Lucigenin-Induced Chemiluminescence in Human Neutrophils in the Process of Adherence and Chemotactic Migration Measured in a Modified Boyden Chamber System

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KATO, T., TERUI, T. and TAGAMI, H. Lucigenin-Induced Chemiluminescence in Human Neutrophils in the Process of Adherence and Chemotactic Migration Measured in a Modified Boyden Chamber System. Tohoku J. exp. Med., 1987, 151 (4), 409-417 — To answer the question of whether an oxidative burst in neutrophils occurs in the process of adherence and chemotactic migration, we estimated their lucigenin-induced chemiluminescence (CL) responses in a specially devised Boyden chamber that utilized a vial for CL measurement. Neutrophils placed in the upper compartment immediately after isolation induced lucigenin-dependent CL even in the absence of chemotactic factors in the lower chamber, which was completely scavenged by superoxide dismutase at a concentration of 100 μg/ml. This response was suggested to be caused by neutrophils in the process of adherence to a filter. When formyl peptide (FMLP) or zymosan-activated serum (ZAS) was placed in the bottom compartment, they were effective to induce CL in the neutrophils already adhered to the membrane filter. These chemotactic factors induced light emission in neutrophils maximally at the concentration of 10^{-7} M for FMLP and 5% for ZAS in the reaction mixture without any more increase in CL being observed above these concentrations. This makes a sharp contrast to the findings observed in the so far used unphysiological experimental system, in which neutrophils are abruptly exposed to chemotactic factors likewise in present study. When FMLP or ZAS was directly added to the cells in an upper compartment, CL increased dose-dependently from 10^{-9} to 10^{-5} M for FMLP, and from 5 to 50% for ZAS. These findings suggest that our method is potentially useful because it enables to assess the respiratory burst of neutrophils in the process of adherence and in that of chemotaxis in a way that closely simulates the in vivo situations. Furthermore, we can expect that they become a hopeful simple and rapid alternative for the tedious conventional assays for cell adherence and chemotaxis.

— lucigenin; chemiluminescence; cell adherence; chemotaxis

Among the methods for monitoring oxygen activation of neutrophils chemiluminescence (CL) measurements appear to be one of the most simple and rapid assay (Fischer et al. 1983). The intensity of emitted photons is generally low; it can, however, be amplified by several orders of magnitude, when

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luminogenic substances such as luminol (Allen and Loose 1976), lucigenin (Allen 1981), or Cypridina luciferin analog (Sugioka et al. 1986) are added to cell suspension. Luminol is well known to be oxidized to aminophthalic dianion which in turn emits a light, and superoxide and myeloperoxidase-\( \text{H}_2\text{O}_2\)-halide system are involved in luminol-dependent CL, whilst the generation of superoxide and probably also the production of \( \text{H}_2\text{O}_2 \) participate lucigenin-dependent CL.

The adherence of neutrophils to the wall of blood vessels with subsequent chemotactic migration toward chemotactic factors is an initial cellular response in various acute inflammatory changes. Besides attracting neutrophils, chemotactic factors exert numerous effects on the metabolic and secretory functions of neutrophils, such as the formation of reactive oxygen species and the release of lysosomal enzymes, both of which cause damaging effects on the surrounding tissues (English and Lukens 1983). Therefore, most neutrophils found at the site of inflammation are presumed to be in a highly activated state as a result of the exposure to chemotactic factors. A premature activation of oxidative metabolism in neutrophils has been thought to start only after they have moved up a certain gradient of chemotactic factors (Wilkinson 1983). However, in the present study we offer evidence that CL is measurable in neutrophils even during chemotactic migration with our newly developed method using a specially devised Boyden chamber that simulates the in vivo situations more closely than the conventional experimental system. Additionally we have obtained evidence that CL is also measurable even in the neutrophils adhering to a polycarbonate filter of the Boyden chamber. The availability of this Boyden chamber system was discussed as a useful tool for the future study in this field.

**Materials and Methods**

**Chemical media.** Dulbecco’s modified Eagle’s medium (Flow Lab., Tokyo) containing 25 mM HEPES at pH 7.4 (CL medium) was used for the dilution of samples and chemicals. Lucigenin (Sigma, St. Louis, MO, USA) was dissolved in phosphate buffered saline (PBS) at a concentration of 5.1 mg/ml, supplemented with 8 μl of triethylamine (Tokyo Kasei, Tokyo) per ml. Formyl-methionyl-leucyl-phenylalanine (FMLP), zymosan, superoxide dismutase (SOD) and catalase were also obtained from Sigma, St. Louis, USA. FMLP was dissolved in 1N NaOH at a concentration of \( 10^{-4} \) M and diluted in CL medium.

**Preparation of neutrophils.** Fresh heparinized venous blood (20 IU/ml blood) of healthy donors (3.5 ml) was carefully overlaid on 3 ml of Ficoll-Isopaque solution (Mono-poly resolving medium, Flow Lab., Tokyo) and centrifuged at 300 g for 30 min at room temperature. The layer of neutrophils was washed once in CL medium, and cell suspension in CL medium supplemented with 5% of autologous plasma was adjusted at 2 × 10⁶ per ml.

**A modified Boyden chamber.** A siliconized or nonsiliconized glass tube (4 mm in diameter, 20 mm in length) was used as an upper compartment of modified Boyden chamber. Its bottom surface was attached with a non-siliconized 3.0 μm pore sized polycarbonate filter (Bio-Rad Lab., Tokyo) using Eukit, a mounting reagent (O. Kindler, Freiburg, FRG). In some experiments a siliconized larger glass tube (6 mm in diameter, 20 mm in length) was used as an upper compartment. A vial utilized for CL measurement (10 mm in diameter, 4.8 mm in length) itself was used as a lower compartment of Boyden chamber (Fig. 1).

**Preparation of zymosan-activated serum (ZAS) and opsonized zymosan.** 10 mg of
zymosan was incubated in 1 ml of normal fresh serum at 37°C for 30 min, and centrifuged at 300 g for 10 min. Supernatant was promptly inactivated by heating at 56°C for 30 min and this was used as ZAS. Sediment was washed once and resuspended in CL medium at the concentration of 50 mg/ml as an opsonized zymosan.

**CL assay.** For the measurement of CL, a Biolumat (LB9500, Berthold, Wildbad, FRG) was used. A round-bottomed vial containing an upper cellular compartment inside was placed into a Biolumat chamber at 37°C, and CL was continuously monitored using a computer (PC8801 mk II SR, NEC, Tokyo). Peak intensity was used as an index of CL.

**CL measurement in adhering neutrophils.** Prior to measurement of CL a cell suspension was mixed with 20 µl per ml of lucigenin. 200 µl of cell suspension containing 4 x 10⁵ cells was placed in a glass tube with the polycarbonate filter, which was placed in a vial containing 200 µl of medium for the measurement of CL.

**CL of chemotactically locomoting neutrophils.** A top compartment containing a cell suspension (4 x 10⁵ cells/200 µl) was incubated at 37°C for 40 min for the adherence of the cells to the filter. Thereafter it was placed into a vial containing various concentrations of chemoattractants and CL was measured in CL analyser.

**CL of neutrophils with the direct stimulation of chemotactic factors.** CL was also measured after the direct addition of chemotactic factors to the top compartment. A top compartment containing a cell suspension (4 x 10⁵ cells/100 µl) was incubated at 37°C for 40 min. Thereafter, various concentrations of chemotactic factors were added to cell suspension in a final volume of 200 µl and the upper chamber was placed into a vial containing 200 µl of CL medium.

**RESULTS**

**CL of neutrophils during the process of cell adherence.** CL was evident promptly after the setting of the modified Boyden chamber into Biolumat and it reached a maximum after 15 to 20 min. When the diameter of the siliconized glass tube was increased to 6 mm, CL response increase approximately twice as compared with that obtained with the 4-mm-diameter siliconized tube. Their peak intensities in 4-mm- and 6-mm-diameter-tube were 11.66 ± 1.41 x 10⁴ cpm and 19.65 ± 5.10 x 10⁴ cpm per 10⁵ cells, respectively.

The CL responses were almost the same whether measurement was done in the
Fig. 2. CL response curve of neutrophils in the process of adherence. There is no significant difference in response between non-siliconized glass tube (•—•) and siliconized one (■—■) as long as they were equipped with non-siliconized polycarbonate filter. When a siliconized tube of which bottom surface was attached with a siliconized polycarbonate filter was used, CL dramatically decreased (○—○). CL measurement of neutrophils under the presence of non-siliconized tube without any filter induced only slight light emission (▲—▲).

Fig. 3. CL response curve of neutrophils adhering to the polycarbonate filter (●—●). SOD almost completely suppressed the response at concentration of 100 µg/ml (▲—▲), while catalase scavenged about 50% of CL at a concentration of 100 µg/ml (■—■).
siliconized glass tubes or in non-siliconized ones as long as they were equipped with a non-siliconized filter. However, the response decreased dramatically when a siliconized filter was used instead of non-siliconized one (Fig. 2). There was only slight CL of neutrophils when a non-siliconized glass tube without any attached filter was simply placed in a CL vial (Fig. 2.)

When SOD was added at a concentration of 100 $\mu$g/ml, CL was suppressed completely, whereas catalase at a concentration of 100 $\mu$g/ml only partially decreased the CL response of adhering neutrophils (Fig. 3).

CL responses in chemotactically migrating neutrophils. When FMLP was placed in the lower compartment, it distinctively induced CL in the neutrophils already adhered to a filter. CL reached a maximum within 15 min under the

![CL response curve to FMLP](image)

Fig. 4. CL response curve of neutrophils in the process of chemotactic migration to FMLP.

![CL responses under the stimulation of FMLP](image)

Fig. 5. CL responses of neutrophils under the stimulation of FMLP. CL responses in the presence of FMLP placed in the lower compartment (●—●) or in the upper compartment (■—■). a, number of determinations = 14; b, number of determinations = 3; c, number of determinations = 7; bar, standard deviation.
stimulation of chemotactic factors for all the concentrations examined (Fig. 4). The maximum CL responses was observed at the concentration of $10^{-7}$ M of FMLP, and CL decreased somewhat at higher concentrations of FMLP (Fig. 5). When remaining non-adherent cells were removed beforehand by washing of top compartment with medium, similar results were observed. CL responses under the chemotactic stimulation of ZAS were similar to those with FMLP (Fig. 6). Addition of SOD and catalase also suppressed these CL responses (Fig. 7).

Even neutrophils that adhered to the filter after simple preincubation for 40 min induced CL slightly without the stimulation of FMLP (Fig. 5), but its response was significantly lower than that observed in the presence of the chemotactic factors.

Fig. 6. CL responses of neutrophils under the stimulation of ZAS. CL response in the presence of ZAS in the lower compartment (number of determinations = 8). bar, standard deviation.

Fig. 7. Influence of SOD and catalase on CL of neutrophils chemotactically migrating to ZAS.
Opsonized zymosan placed in the lower compartment did not induce CL (data not shown). When a siliconized filter was used instead of a conventional non-siliconized one in the same experimental system, there was no measurable response even under the stimulation of the chemoattractants (data not shown).

When FMLP was added to the cell suspension in the upper compartment, CL responses increased dose-dependently in the concentration range from $10^{-8}$ to $10^{-5}$ M (Fig. 5). The effect of direct addition of ZAS on neutrophils was similar to that with FMLP. Each peak intensity with the stimulation of ZAS at the concentrations of 2%, 5%, 20%, and 40% in reaction mixture was 81.70, 168.48, 248.70, and 374.50 cpm $\times 10^{-3} \times 10^{-5}$ neutrophils, respectively.

**DISCUSSION**

The micropore filter system using a Boyden chamber is regarded as an in vitro model closely simulating the in vivo cell migration toward the inflammatory site. However, the oxidative metabolism of neutrophils has never been studied in this system due to technical difficulty. We have overcome this difficulty by devising a specially modified chamber system to study the oxidative metabolic activity of neutrophils in the situations closely simulating those occurring in vivo.

The present studies demonstrated that simple placement of neutrophils alone in the top compartment of the Boyden chamber induced lucigenin-dependent CL spontaneously. This response was evidently greater than that noted in the system in which neutrophils were placed in a top compartment that consisted of only a glass tube without any attached filter, suggesting that its spontaneous CL in neutrophils is induced in the process of adherence to the polycarbonate filter. This is further corroborated by the finding that such CL decreased greatly by prior siliconization of the filter. It is well known that neutrophils generate superoxide radical during adherence to glass surface (Yanai and Quie 1981), Milipore-filter (Dahinden and Fehr 1983) and nylon fiber (Clifford et al. 1984). Although Yanai and Quie reported that CL of neutrophils adhering to the surface of glass vial was suppressed with the addition of protein such as gelatin, human and fetal calf serum, and albumin, we could not find any effects of plasma on the spontaneous CL of adhering neutrophils to a non-siliconized polycarbonate filter. A similarity between neutrophil adherence to nylon fiber and that to monolayers of endothelium in tissue culture in the presence of plasma was demonstrated by MacGregor et al. (1978). Thus, nylon fiber has been generally used for the measurement of adherence (Rasp et al. 1981; Clifford et al. 1984). Our present study suggests the availability of the polycarbonate filter for such analytical study of neutrophil adherence to endothelium.

In our previous studies we found that both FMLP and C5a were maximally active chemotactically at the concentration of $10^{-7}$ M, and that its activity diminished at concentrations above or below this concentration (Kato et al. 1986; Terui et al. 1986). Such maximum leukocyte chemotaxis has been noted to occur
at a much lower concentration than that causes a prominent respiratory burst (Wilkinson 1983). We also observed in the present study that CL in neutrophils increased dose-dependently, when FMLP or ZAS was directly mixed with neutrophils in the upper compartment. Therefore, it has been thought that the locomotor response is more sensitive to stimulation by chemotactic peptide than the metabolic response and that both processes to be dissociable (Becker et al. 1979). However, such an assumption has been made based on the observations performed in the in vitro system quite unphysiological from the view point of the in vivo situation, i.e. sudden direct exposure to high concentrations of chemotactic factors without any previous exposure to a gradually increasing gradient of chemotactic factors. Our present results demonstrated for the first time that the oxidative metabolism that can be evaluated by lucigenin-dependent CL takes place in the neutrophils in the process of maximum chemotactic locomotion. Moreover, it is noteworthy that we found no further increase in CL response above the optimal concentration for chemotaxis, although a distinctive desensitization was not noted.

We think that the CL measurement performed in such a fashion can take the place of the so far used time-consuming and tedious conventional chemotactic assay as a simple and rapid alternative.

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