Lancet **ii**: 1164-1165.
12. **Kiyotani, K.** 1984. Calcium-independent neuraminidase activity of influenza B/Kanagawa/3/76 virus. Medicine and Biology **109**: 83-86 (in Japanese).
13. **Kiyotani, K., Takei, N. and Matsuo, Y.** 1984. Fluorometric measurement of neuraminidase activity of influenza viruses. Hiroshima J. Med. Sci. **33**: 287-292.
14. **Klebanoff, S.J. and Hamon, C.B.** 1972. Role of myeloperoxidase-mediated antimicrobial systems in intact leukocytes. J. Reticuloendothel. Soc. **12**: 170-196.
15. **Klebanoff, S.J.** 1975. Antimicrobial mechanisms in neutrophilic polymorphonuclear leukocytes. Semin. Hematol. **12**: 117-142.
16. **Mills, E.L., Debets-Ossenkopp, Y., Verbrugh, H.A. and Verhoef, J.** 1981. Initiation of the respiratory burst of human neutrophils by influenza virus. Infect. Immun. **32**: 1200-1205.
17. **Noseworthy, J.Jr., Korchak, H. and Karnovsky, M.** 1972. Phagocytosis and the sialic acid of the surface of polymorphonuclear leukocytes. J. Cell. Physiol. **79**: 91-96.
18. **Ruutu, P., Vaheri, A. and Kosunen, T.U.** 1977. Depression of human neutrophil motility by influenza virus in vitro. Scand. J. Immunol. **6**: 897-905.
19. **Sellers, T.F., Achulman, J., Bouvier, C., McCune, R. and Kilbourne, E.D.** 1961. The influence of influenza virus infection on exogenous staphylococcal and endogeneous murine bactreial infection in the bronchopulmonary tissue of mice. J. Exp. Med. **114**: 237-256.
20. **Tobita, K., Sugiura, A., Enomoto, C. and Furuyama, M.** 1975. Plague assay and primary isolation of influenza A viruses in an established line of cannine kidney cells (MDCK) in the presence of trypsin. Med. Microbiol. Immunol. **162**: 9-14.
21. **Tsan, M-F. and McIntyre, P.A.** 1976. The requirement for membrane sialic acid in the stimulation of superoxide production during phagocytosis by human polymorphonuclear leukocytes. J. Exp. Med. **143**: 1308-1316.
22. **Young, L.S., Laforce, M., Head, J.J., Fealy, B.S. and Bennet, J.V.** 1972. A simultaneous outbreak of meningococcal and influenza infections. N. Eng. J. Med. **287**: 5-9.