Cell adhesion markedly increases lucigenin-enhanced chemiluminescence of the phagocyte NADPH oxidase

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Abstract

Lucigenin-enhanced chemiluminescence (LECL) is widely used for the detection of reactive oxygen species released from various cells and mitochondria. However, the LECL response varies depending on cell species and assay conditions at least in part by unknown factors. Here we report that cell adhesion is an important factor for increasing LECL of tetradecanoylphorbol acetate (TPA)-stimulated human neutrophils. More than 90% LECL remained even after complete removal of the cell suspension 10 min after TPA stimulation, and ~22.5% of neutrophils were adhered to the reaction tube. These results indicate that LECL by an adhering neutrophil is ~45X higher than that by a non-adhering neutrophil. LECL by leukocyte adhesion deficiency neutrophils was one-fifth of that by normal neutrophils and completely disappeared when the cell suspension was removed, confirming that LECL depends highly on cell adhesion. The oxidase activity of adhering neutrophils measured after permeabilization with Renex 30 together with NADPH addition was similar to that of non-adhering neutrophils, indicating that lucigenin and cell adhesion do not enhance the oxidase activity. Based on these findings, we propose that a mixture of adhering and non-adhering neutrophils can

be used for simultaneous screenings of adhering activity and the oxidase activity of

neutrophils.

Introduction

Superoxide is produced by the phagocyte NADPH oxidase of peripheral neutrophils, eosinophils, monocytes, and B lymphocytes. When neutrophils phagocytose bacteria or are activated by various artificial stimulators such as tetradecanoylphorbol acetate (TPA) or the bacteria-derived peptide, formyl-methionyl-leucyl-phenylalanine (fMLP), they produce superoxide (Kobayashi *et al*. 1990 and 1995; Kuribayashi *et al*. 1995; Sakamoto *et al*. 2006; Minakami & Sumimoto 2006). The significance of the oxidase in host defense is exemplified by recurrent and life-threatening infections that occur in patients with chronic granulomatous disease (CGD), whose phagocytes are genetically defective in the oxidase (Kuribayashi *et al*. 1995; Roos D *et al*. 1996).

Neutrophils of patients with leukocyte adhesion deficiency (LAD), another neutorphil disorder caused by a genetic defect in CD18 (β2-integrin), have a normal amount of NADPH oxidase to produce superoxide (Nauseef *et al*. 1986). These leukocytes do not express LFA-1, CR3, and gp150/95 on the plasma membrane as CD18 is their common subunit (Springer *et al*. 1984; Graham *et al*. 1989; Arnaout 1990), and therefore, do not adhere to ligands such as ICAM-1/2 and to artificial adhesion surfaces such as plastics

(Patarroyo *et al*. 1990; Nagahata *et al*. 1995).

Superoxide produced by neutrophils can be either detected by cytochrome *c* reduction, NBT reduction, or chemiluminescence (CL) responses (Baehner & Nathan 1968; Minkenberg & Ferber 1984; Kuribayashi *et al*. 2002) or calculated from cyanide-insensitive O_2 consumptions and compound III formation of peroxidases (Makino *et al*. 1986). Lucigenin-enhanced CL (LECL) is widely used because it is one of the most sensitive and convenient methods to detect reactive oxygen species (ROS) released from various cells and mitochondria (Minkenberg & Ferber 1984; Bhunia *et al*. 1997; Caldiz *et al*. 2007). It has been shown that LECL of TPA-stimulated neutrophils is abolished by superoxide dismutase (SOD) and is not seen with CGD neutrophils (Kuribayashi *et al*. 1995). However, the exact mechanism of the lucigenin response to superoxide is unknown.

In the present study, we demonstrate that LECL of neutrophils is highly increased by adhesion, and therefore, reflects both the phagocyte NADPH oxidase activity and adhesion efficiency. Thus, conventional LECL assay using a mixture of adhering and non-adhering cells can be adopted for screening disorders presenting at least one of two

functions: adhesion and the oxidase activity.

Results

LECL is highly dependent on adhering cells

Neutrophils were suspended in CL probe-containing assay buffers, poured into plastic tubes, and stimulated by TPA 3 min later. The LECL of the tube was still more than 90% of the original activity after the cell suspension was removed by pipetting 10 min after TPA stimulation, and steeply increased for an additional 10 min reaching 4X high LECL in a fresh medium (Fig. 1A, solid line). Most of the disappeared LECL, ~10%, was recovered in a new tube containing transferred cell suspension (Fig. 1A and B, dashed line). Neither the remaining high CL nor its steep increase was observed in the initial tube when the CL probe was luminol (Fig. 1C), Diogenes (Fig. 1D), or Cypridina luciferin analog (CLA) (data not shown). Neutrophils remaining in the initial tube were microscopically observed to be adhering to the surface of the tube (data not shown). Accordingly, the high LECL remaining in the initial tube suggested that lucigenin in particular, increased either the number of adhering cells or the phagocyte NADPH oxidase activity of the adhering cells.

Lucigenin dose not increase adhering neutrophils

To clarify the adhesion efficiency by lucigenin, we counted the number of cells removed by pipetting (Table 1). The removed percentage of the cells in the presence of lucigenin (77.5 %) was the same as that in the absence of lucigenin or in the presence of other CL probes. Therefore, the high LECL remaining in the initial tube was not due to the increased population of adhering cells but to the adhesion-specific increase of LECL. The ratio of the TPA-stimulated LECL of an adhering cell to that of a non-adhering cell was 45.1 at the time of removal of non-adhering cells, which was significantly higher when compared with ~1 for other two CL probes (Table 2). This ratio reached at least 100X at 20 min after TPA addition (Fig. 1A, solid line).

Adhesion is essential for LECL

To ascertain the importance of adhesion for high LECL, two series of experiments were designed. We artificially increased adhering cell numbers by increasing the surface area for adhesion in one experiment (Fig. 2), and decreased the numbers of adhering cells using non-adhesive neutrophils derived from a CD18-deficient LAD patient (Fig. 3) in

the other. The surface area of a tube for adhesion was increased by providing either

another piece of tube bottom (Fig. 2, tube 2) or the fragments of a cracked tube (tube 3).

LECL of tube 2 was twice that of tube 1 with no plastic pieces, despite of the same

numbers of cells in the two tubes. The highest LECL of neutrophils among the three

tubes was observed in tube 3 (Fig. 2, curve 3).

In contrast, when adhering neutrophils were absent as shown by LAD cells (Fig. 3, A

(LAD) and C), the LECL of LAD neutrophils at maximal intensity was approximately

one-fifth of that of normal neutrophils (Fig. 3A), and completely disappeared from the

original assay tube after transferring the cell suspension to a second new one (Fig. 3C,

left). Essentially the same results were observed when normal neutrophils were

incubated in non-adhesive siliconized tubes (data not shown). The specific LECL

activity of a normal TPA-stimulated non-adhering neutrophil of the second tube was 1.3

x 10^{-2} cpm/cell (Fig. 3B, position indicated by open arrowhead), which was comparable

with the corresponding specific activity of LAD cells (1.5 x 10^{-2}, Fig. 3C, open

arrowhead). These data imply that non-adhering normal neutrophils and non-adhesive

LAD neutrophils exhibit similar LECL, which is consistent with the observations that

TPA-stimulated LAD neutrophils consumed and released similar amounts of oxygen

molecules and superoxide anions, respectively, as normal neutrophils (Table 3).

Lucigenin does not increase the NADPH oxidase activity of adhering neutrophils

The TPA-stimulated phagocyte NADPH oxidase would not commonly be activated by

adhesion as adhering cells exhibited no increased CL response when luminol, Diogenes,

or CLA was used as a CL probe (Fig. 1 and data not shown). Therefore, we examined

whether lucigenin in particular increased the NADPH oxidase activity of adhering

neutrophils by assaying the LECL of TPA-stimulated neutrophils completely dependent

on their oxidase activity.

Prior to the assay of the oxidase activity expressed by LECL, neutrophils were

permeabilized by Renex 30 to remove NADPH, the electron-donating substrate of the

oxidase, and NADPH was then supplemented exogenously (Fig. 4A, no 1). The LECL

which appeared after NADPH addition was exclusively dependent on the phagocyte

NADPH oxidase as no significant CL was observed when neutrophils had not been

stimulated by TPA (Fig. 4A, no 3) nor when NADH was used as an electron-donating

substrate (Fig. 4A, no 2). Furthermore, neutrophils of a CGD patient exhibited no TPA- and NADPH-dependent LECL (Fig. 4A, no 4). This NADPH oxidase activity was proportional to the number of neutrophils applied (Fig. 4B). The high LECL of adhering cells was followed by a low phagocyte NADPH oxidase activity (Fig. 4C, upper panel). In contrast, the low LECL observed in stimulated non-adhering neutrophils was followed by a high oxidase activity. These high and low NADPH oxidase activities were 7.0×10^6 and 1.9×10^6 cpm, respectively, and corresponded to the high (77.5%) and low (22.5%) populations of non-adhering and adhering neutrophils (Table 1), respectively. The actual specific NADPH oxidase activities of adhering and non-adhering cells were 21.5 ± 2.0 cpm/cell (n=3) and 24.8 ± 5.4 cpm/cell (n=3), respectively. Furthermore, the phagocyte oxidase activities reflected by LECL were consistent regardless of whether lucigenin was added before or after the cells were permeabilized by Renex 30 (Fig. 4C, lower panel). Accordingly, lucigenin did not augment the oxidase activity of adhering cells in particular. These data clearly indicated that the oxidase activity of neutrophils was not increased by either adhesion or by lucigenin. Therefore, increased LECL of adhering neutrophils is independent of the oxidase activity *per se*, but dependent on a

certain chemical process occurring under CD-18-dependent adhering situation.

Discussion

Although lucigenin is widely used to detect ROS derived from NADPH oxidase and mitochondrial membranes, the exact mechanism underlying the responses of lucigenin to ROS remained elusive. In the present study, we have shown that the LECL of the phagocyte NADPH oxidase is largely dependent on cell adhesion. When the cell suspension with luminol, Diogenes or CLA as a CL probe is removed, the CL activity is mostly disappeared as expected (Fig. 1B and C and Fig. 3C). However, LECL continues after the reaction buffer has been removed (Fig. 1A). Reaction tubes possessing larger surface areas have higher LECLs (Fig. 2), confirming that the LECL of the phagocyte NADPH oxidase is highly increased by adhesion.

The present study shows that the LECL derived from adhering neutrophils is augmented. At first, there was a possibility that LECL from cell suspension is deeply reduced through assay buffer, and with the result that LECL from adhering neutrophils becomes higher relatively. If so, artificial surroundings that LECL from adhering cells reaches photon detector through the buffer must attenuate the CL. As shown in curve 2 in Fig. 2, LECL released from cells adhering to another bottom sank in suspension

buffer is as high as that to the original one, suggesting that the CL from the additional

bottom reaches the photon detector through the suspension buffer without any

quenching. Thus, CL generated by neutrophils in suspension has not been attenuated by

an absorption due to the suspension buffer and/or cells themselves, but the LECL is

augmented by the adhesion of cells. Furthermore, most of the LECL by neutrophils

stimulated with fMLP remains after the cell suspension is removed (data not shown),

suggesting that lucigenin together with adhesion has a specific mechanism to elevate CL

regardless of the stimuli.

In this report, we have shown that lucigenin itself has a unique mechanism to enhance

CL generated by adhering cells. Microenvironments enclosed by tube, cell membranes

containing CD18 dependent adhesive molecules, and some secreted proteins might be

important for LECL by superoxide. From our previous report (Kuribayashi *et al*. 1995)

and our unpublished data, eosinophils, but not B lymphocytes, behave in the same

manner as neutrophils, suggesting that some proteins derived from granules commonly

shared by both neutrophils and eosinophils are necessary to enhance LECL, or the

ability of B lymphocytes to adhere to the tube is weak as seen with LAD neutrophils.

The exact mechanism by which lucigenin augments CL by adhering cells has been remained unknown even after several experiments. We considered two major possibilities: One was attributed to the uneven distribution of the primary products (pP) and logarithmic interaction among them, assuming LECL to be reflecting the forming velocity of the final products (fP) giving an equation ($v = d[fP]/dt = k[pP]^m$). The other was attributed to interactions of lucigenin intermediates catalyzed by lysosomal enzymes working in strict areas observable only in adherent neutrophils. Concerning to the former possibility, xanthine-xanthine oxidase system gave at most only 2 for m, which is far less than the value observed ($>32=2^5$, namely m>5). Concerning to the latter possibility, we used cyanide, azide, SOD, and mannitol as inhibitors, but failed to observe any sufficiently different effects of them between adherent and non-adherent neutrophils. Even if the precise mechanism of the dramatic increase in LECL of the phagocyte NADPH oxidase is in a black box, it can be noted that our present results have demonstrated an example exhibiting the same amount of energy source, here NADPH, can emit almost two order-high light under certain conditions.

To use the LECL assay for accurate evaluation of the phagocyte NADPH oxidase

activity, the assay should be performed under the conditions where either adhesion is

complete or adhesion is completely blocked. However, a conventional LECL assay

using a mixture of adhering and non-adhering cells can be adopted for screening

disorders such as disability of adhesion known as LAD and NADPH oxidase as CGD.

Experimental procedures

Reagents

Lucigenin (bis-*N*-methylacridinium nitrate), luminol, cytochrome *c*, SOD, fMLP and

TPA were purchased from Sigma. Lucigenin obtained from Boehringer Ingelheim was

also examined to confirm that from Sigma. CLA was obtained from Tokyo Kasei Kogyo

(Japan). Diogenes was obtained from National Diagnostics (USA).

Cell preparation

Peripheral blood was obtained from healthy volunteers, a CGD, and an LAD patient

after informed consent was obtained. Neutrophils were purified from peripheral blood

as previously described (Yamauchi *et al*. 2001). Briefly, the cells were isolated by a

combination of dextran sedimentation, Ficoll-Conray gradient centrifugation, and

hypotonic treatment, and suspended to 10^7 cells /ml in HEPES-buffered saline (120 mM

NaCl, 5 mM KCl, 5 mM glucose, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, and 17 mM HEPES pH

7.4).

CL assay

The signal of ROS was made chemiluminescent by one of the following CL probes: lucigenin (100 µM), Diogenes (1/100X dilution from stock solution according to the manufacturer's protocol), luminol (1 mM), or CLA (1 µM). For typical measurements of LECL, 5×10^5 neutrophils were incubated in 0.5 ml of HEPES-buffered saline including BSA (0.03%) and lucigenin for 3 min before TPA (200 ng/ml) addition, and CL was monitored for 30 min by an automatic luminescence analyzer LB9505 or LB9505C (Berthold, Japan). Other LECL protocols were described in figure legends.

Cytochrome c reduction and O$_2$ consumption assays

Superoxide production by neutrophils was determined by a SOD-sensitive cytochrome c reduction (Kuribayashi *et al*. 2002). Typically, 1.0×10^6 cells were suspended in 1 ml of HEPES-buffered saline containing cytochrome c (75 µM), then preincubated at 37°C for 3 min before the addition of TPA. The reduction of cytochrome c was measured using a UV-3000 (SHIMADZU, Japan), and superoxide production was calculated from the absorbance difference at 550-540 nm using an absorption coefficient of 21,000 M^{-1}.

cm^{-1}. The reduction was halted by the addition of SOD (0.1 mg/ml) to confirm the reduction to be done by superoxide. Oxygen-consuming activity was determined potentiometrically using a Clark-type electrode as described previously (Makino *et al*. 1986).

Counting neutrophils in suspension

Neutrophils (5×10^5 cells in the same tube with CL assay) were incubated in HEPES-buffered saline with or without CL probes for 3 min before TPA addition. When TPA was added to the reaction buffer, the cell number was first counted by light microscopy (PRIMO STAR, Carl Zeiss, Japan) to confirm that the numbers were not significantly different from the original ones. The suspended cells were again counted 10 min later, and the ratio of the cell numbers before and after TPA stimulation was calculated.

NADPH oxidase activity assay

This assay was performed as previously detailed with some modifications (Nakamura *et*

al. 1981; Shiibashi & Iida 2001). Briefly, 10 min after TPA stimulation with lucigenin, the neutrophils were permeabilized by Renex 30 (0.015%), and the subsequent oxidase activity with NADPH (150 µM) was monitored at 26°C.

Acknowledgements

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Figure legends

Figure 1 Effect of removal of suspension buffer from an assay tube on CL. Neutrophils

(5.0×10^5) were preincubated in 0.5 ml reaction buffer containing lucigenin (A) and (B),

luminol (C), or Diogenes (D) for 3 min prior to addition of TPA (200 ng/ml) ($\downarrow$), and

CL was monitored by LB9505C. Reaction buffer containing non-adhering neutrophils

was removed from each tube at time point 13 min indicated as solid line, and transferred

to a new tube indicated as dashed line. New buffer containing CL probe and TPA was

poured into former each tube, and the CL was further indicated as solid line. Note

enlargement of (A) was indicated in (B) to clearly show the difference of LECL around

time point 13 min.

Figure 2 Effect of adherent area on CL. A suspension of neutrophils (3.6×10^5) with

lucigenin stimulated by TPA was divided equally into three tubes, and CL was

monitored by LB9505. Superoxide production by the original tube was detected in tube

1. To provide additional surface area for neutrophil adhesion, tube 2 contained another

bottom which was removed at the indicated time ($\blacktriangledown$). Tube 3 contained broken

fragments made of an identical tube.

Figure 3 Comparison of LECL by control or LAD neutrophils. (A) Normal (Cont) or LAD neutrophils (2.0×10^5) were stimulated by TPA at the indicated time (↓), and CL was monitored by LB9505. Normal (B) or LAD (C) neutrophils were stimulated by TPA (↓). Neutrophils in suspension were removed from the original tube at the indicated time (▼) in the left panels and placed into another tube (▽) in the right panels. Note that the vertical scales are different in (B) and (C).

Figure 4 NADPH oxidase activity assay for adhering and non-adhering neutrophils. (A) Control neutrophils (5.0×10^4) or CGD ones (no 4) were preincubated in 0.5 ml reaction buffer for 3 min prior to addition of TPA (↓) or buffer (no 3), and CL was monitored by LB9505C at 26°C. Renex 30 was added at time point 13 min followed by NADPH (150 µM) or NADH (no 2) addition. (B) Effect of cell numbers on LECL. Renex 30 was added 10 min after TPA stimulation, and LECL after NADPH addition was monitored. Note enlargement of time scale was indicated to clearly show the oxidase activity after

NADPH addition. (C) Comparison of oxidase activity between adhering and

non-adhering cells. Neutrophils (5.0 x 10^5) were stimulated by TPA (↓) at time point 3

min, and LECL was monitored. The reaction buffer containing non-adhering neutrophils

was removed from the original tube at time point 13 min indicated as open circle and

transferred to a new tube indicated as closed circle. New buffer containing CL probe

and TPA was poured into the original tube in upper panel. Renex 30 (↓) (together with

lucigenin in the lower panel) and NADPH (▲) was added into both tubes.

Table 1. Cell numbers of neutrophil suspensions 10 min after TPA stimulation

CL probe	10 min
–	76.4 ± 4.3
Luminol	81.8 ± 6.1
Diogenes	76.0 ± 5.4
Lucigenin	77.5 ± 7.3

Each value is the mean ± S.D. obtained from five assays where the number of cells represents 100 at time point 0 min of TPA addition.

Table 2. Relative CL of an adhering neutrophil compared with a non-adhering cell

defined as 1.

Luminol	1.04 ± 0.42
Diogenes	0.97 ± 0.18
Lucigenin	45.1 ± 17.1

Each value is the mean $\pm$ S.D. of (cpm for adhering cells / part of adhering cells in total

cell number) divided by (cpm for non-adhering cells / part of non-adhering cells in total

cell number) obtained from five assays. Thus, activities of a non-adhering cell were,

therefore, independently assumed to be 1 in individual conditions.

Table 3. O$_2$ consumption and cytochrome *c* reduction by TPA-stimulated normal and

LAD neutrophils

	O$_2$ consumption	Cytochrome *c* reduction
Control	4.5 ± 0.3	4.9 ± 0.4
LAD	4.4 ± 0.1	6.0 ± 0.6

Each value (nmol/min/10^6 neutrophils) is the mean ± S.D. obtained from three assays.
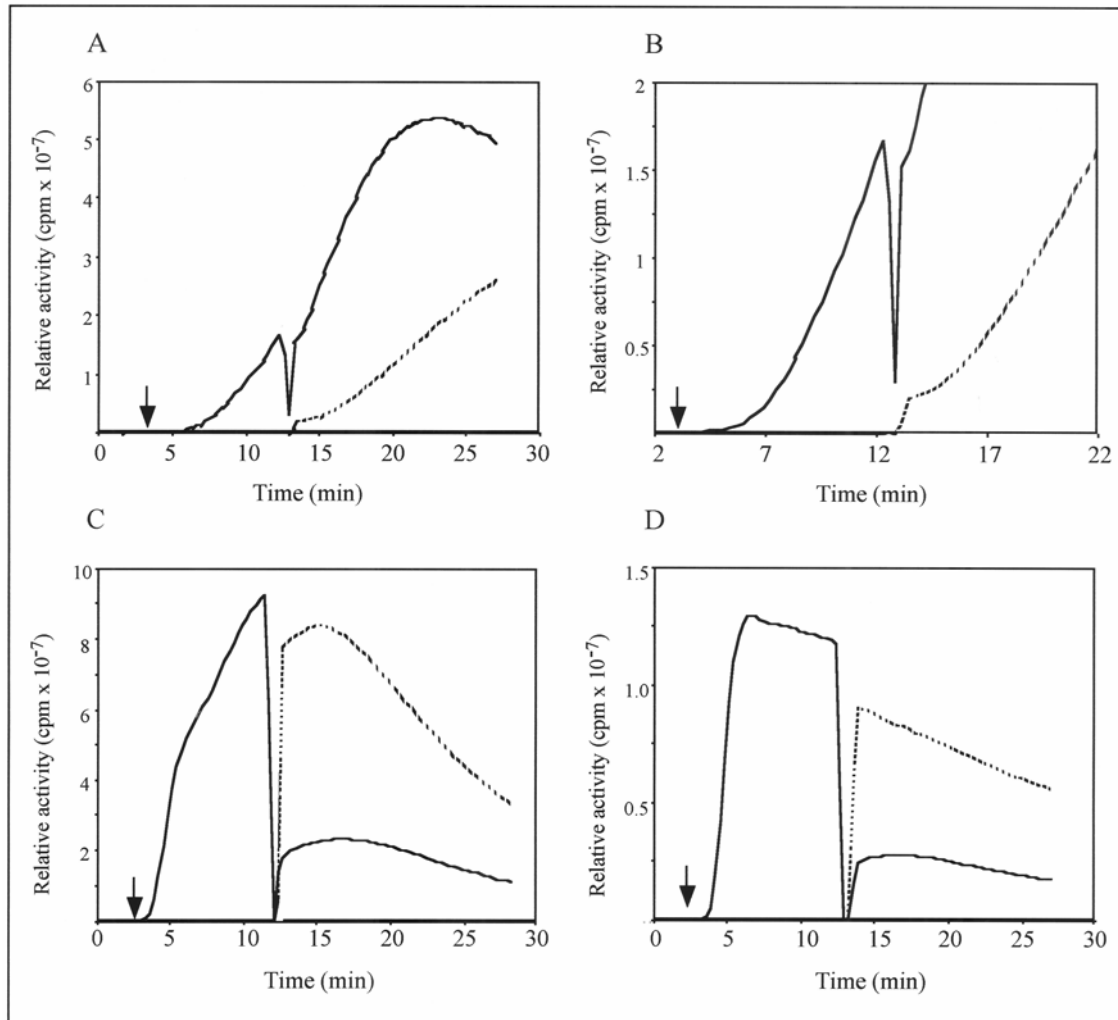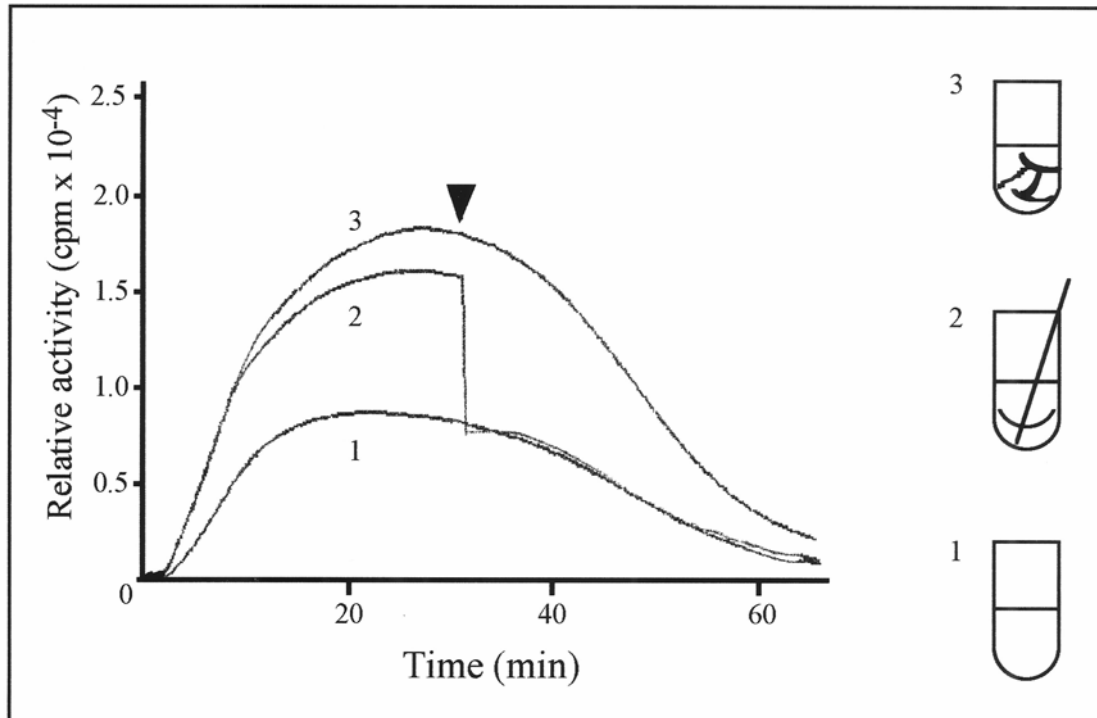
Fig.1

Fig. 2

Fig. 3

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