Chemiluminescence in neutrophils and Lettré cells induced by myxoviruses

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Luminol-mediated chemiluminescence in neutrophils is stimulated by Sendai virus and by influenza virus; Lettré cells also exhibit chemiluminescence (less than 10% of that of neutrophils), which is stimulated by Sendai virus and by influenza virus. Virally induced permeability changes are not responsible for chemiluminescence, since (i) extracellular Ca^{2+} inhibits permeability changes but stimulates chemiluminescence, and (ii) influenza virus, which induces permeability changes at pH 5.3 but not at pH 7.4, induces chemiluminescence at either pH. Other agents [zymosan, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine, 4-phorbol 12-myristate 13-acetate (phorbol ester), A23187] likewise induce chemiluminescence in the absence of permeability changes.

The interaction of viruses with leucocytes is interesting because a primary exposure to influenza (Abramson *et al.*, 1982*a,b*) or other (Faden *et al.*, 1979) viruses might impair the ability of leucocytes to destroy a subsequent bacterial challenge; such impairment would explain the establishment of a secondary bacterial infection after a primary viral infection of, for example, the respiratory tract (Larson & Blades, 1976). The chemiluminescent response initiated by Sendai virus (Peterhans, 1979, 1980) has been suggested (Peterhans *et al.*, 1983; Kolbuch-Braddon *et al.*, 1984) to be due to the increased permeability of cells to ions and low- M_r compounds known to be induced when Sendai virus fuses with susceptible cells (Fuchs & Giberan, 1973; Pasternak & Micklem, 1973; Okada *et al.*, 1975). On the other hand Ca^{2+} , which blocks permeability changes (Pasternak & Micklem, 1974; Impraim *et al.*, 1979, 1980; Masuda & Goshima, 1980; Micklem & Pasternak, 1982), stimulates virally induced chemiluminescence (Semadeni *et al.*, 1984). In order to resolve this apparent paradox, we have measured the effects of Sendai virus on chemiluminescence and permeability changes simultaneously, in the presence and absence of Ca^{2+} ; since influenza virus at pH 5.3 induces permeability changes similar to those induced by Sendai virus at pH 7.4 (Patel &

Pasternak, 1983, 1985), we also examined the effects of influenza virus (at pH 5.5 and 7.4). Because permeability changes have been best characterized in Lettré cells (reviewed by Poste & Pasternak, 1978; Pasternak & Micklem, 1981; Pasternak, 1984), we tested these cells for the chemiluminescent response and found them to be active. Our results with neutrophils and Lettré cells are consistent in showing that the induction of chemiluminescence does not depend on non-specific permeability changes.

Methods and materials

Sendai virus ('3-day'; late-harvest variety), influenza virus (strain X47) (Patel & Pasternak, 1983) and Lettré cells (Bashford *et al.*, 1983) were prepared as previously described. Zymosan A from *Saccharomyces cerevisiae* (Sigma Chemical Co.) was boiled for 10 min and then opsonized by the method of Faden *et al.* (1979). The chemotactic peptide fMet-Leu-Phe, 4-phorbol 12-myristate 13-acetate (phorbol ester) and the Ca^{2+} ionophore A23187 were obtained from Sigma Chemical Co.

Neutrophils were prepared from fresh human 'buffy-coat' material by the method of Segal *et al.* (1980). All steps were carried out at room temperature unless indicated otherwise. A 100 ml sample of buffy coat was mixed with 100 ml of phosphate-buffered saline (137 mM-NaCl/8 mM- Na_2HPO_4 /2.7 mM-KCl/1.5 mM- KH_2PO_4 , pH 7.4) containing dextran (Sigma; 1%) and heparin (Sigma; 500

Abbreviations used: fMet-Leu-Phe, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; phorbol ester, 4-phorbol 12-myristate 13-acetate; HAU, heamagglutinating unit(s).

units) and red cells allowed to sediment for 45 min. The leucocyte-enriched supernatant was diluted with an equal volume of phosphate-buffered saline and centrifuged for 5 min at 600g. The pellet was suspended in Hepes-buffered saline (150 mM-NaCl/5 mM-KCl/5 mM-Hepes/1 mM-MgCl₂, pH 7.4) containing 0.1 mM-EGTA and layered over Ficoll/Hypaque (Pharmacia) and centrifuged for 20 min at 1000g at 37°C. The pellet was suspended in 20 ml of distilled water in order to lyse residual red cells and immediately (within approx. 10 s) mixed with 20 ml of 1.8% (w/v) saline. The suspension was centrifuged for 5 min at 250g, washed in medium 199 (Flow Laboratories) and suspended (at approx. 3×10^8 – 5×10^8 cells/ml) in medium 199 and kept at room temperature.

Chemiluminescence was measured by diluting cells into 3 ml of buffer consisting of 150 mM-NaCl, 5 mM-KCl, 1 mM-MgCl₂, 5 mM-glucose, 5 mM-Hepes, pH 7.4 or pH 5.5, and 0.2 μ M-luminol in scintillation vials. The final cell density was 10^5 – 10^7 neutrophils/ml or 2×10^6 – 6×10^6 Lettré cells/ml. Neutrophils and macrophages were identified in cell preparations by conventional microscopy after staining with Giemsa/Jenner, Leischmann or haematoxylin/eosin. A typical preparation of Lettré cells contained approx. 0.03% neutrophils and <0.02% macrophages. Vials were kept at 37°C in the dark for at least 1 h before addition of virus or other agent, and then transferred to an LKB liquid-scintillation counter operating with the coincidence selectors removed. As soon as the chemiluminescence had been recorded, vials were re-placed at 37°C. Under the conditions used, neither neutrophils nor Lettré cells showed appreciable endogenous (or stimulated) response in the absence of luminol: thus neutrophils (2×10^5 /ml) gave 80 c.p.m. without luminol and 10000 c.p.m. with luminol; in the presence of Sendai virus (15 HAU/ml) these values were 100 c.p.m. and 900000 c.p.m. respectively. Lettré cells (6×10^6 /ml) gave 70 c.p.m. without luminol and 1900 c.p.m. with luminol; in the presence of Sendai virus (15 HAU/ml), these values were 80 c.p.m. and 7000 c.p.m. respectively.

Intracellular Na⁺ and K⁺ were measured as previously described (Bashford *et al.*, 1983); an increase in the ratio of Na⁺ to K⁺ was taken to indicate increased permeability. Measurement of ⁸⁶Rb⁺ or ³H loss from cells prelabelled with ⁸⁶Rb⁺ or [³H]choline [which is converted into phospho-[³H]choline, which leaks out only of virally affected cells (Pasternak & Micklem, 1973; Poste & Pasternak, 1978; Impraime *et al.*, 1980) and is a useful measure of the creation of an approx. 1 nm pore (Wyke *et al.*, 1980)] confirmed that ion changes provide a reasonable measure of virally induced permeability changes. Lactate dehydro-

genase was measured by the method described by Varley (1969).

Results

Experiments with neutrophils

A typical response to the addition of Sendai virus is illustrated in Fig. 1 (left-hand panels): an increase in chemiluminescence is induced at pH 7.4, but not at pH 5.5. In contrast, influenza virus induces a response at pH 7.4 or pH 5.5. The possibility that viruses induce chemiluminescence extracellularly, from H₂O₂ produced by cells, was examined. First, chemiluminescence in the absence of cells was measured: H₂O₂ (100 μ M) plus luminol gave 13000 c.p.m.; in the presence of Sendai virus (15 HAU/ml) or foetal-calf serum (1%) this was 20000 c.p.m. and >800000 c.p.m. respectively. The addition of catalase (1 μ g/ml) reduced chemiluminescence in the absence or presence of Sendai virus to 140 and 600 c.p.m. respectively within 30 min. In the presence of Lettré cells, luminol +/– catalase gave 350 c.p.m.; the addition of Sendai virus +/– catalase increased this to 2500 c.p.m.; the addition of 1% foetal-calf serum gave 350 c.p.m. The addition of peroxidase (which increases H₂O₂-stimulated luminol chemiluminescence in the absence of cells 1000-fold) gave 310 and 2600 c.p.m. in the absence and presence of Sendai virus respectively. We conclude that the effects of Sendai or influenza virus on luminol-stimulated chemiluminescence in neutrophils or Lettré cells cannot be ascribed to a leakage of H₂O₂ +/– peroxidase from cells.

Sendai virus induces a permeability change at pH 7.4, but not at pH 5.5; influenza virus induces a permeability change at pH 5.5, but not at pH 7.4 (see Table 1 and Fig. 3, below). Since permeability changes are likely to be a consequence of virus–cell fusion (Wyke *et al.*, 1980), which in the case of Sendai virus occurs maximally at neutral pH but in the case of influenza occurs maximally at pH values below 6 (Huang *et al.*, 1981; Lenard & Miller, 1981), this is the expected result.

Permeability changes in neutrophils induced by Sendai virus are inhibited by extracellular Ca²⁺ (Fig. 2, left panel). The sensitivity to Ca²⁺ is intermediate between that of Lettré cells (Fig. 2, right panel), which are more sensitive, and human erythrocytes (Bashford *et al.*, 1984), which are less sensitive. Chemiluminescence, on the other hand, is increased by extracellular Ca²⁺ (Fig. 2, left panel), as previously reported (Semadeni *et al.*, 1984).

Since the concentration of influenza virus used to elicit the chemiluminescent response shown in Fig. 1 was some 100-fold greater than that of Sendai virus, it is possible that a slight per-

Fig. 1. Stimulation of chemiluminescence by myxoviruses

Neutrophils ($10^7/\text{ml}$; left-hand panels) or Lettré cells ($7 \times 10^6/\text{ml}$; right-hand panels) were incubated at pH 7.4 (upper panels) or pH 5.5 (lower panels) and chemiluminescence measured as described in the Methods and materials section. At the times indicated by the arrow, Sendai virus (15HAU/ml; open squares), influenza virus (1200HAU/ml; open circles) or buffer (closed circles) was added to vials as shown.

Table 1. Induction of chemiluminescence in neutrophils and Lettré cells

Neutrophils ($2 \times 10^5/\text{ml}$) or Lettré cells ($6 \times 10^6/\text{ml}$) were incubated in Ca^{2+} -free medium as described in Methods and materials section for 65–80 min before addition of agent. In the case of fMet-Leu-Phe and ionophore A23187, the medium contained 2 mM-Ca^{2+} . Chemiluminescence and permeability change were measured as described in the Methods and materials section. The values given are means for two or more experiments, and are relative to the values in the absence of added agent (=100). Abbreviation: n.d., not determined.

Agent	Concn.	Chemiluminescence				Permeability change			
		Neutrophils		Lettré cells		Neutrophils		Lettré cells	
		pH 7.4	pH 5.5	pH 7.4	pH 5.5	pH 7.4	pH 5.5	pH 7.4	pH 5.5
Sendai virus	15HAU/ml	5400	<150	600	<100	250	<120	300	<120
Influenza virus	1200HAU/ml	6000	5100	300	300	<120	270	<120	200
Zymosan	0.3mg/ml	1800	<150	500	<150	<120	<120	<120	<120
fMet-Leu-Phe	6 μM	1600	<150	600	200	<120	<120	<120	<120
Phorbol ester	5 μM	1500	1100	6000	400	<120	<120	<120	<120
A23187	10 μM	1400	n.d.	600	n.d.	<120	<120	<120	<120

meability change at pH 7.5 does underly the chemiluminescent response. Fig. 3 (left panel) shows this not to be the case, in that increasing amounts of influenza virus increase chemiluminescence, but have no effect on cellular permeability. Other agents that induce a chemi-

luminescent response in neutrophils, such as zymosan, the chemotactic peptide fMet-Leu-Phe, phorbol ester and the Ca^{2+} ionophore A23187, are without effect on cellular permeability to ions (Table 1). Note that phorbol ester, unlike zymosan or fMet-Leu-Phe, is also active at pH 5.5.

Experiments with Lettré cells

Lettré cells (a cell line derived from a mouse mammary tumour; Lettré *et al.*, 1972) were found to respond to Sendai or influenza virus in the same way as neutrophils (Fig. 1, right-hand panel). The magnitude of stimulation by virus, as well as chemiluminescence in the absence of virus, is less in Lettré cells than in neutrophils. Since Lettré cells are grown intraperitoneally as ascites fluid in mice, it is possible that the observed chemiluminescence is actually due to contaminating phagocytic cells, rather than to Lettré cells themselves. Although macrophages and neutrophils account for less than 0.05% in a typical preparation, there are approx. 5% of other 'large cells' present. Treatment with serum on plastic dishes reduced this value to approx. 1.4%, with little loss of chemiluminescence response (Table 2). The suspension

before treatment contained 8% Trypan Blue-including cells, and 4% Trypan Blue-including cells after treatment. The amount of lactate dehydrogenase that leaked out of Lettré cells during 2 h of incubation was 10% (not increased by Sendai virus). From these data and from the appearance of stained preparations, it is concluded that the 'large cells' present in Lettré-cell suspensions are probably swollen Lettré cells. Since Lettré cells are easier to prepare in large numbers than neutrophils, they should prove useful for further studies of the chemiluminescence response. That chemiluminescence is not confined to circulating phagocytes has been demonstrated by the response of spleen (Peterhans, 1979, 1980; Koppel *et al.*, 1984), and thymus (Kohlbuch-Bradden *et al.*, 1984) cells, as well as platelets (Mills *et al.*, 1978), liver (Wefers *et al.*, 1984) and pancreatic islets (Asayama *et al.*, 1984).

Fig. 2. Effect of Ca^{2+} on Sendai-virus-induced chemiluminescence and permeability changes

Neutrophils ($5 \times 10^6/\text{ml}$; left-hand panel) or Lettré cells ($5 \times 10^6/\text{ml}$; right-hand panel) were incubated for 70–80 min at pH 7.4 in the presence of CaCl_2 as indicated. Sendai virus (15 HAU/ml) was added and the peak chemiluminescence (open squares) and final intracellular ion content (closed squares) during the subsequent 20 min measured. The values shown are percentages of the chemiluminescence and ion ratio (Na^+/K^+) of cells to which buffer was added instead of virus.

Fig. 3. Influenza-virus-induced chemiluminescence and permeability changes

Neutrophils ($4 \times 10^6/\text{ml}$; left-hand panel) or Lettré cells (5×10^6 ; right-hand panel) were incubated for 80–85 min at pH 7 before the addition of influenza virus as indicated. Peak chemiluminescence (open circles) and final intracellular ion content (closed circles) during the subsequent 20 min was measured. The values shown are percentages of the chemiluminescence and ion ratio (Na^+/K^+) of cells to which buffer was added instead of virus.

Table 2. Effect of pretreatment on chemiluminescence by Lettré cells

The chemiluminescence response of Lettré cells [$(4\text{--}6) \times 10^6/\text{ml}$] to Sendai virus at pH 7.4 was measured as described in the Methods and materials section, before or after exposure [at $(1.7\text{--}2.6) \times 10^8/\text{ml}$] to 10% foetal-calf serum in plastic dishes for 30 min at 37°C . In each case cells were then incubated for 50–85 min before addition of Sendai virus. Foetal-calf serum (10%) alone was without effect on chemiluminescence. The 'large cell' content of the cell suspension was assessed microscopically. Contamination by other cells was less than 0.05%. The results of four separate experiments are shown.

Expt. no. ...	Content of large cells (% of all cells)				Chemiluminescence (% of control)			
	1	2	3	4	1	2	3	4
Before treatment	5.6	4.4	6.9	3.3	240	350	710	1100
After treatment	1.2	1.5	2.2	0.7	160	330	570	1400

Virus-induced chemiluminescence in Lettré cells is, like that in neutrophils, increased by extracellular Ca^{2+} , whereas permeability changes are inhibited (Fig. 2, right panel). The response to influenza virus is also similar to that of neutrophils: chemiluminescence is induced at pH 7.4 as well as at pH 5.5, whereas permeability changes are induced only at pH 5.5 (Fig. 3, right panel, and Table 1; Patel & Pasternak, 1983). Although Lettré cells respond to the same agonists as neutrophils, the magnitude of the response is different (Table 1). Thus Lettré cells respond less well than neutrophils to zymosan, but better than neutrophils to phorbol ester (at pH 7.4). In no case could a permeability change be detected (Table 1). Note that fMet-Leu-Phe, which depolarizes neutrophils (Seligman *et al.*, 1980; Tatham *et al.*, 1980; Kuroki *et al.*, 1982), does not affect the membrane potential of Lettré cells (C. L. Bashford, unpublished work).

Discussion

The present results clearly indicate that influenza-virus-induced chemiluminescence, previously shown to occur in neutrophils at neutral pH (Mills *et al.*, 1981; Abramson, 1982a,b), is no greater at pH 5.5 than at pH 7.4, despite its permeabilizing action at the lower pH (Patel & Pasternak, 1983). The fact that influenza virus is so much less active than Sendai virus (Peterhans, 1980; the present paper) cannot therefore be explained on the basis of a lesser ability to induce a permeability change at neutrality. Moreover, other agents that stimulate chemiluminescence as a result of a receptor-binding event at the leucocyte cell surface, such as fMet-Leu-Phe (Abramson *et al.*, 1982b; Della Bianca *et al.*, 1983; Hallett & Campbell, 1983), induce chemiluminescence in the absence of a detectable permeability change (Table 1). Since there is good evidence that an increase in intracellular Ca^{2+} accompanies receptor-mediated chemiluminescence (Hallett & Campbell, 1982, 1983), which can be induced by ionophore A23187 in the presence of extracellular Ca^{2+} (Wilson *et al.*, 1978; Wrogemann *et al.*, 1978; Hallett *et al.*, 1981; de Chatelet & Shinsky, 1982; Hallett & Campbell, 1983; Ozaki & Kume, 1984; the present paper), the origin of that increase becomes, like the increase in intracellular Ca^{2+} following receptor-mediated events in other cell types (Michell, 1975, 1982; Berridge, 1981), an important question for further study. The present results show that the increase is unlikely to be due to the formation of a non-specific pore. Whether this is true of the increase in intracellular Ca^{2+} that accompanies the stimulation of polymorphs by

complement (Hallett *et al.*, 1981), the terminal factors of which resemble myxoviruses in certain regards (Bashford *et al.*, 1984; Pasternak *et al.*, 1985a,b) remains to be seen.

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