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Oxygen uptake associated with Sendai-virus-stimulated chemiluminescence in rat thymocytes contains a significant non-mitochondrial component

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Sendai virus (150 haemagglutinating units/10^6 cells) stimulates rat thymocytes incubated in medium containing 5 mM-glucose at 37°C to produce luminol-dependent chemiluminescence and a simultaneous increase in O_2 consumption of 40%. Stimulation of thymocytes with Sendai virus is accompanied by reduction of exogenous acetylated ferricytochrome *c*, which is inhibited by superoxide dismutase, and the quantitative conversion of ferricyanide to ferrocyanide, which is not. Replacement of air in the gas space with N_2 inhibits the chemiluminescent response by 97% but does not prevent the virus-stimulated reduction of ferricyanide. The non-permeant ferricyanide anion (2 mM) also inhibits the chemiluminescent response to Sendai virus, its accompanying 'extra' O_2 uptake and the reduction of acetylated ferricytochrome *c* without affecting the basal respiration of the cells. Thymocytes in which the basal O_2 consumption has been stimulated maximally with dinitrophenol (10 μM) or inhibited completely with antimycin A (0.1 μM) respond to Sendai virus with an additional increment of ferricyanide-inhibitable O_2 consumption. The chemiluminescent response to virus is not inhibited by concentrations of antimycin A that block the basal respiration completely. We suggest that a portion of the increased O_2 uptake induced by Sendai virus is involved in the non-mitochondrial reduction of O_2 to $O_2^{-\cdot}$ at the cell surface where the non-permeant ferricyanide anion inhibits $O_2^{-\cdot}$ formation by acting as an alternative high-affinity electron acceptor to O_2.

The adsorption and penetration of Sendai virus in mouse spleen cells (Peterhans, 1979, 1980; Peterhans *et al.*, 1983) and bovine polymorphonuclear leucocytes (Weber *et al.*, 1983) is accompanied by a transient increase in luminol-dependent chemiluminescence that lasts for several minutes. The cellular origin of the chemiluminescent signal in spleen cells, and its biochemical mechanism, is not known with certainty. The rat thymus also contains a subpopulation of cells that generate a short-lived burst of luminol-dependent chemiluminescence in response to the polyclonal mitogen concanavalin A (Wrogemann *et al.*, 1978; Hume *et al.*, 1981), but there are no reports in the literature indicating whether this response can be evoked by virus. The response of the thymocytes to concanavalin A is accompanied by an increase in O_2 consumption that is susceptible, like the chemiluminescent burst itself, to inhibition by concentrations of the non-permeant ferricyanide anion that are too low to inhibit the basal respiration of the cells (Weidemann *et al.*, 1982). The inhibition of the response by ferricyanide suggests that an increment of the 'extra' O_2 uptake may be of non-mitochondrial origin, the non-permeant ferricyanide anion competing with molecular O_2 at the external surface of the plasma membrane for electrons of intracellular origin that are normally involved in reducing extracellular O_2 to $O_2^{-\cdot}$ (Weidemann *et al.*, 1982).

The present experiments were undertaken to find out whether Sendai virus induces luminol-dependent chemiluminescence in rat thymocytes and, if it does, to investigate the mechanism involved. In particular, the ability of Sendai virus

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to induce 'additional' O_2 consumption at a non-mitochondrial site under conditions where the cells can be stimulated to reduce exogenous acetylated ferricytochrome *c* in a superoxide-dismutase-inhibitable manner can be taken as firm evidence that $O_2^{-\cdot}$ is produced. The site of $O_2^{-\cdot}$ production can be assumed to be at the external surface of the cell if the non-permeant ferricyanide anion, acting as an alternative electron acceptor to O_2, is reduced in the presence of virus. The involvement of mitochondrial processes in $O_2^{-\cdot}$ production can be eliminated if both luminol-dependent chemiluminescence and additional O_2 consumption can be initiated by Sendai virus in thymocytes in which the basal O_2 consumption has been stimulated maximally with dinitrophenol or inhibited completely with antimycin A.

Experimental

Materials

Sendai virus was grown in the allantoic cavities of 11-day-old chicken embryos maintained at 37°C. The allantoic fluid was harvested 60h after inoculation. Cellular debris was removed by centrifuging the allantoic fluid for 10min at 5000*g* and the virus was pelleted by centrifugation at 25000*g* for 2.5h. Further purification was achieved by layering the resuspended virus onto a 10–60% (w/w) sucrose gradient buffered with Hepes [4-(2-hydroxyethyl)piperazine-ethane sulphonic acid] (10mM; pH7.6) and centrifuging at 25000rev./min in a SW 27 rotor for 50min. Virus bands were collected, diluted with phosphate-buffered saline and pelleted by centrifugation in the same rotor at 25000rev./min for 60min. The virus was resuspended in phosphate-buffered saline, divided into small aliquots, freeze-dried and stored at −70°C. For use in the experiments described, aliquots of freeze-dried virus were rehydrated and used within 1 week of reconstitution. Freeze-drying had no significant effect on the chemiluminescence-inducing capacity of the virus. Haemagglutinating capacity was determined by adding chicken erythrocytes [10μl of 5% (v/v)] to doubling dilutions of the virus suspension in saline (100μl).

Luminol (5-amino-2,3-dihydrophthalazine-1,4-dione), from Sigma, St. Louis, MO, U.S.A., was solubilized by sonicating 2mg in 1.0ml of phosphate-buffered saline containing 8μM-triethylamine (Hume *et al.*, 1981). Potassium ferricyanide (AnalaR) was obtained from BDH, antimycin A and 1,10-phenanthroline monohydrate from Sigma, horse heart cytochrome *c* from Boehringer Mannheim and dinitrophenol from E. Merck. Stock solutions of dinitrophenol and antimycin A were prepared in 99.5% ethanol. Appropriate control experiments were carried out to test the effect of the solvent ethanol on the response under investigation: at the concentrations used, ethanol had no significant effect on either chemiluminescence or O_2 consumption. H_2O_2 was a Unilab product supplied by Ajax Chemicals, Sydney, Australia. High purity N_2 gas was from C.I.G., Canberra, Australia. All other reagents used were of analytical grade.

Methods

Rat thymocytes from 6–7-week-old female outbred Wistar rats were prepared by teasing the isolated thymuses with sterile needles [18 gauge, 1½in (3.8cm)] in phosphate-buffered saline (pH7.2) containing 5mM-glucose and, after two washings in the same medium, resuspending the isolated cells at 2×10^7 cells/ml (Culvenor & Weidemann, 1976). Prior to teasing, the thymuses were dissected free of perithymic lymph nodes and non-thymic connective tissue. Cell viability, as assessed by Trypan Blue exclusion, was always higher than 90%. Differential counting to identify the minor subpopulations of non-lymphoid cells present in the thymocyte preparation was carried out on cell smears prepared using the cytocentrifuge (Shandon–Southern Cytospin) and stained with Diff-Quik solutions.

Luminol-dependent chemiluminescence was measured at 37°C in two different instruments: (i) in a modified, temperature-controlled Packard Tricarb liquid-scintillation counter set in the off-coincidence mode, using 5.0ml samples (2×10^7 cells/ml) in plastic liquid-scintillation vials (Hume *et al.*, 1981); and (ii) in an LKB Wallac 1251 Luminometer, set in both the peak and integrating modes, using 1.0ml samples (2×10^7 cells/ml) in the polystyrene tubes (Clinicon 2174–086) supplied with the instrument. Luminol (0.1mM final concentration) was preincubated with the dark-adapted cell suspension for 10min before the addition of Sendai virus (150 haemagglutinating units/10^6 cells), which was accompanied by gentle swirling. Light emission began within 5–10s of adding the virus and its rate was recorded continuously for 2–5min. For comparative purposes, the total chemiluminescence produced during the first 2min of incubation with the virus minus the total background chemiluminescence observed during the 2min preceding the virus addition was the standard measurement employed. Where appropriate, continuous agitation was applied to the virus/cell mixture during the measurement of light output in the LKB instrument. Sendai virus added in the absence of cells did not produce an increase in luminol-dependent chemiluminescence above the background rate.

Oxygen consumption by suspensions of isolated

thymocytes (10^8 cells in 2.0ml of phosphate-buffered saline containing 5mM-glucose) subjected to gentle, continuous stirring was measured at 37°C in a Clark-type oxygen electrode (Reed, 1972).

Ferricyanide disappearance and ferrocyanide production were measured in a discontinuous assay that involved incubating cells (1×10^8/ml) at 37°C in phosphate-buffered saline containing 5mM-glucose and 0.5mM-potassium ferricyanide in a total volume of 10ml. Samples (1.0ml) were withdrawn into chilled centrifuge tubes (0°C) at the time intervals indicated in the legends before and after Sendai virus addition and cells were removed by centrifugation at 800*g* for 12min. The concentration of ferricyanide in the cell-free supernatant was determined by measuring the A_{420} ($\varepsilon 1000 M^{-1} \cdot cm^{-1}$) (Clark *et al.*, 1981) and ferrocyanide formation was assessed as its *o*-phenanthroline derivative at 510nm ($\varepsilon 10500 M^{-1} \cdot cm^{-1}$) as described by Avron & Shavit (1963).

Superoxide ($O_2^{-\cdot}$) production was assessed by the ability of Sendai-virus-stimulated cells to reduce acetylated ferricytochrome *c* in the absence but not the presence of superoxide dismutase. Ferricytochrome *c* was acetylated according to the procedure of Azzi *et al.* (1975) and the extent of its reduction in an incubation medium containing 10^8 cells/ml was determined discontinuously as described above for ferricyanide reduction. The glucose-containing incubation medium was supplemented with 22μM acetylated ferricytochrome *c* and superoxide dismutase (10μg/ml) as indicated in the legends. The absorbance change at 550nm was recorded in the cell-free supernatant obtained after removing the cells by centrifugation, and the rate of ferricytochrome *c* reduction was calculated based on a molar difference absorption coefficient for cytochrome *c* of $19.7 \times 10^3 M^{-1} \cdot cm^{-1}$.

Results

When Sendai virus (150 haemagglutinating units/10^6 cells) was added to a suspension of rat thymocytes incubated at 37°C, the cells responded by producing an intense burst of luminol-dependent chemiluminescence (Fig. 1*a*). The intensity, duration and slow decay of the response resembled that induced by ionophore A23187 (Wrogemann *et al.*, 1978) rather than the transient peak evoked by concanavalin A (Hume *et al.*, 1981). Since intracellular Ca^{2+} is known to accumulate in mouse thymocytes stimulated with A23187 (Tsien *et al.*, 1982) and in human erythrocyte ghosts treated with Sendai virus (Hallett *et al.*, 1982), a sustained increase in cytoplasmic free Ca^{2+} due to alterations in membrane permeability brought about by Sendai virus binding (Pasternak & Micklem, 1981) might be the signal that initiates and maintains the prolonged chemiluminescent response.

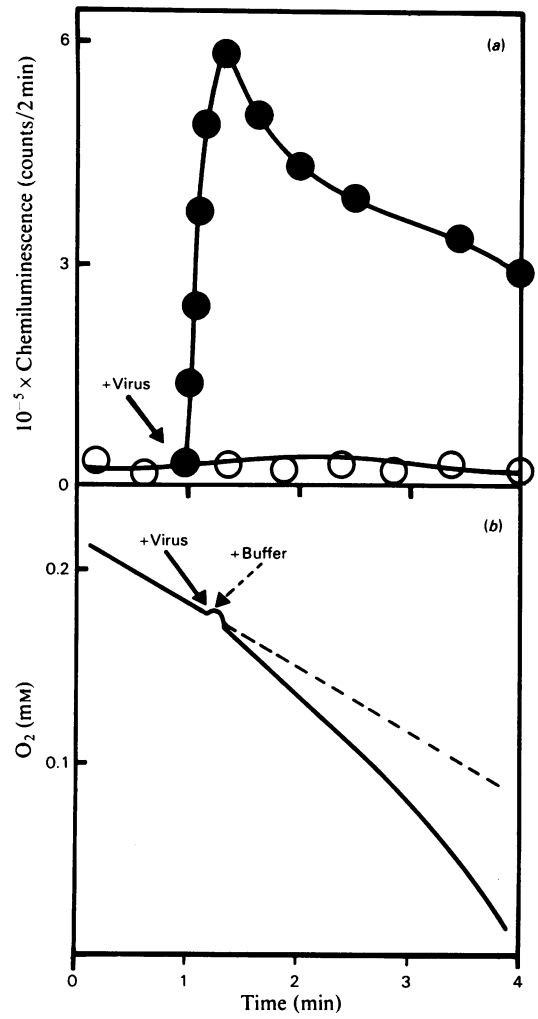

Fig. 1. *Chemiluminescence and additional O_2 consumption induced in rat thymocytes by Sendai virus*

(*a*) Luminol-dependent chemiluminescence of 1.25×10^8 rat thymocytes, measured in a liquid-scintillation counter as described in the Methods section, was initiated by the addition of Sendai virus (150 haemagglutinating units/10^6 cells) (●) or phosphate-buffered saline (pH7.2) (○) 10min after luminol addition. (*b*) Typical oxygen-electrode trace, showing the effect of addition of Sendai virus (150 haemagglutinating units/10^6 cells) (continuous line) or phosphate-buffered saline (pH7.2) (broken line) on O_2 consumption of rat thymocytes (10^8 cells suspended in 2.0ml of phosphate-buffered saline containing 5mM-glucose).

Fig. 1(*b*) shows that addition of Sendai virus to the cells was accompanied by an increase of $41 \pm 2\%$ ($n = 9$) in O_2 uptake that continued

throughout the period of intense chemiluminescence. This observation raises the possibility that the two coincident phenomena might be directly related, since oxygen radicals are the most likely source of luminol-dependent chemiluminescence. Gentle purging of the cell suspension and gas space for 2min with 100% N_2, but not with air, reduced the chemiluminescent response to virus by more than 97% (Table 1). The absolute requirement for oxygen argues that its reduction is an essential step in the production of the luminol-reactive species. Since purging is unlikely to have brought about complete exhaustion of oxygen from the vial, the affinity of the oxygen-consuming step for its substrate must be relatively low, like that described for the 'respiratory burst' of rat polymorphonuclear leucocytes responding to phagocytic and chemotactic stimuli (Edwards *et al.*, 1984).

Superoxide anions ($O_2^{-\cdot}$) are formed and additional O_2 is consumed during the appearance of luminol-dependent chemiluminescence in stimulated polymorphonuclear leucocytes (Rosen & Klebanoff, 1976). Fig. 2 shows that addition of Sendai virus brought about the reduction of acetylated ferricytochrome *c* by rat thymocytes with a time course that coincided with the generation of luminol-dependent chemiluminescence. That $O_2^{-\cdot}$ is the species mainly responsible for this reduction is indicated by the sensitivity of the response to superoxide dismutase (Fig. 2). Since added ferricyanide (0.5mM) completely blocked the reduction of cytochrome *c* in the presence of Sendai virus (Fig. 2), it is possible that it acted as an alternative high-affinity electron acceptor to O_2, thereby preventing $O_2^{-\cdot}$ from being formed. Consistent with this view, Fig. 3 shows that >90% of the Sendai virus-induced chemiluminescence was indeed inhibited by addition of ferricyanide (2mM) to the cells ($K_i = 0.2$mM). Ferricyanide is a potent amplifier of the chemiluminescence generated by the chemical reaction between luminol (0.1mM) and H_2O_2 (1.5mM) whether unstimulated thymocytes (2×10^7 cells/ml) are present in the incubation medium or not (J. Ryan, M. E. Kolbuch-Braddon & M. J. Weidemann, unpublished work), so its blockade of thymocyte chemiluminescence must be due either to the failure of the cells to produce $O_2^{-\cdot}$ or to the chemical reoxidation of $O_2^{-\cdot}$ to O_2 in the presence of ferricyanide.

The likelihood that the triply negatively charged ferricyanide anion does not penetrate into the cytoplasmic space of thymocytes, as is the case with solid liver tissue and isolated hepatocytes (Clark *et*

Table 1. *Effect of O_2 displacement on Sendai virus-induced chemiluminescence in rat thymocytes*

Rat thymocytes (2.5×10^7 cells/ml) suspended in 5.0ml of phosphate-buffered saline containing 5mM-glucose, and the gas space above the cells in screw-capped plastic liquid-scintillation vials, were purged gently for 2min with 100% N_2 or air. The caps were replaced tightly and the chemiluminescent responses of the purged and control cells to N_2-purged Sendai virus (150 haemagglutinating units/10^6 cells) were determined at 37°C as described in the Methods section. Positive pressure of the appropriate gas was applied to the vial during the introduction of the virus sample. Results are expressed as mean counts/2min (±S.E.M.) for the number of observations indicated in parentheses.

Experimental conditions	Chemiluminescence (counts/2min)	% of control value
Control	608343±51160 (14)	100
N_2-purged	19999±6024 (11)	3.3
Air-purged	552667±53324 (4)	90.9

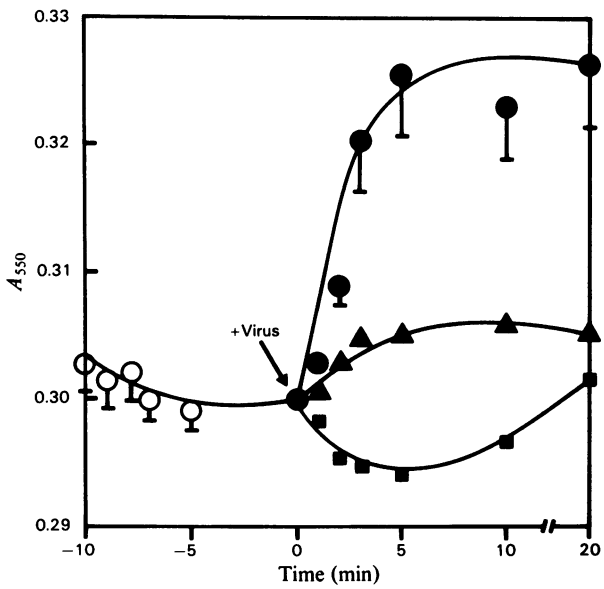

Fig. 2. *Reduction of ferricytochrome c by rat thymocytes upon addition of Sendai virus*

Rat thymocytes (10^8 cells/ml) were incubated at 37°C in the presence of glucose (5mM), acetylated ferricytochrome *c* (22μM) and the inhibitors listed below. Absorbance changes at the time intervals indicated were measured at 550nm in the cell-free supernatant obtained after centrifugation (see the Methods section) from samples taken before (○) and after (●) addition of Sendai virus (150 haemagglutinating units/10^6 cells) as indicated by the arrow. Absorbance changes in virus-treated samples containing superoxide dismutase (10μg/ml) (▲) and ferricyanide (0.5mM) (■) from the beginning of the incubation period were measured in the same way. Results are expressed as mean absorbance changes ±S.E.M. (three experiments) or as the averages of duplicate estimations (two experiments).

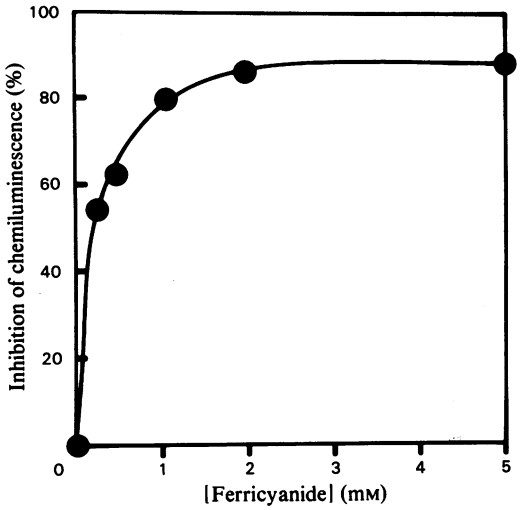

Fig. 3. *Inhibition of Sendai-virus-induced chemiluminescence by ferricyanide*

Rat thymocytes (2.5×10^7 cells/ml) suspended in phosphate-buffered saline containing 5mM-glucose and 0.1mM-luminol were treated with potassium ferricyanide at the concentrations shown 2min before Sendai virus addition. The chemiluminescence induced by Sendai virus (150 haemagglutinating units/10^6 cells) was measured at each ferricyanide concentration for 2min after virus addition. Each point represents the percentage inhibition of the control response by the designated concentration of ferricyanide.

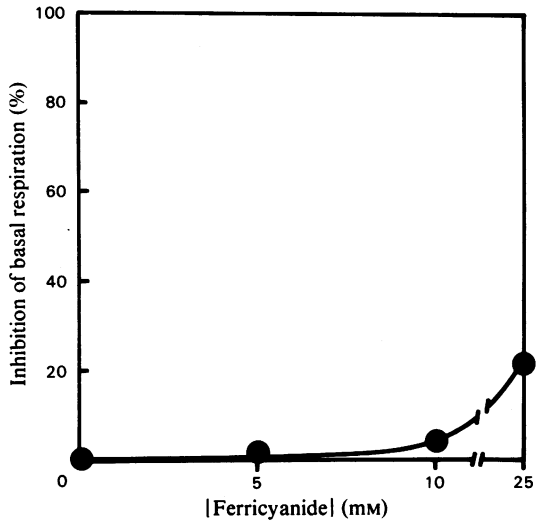

Fig. 4. *Inhibition of basal respiration of rat thymocytes by ferricyanide*

O_2 consumption of 10^8 rat thymocytes oxidizing 5mM-glucose was monitored in the presence of potassium ferricyanide at the concentrations indicated by using the Clark O_2 electrode. The results are expressed as percentage inhibition of the basal O_2 consumption brought about by the concentration of ferricyanide tested.

al., 1981), provides an opportunity to test whether O_2 is reduced to $O_2^{-\cdot}$ at the external surface of the cell. The failure of ferricyanide, even at 10mM, to inhibit the basal O_2 consumption of intact unstimulated thymocytes (Fig. 4), in spite of its ability to inhibit the O_2 consumption of isolated thymus mitochondria [$K_i = 2.25 \pm 0.02$mM ($n = 3$); M. E. Kolbuch-Braddon & M. J. Weidemann, unpublished work], confirms that it is excluded from the cytoplasmic compartment occupied by these organelles. When added 2min before Sendai virus, ferricyanide (2mM) blocked more than 66% of the 'extra' O_2 consumption normally associated with virus addition (Fig. 5). When added 2min after Sendai virus, it reversed the stimulation of O_2 consumption and restored the basal rate. Cells treated with ferricyanide (2mM) for 10min, then pelleted and resuspended in an incubation medium free of ferricyanide, responded to Sendai virus with a normal 40% increase in O_2 consumption, confirming that the inhibitory action of ferricyanide is fully reversible (M. E. Kolbuch-Braddon & M. J. Weidemann, unpublished work). Fig. 6 demonstrates that the slow basal rate of ferricyanide reduction to ferrocyanide observed under aerobic conditions was stimulated several-fold by

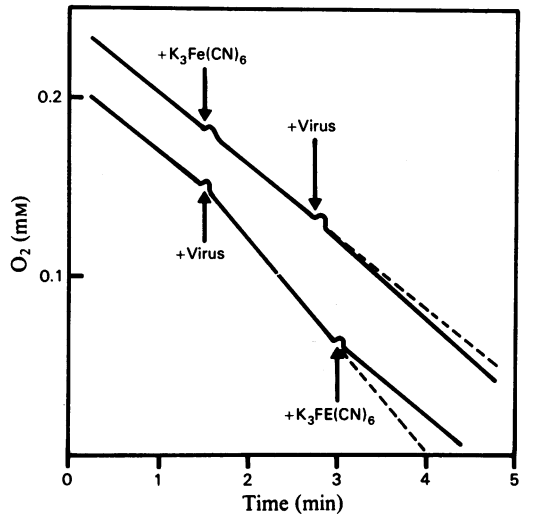

Fig. 5. *Effect of ferricyanide on the additional O_2 consumption induced by Sendai virus*

O_2 electrode traces are presented which show the effect of reversing the order of addition of potassium ferricyanide (2.0mM) and Sendai virus (150 haemagglutinating units/10^6 cells) on the O_2 consumption of rat thymus lymphocytes (5×10^7 cells/ml). Broken lines showing extensions of the previous 2min rates are presented to emphasize deviations from these values; the degree of deviation in each case represents the mean of three separate experiments.

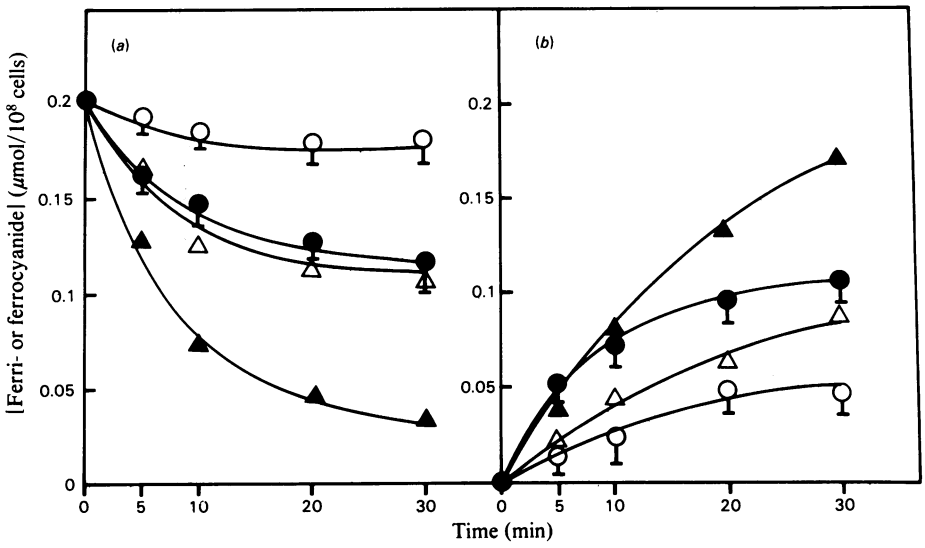

Fig. 6. *Effect of Sendai virus on the rate of ferricyanide reduction by rat thymocytes incubated under aerobic and anaerobic conditions*

Rat thymocytes (10^8 cells/ml) were incubated at 37°C in the presence of glucose (5 mM) and potassium ferricyanide (0.5 mM). Under aerobic conditions, either Sendai virus (●) (150 haemagglutinating units/10^6 cells) or an equivalent volume of buffer (○) was added at zero time and the concentrations of ferricyanide (*a*) and ferrocyanide (*b*) were measured in the cell-free supernatants obtained after centrifugation as described in the Methods section. Identical results were obtained when superoxide dismutase (10 μg/ml) was included in the incubation medium. Anaerobic conditions were established by purging the incubation medium and gas space with 100% N_2 throughout the incubation period as described in the legend to Table 1. In this case the concentrations of ferricyanide (*a*) and ferrocyanide (*b*) were measured at each time interval in Sendai-virus-treated (▲) and control (△) incubations. Results are expressed as μmol of ferri- or ferrocyanide/10^8 cells and represent means ± S.E.M. ($n = 5$) for the aerobic incubations and a typical experiment for the anaerobic incubation.

Sendai virus during the period that coincided with ferricyanide inhibition of chemiluminescence and 'extra' O_2 consumption. Superoxide dismutase added at the concentration that blocked virus-induced ferricytochrome *c* reduction (Fig. 2) had no significant effect on ferricyanide disappearance or ferrocyanide production in the presence or absence of Sendai virus. Purging the incubation medium and gas space with N_2 throughout the experiment increased the basal rate of ferricyanide reduction but failed to block the virus-stimulated increment (Fig. 6). Under both aerobic and anaerobic conditions, a similar increment of ferricyanide disappearance in the presence of Sendai virus could be accounted for, quantitatively, as ferrocyanide production under conditions where there was no significant net change in the total ferricyanide plus ferrocyanide concentration in the incubation medium (Fig. 6). Taken together, these results indicate that a portion, at least, of the 'extra' O_2 taken up is converted to $O_2^{-\cdot}$ at the external surface of the plasma membrane where the non-permeant ferricyanide anion acts as an alternative high-affinity electron acceptor to O_2. They exclude the possibility that $O_2^{-\cdot}$ is the immediate electron donor that reduces ferricyanide, since $O_2^{-\cdot}$ is not formed under N_2.

The uncoupling of mitochondrial oxidative phosphorylation with dinitrophenol increased the basal rate of O_2 consumption by intact thymocytes 2.5-fold (Fig. 7). Pretreatment of the cells for 2 min with a concentration of dinitrophenol (10 μM) that maximized this effect (Fig. 7) caused less than 50% inhibition of Sendai virus-induced chemiluminescence (Fig. 8), approx. 30% of which could be accounted for by the quenching of the chemiluminescence produced by the excitation of luminol with H_2O_2 (Fig. 8). The addition of Sendai virus caused a further increase in the rate of O_2 consumption by the dinitrophenol-treated cells equivalent to $37.4 \pm 1.2\%$ of the original basal rate ($n = 13$, mean ± S.E.M.). The addition of ferricyanide (2 mM) to dinitrophenol-treated cells either blocked the effect of the virus or restored the O_2 consumption of virally-stimulated cells to its pre-viral rate (Fig. 9). The ability of the virus to stimulate the O_2 uptake of cells that are already maximally stimulated with dinitrophenol supports the view that the ferricyanide-sensitive increment of the 'extra' O_2 consumption is partly of non-mitochondrial origin.

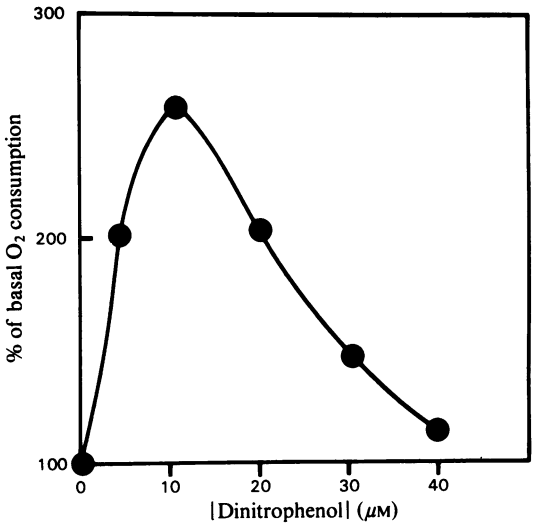

Fig. 7. *Concentration-dependence of dinitrophenol-stimulated basal respiration of rat thymocytes*
The O_2 consumption of 10^8 rat thymocytes was measured as described in the Methods section. The dose–response to dinitrophenol was determined by treating the cells with various concentrations of the uncoupler and measuring the corresponding percentage increase in O_2 consumption above the unstimulated basal rate, expressed as 100%.

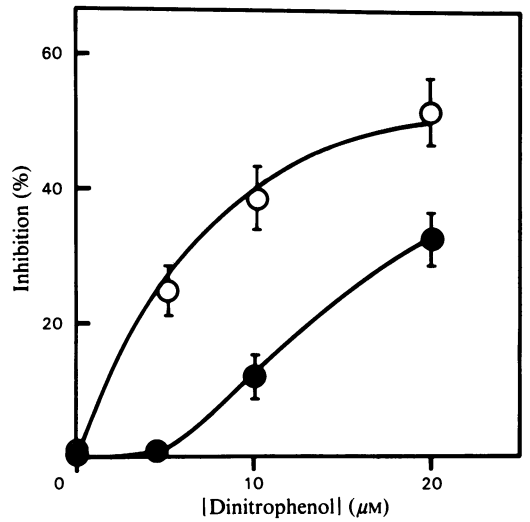

Fig. 8. *Effect of dinitrophenol on the chemiluminescence due to Sendai virus addition to rat thymocytes* (○) *and that due to the chemical reaction between* H_2O_2 *and luminol* (●)
(○) Rat thymocytes (2.5×10^7 cells/ml) suspended in 1.0ml of phosphate-buffered saline were treated with dinitrophenol at the concentrations shown for 2min before virus additions. Chemiluminescence induced by Sendai virus (150 haemagglutinating units/10^6 cells) was measured at each dinitrophenol concentration in an LKB-Wallac 1251 Luminometer, with continuous stirring, as described in the Methods section. (●) The effect of dinitrophenol on the chemiluminescence associated with the chemical reaction between H_2O_2 (1.5mM) and luminol (0.1mM) in 1.0ml of phosphate-buffered saline was measured in a liquid-scintillation counter as described in the Methods section. The results in each case are expressed as percentage inhibition by dinitrophenol of the response under investigation and represent the means ± S.E.M. for three separate experiments.

More direct proof that this is the case was obtained when Sendai virus was used to stimulate the O_2 consumption of thymocytes pretreated with the electron transport chain inhibitor antimycin A. This inhibitor caused complete cessation of the basal O_2 consumption at 0.1 μM (Fig. 10); even at 0.01 μM the inhibition produced was immediate and more than 95% complete ($K_i = 0.004\,\mu M$). Antimycin A caused no significant inhibition of virus-stimulated chemiluminescence within this concentration range (i.e. only 0.3% at 0.1 μM), although some inhibition (24 ± 1%) was observed when its concentration was raised to 1 μM. These observations are incompatible with the direct participation of mitochondrial O_2 consumption in Sendai virus-induced chemiluminescence. When the basal respiration was inhibited by antimycin A (0.01 μM), Sendai virus initiated a low rate of O_2 consumption equivalent to 10 ± 0.2% of the basal rate. The antimycin A-insensitive O_2 consumption was observed whether the inhibitor was added 2min before or 2min after the treatment of the cells with virus (Fig. 11). Table 2 shows that cells stimulated with dinitrophenol prior to the addition of antimycin A (1 μM) responded to virus addition with a measurable O_2 consumption not significantly different to that seen with antimycin A alone.

Discussion

The luminol-dependent chemiluminescence induced by Sendai virus in mouse spleen cells, which is one of the earliest measurable responses to receptor occupation, is triggered by two virus envelope spike proteins (haemagglutinin–neuraminidase and fusion glycoproteins) (Peterhans, 1979, 1980; Peterhans *et al.*, 1983). The observations reported here show that the rat thymus also contains Sendai-virus-responsive cells in which the early burst of chemiluminescence is accompanied by a significant increase in O_2 uptake and production of $O_2^{-\cdot}$. Enhanced O_2 consumption with a duration matching that of the episode of chemiluminescence has also been observed in rat thymocytes stimulated with concanavalin A or ionophore A23187 (Weidemann *et al.*, 1982).

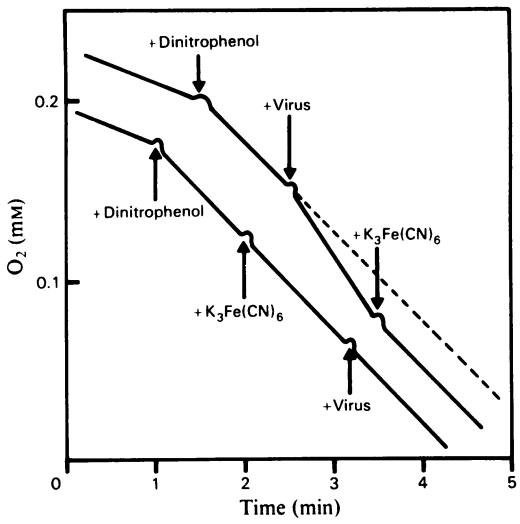

Fig. 9. *Differential effects of dinitrophenol, ferricyanide and Sendai virus on the O_2 consumption of rat thymocytes*
Oxygen-electrode traces (summarizing three separate experiments) are presented showing the effects of reversing the order of addition of Sendai virus (150 haemagglutinating units/10^6 cells) and potassium ferricyanide (2mM) on the O_2 consumption of rat thymocytes (5×10^7 cells/ml) stimulated with an optimum concentration of dinitrophenol (10μM). For other experimental conditions and symbols, see Fig. 5.

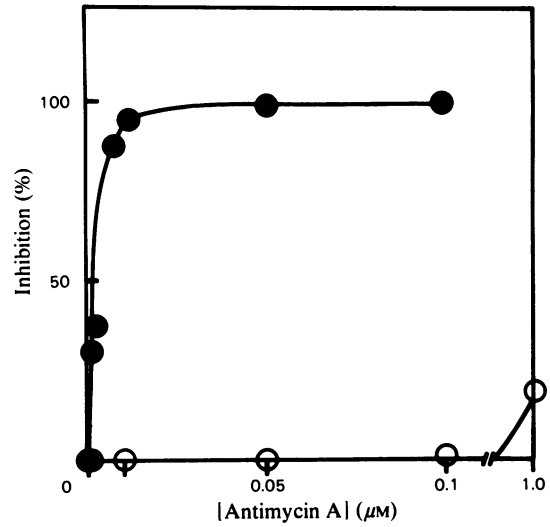

Fig. 10. *Effect of antimycin A on Sendai virus-induced chemiluminescence* (○) *and the basal O_2 consumption* (●) *of rat thymocytes*
The basal O_2 consumption of rat thymocytes (●) and their chemiluminescent response to Sendai virus (150 haemagglutinating units/10^6 cells) (○) were determined, separately, in the presence of antimycin A at the concentrations indicated on the abscissa. The experimental conditions were similar to those described for Figs. 3 and 4 except that antimycin A was used instead of ferricyanide. The results are expressed as percentage inhibition of the appropriate parameter by the designated concentration of antimycin A.

The original intention of the present experiments was to find out whether the generation of luminol-reactive oxygen metabolites and net consumption of additional O_2 by rat thymocytes stimulated by Sendai virus are related phenomena. A secondary aim was to investigate the mechanism responsible for the O_2-dependent chemiluminescent burst. Under conditions where antimycin A, which blocks mitochondrial electron transport between cytochromes *b* and c_1 (Kaniuga *et al.*, 1969), totally inhibits the basal O_2 consumption of thymocytes (Fig. 10), virus addition still induces chemiluminescence (Fig. 10) and causes O_2 uptake to be resumed at a rate equivalent to 10% of the basal respiration (Fig. 11). Because the chemiluminescence is virtually uninhibited by antimycin A at 0.1μM, this experiment rules out the possibility that mitochondrial electron transport participates in the formation of luminol-reactive species and, at the same time, defines a proportion of the virus-induced O_2 uptake that is incontrovertibly non-mitochondrial. That this amounts to only 25% of the total additional O_2 uptake indicates that virus binding activates both mitochondrial and non-mitochondrial processes, although only the non-mitochondrial component appears to be linked obligatorily to the generation of luminol-reactive metabolites. The ability of Sendai virus to induce increased O_2 consumption in cells already stimulated to respire maximally as a result of uncoupling with dinitrophenol (Fig. 9) supports this general conclusion. The discrepancy between the additional O_2 consumption induced by virus in cells treated with dinitrophenol alone (Fig. 9) or dinitrophenol plus antimycin A (Table 2) suggests that O_2 consumption of mitochondrial origin may still be induced by virus in uncoupled cells that retain a competent mitochondrial electron transport chain.

The enhanced reduction of acetylated ferricytochrome *c* by thymocytes upon stimulation with Sendai virus, and its inhibition by superoxide dismutase or ferricyanide (Fig. 2), supports the view that $O_2^{-\cdot}$ production accounts for a significant portion of the non-mitochondrial O_2 consumption. If, as in phagocytic cells (Fantone & Ward, 1982), O_2 is reduced to $O_2^{-\cdot}$ by a plasma-membrane-spanning NAD(P)H oxidase that receives electrons from the intracellular pool of nicotinamide nucleotides reduced during glucose metabolism, the simplest interpretation of the

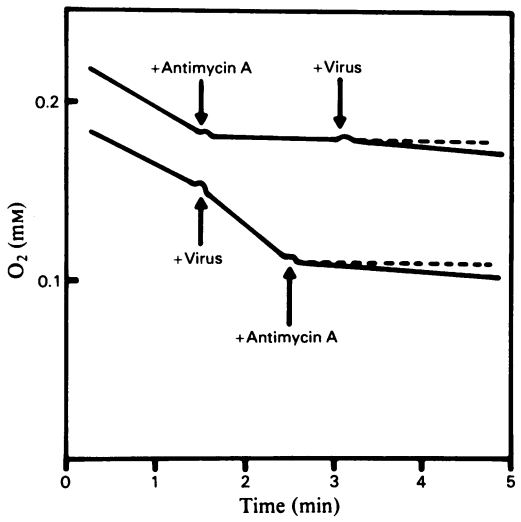

Fig. 11. *Effect of antimycin A on the additional O_2 consumption induced in rat thymocytes by Sendai virus*
Typical oxygen electrode traces are presented which show the effect of reversing the order of addition of antimycin A (0.1 μM) and Sendai virus (150 haemagglutinating units/10^6 cells) on the oxygen consumption of rat thymocytes (5×10^7 cells/ml). For other conditions and symbols, see Fig. 5.

Table 2. *Effect of dinitrophenol and antimycin A on Sendai-virus-induced O_2 consumption by rat thymocytes*
The O_2 consumption of 10^8 rat thymocytes was determined as described in the Methods section in the presence of dinitrophenol (10 μM) and Sendai virus (150 haemagglutinating units/10^6 cells) in various combinations as listed below. The results are expressed as mean percentages of the unstimulated basal rate ± S.E.M., with the numbers of observations given in parentheses.

Experimental conditions	Oxygen consumption (% of basal rate)
Control (no additions)	100 (9)
+10 μM-Dinitrophenol	227 ± 6.4% (9)
+10 μM-Dinitrophenol +1 μM-antimycin A	2.2 ± 0.9% (9)
+10 μM-Dinitrophenol +1 μM-antimycin A +Sendai virus	13.5 ± 2.9% (9)

effect of the non-permeant ferricyanide anion is that it acts as a high-affinity electron acceptor, capable of competing with O_2 at the external cell surface. The reduction of ferricyanide to ferrocyanide in response to Sendai virus addition (Fig. 6) under conditions where $O_2^{-\cdot}$ production (Fig. 2), 'extra' O_2 uptake and the formation of luminol-reactive metabolites (Fig. 3) are all blocked by ferricyanide supports this view. The failure of both superoxide dismutase and anaerobiosis to inhibit the virus-induced ferricyanide reduction (Fig. 6) indicates that $O_2^{-\cdot}$ is not the electron donor and the source of regenerated O_2, and that ferricyanide and molecular O_2 must have a common electron donor, at the external cell surface, for which they compete. The possibility that ferricyanide has an indirect effect on the cells, mediated perhaps by a blockade of the specific virus–receptor interaction at the plasma membrane surface or the inhibition of Ca^{2+} entry, cannot be entirely ruled out, although this interpretation would not explain why ferricyanide is reduced during Sendai virus stimulation under both aerobic and anaerobic conditions (Fig. 6) nor the fact that it blocks both chemiluminescence and 'extra' O_2 consumption when stimuli as diverse as concanavalin A, La^{3+} ions and ionophore A23187 are used (Weidemann *et al.*, 1982).

We have suggested previously (Hume *et al.*, 1981) that the O_2 consumed extramitochondrially during the lipoxygenation of arachidonic acid by rat thymus cells stimulated with concanavalin A may contribute directly to activated oxygen production as a result of the conversion of unstable hydroperoxy intermediates of arachidonic acid to their hydroxy fatty acid products (Sugioka & Nakano, 1976; Cadenas *et al.*, 1980; Smith & Weidemann, 1980). It is possible, since 20% of the chemiluminescent response of thymocytes to Sendai virus survives prolonged glucose starvation (M. Clare Johnson & M. J. Weidemann, unpublished work), that reactive intermediates from this glucose-independent source may contribute to ferricyanide reduction at the cell surface, especially since, in mouse spleen cells responding to Sendai virus, the magnitude of the chemiluminescent response is reduced strongly, in a concentration-dependent manner, by 2-deoxyglucose and inhibitors of the phospholipase A_2 and lipoxygenase reactions, but not by cyclo-oxygenase inhibitors (Semadeni *et al.*, 1984). 2-Deoxyglucose, in addition to inhibiting D-glucose transport competitively, inhibits the formation of hydroxyeicosatetraenoic acids (Walker & Parish, 1981) and leukotrienes (Ziltener *et al.*, 1983) in neutrophilic and eosinophilic leucocytes, respectively. An alternative explanation for the sensitivity of Sendai virus-induced chemiluminescence to lipoxygenase inhibitors is that an intermediate or product of the lipoxygenase pathway may be an essential positive modulator of the $O_2^{-\cdot}$ generating NAD(P)H oxidase.

In fact, it was recently demonstrated that the chemiluminescence-negative polymorphs from patients with chronic granulomatous disease synthesize lipoxygenase products (Feinmark *et al.*, 1983; Henderson & Klebanoff, 1983). Furthermore, no correlation was found between the

amount of light emitted and amount of leukotrienes formed in horse eosinophils and neutrophils (P. A. Chavaillaz & A. Jörg, personal communication) and in bovine polymorphs (M. Grob, A. Jörg & E. Peterhans, unpublished work), suggesting that lipoxygenase may not be a significant source of chemiluminescence emitted by intact cells.

The present experiments do not allow us to identify positively the individual or interacting cell type(s) within the thymus that produce $O_2^{-\cdot}$ in response to virus. Freshly-prepared thymocyte suspensions contain only 0.3% neutrophils plus eosinophils, which is an insufficient number, by 5–8-fold, to account for the 'extra' O_2 consumed by thymocytes stimulated with Sendai virus in the presence of antimycin A (Fig. 11); and in 12–18h thymocyte cultures *in vitro*, from which these short-lived granulocytes have virtually disappeared, the maturation of immature thymocytes in the presence of thymic macrophages increases the chemiluminescent responsiveness of the cell suspension to concanavalin A 10-fold (Wrogemann *et al.*, 1980; Hume *et al.*, 1981). The subpopulations of macrophages and interdigitating cells within the thymus (Duijvestijn *et al.*, 1983) represent a more significant source of non-lymphoid cells (i.e. 5.5% of the total population). We have argued, in the case of concanavalin A stimulation, that thymocyte chemiluminescence can be distinguished from that of macrophages on the basis of its insensitivity to inhibition by superoxide dismutase and its sensitivity to catalase (Wrogemann *et al.*, 1978; Mookerjee *et al.*, 1980; Hume *et al.*, 1981). Furthermore, thioglycollate-elicited rat peritoneal macrophages that respond strongly to zymosan produce little chemiluminescence in response to Sendai virus (i.e. only 42% above the basal rate) and no concomitant metabolic burst (Alaudeen, 1982). However, thymic macrophages may be different from the peritoneal macrophages elicited by thioglycollate, and the question of the cellular origin of chemiluminescence in thymocyte suspensions must remain open.

The intensity of the chemiluminescent response of thymus and spleen cells to Sendai virus raises the general question of its biological significance. In host cells that respond to virus binding by producing activated oxygen, the fusion of the viral envelope with the plasma membrane of the host cell may be facilitated by the propagation of lipid peroxidation chain reactions through the respective lipid bilayers. The reduced species of oxygen produced are potentially damaging to the integrity of the virus itself or to virus-infected cells that display the viral antigens (Belding *et al.*, 1970; Jones, 1982). In either case, the intensity of the reaction may be sufficient to inflict oxidative damage on uninfected 'bystander' cells that make contact with virus-stimulated immunocytes. It is interesting, in this context, that Alsheikhly *et al.*, (1983) have demonstrated that the purified haemagglutinin–neuraminidase glycoprotein of Sendai virus augments the cytotoxicity of human peripheral blood lymphocytes to non-infected T24 target cells.

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