Effects of glucose, anoxia, and adriamycin on the chemiluminescence of Ehrlich Ascites cells

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Glucose and anoxia accelerate the photocount due to luminescence of Ehrlich Ascites cells. Adriamycin also has this effect if glucose is present. Comparison with a chemical standard combined with estimates of cellular and population transmittance yield a photon generation rate of at least $10.s^{-1}.cell^{-1}$ in the presence of 10^{-2} M glucose, and twice this with anoxic conditions or 10^{-5} M adriamycin. Effects of adriamycin on Ehrlich Ascites cell respiration may depend on the presence of glucose.

Chemiluminescence *Antibody* *Ehrlich Ascites* *Adriamycin* *Anoxia* *Glucose*

1. INTRODUCTION

Adriamycin (ADM), an anticancer drug, uncouples oxidative phosphorylation, depresses ubiquinone Q10 concentration, and increases lipid peroxidation in rat heart mitochondria [1], increases cellular levels of lipoperoxide [2], and reduces horse heart ferricytochrome *c*, in the absence of superoxide dismutase, with concomitant production of the ADM semiquinone free radical [3]. It increases UDP glucuronyl transferase activity by 450%, microsomal lipid peroxidation 6-fold, and decreases cytochrome P450 activity by 85%, cytochrome P450-dependent monooxygenation by 70%, and glucose 6-phosphate activity by 80% in rat liver microsomes [4]. ADM produces DNA cleavage in a reaction mediated by molecular oxygen after reduction to a semiquinone free radical by NADPH cytochrome P450 reductase [5]. When ADM is fixed to a solid support in a chromatography column, tumor cell killing is observed on their passage through the column so it is not necessary for ADM to enter the cell to exert its effect [6]. When it does enter the cell the ADM semiquinone becomes firmly attached to the microsomal fraction [7]. ADM is less toxic to hypoxic than to aerobic sarcoma cells [8] and its ESR spectra in aerobic microsomes appear only after a lag which is proportional to the oxygen concentration [9]. A recent study of ADM delivery with antibody conjugates [10] has made it especially important to investigate the cytotoxic mechanism of the drug.

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Abbreviations: ADM, adriamycin; UDP, uronyl diphosphate; CL, chemiluminescence; EAC, Ehrlich Ascites cells

2. METHODS

The photon counter employed has been described in [11]. The cuvette was attached to the light collector by a brass tube slotted at the front for insertion of an aluminum flange which was attached to the cuvette. This flange had 4.7 cm square outer dimensions and a 4.46 cm diameter center hole, which was glued with silicone cement to the window end of the 4.45 cm diameter by 9 cm long aluminum tube serving as cuvette. The 0.16 cm thick quartz window and aluminum disc rear wall of the cuvette were attached with silicone cement.

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A hole in the top of the cuvette provided access for a no.6 rubber stopper. This in turn was bored to accept a full-size Clark-type oxygen electrode and a small diameter plastic tube to serve as an inlet for reactant injection syringes. The quantum efficiency of the apparatus was found to be 2.4×10^{-3} by the luminol standard reaction [12]. A shroud of doubled, rubberized darkroom drapery cloth was fitted to the adaptor. Reactant additions were made by insertion of syringes through the cloth and into the plastic guide tube. Anaerobic conditions were achieved by wrapping the cuvette with several layers of parafilm.

Ehrlich Ascites cells (EAC) were maintained by passage in purebred white mouse peritoneal cavities where they were grown for 7 days. After harvesting they were washed twice in Ringers solution and resuspended in 100 ml Ringers. The cells were counted in a hemocytometer and checked for viability after some experiments by trypan blue exclusion, and oxygen uptake. The ADM (Sigma) was prepared freshly for each experiment. A small magnetic stirrer was used to keep the cells from settling during each experiment. It made no detectable contribution to the signal. The temperature was maintained at 27 ± 0.5°C.

3. RESULTS

All results are summarized in table 1. The background luminescence due primarily to events in Ringers buffer was about 5 cps greater than dark count in fig.1 (▲). The average fluctuation of background including dark count was 1.4 cps based on 12 expt. After slowly injecting EAC, the count rate remained the same in the experiment shown in fig.1. The average increase over background in 9 expt was 0.7 ± 1.7 cps. Injecting glucose to a final concentration of 1×10^{-2} M resulted in an increase of count rate to 10 cps over background. In 9 expt the average of the plateau count rate over background was 7.3 ± 3.8, and the average maximum value was 8.9 ± 3.3. Injecting ADM to a final concentration of 1×10^{-5} M raised the count rate to about 18 cps over background. The average plateau value, in excess of background, produced by ADM was 12.2 ± 6.2 based on 3 expt. The average maximum was 13.9 ± 5.5. Addition of ADM prior to glucose resulted

Table 1

n	Condition	Average maximum	Average plateau
9	Glucose	8.9 ± 3.3	7.4 ± 3.9
4	Glucose + anoxia	15.8 ± 3.7	12.8 ± 2.4
3	Glucose + ADM	14.0 ± 5.5	12.2 ± 6.2
2	ADM	4.1 ± 0.3	2.2 ± 0.3
2	ADM + glucose	9.6 ± 0.1	8.4 ± 0.5

n = number of experiments, values = cps over background

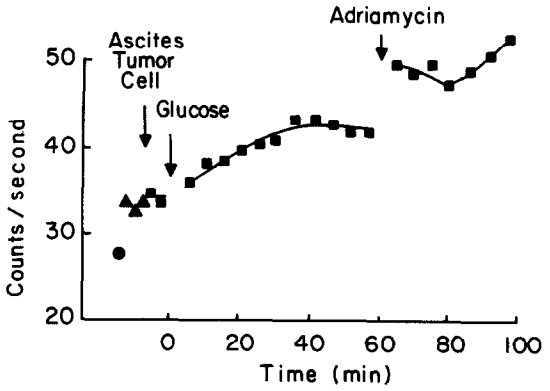

Fig.1. Effects of 10^{-2} M glucose and 10^{-5} M ADM on CL of 10^6–10^7 EAC: Dark count (●); background (▲); ascites cell (■); post glucose and adriamycin (■, as indicated by arrows) signals.

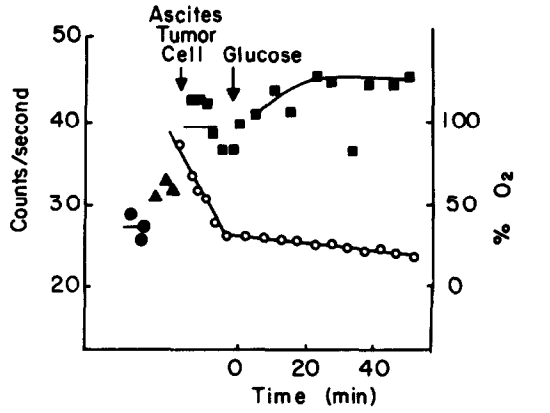

Fig.2. Effects of glucose on % oxygen saturation (○) and EAC CL (symbols as in fig.1).

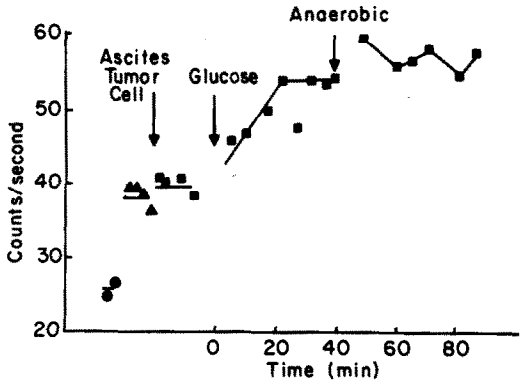

Fig.3. Effects of glucose and anoxia on EAC CL (symbols as in fig.1).

in an average plateau of 2.3 ± 1.5 and an average maximum of 4.1 ± 0.3 cps greater count rates than background in 2 expt. Subsequent addition of glucose produced an average maximum of 9.6 ± 0.1 and an average plateau of 8.4 ± 0.8 cps over background.

Fig.2 shows a similar experiment in which the EAC produced luminescence exceeding that of the buffer by 4 ± 0.4 cps. Again, addition of glucose produced a slow increase in count rate (typically 20 min to plateau). The oxygen electrode trace (○) indicates a sharp break at the point of glucose injection indicating much lower oxygen utilization rates by the cells after that point. In fig.3 the luminescence increase and plateau after supplementing Ringers buffer with 10^{-2} M glucose is again apparent and the results of subsequently making the system anaerobic are shown. Four such experiments produced an average plateau of 12.8 ± 2.4 and an average maximum of 15.8 ± 3.7 in excess of background. Glucose alone added to buffer in absence of EAC, produced an average maximum of 4.6 ± 0.5 and an average of 2 ± 0.3 cps over background. ADM at a final concentration of 10^{-5} M reduced the background count rate 3.1 ± 1.4 cps.

4. DISCUSSION

The addition of glucose to EAC results in an increase in CL count rates over background. This is accompanied by decreasing O_2 concentrations in the cuvette and probably by acidification of the medium. The excretion of acid by EAC metabolizing glucose has been studied in [13], among others. The 40-ml aliquot in the cuvette employed in these experiments minimized the effects of acidification, although Ringers solution is a very weak buffer. The count rate change of +7 cps caused by glucose represents an intensity increase of about 3000 photons/s from the cuvette based on the luminol calibration described in section 2. If reabsorption by cells other than the emitter diminished the intensity by a factor of 100 (i.e., 1% of the light emitted by the cells hit the photocathode) an average of 0.1 photon/s was emitted by each cell. If the photons had 1% probability of escaping the emitter cell after generation, then photons are produced at the rate of 10 $s^{-1}.cell^{-1}$. This is consistent with an enzyme reaction with relatively slow turnover and high quantum yield (photons generated/reaction) or a fast turnover (e.g., 1000/s) and lower quantum yield.

Both anoxia and adriamycin additions produced an intensification of about twice the value achieved by glucose addition for an emission rate of 18 photons.$s^{-1}.cell^{-1}$. Intensification due to anoxia may result from reduction of cellular oxygen supplies by the abnormally reduced metal and flavin centers [14]. The very small increase of count rate due to the addition of adriamycin alone indicates that glucose plays some role in ADM-mediated generation of CL. This could be due to some transport effect, lowering of oxygen concentrations facilitating the radical species of ADM, activation of some metabolic pathway which potentiates ADM toxicity, or enhancement of reduced cellular conditions and increased concentrations of reduced oxygen species which are potentiated by the presence of ADM.

Glucose-mediated transport is well established for many chemicals. High glucose concentrations, acidification of the medium, anoxia and CL intensity increase are all features of hexose monophosphate shunt activation [15,16]. This could produce emission from CO_2 derivatives [17], $HO^{\cdot}$ [18,19], singlet oxygen or excited state ketones via the Russell mechanism [20], or other excited state carbonyls via dioxetane reactions [21]. Since ADM is found complexed with intracellular membranes [7] its effect on them may render them more susceptible to attack by reduced oxygen species. Killing of EAC by external ADM

[6], if it occurs via reduced oxygen species as proposed by those authors, must be much less efficient in the absence of glucose than in its presence. Alternatively, internal drug might be much more efficient although external drug does have some toxicity. The CL signal is much more intense if glucose is present with the ADM and takes several minutes to achieve maximum intensity. Both these conditions indicate internalization of the drug.

Tumor cells of the age employed in this experiment have unusually high concentrations of free radicals, most of which are in the mitochondria [22]. In a study of mitochondrial and EAC respiratory inhibition by ADM and other agents it was found that ADM inhibition of mitochondrial respiration is much greater than inhibition of EAC respiration [23]. Based on the CL response of EAC to ADM with and without glucose this was due to the absence of glucose in the medium employed in the EAC experiments.

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