Concanavalin A-induced chemiluminescence in rat thymus lymphocytes

Its origin and role in mitogenesis

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1. The luminol-dependent chemiluminescence of rat thymocytes responding to concanavalin A can be resolved into glucose-dependent and glucose-independent portions. 2. The glucose-dependent portion, supported by D-glucose and D-mannose oxidation, is inhibited by catalase (200 µg/ml), amobarbital (1 mM) and hexose analogues that block D-glucose uptake. Thus concanavalin A may activate, transiently, an NAD(P)H oxidase that utilizes reducing equivalents derived from the oxidation of exogenous glucose to give dismutation products of O_2^- (including H_2O_2) as its major products. 3. The glucose-independent portion is inhibited by eicosa-5,8,11,14-tetraenoic acid but not by indomethacin. It may therefore be associated with the conversion of hydroperoxy intermediates of arachidonic acid metabolism to hydroxy products by the lipoxygenase pathway. 4. Preincubation of thymocytes for 18 h in serum-free medium enhances the subsequent chemiluminescent response to concanavalin A severalfold and evokes the response at a lower threshold concentration. The incorporation of [3H]thymidine by preincubated cells is similarly enhanced at low doses of concanavalin A, whereas the response to optimal doses is unaltered. 5. Catalase does not inhibit the enhanced incorporation of [3H]thymidine obtained in response to concanavalin A, but instead amplifies the response to low doses in the same manner as preincubation.

Rat thymocytes respond to concanavalin A, in the presence of luminol, with a short-lived burst of chemiluminescence that is partly inhibited by added catalase (Weidemann *et al.*, 1977; Wrogemann *et al.*, 1978, 1980). These properties indicate that the chemiluminescence observed is not due to the direct emission of photons but is attributable mainly to the production of H_2O_2 . Additional work has shown (Wrogemann *et al.*, 1980) that preincubation of rat thymocytes for more than 6 h in serum-free medium without mitogen enhances severalfold their chemiluminescent responsiveness to concanavalin A. The present work extends these observations, considering first the nature of the metabolic processes that may give rise to luminol-reactive products, and secondly the relevance of these immediate metabolic changes to the later events of T-cell mitogenesis.

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Experimental

Materials

Dulbecco's modified Eagle's minimal essential medium was buffered with 20 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], pH 7.2, for chemiluminescence experiments. For cell culture experiments the medium was Dulbecco's modified Eagle's minimal essential medium supplemented with 15% horse serum and 50 µM-2-mercaptoethanol. All components of these media were obtained from Gibco Biocult, Paisley, Scotland, U.K. Concanavalin A was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Luminol (5-amino-2,3-dihydrophthalazine-1,4-dione), obtained from Sigma Chemical Co., St. Louis, MO, U.S.A., was prepared for use as follows: a sample (2 mg) was added to 1 ml of phosphate-buffered saline (Krebs & de Gasquet, 1964), pH 7.2, containing 8 µM-triethylamine, sonicated until clear, and stored frozen in convenient portions. Eicosa-5,8,11,14-tetraenoic acid, a gift from Dr. B. Peskar of the Pharmakologisches Institut, University of

Freiburg, Freiburg, Germany, was dissolved in dimethyl sulphoxide (55%) at a final concentration of 1.4 mM, sonicated and stored frozen. Catalase (thymol-free), prostaglandin E_1 , indomethacin and amobarbital [5-ethyl-5-(3-methylbutyl)pyrimidine-2,4,6(1*H*,3*H*,5*H*)-trione] were obtained from Sigma. Other reagents used were of analytical grade.

Methods

Male outbred Wistar or male inbred Buffalo strain rats, 6–8 weeks of age, were killed with a blow to the head followed by cervical dislocation. The thymus glands were removed under sterile conditions and thymocyte suspensions were prepared in Eagle's/Hepes medium, pH 7.2, by teasing the thymus apart with needles (Hume & Weidemann, 1978).

In chemiluminescence experiments, cells (5×10^6 – 2×10^7 /ml) were incubated in plastic scintillation vials in 5 ml of medium. Luminol (0.05 ml of the solution described above) was added 10 min before addition of concanavalin A. Chemiluminescence was monitored at 37°C in a temperature-controlled Packard Tricarb liquid-scintillation counter set in the off-coincidence mode. Peak or total chemiluminescence was calculated as described by Wrogemann *et al.* (1980).

For assays of thymidine incorporation, cells (5×10^6 /ml) were cultured at 37°C in 0.2 ml microtitre wells. [3 H]Thymidine (1 μ Ci, 10 μ M) was added to each well after the desired time and the cells were harvested 12 h later on to filter paper with a Skatron automatic harvester. The filter discs were transferred to scintillation vials and assayed for radioactivity incorporated.

Results and discussion

The nature of thymocyte chemiluminescence

Omission of glucose diminished the chemiluminescent response of rat thymocytes to concanavalin A by 45% compared with that of control cells suspended in medium containing 5 mM-glucose (Table 1). The response could be completely restored, however, if the cells were treated with glucose (5 mM) for a period equivalent to that of the original starvation (1–2 h). Its reappearance excludes the possibility that the effect of glucose omission is due to cell mortality, which becomes significant (on the basis of Trypan Blue exclusion) only after prolonged glucose starvation (>6 h). The apparent ' K_m ' for D-glucose, to which the cells responded hyperbolically, was identical with that reported for D-glucose transport into rat thymocytes ($0.37 \text{ mM} \pm 0.04$; three determinations; Yasmeen *et al.*, 1977). 3-*O*-Methyl-D-glucose (10 mM), which shares the hexose-uptake pathway with D-glucose in rat thymocytes (Feldman *et al.*, 1974; Yasmeen *et al.*, 1977), inhibited the glucose-depen-

Table 1. *Effect of glucose deprivation, eicosa-5,8,11,14-tetraynoic acid (ETYA), indomethacin and prostaglandin E_1 on chemiluminescence produced by rat thymocytes in response to concanavalin A*

Chemiluminescence was measured in the presence of luminol as described under 'Methods' for 5 min before and 5 min after addition of concanavalin A (200 μ g/ml). The net increase in chemiluminescence evoked by concanavalin A was determined by difference. The value obtained in the presence of 5 mM-glucose without solvents or inhibitors present (24860 ± 3080 counts/5 min per 10^7 cells) was taken as 100% and all other results are expressed as a percentage of this value $\pm$ S.E.M. (number of separate cell preparations tested given in parentheses). Cells deprived of glucose for a short period (<30 min) were resuspended in glucose-containing or glucose-free Eagle's/Hepes medium and the effect of glucose deprivation was studied by comparing the deprived cells with matched glucose-containing controls.

Additions	Presence (+) or absence (–) of glucose (5 mM)	Chemi- luminescence (%)
None	+	100 (5)
Dimethyl sulphoxide (0.05%)	+	86.6 ± 3.8 (5)
Dimethyl sulphoxide (0.05%) + ETYA (1.5 μ M)	+	48.3 ± 5.5 (5)
Indomethacin (10 μ M)	+	98.1 ± 2.2 (3)
Prostaglandin E_1 *	+	36.7 ± 3.7 (4)
None	–	43.2 ± 6.6 (5)
Dimethyl sulphoxide (0.05%)	–	44.9 ± 6.7 (5)
Dimethyl sulphoxide (0.05%) + ETYA (1.5 μ M)	–	23.4 ± 4.8 (5)

* Prostaglandin E_1 was dissolved in ethanol rather than dimethyl sulphoxide; the addition of an equivalent volume of ethanol alone had no significant effect on the chemiluminescence of control cells.

dent chemiluminescence at low D-glucose concentrations (up to 5 mM), but failed to do so when the concentrations of the two sugars were equal at 10 mM (result not shown). Thus sugar uptake via the glucose carrier, with its characteristically low affinity for the competitive analogue 3-*O*-methyl-glucose ($K_m = 4.6 \text{ mM}$; Yasmeen *et al.*, 1977), appears to be an essential component of the D-glucose-dependent chemiluminescent response to concanavalin A. Of a wide range of sugars tested (D-mannose, L-mannose, D-galactose, D-fructose, D-fucose, L-fucose, D-ribose, α -L-rhamnose, maltose, lactose, sucrose, cellobiose and raffinose), only D-mannose and maltose significantly substituted for

D-glucose in supporting the chemiluminescent response. Thus its specificity appears to be confined to D-glucose and D-mannose, uniformly labelled samples of which are metabolized by rat thymocytes at equivalent rates in the presence of concanavalin A (R. M. Taylor & M. J. Weidemann, unpublished work). Maltose is hydrolysed to D-glucose by an endogenous disaccharidase at a rate sufficient to account for its support of chemiluminescence.

Approx. 65% of the concanavalin A-stimulated chemiluminescence observed in the presence of 5 mM-glucose is inhibited by catalase at a concentration of 200 $\mu\text{g/ml}$ (Wrogemann *et al.*, 1978). In contrast, the response of glucose-starved cells was inhibited less than 20% by catalase over the concentration range 100–500 $\mu\text{g/ml}$. This indicates that although H_2O_2 is the major product of the glucose-dependent portion of the response, other active oxygen species may participate in the glucose-independent portion. Polymorphonuclear leucocytes produce H_2O_2 as a result of the dismutation of O_2^- generated by the activation of a cyanide-insensitive NAD(P)H oxidase located within the plasma membrane that transfers single electrons to oxygen (Briggs *et al.*, 1975; Hafeman & Lucas, 1979). The oxidation of glucose via the glycolytic or pentose-phosphate pathways is the most likely

source of reducing equivalents for this reaction. The inhibitors amobarbital, phenylbutazone (Holmes & Good, 1972; Hafeman & Lucas, 1979) and atebirin (Crane & Löw, 1976) have all been used to block electron transfer from reduced nicotinamide nucleotides to oxygen and, thereby, to inhibit cyanide-insensitive O_2^- production. Phenylbutazone and atebirin both strongly inhibited the chemiluminescence produced during the oxidation of luminol by H_2O_2 *in vitro* (Fig. 1a) and were therefore unsuitable compounds for investigating the source of lymphocyte chemiluminescence. Amobarbital had no effect on the chemiluminescence associated with the chemical oxidation of luminol, but strongly inhibited the cellular response evoked by concanavalin A in the presence of glucose (Figs. 1a and 1b). Thus, as in polymorphonuclear leucocytes, the glucose-dependent chemiluminescence may involve the activation of an enzyme capable of generating O_2^- and its dismutation products (H_2O_2 , singlet oxygen and $\text{OH}^\bullet$ radicals). Amobarbital also blocks the antibody-dependent lysis of adenocarcinoma cells by lymphocytes (Hafeman & Lucas, 1979), indicating that O_2^- production may be involved, as it is in the bactericidal action of phagocytes (Klebanoff *et al.*, 1979), in lymphocyte-mediated cytotoxicity. Furthermore, the 'lethal-hit' stage of T-cell-mediated

Fig. 1. Effect of amobarbital on the chemiluminescent response of fresh and 18 h-preincubated rat thymocytes to concanavalin A

(a) The effect of amobarbital (●), phenylbutazone (○) and atebirin (Δ) on the chemiluminescence produced by adding H_2O_2 (7.9 mM final concn.) to 5.0 ml of cell-free Eagle's/Hepes medium containing luminol at the concentration normally used to detect lymphocyte chemiluminescence. (b) Freshly prepared rat thymocytes (2×10^7 cells/ml) (●) and thymocytes preincubated in Eagle's/Hepes medium at 37°C for 18 h (○) were treated with luminol 10 min before, and with amobarbital (at the concentrations shown) 2 min before, concanavalin A addition. The chemiluminescent response to concanavalin A (40 $\mu\text{g/ml}$) was determined as described under 'Methods' and in the legend to Table 1.

cytotoxicity is only expressed when glucose is present in the medium (MacLennan & Goldstein, 1978).

It is possible that the chemiluminescence observed in the absence of glucose may be associated, as it is in macrophages, with the formation of intermediates of arachidonic acid metabolism via the lipoxygenase pathway (Weidemann *et al.*, 1980; Smith & Weidemann, 1980). Fig. 2 shows the effect of adding an arachidonic acid analogue (eicosa-5,8,11,14-tetraynoic acid) that inhibits both the cyclo-oxygenase and lipoxygenase reactions (Flower, 1974) on concanavalin A-induced chemiluminescence. The analogue (0.3–1.5 μM) inhibited the concanavalin A-stimulated response, but not the basal chemiluminescence, to a much greater extent than did its solvent dimethyl sulphoxide. Table 1 shows that the inhibitory effects of eicosa-5,8,11,14-tetraynoic acid addition and glucose omission were substantially additive, whereas the cyclo-oxygenase inhibitor indomethacin (10 μM) had no significant effect. Thus the inhibition by eicosa-5,8,11,14-tetraynoic acid appears to be associated with a blockade of the lipoxygenase rather than the cyclo-oxygenase pathway. The lipoxygenase pathway is known to be activated in peripheral blood lymphocytes in response to mitogens (Kelly & Parker, 1979; Kelly *et al.*, 1979; Parker *et al.*, 1979). It generates hydroxy fatty acid products by way of hydroperoxy intermediates that, while undergoing breakdown, produce chemiluminescence (Sugioka & Nakano, 1976; Cadenas *et al.*, 1980). A portion of the chemiluminescence may be due to the presence of $\text{OH}^\bullet$ formed during the interaction of the lipid hydroperoxide intermediates with O_2^- (Babior, 1978). The hydroxy end-products of the pathway are involved in regulating the spontaneous migration of lymphocytes (McCarty & Goetzl, 1979) and in attracting neutrophilic and eosinophilic polymorphonuclear leucocytes to sites of lymphocyte stimulation by positive chemotaxis (Turner *et al.*, 1975; Goetzl *et al.*, 1977; Lynn & Mukherjee, 1978).

Fig. 3 shows that the dose-dependence of the chemiluminescent response of freshly-prepared thymocytes to concanavalin A is essentially identical with that for mitogen-stimulated glucose transport (Yasmeen *et al.*, 1977), lactate production (Culvenor & Weidemann, 1976) and [^3H]thymidine incorporation (see Fig. 5 below) in rat thymocytes. There is some evidence of positive co-operativity in the dose-response curve (Hill coefficient = 1.5).

Effect of thymocyte preincubation of chemiluminescence and the mitogenic response to concanavalin A

Incubation of thymocytes for 18 h in the absence of mitogen led to a massive enhancement of the chemiluminescent response to concanavalin A (Fig.

Fig. 2. Effect of eicosa-5,8,11,14-tetraynoic acid (ETYA) on concanavalin A-induced chemiluminescence in rat thymocytes

Freshly prepared rat thymocytes (10^7 cells/ml) were incubated in 5 ml of Eagle's/Hepes medium containing 5 mM-glucose. Eicosa-5,8,11,14-tetraynoic acid or dimethyl sulphoxide (DMSO; 55%) were added, at the concentrations indicated, at 12 min and luminol at 10 min before addition of concanavalin A (40 $\mu\text{g}/\text{ml}$). Chemiluminescence in response to concanavalin A addition was measured in the presence of dimethyl sulphoxide ($\circ$) or eicosa-5,8,11,14-tetraynoic acid ($\bullet$) as described under 'Methods'. Basal chemiluminescence in the presence of dimethyl sulphoxide (Δ) or eicosa-5,8,11,14-tetraynoic acid ($\blacktriangle$) alone was determined under identical experimental conditions, in the absence of added concanavalin A. Eicosa-5,8,11,14-tetraynoic acid in the concentration range added had no inhibitory effect on the chemiluminescence generated by adding H_2O_2 to luminol.

4). The best responses corresponded consistently with the appearance of a second peak, or shoulder, of chemiluminescence characteristic of the interaction of concanavalin A with endogenous thymic macrophages (Wroegemann *et al.*, 1978, 1980). The

Fig. 3. Concanavalin A concentration-dependence of chemiluminescence in fresh and 18h-preincubated rat thymocytes

The dose response of freshly prepared (●) or 18h-preincubated (○) rat thymocytes to concanavalin A was determined by treating cells with luminol 10 min before concanavalin A and measuring the increase in chemiluminescence brought about by the lectin during the 5 min period after its addition at various concentrations. The mean results for three separate preparations of fresh cells and four preparations of preincubated cells are given. h is the Hill coefficient (mean $\pm$ S.E.M.) determined for the appropriate cell preparation.

optimum dose of concanavalin A was unaltered in the 18h-preincubated cells, but they had become more responsive at low concentrations and were inhibited at high concentration (Fig. 3). Preincubation also caused a significant shift in the sensitivity of the concanavalin A response to inhibition by amobarbital (Fig. 1).

Fig. 4. Time course of the chemiluminescent response of fresh or preincubated cells to concanavalin A

Concanavalin A (40 μ g/ml) was added at zero time (i.e. 10 min after luminol addition) to 2×10^7 cells/ml of either freshly prepared cells (▲), cells preincubated for 6 h (○) or cells preincubated for 12 h (●). The subsequent chemiluminescent response was determined as described under 'Methods'. The results are from a typical experiment in which all of the cells used were from a single preparation obtained from three pooled thymus glands.

Goodwin *et al.* (1977, 1978) have shown that preincubation of lymphocytes before mitogen treatment leads to the death of prostaglandin-producing suppressor cells and that added indomethacin mimics the effect of preincubation in a non-additive manner. Such a mechanism could account, theoretically, for the enhanced chemiluminescence observed in the present work, since the response of fresh cells to concanavalin A is strongly inhibited by prostaglandins of the E-series (see Table 1). However, indomethacin (10 μ M) present during the 18h-preincubation period had no effect on the subsequent chemiluminescent response and we could find no evidence that the sensitivity of the reaction to added prostaglandin E_1 was altered during preincubation (result not shown).

Finally, we attempted to establish whether the amplified chemiluminescence observed in preincubated cells is associated with an enhanced mitogenic response and whether the inhibition of

Fig. 5. Effect of preincubation on the time course and concentration-dependence of concanavalin A-induced [^3H]thymidine incorporation by rat thymocytes

Rat thymocytes, either freshly-prepared (O) or preincubated in plastic scintillation vials for 12 h (●) as for the chemiluminescence assays, were cultured in microtitre plates (at 5×10^6 cells/ml in 0.2 ml) at three different concentrations of concanavalin A: (a) $3 \mu\text{g/ml}$; (b) $10 \mu\text{g/ml}$; and (c) $40 \mu\text{g/ml}$. [^3H]Thymidine ($1 \mu\text{Ci}$, $10 \mu\text{M}$) was added 12 h before the elapse of the total culture time for each cell sample when the cells were harvested and counted for radioactivity as described under 'Methods'. The results presented are expressed as [^3H]thymidine radioactivity (c.p.m.) incorporated and are the means of five separate experiments.

chemiluminescence by catalase would inhibit mitogenesis. Cells preincubated overnight and cultured subsequently in fresh medium containing concanavalin A incorporated larger amounts of [^3H]thymidine than did fresh cells at low doses of mitogen, whereas the response at the optimum concentration was unaffected (Fig. 5). In addition, the onset of [^3H]thymidine incorporation occurred approx. 12 h earlier in the preincubated cells at all concanavalin A doses. Incubation with catalase amplified, rather than abolished, the response to low doses of concanavalin A; it did not alter the response at the optimum dose, however, and in this sense mimicked preincubation (Fig. 6). It may be significant in this context that catalase inhibits prostaglandin synthesis in other types of cells, including macrophages (Weidemann *et al.*, 1980; Vargaftig *et al.*, 1975). Prostaglandins may not directly inhibit enhanced [^3H]thymidine incorporation in lymphocytes that respond to low doses of concanavalin A, but may inhibit, instead the production of stimulatory soluble mediators by macrophages (Rosenstreich & Mizel, 1978). Persson *et al.* (1978a,b) have suggested that the T-cells that respond to low doses of concanavalin A are a subpopulation uniquely dependent on the presence of macrophages. The ability of low concentrations of concanavalin A to stimulate the spleen plaque-forming cell response suggests that the responsive cells may be helper

T-cells (Sjöberg *et al.*, 1973; Dutton, 1973; Watson *et al.*, 1973).

The failure of catalase to inhibit [^3H]thymidine incorporation (Fig. 6) indicates that there is no obligatory link between H_2O_2 production and RNA synthesis. We have shown previously that the activation of glycolysis by mitogens is neither a necessary nor sufficient precondition for the progression of lymphocytes into the S-phase of the cell cycle (Hume *et al.*, 1978). The proliferative step is initiated by a physiological mitogen, T-cell growth factor, that will only stimulate lymphocytes rendered responsive to it as a result of the initiation of cell cycling by an antigenic or mitogenic stimulus (Larsson *et al.*, 1980). It remains possible that, among the early metabolic events initiated by mitogens, H_2O_2 production and the activation of glycolysis are causally related (Hume & Weidemann, 1980). Added H_2O_2 stimulates thymocyte 3-O-methylglucose transport in a manner that is not additive with that induced by concanavalin A (Hume *et al.*, 1979). Furthermore, there is evidence that the activation of the glucose carrier by mitogens involves the oxidation of membrane thiol groups (Hume & Weidemann, 1978), a process in which endogenously generated H_2O_2 may participate.

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Fig. 6. Effect of catalase on concanavalin A-induced [^3H]thymidine incorporation by rat thymocytes

Freshly-prepared rat thymocytes were cultured (at 5×10^6 cells/ml in 0.2 ml) at the concanavalin A concentrations given in the presence (●) or absence (○) of catalase (200 µg/ml) for 24 h before [^3H]thymidine addition. They were harvested and counted for radioactivity after a further 12 h as described under 'Methods'. The results are the means for five separate experiments and are expressed as [^3H]thymidine radioactivity incorporated (c.p.m.).

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