INFECTION AND IMMUNITY, June 1979, p. 673-678
0019-9567/79/06-0673/06$02.00/0

Vol. 24, No. 3

Effect of Viruses on Luminol-Dependent Chemiluminescence of Human Neutrophils

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Received for publication 2 March 1979

The effects of Newcastle disease, herpes simplex, vaccinia, encephalomyocarditis, vesicular stomatitis, and reoviruses on in vitro function of neutrophils were studied in Ficoll-Hypaque-separated polymorphonuclear leukocytes (PMN) employing the technique of luminol-dependent chemiluminescence. Newcastle disease, herpes simplex, vaccinia, and reoviruses depressed chemiluminescence by 98, 65, 46, and 29%, respectively, while encephalomyocarditis and vesicular stomatitis viruses had no inhibitory effect. None of the viruses affected phagocytosis or PMN viability. These observations suggest significant alteration of neutrophil function by interaction with several viruses in in vitro settings. It is suggested that similar changes in PMN function may occur during in vivo viral infection.

A variety of viruses have been shown to affect neutrophil functions in vitro. Influenza virus, which has been studied most widely, inhibits chemotaxis, phagocytosis, and intracellular killing (3, 8, 9, 11). Herpesvirus (HV), measles virus, respiratory syncytial virus (RSV), mumps virus, varicella-zoster virus, and cytomegalovirus have also been shown to suppress chemotaxis, whereas mumps virus limits phagocytosis and RSV may interfere with intracellular killing of bacteria (3, 9; B. H. Park, Y. Chiba, R. Ramirez, and P. L. Ogra, Fed. Am. Soc. Exp. Biol. **36:** 1189, abstr. 4865, 1977). The mechanisms by which viruses affect neutrophil functions are not well understood. Unfortunately, many of the in vitro tests do not lend themselves to the study of virus-neutrophil interaction. These tests are often cumbersome, require large numbers of neutrophils, and measure single points in time. Chemiluminescence, a recently developed assay of neutrophil function, may provide several advantages over earlier assay systems because it is simple to perform and permits observations over extended periods. This assay is based on the ability of neutrophils to emit flashes of light during phagocytosis (2). More recent studies suggest that phagocytosis may not be prerequisite for the production of chemiluminescence and that perturbation of the cellular membrane alone is capable of initiating the reaction (5, 6). Since viruses attach to membrane receptors on cell surfaces, it seemed reasonable to expect that viruses would interfere with chemiluminescence. The present studies were designed to examine the effects of Newcastle disease virus (NDV), HV, vaccinia virus (VV), encephalomyocarditis virus (EMCV), vesicular stomatitis virus (VSV), and reovirus on chemiluminescence of human neutrophils.

Luminol, a cyclic hydrazide, markedly enhances chemiluminescence (1, 4). Although luminol-dependent chemiluminescence has recently been introduced as a simple and inexpensive means to study neutrophil function, factors which affect such chemiluminescence in in vitro settings have not been clearly defined. This report also summarizes the effect of various factors on luminol-dependent chemiluminescence.

MATERIALS AND METHODS

Preparation of neutrophils. Ten milliliters of blood were collected from normal healthy adult volunteers in a syringe containing 200 U of sodium heparin (The Upjohn Co., Kalamazoo, Mich.). Two milliliters of phosphate-buffered saline were added to the blood before it was layered over a dextran-Hypaque mixture, and the leukocyte-rich plasma fraction was centrifuged over a column of Ficoll-Hypaque (Pharmacia, Piscataway, N.J.) for 10 min at 800 rpm in a siliconized glass tube. The neutrophil-erythrocyte pellet was resuspended in 5 ml of ice-cold hypotonic saline, mixed, and combined with an equal volume of hypertonic saline. The cell suspension was centrifuged at 800 rpm for 10 min, and the cells were resuspended in Hanks balanced salt solution (HBSS; Grand Island Biological Co., Grand Island, N.Y.) in a concentration of 10^6 polymorphonuclear leukocytes (PMN) per ml unless stated otherwise. The PMN were 97% pure with viability greater than 98% as tested by trypan blue dye exclusion.

Preparation of luminol. Luminol, 5-amino-2,3-dihydro-1,4,-phthalazinedione (Sigma, St. Louis, Mo.),

was dissolved in dimethyl sulfoxide in a 1 M concentration. This preparation was diluted to 2×10^{-6} M concentration in HBSS and was used throughout the experiments unless stated otherwise.

Preparation of zymosan. Zymosan (International Chemical and Nuclear, Plainview, N.Y.) was prepared by boiling 100 mg of zymosan in 10 ml of barbital buffer for 1 h. The zymosan was centrifuged at 2,400 rpm for 30 min and resuspended in 2 ml (50 mg/ml) of barbital buffer as the stock preparation. The zymosan was opsonized in the donor's fresh serum by incubation at 37°C for 0.5 h. The zymosan suspension was centrifuged at 1,800 rpm for 10 min and resuspended in HBSS at a concentration of 4 mg/0.6 ml. The zymosan was kept in the dark until used.

Preparation of viruses. The Herts strain of NDV was prepared in embryonated hen eggs that had been injected by the allantoic route and titrated to 8.7×10^7 plaque-forming units (PFU) per ml when assayed in chicken embryo fibroblasts. VV was prepared in human foreskin fibroblasts and titrated to 5.5×10^6 PFU per ml when assayed in the same cell line. HV was also prepared in human foreskin fibroblasts and titrated to 10^8 PFU per ml. Reovirus, EMCV, and VSV were prepared in mouse L929 fibroblasts and titrated to 2.1×10^8, 4.5×10^8, and 7.0×10^7 PFU per ml, respectively, when assayed in mouse fibroblasts. The numbers of PFU of NDV, VV, HV, reovirus, EMCV, and VSV used in the chemiluminescence assays were 3.5×10^7, 2.2×10^6, 4×10^7, 4.4×10^7, 1.8×10^8, and 2.8×10^7, respectively. Heat inactivation of viruses was accomplished by boiling at 100°C for 5 min.

Preparation of control media. In each experiment with the viruses, appropriate controls were included. These consisted of PMN treated with uninfected tissue culture prepared in the same way as the virus pools, allantoic fluid harvested on day 16 of incubation, human foreskin fibroblasts frozen and thawed in minimal essential medium (Grand Island Biological Co.) with 2% fetal calf serum, and mouse fibroblasts frozen and thawed in minimal essential medium with 2% fetal calf serum.

Chemiluminescence assay. Chemiluminescence was measured in a liquid scintillation spectrometer (Nuclear Chicago, Des Plains, Ill.) at 8°C. Before the use of luminol, the machine was set to count out of coincidence, but later the machine was set to count in coincidence. Plastic scintillation vials (Fischer Scientific Co., Rochester, N.Y.) were dark adapted for at least 24 h before use. The experiments were conducted in a darkened room. One milliliter of PMN was added to 3.4 ml of HBSS with luminol in each counting vial. The vials were counted for 0.2 min to obtain background counts. When counted out of coincidence with HBSS minus luminol, the background counts varied between 1,000 and 3,000. When counted in coincidence with HBSS plus luminol, the background counts were less than 10. Between counting periods, the vials were kept at room temperature. In later experiments, the vials were kept on a shaker in a 37°C water bath. After the background counts stabilized, 0.6 ml of zymosan was added and the vials were counted at 10-min intervals. To ascertain that the sensitivity of the photomultiplier tubes did not change during the course of several experiments, we counted standard samples of ^{14}C (control) with each experiment. There was less than a 2% change from the highest to the lowest counts over several months. Other controls included HBSS alone, normal PMN alone, opsonized and unopsonized zymosan alone, and normal PMN with unopsonized zymosan.

In experiments with viruses, 1 ml of PMN suspension (10^6 PMN) was incubated with either 0.4 ml of virus preparation or control media in a scintillation vial at 37°C for 0.5 h. Slides were prepared from each vial and examined for changes in cell morphology and estimates of phagocytosis.

Blocking of the NDV-specific effect on chemiluminescence was carried out by incubating 0.4 ml of a 1:4 dilution of NDV-specific antisera (National Institute of Allergy and Infectious Diseases, Bethesda, Md.) with NDV at room temperature for 0.5 h before conducting the assay.

The results are presented as chemiluminescence curves and as percent inhibition of peak chemiluminescence response of experimental groups compared to the controls. The paired t test was used to evaluate the statistical significance of the effects of virus on chemiluminescence.

Phagocytosis. At the end of the chemiluminescence assay, 1 drop from each of the scintillation vials was placed on a microscope slide and allowed to dry. The slides were then fixed in ethanol for 5 min and then stained with methylene blue for 10 min. The relative proportion of neutrophils with ingested zymosan particles of the virus-treated specimens was compared to the controls.

Viability studies. Trypan blue dye exclusion was used to determine the viability of PMN.

RESULTS

Effect of neutrophil numbers on chemiluminescence. Many factors, including the number of neutrophils, were found to affect luminol-dependent chemiluminescence. Initial studies demonstrated that 10^7 PMN were required to produce chemiluminescence with opsonized zymosan in the absence of luminol. When 10^6 PMN were used, insufficient chemiluminescence resulted (Fig. 1). In an effort to decrease the number of PMN needed in the assay system, the chemiluminescent response with different concentrations of PMN was studied in HBSS with 2×10^{-6} M luminol. Significant responses were observed with PMN ranging in concentration from 10^4 to 10^7 per ml (Fig. 2). For the sake of uniformity, all subsequent studies were conducted with 10^6 PMN/ml.

Effect of various luminol concentrations on chemiluminescence. The effect of various dilutions of luminol on chemiluminescence is presented in Fig. 3. The addition of luminol markedly enhanced the response of neutrophils. Higher responses were observed with 4.8×10^{-5} M luminol, and the degree of chemiluminescence manifested a significant decline with decreasing concentrations of luminol. In all subsequent ex-

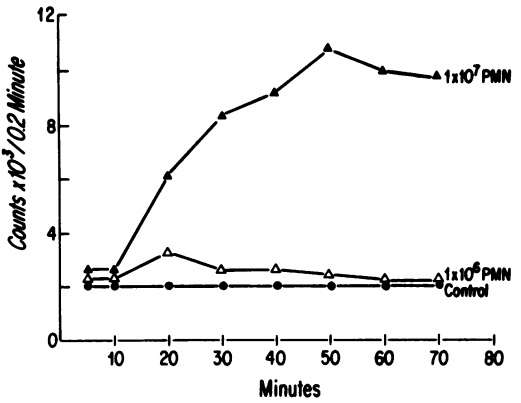

FIG. 1. *Zymosan-induced chemiluminescent responses of 10^7 and 10^6 PMN in media without luminol.*

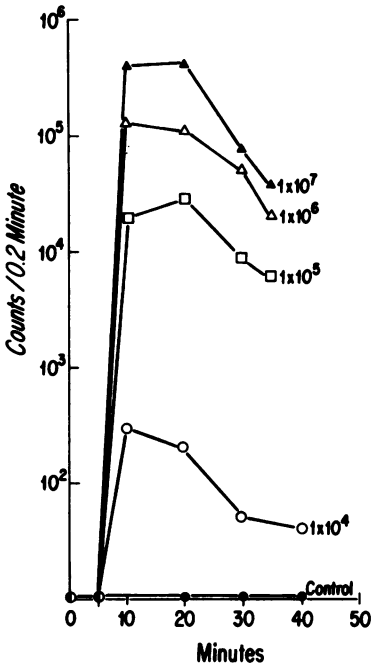

FIG. 2. *Zymosan-induced chemiluminescent responses of 10^4 to 10^7 PMN in media with 2×10^{-6} M luminol.*

periments, 2×10^{-6} M luminol was used. With luminol, the scintillation spectrometer was set to count in coincidence.

Effect of various amounts of zymosan on luminol-dependent chemiluminescence. Stock suspensions of zymosan were prepared prior to the performance of a series of experiments. The number of particles of zymosan per vial used in different experiments varied from 10^8 to 10^9. Figure 4 demonstrates the effect of various ratios of zymosan to PMN on chemiluminescence in 2×10^{-6} M luminol. As the ratio declined, the height of the chemiluminescence response declined and the temporal pattern of the initial appearance of the response appeared to be delayed. The decline of the luminol-dependent chemiluminescence was more gradual

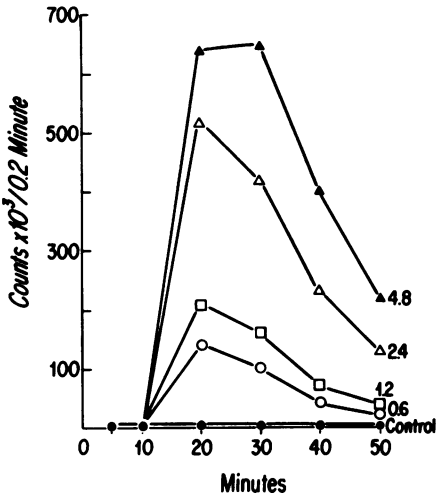

FIG. 3. *Zymosan-induced chemiluminescent response of 10^6 PMN in 0.6×10^{-5} to 4.8×10^{-5} M luminol.*

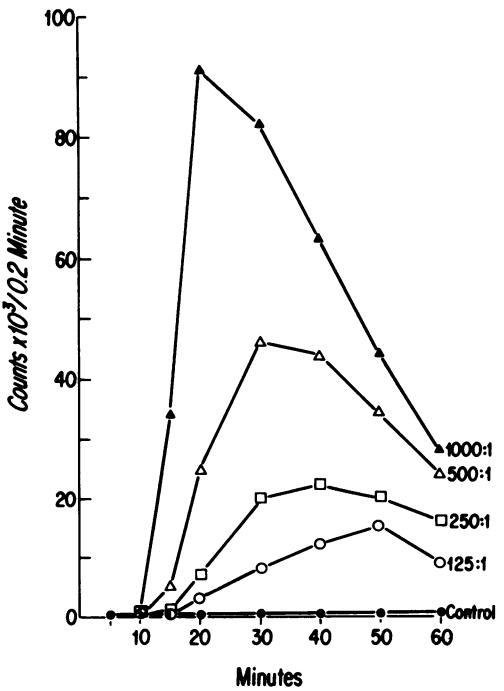

FIG. 4. *Chemiluminescent response of 10^6 PMN in 2×10^{-6} M luminol with PMN:zymosan ratios of 1:125 to 1:1000.*

at lower ratios. Controls such as HBSS, PMN alone, or unopsonized and opsonized zymosan alone produced no chemiluminescence. Neutrophils with unopsonized zymosan generated a small degree of chemiluminescence, reaching a peak value of <500 counts per 0.2 min.

Effects of viral control media on luminol-dependent chemiluminescence. Uninfected cultures of human foreskin fibroblasts and L-cell monolayers had no effect on chemiluminescence when compared to HBSS. In contrast, allantoic fluid had a markedly inhibitory effect, suppressing chemiluminescence by 40% when compared to HBSS control.

Effect of NDV on luminol-dependent chemiluminescence. NDV had a markedly suppressive effect on chemiluminescence (Fig. 5). In each of four experiments, the NDV-treated neutrophils produced <5% of the chemiluminescence produced by the controls ($P < 0.05$). The representative pattern of chemiluminescence observed with NDV in one experiment is presented in Fig. 6a.

Each scintillation vial used in an NDV assay contained 2×10^8 PFU of NDV and 10^6 PMN for a multiplicity of infection (MOI) of 200. As the preparation of virus was diluted, the inhibitory effect decreased (Fig. 6b). At a dilution of 1:16 or an MOI of 12.5, the inhibitory effect was 50% of the undiluted preparation.

To test whether the NDV-associated inhibition of chemiluminescence was virus specific and not due to a constituent of allantoic fluid, NDV was preincubated with specific antisera before the interaction with PMN. The antibody effectively neutralized the inhibitory effect of NDV (Fig. 6c). Although complete inhibition of chemiluminescence was observed with NDV alone, the peak chemiluminescence response with antibody was found to be over 70% of that observed in the uninfected allantoic fluid control.

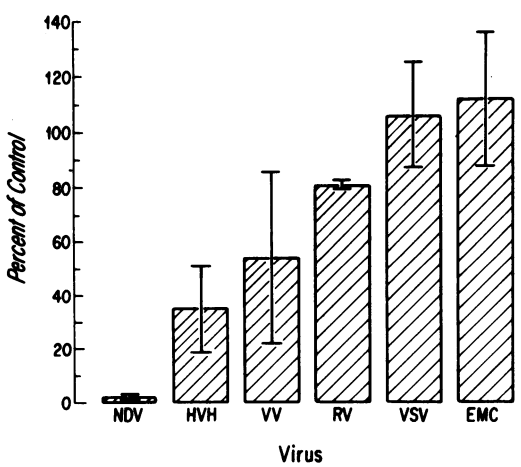

FIG. 5. *Effect of NDV, HVH (HV), VV, RV (reovirus), VSV, and EMC (EMCV) on luminol-dependent chemiluminescence. NDV suppressed chemiluminescence 98% ($P < 0.05$), HV 65% ($P < 0.1$), VV 46% ($P < 0.1$), and reovirus 29% ($P > 0.1$). VSV and EMCV had no inhibitory effect.*

Effect of other viruses on luminol-dependent chemiluminescence. The other viruses tested had less appreciable effects on chemiluminescence than did NDV (Fig. 5). The suppression of chemiluminescence with HVH, VV, and reovirus was 65, 46 and 29%, respectively, and EMCV and VSV had no observable effect on the chemiluminescence. In all cases, microscopic examination of the mixture of virus-treated PMN and zymosan demonstrated significant phagocytosis when compared to the controls, despite depressed chemiluminescence.

Effect of heat-inactivated viruses on luminol-dependent chemiluminescence. Heat inactivation (boiling for 5 min) decreased the inhibitory effect of NDV and reovirus. In two experiments, the inhibition of chemiluminescence obtained with heat-inactivated NDV was only 6% compared to 95% by the live virus preparation. Heat inactivation of reovirus also produced similar results in that inhibitory effect of the virus disappeared with heat treatment (2 versus 39%).

Effect of MOI on luminol-dependent chemiluminescence. A comparison of the MOI of the viruses studied to the percent inhibition of chemiluminescence achieved is presented in Table 1. CMCV, which had the largest MOI (450), had no inhibitory effect, whereas VV, which had the lowest MOI (5.5), had a moderate inhibitory effect. NDV had an MOI between the two extremes of EMCV and VV, and it produced the most inhibition, 98%.

Effect of viruses on PMN viability. Viability studies were done on neutrophils after 0.5 h of incubation with either virus or uninfected cell culture controls. None of the viruses which inhibited chemiluminescence had any demonstrable effect on the viability of the neutrophils (Table 2).

DISCUSSION

The observations reported here suggest that NDV, VV, HV, and possibly reovirus had an inhibitory effect on the luminol-dependent chemiluminescence of human neutrophils. NDV had the most significant effect, inhibiting chemiluminescence by 98%. The mechanism(s) underlying the NDV-induced inhibition of PMN chemiluminescence cannot be explained on the basis of the present data. For example, NDV did

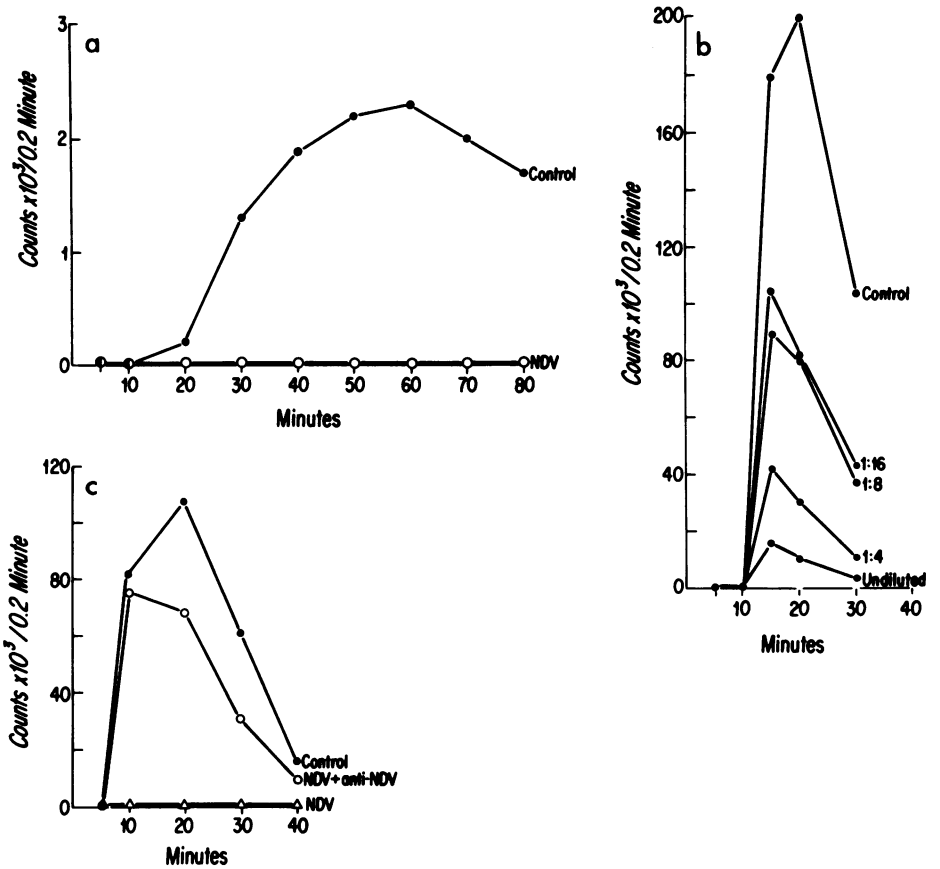

FIG. 6. *(a) Luminol-dependent chemiluminescent response of neutrophils treated with NDV compared to allantoic fluid control. (b) Effect of decreasing MOI on luminol-dependent chemiluminescence with NDV. (c) Effect of NDV-specific antisera on NDV inhibition of luminol-dependent chemiluminescence.*

TABLE 1. *Relationship of MOI to inhibition of chemiluminescence*

Virus	MOI	% Inhibition
NDV	200	98
HV	100	65
VV	5.5	46
Reovirus	210	29
EMCV	450	0
VSV	70	0

TABLE 2. *Percent viability of PMN after 0.5 h of incubation with virus or control media*

Virus	Control media	Virus
NDV	97	90
HV	92	93
VV	95	91
Reovirus	98	96

not affect PMN viability or phagocytosis as measured by microscopic examination of methylene blue-stained smears of PMN and zymosan. However, it is possible that a more sensitive technique for evaluating phagocytosis might have detected some degree of inhibition. The presence of relatively good phagocytosis would suggest that NDV interfered with another step in oxidative metabolism. It also became obvious that heat-inactivated NDV was unable to inhibit chemiluminescence. The possibility that a nonspecific metabolic product(s) formed in allantoic fluid during propagation of the virus pool could inhibit chemiluminescence was excluded by the experiments in which NDV-specific antibody neutralized the effect of the virus. Studies from other laboratories have shown that NDV can interfere with the homing mechanisms of lymphocytes via a surface antigen, the neuraminidase (14). This enzyme is believed to destroy cellular surface components necessary for migration (13, 14). The neuraminidase of bacterial origin has also been shown to inhibit superoxide production when measured enzymatically, while not affecting phagocytosis (12). It seems reason-

able to hypothesize that NDV neuraminidase might function similarly to inhibit superoxide generation as measured with chemiluminescence. Further studies are needed to test this possibility.

The mechanism(s) of chemiluminescence inhibition of HVH, VV, and reovirus cannot be attributed to neuraminidase since they lack the enzyme. However, like NDV, these viruses did not affect viability of the neutrophils or interfere with phagocytosis. Additional studies have suggested that HV also depresses neutrophil mobility (10). Studies with reovirus infections in mice further suggest that reovirus may interfere with PMN function and increase the susceptibility of the mice to *Staphylococcus aureus* infection (7). Although these observations imply that HV, VV, and reovirus interfere with neutrophil function as measured by several different techniques, the precise mechanism of interference remains to be defined.

This study also demonstrates that luminol-dependent chemiluminescence may provide a sensitive technique to study the effects of viruses on neutrophil function. Its greatest advantage is in the small number of neutrophils required. However, the assay system is also subject to a variety of factors that need to be carefully controlled in the application of this test.

ACKNOWLEDGMENTS

These studies were supported in part by Public Health Service research grants from the National Institute of Arthritis, Metabolism, and Digestive Diseases (AM 17050) and the National Institute of Child Health and Human Development (HD 10088), and by grant RR-05493 from the Biomedical Research Support Grant Program, Division of Research Resources, National Institutes of Health.

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