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Pattern of Formylmethionyl-Leucyl-Phenylalanine-Induced Luminol- and Lucigenin-Dependent Chemiluminescence in Human Neutrophils

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The stimulation of neutrophils by formylmethionyl-leucyl-phenylalanine results in a bimodal luminol-dependent chemiluminescence pattern. We observed, however, only a single peak chemiluminescence pattern in the response to the peptide when we used lucigenin as an amplifying substance. We suggest that lucigenin, the larger molecule, (510 daltons; luminol is 177 daltons) only exerts an extracellular effect.

When polymorphonuclear leukocytes (PMNL) and chemotactic factors interact, the cells produce chemiluminescence (CL) linked to activation of the oxidative metabolism (11). It has been shown by many investigators that addition of luminol to PMNL can amplify the CL response (2, 14). Previous studies have shown that PMNL stimulated with chemotactic factors exhibit a bimodal pattern of luminol-enhanced CL (7, 15). Another compound, lucigenin, has also proved to amplify the CL response in PMNL (1, 16), and the aim of the present study was to compare the luminol- and the lucigenin-dependent CLs emitted from PMNL in response to a chemoattractant.

PMNL were obtained from human blood-EDTA by the method of Bøyum (4). Residual erythrocytes were removed by hypotonic lysis, and the PMNL were washed and suspended to 10^7/ml in Krebs-Ringer phosphate buffer (KRG). Luminol, lucigenin, and the chemotactic peptide formylmethionyl-leucyl-phenylalanine (fMLP) were obtained from Sigma Chemical Co., St. Louis, Mo. CL was measured at ambient room temperature in a modified luminometer (LKB, Stockholm, Sweden). Samples for CL were obtained by adding 0.1 ml of PMNL suspension to disposable 4-ml polypropylene tubes containing 0.4 ml of Krebs-Ringer phosphate buffer KRG with luminol or lucigenin. The tubes were placed in the luminometer and allowed to stand until a stable background was obtained (less than 5 min). To activate the system, 0.1 ml of fMLP (10^{-7} M) was added. The tubes were stirred, and the light emission was recorded. Since no labeled luminol or lucigenin was available to us, we used polyethylene glycol (PEG) as a permeability marker. To assess the permeability properties, the cells were incubated with a mixture of PEG 400 (2% [wt/vol]) and PEG 1000 (5% [wt/vol]). After 6 h at 4°C, the PMNL were washed, freeze-thawed five times, mixed with trichloroacetic acid (50%) for 10 min at room temperature, and centrifuged for 10 min (1,500 × *g*). Supernatant (5 ml) was extracted with 5 ml of chloroform, dried under N_2, and reconstituted with 5 ml of distilled H_2O. The sample was then treated as described previously for extraction of PEGs from urine (10) with a high-pressure liquid chromatograph (reversed phase, Nucleosil C_8 column) for separation and a refractive index interferometer (HSRI 931; Tecator, Sweden) for quantification of PEG molecular weight species.

When PMNL were exposed to fMLP in the presence of luminol, the cells responded and produced CL. When the time course of CL emission was studied, an initial peak was found within 2 min, and a second peak was found ca. 10 min after stimulus addition (Fig. 1). In a CL response, luminol, which is a rather small molecule (177.2 daltons), acts as a bystander substrate for the oxidative species generated during activation of the PMNL. From CL data obtained with cells from patients with myeloperoxidase (MPO) deficiencies, it could be concluded that the luminol-dependent CL is totally dependent on the MPO-H_2O_2 system (7, 9, 13). The presence of MPO in the extracellular fluid is, however, not sufficient to obtain a normal CL response from MPO-deficient PMNL, despite a pronounced $H_2O_2^-$ production (7). Thus, the reaction leading to luminol-dependent light emission from PMNL takes place both intra- and extracellularly, and it has been shown that the first peak of the fMLP-induced response in normal PMNL is a result of extracellular reactions and the second peak is a result of intracellular reactions of the MPO-H_2O_2 system (3, 5).

When PMNL were exposed to fMLP in the presence of lucigenin, the cells responded and produced CL. When the time course of CL emission was studied, only one peak was found (Fig. 2) corresponding to the initial extracellular peak of the luminol-dependent CL. The mechanisms for CL, as recognized by the use of luminol and lucigenin, could differ, and the lack of a second peak with lucigenin might reflect different pathways of amplification. Lucigenin, which is a larger molecule (510.5 daltons) than luminol, has also been proposed to amplify the CL response by an MPO-independent mechanism, probably related directly to the PMNL production of O_2^- (1, 16). This could, however, not explain the difference in time course obtained in lucigenin and luminol systems, since most of the cellularly produced H_2O_2 (measured in luminol-dependent systems) has O_2^- (measured in lucigenin-dependent systems) as its precursor and is formed by spontaneous dismutation of O_2^- (12).

The appearance of a bimodal response is not a result of a slow diffusion of luminol into the cells, since the second peak is reached after ca. 10 min even when extracellular luminol is removed before stimulus addition (3). But to detect a CL response of intracellular origin, the amplifying substance has to get into the cells. From the permeability data obtained with the mixture of PEG 400 and PEG 1000, it could be concluded that molecules larger than 370 daltons are excluded from the cells (Fig. 3). The experiment with

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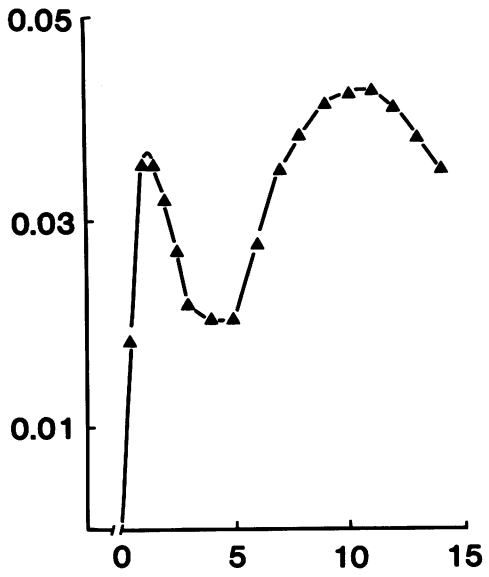

FIG. 1. Time trace of chemiluminescence emitted from PMNL in the presence of luminol (10^{-5} M), when exposed to fMLP. Abscissa, time of study (min); ordinate, chemiluminescence (mV).

PEGs was done at 4°C to distinguish permeability properties of the PMNL membrane from other processes, e.g., pinocytosis, that could have resulted in transfer of PEG molecules into the cells. Since bulk movement of extracellular fluid (pinocytosis) into PMNL does not occur to any appreciable extent (6, 8), pinocytic activity was probably not very important even at room temperature. Furthermore, bulk movement of fluid would result in equal uptake of luminol and lucigenin and, therefore, similar PMNL CL response. The explanation for the difference between luminol and lucigenin systems could thus be that the cells are almost impermeable to lucigenin. The lack of a second peak in the lucigenin-dependent PMNL response could thus be due to

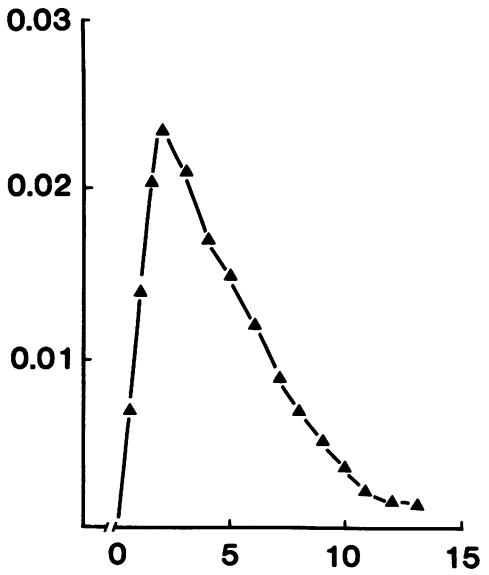

FIG. 2. Time trace of chemiluminescence emitted from PMNL in the presence of lucigenin (2×10^{-4} M), when exposed to fMLP. Abscissa, time of study (min); ordinate, chemiluminescence (mV).

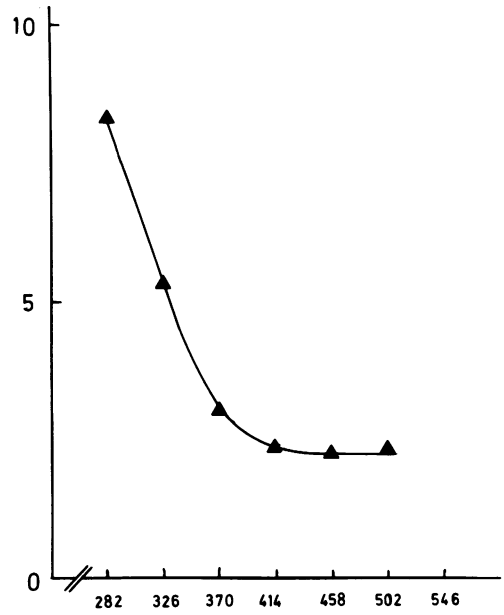

FIG. 3. Permeation of differently sized PEGs (PEG 400 and PEG 1000; 282 to 1250 daltons) into PMNL. Abscissa, size of PEG (daltons); ordinate, percent recovery of PEG molecules per 10 PMNL.

an unavailability of lucigenin intracellularly, although different pathways of amplification rather than nonentry of lucigenin could not be excluded. As judged from experiments with different concentrations of luminol in which the magnitude but not the timing of the second peak was increased as a result of an increased luminol concentration, the magnitude of the second peak is probably limited not only by the generation of H_2O_2 or the amount of available MPO but also by the diffusion of luminol into the cells (5). The lack of a second peak in the lucigenin-dependent response, however, could not be overcome by an increase of the lucigenin concentration. We measured the response in systems with lucigenin concentrations from 10^{-6} to 10^{-3} M without observing any appearance of a bimodal response.

In summary, measurement of CL could be a valuable and simple tool in studying generation of oxidative metabolites in inflammatory cells. Both luminol and lucigenin could be used to amplify the response, but the findings we describe suggest that with lucigenin, only extracellularly located light-generating events are measured, whereas with luminol, both intra- and extracellular events are measured.

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