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Lithium γ-linolenate-induced cytotoxicity against cells chronically infected with HIV-1

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Lithium γ-linolenate (Li-GLA), was evaluated for its activity in selectively killing H9 cells chronically infected with HIV-1_{RF}. After 4 days incubation with Li-GLA approximately 90% of the $H9_{RF}$ cells were non-viable compared to 20% of uninfected H9 cells. The efficacy of the Li-GLA, in preferentially killing HIV infected cells also correlates with lipid peroxidation, as measured by the intracellular thiobarbituric acid-reactive material content. The addition of an antioxidant (vitamin E) to the culture medium reduced the toxicity of Li-GLA. These data indicate that this selective killing effect of cells chronically infected with HIV may be due to the enhanced extent of lipid peroxidation of the added Li-GLA.

HIV; Lipid peroxidation; Syncytium inhibition

1. INTRODUCTION

In recent years an increasing interest has been shown in developing new targets for antiviral chemotherapy. These approaches have included altering plasma membranes of cells so that uninfectious HIV particles are released [1,2], and inducing defective intracytoplasmic vacuolar HIV-1 formation in cells [3]. In this study, we have investigated the selective cell killing effect of the lithium salt of the polyunsaturated fatty acid, gamma linolenic acid (Li-GLA 18:3n-6) on human T-lymphoblastoid cells chronically infected with HIV.

Polyunsaturated fatty acids (PUFAs) have been reported to inactivate Herpesviruses, influenza virus and Sendai virus but were found to have little activity against the non-enveloped viruses, polio, encephalomyocarditis or simian virus 40 [4]. In addition monoolein, monolinolein and γ-linolenyl alcohol were shown to have antiviral activity against the enveloped African swine fever virus. γ-Linolenyl alcohol was the most potent compound tested [5]. A number of saturated and unsaturated fatty acids were subsequently evaluated for their activity against HSV-1 and HSV-2, and it was found that the most efficacious compounds were the fatty acids with one, two or three double bonds; γ-linolenic acid (GLA) was found to have a good selectivity for infected cells [6]. GLA is known to generate lipid peroxides which exert a possible cytostatic effect [7,8]. Hence, a study was set up to investigate the effect of GLA on cells chronically infected with HIV and on cell-to-cell transmission. This report describes the selective activity of Li-GLA, and discusses the possible modes of action of this compound.

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2. METHODS

2.1. *Cell culture with Li-GLA*

H9 cells [9] chronically infected with HIV-1 ($H9_{RF}$) and uninfected H9 cells were maintained in RPMI 1640 medium supplemented with 2 mM L-glutamine, 5% foetal calf serum, 100 μg/ml streptomycin and 100 IU/ml penicillin. Cells were maintained actively dividing by feeding 24 h prior to experiments. Six ml culture tubes were seeded with 2×10^4 H9 or $H9_{RF}$ cells in 3 ml medium supplemented with 10, 15 or 20 μg/ml Li-GLA (Callanish Ltd., Scotland) or an equivalent volume of RPMI medium as a control, and were incubated for four days at 37°C in a 5% CO_2 atmosphere. On days 2, 3 and 4 viable cell counts were determined using trypan blue exclusion. Similar assays were carried out with Li-GLA with the further addition of vitamin E (4 μg/ml) (Sigma Chemical Company, UK). Cells treated with vitamin E only were cultured for comparison.

2.2. *Back titration assay*

One ml volumes of supernatant from $H9_{RF}$ cell cultures treated with Li-GLA for 2, 3 and 4 days were added to one ml volumes containing 2×10^5 C8166 (T-lymphoblastoid) cells. Untreated cultures were included as controls. Cell cultures were incubated at 37°C in a 5% CO_2 atmosphere and observed daily for the presence of syncytia.

2.3. *Cell fusion assay*

H9 and $H9_{RF}$ cells were pretreated with Li-GLA-containing (5 μg/ml) medium or unsupplemented RPMI as a control for three days. The cells were harvested and resuspended in RPMI supplemented with 5% foetal calf serum. Cells were then mixed in combinations of treated and untreated $H9_{RF}$ and H9 cells at a ratio of 1 : 10 respectively and resuspended with 2 ml of culture medium in 24-well plates. The cultures were incubated at 37°C in a 5% CO_2 atmosphere and scored for syncytium formation at 18 h.

2.4. *Lipid peroxidation*

Twenty-five cm^2 culture flasks were seeded with 2×10^6 H9 or $H9_{RF}$ cells in 12.5 ml RPMI medium, supplemented with 5% foetal calf serum, in the presence and absence of 10 and 20 μg/ml Li-GLA. Cultures were incubated at 37°C in a 5% CO_2 atmosphere for three days. Cells were separated from the medium by centrifugation at 2,000 rpm, resuspended in phosphate-buffered saline (PBS), and assayed for thiobarbituric acid-reactive material (TBARM) [10]. Briefly, one ml of 20% trichloracetic acid was added to cells resuspended in 2 ml (PBS) and to two ml of culture medium as control. Two ml of 0.67% thiobarbituric acid was added to each tube and thoroughly mixed. The tubes were incubated for 20 min in a 90°C water bath, cooled and centrifuged at 2,600 rpm for 10 min. The absorbance of the supernatants was measured at 532 nm using PBS as a reference. Absorbance was converted to nanomoles of malonaldehyde-equivalents from a standard curve generated with 1,1,3,3-tetramethoxypropane (Malonaldehyde bis [dimethyl acetal]).

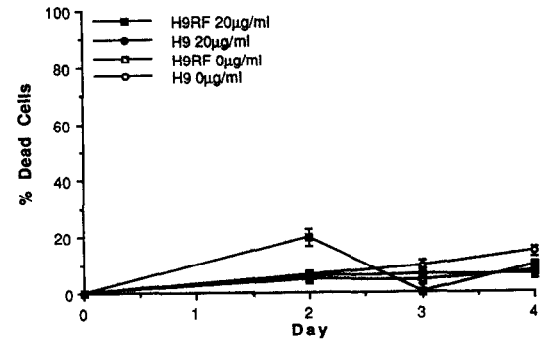

Fig. 2. Effect of Li-GLA on H9 and $H9_{RF}$ cells in the presence of 4 μg/ml vitamin E. Vitamin E alone had no toxic effect on the cells. Each data point on the graph represents the mean of five values ± 1 S.D.

3. RESULTS

The effect of Li-GLA on the viability of H9 and $H9_{RF}$ cells in culture can be seen in Fig. 1. The Li-GLA treated $H9_{RF}$ (chronically infected) cells show much greater cell death than the H9 (uninfected) cells with maximum cell death appearing at day 4 of culture. The addition of vitamin E to Li-GLA treated cultures shows that the cell killing effect is negated and the growth of treated cultures is simular to that of untreated cultures (Fig. 2). Supernatants from Li-GLA treated $H9_{RF}$ cultures which were back-titrated on to C8166 cells showed that at 20 μg/ml there was no release of infectious virus into the culture medium (Table I). Lower concentrations of Li-GLA inhibited virus release to a lesser extent however, viral release was considerably lower than in the untreated controls.

Fig. 3 shows the results of the cell fusion assays which are indicative of cell-to-cell transmission. Mixed cultures of H9 and $H9_{RF}$ cells pretreated with Li-GLA (5 μg/ml) showed a marked reduction in the formation of syncytia as compared to mixed cultures not treated with Li-GLA. Interestingly, cell-to-cell transmission was also reduced in the mixed culture containing pretreated H9 cells and untreated $H9_{RF}$ cells but not noticeably in mixtures containing untreated H9 cells and pretreated $H9_{RF}$ cells. Concentrations of Li-GLA higher than 10 μg/ml are sufficiently toxic to $H9_{RF}$ cells to remove the conditions for syncytium formation.

The thiobarbituric acid test revealed that H9 and $H9_{RF}$ cells treated with Li-GLA produced thiobarbituric acid-reactive materials (TBARM) in a dose-dependent manner, i.e. the greater the concentration of Li-GLA in the culture the more TBARM was produced. The amount of TBARM produced in $H9_{RF}$ cells was much greater than that in H9 cells (Fig. 4).

4. DISCUSSION

The experiments described here were designed to explore the possibility that Li-GLA may have a selective killing effect on HIV-infected cells and, if so, the mechanisms by which it may be acting. Previous studies have shown that tumour cells, which are supplemented with PUFAs are selectively killed at concentrations which do not harm normal cells. Tumour cells produce more superoxide radicals in response to PUFAs than do treated non-tumour cells. Superoxide formation correlates with cell killing and GLA has been identified as one of the most active compounds [8,11]. The data presented here show that the addition of Li-GLA has a significant effect on the H9 cells chronically infected with HIV,

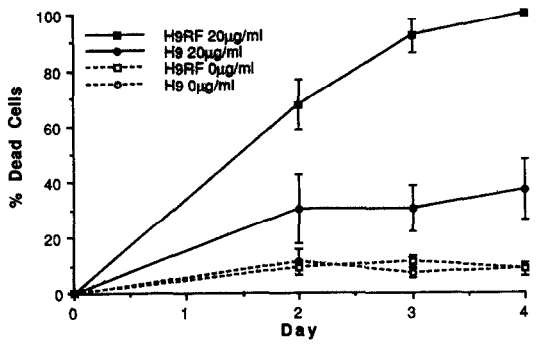

Fig. 1. Selective cytotoxicity of Li-GLA for $H9_{RF}$ cells. Each point on the graph represents the mean of five values ± 1 S.D.

Table I

Syncytium formation in C8166 cells incubated with culture supernatants from $H9_{RF}$ cells treated with Li-GLA

Li-GLA concentration	10^0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
20 μg/ml	−	−	−	−	−	−
15 μg/ml	−	−	−	−	−	−
10 μg/ml	+	+	−	−	−	−
0 μg/ml	+	+	+	+	−	−

+ = syncytium formation, − = no syncytium formation. Syncytium formation was observed daily, this data represents day four of incubation only. Syncytia were beginning to appear in the undiluted control cultures on day three but syncytia were not seen in any of the other cultures at this time.

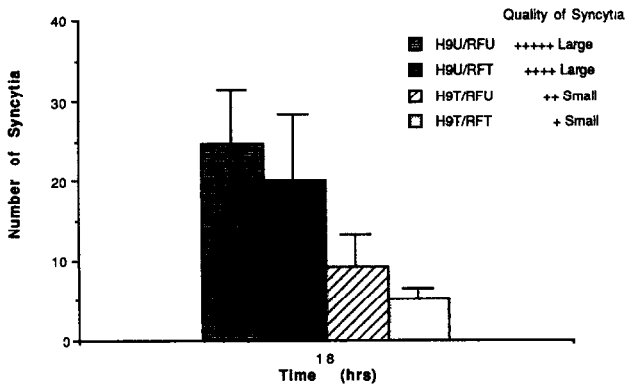

Fig. 3. Fusion assay measuring cell-to-cell transmission of HIV. U, untreated cell cultures; T, cells treated with Li-GLA. This experiment was carried out on two separate occasions and the relative degree of syncytium formation between the experiments was similar. Cultures showing least syncytium formation were cultures of $H9_{RF}$ (treated), H9 (treated) and $H9_{RF}$ (untreated), H9 (treated).

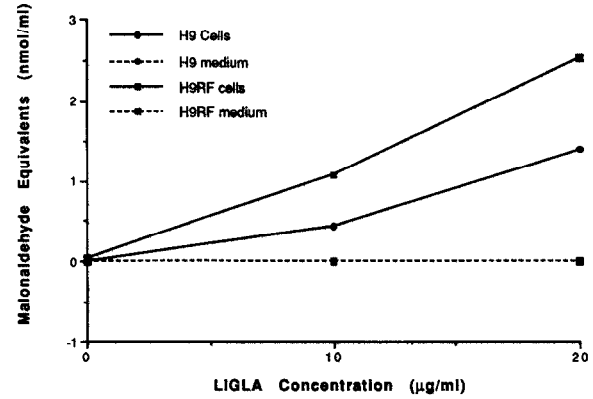

Fig. 4. Production of thiobarbituric acid-reactive material (TBARM) in cells in the presence of Li-GLA. Malonaldehyde-equivalents for each culture were deduced from a standard curve created using 1,1,3,3-tetramethoxypropane standards. Each point on the graph represents the mean of triplicate values.

under the conditions described. Further, the killing capacity of Li-GLA correlates with elevated concentrations of aldehydes and ketones, which are products of lipid peroxidation, as demonstrated by the reaction with thiobarbituric acid. This effect is an indication of enhanced peroxidation within treated cells. Previous work has shown that the ability of PUFAs to kill malignant cells is inhibited by vitamin E, indicating that generation of lipid peroxides is involved in this process [8]. The possibility that the same mechanism is at work in the destruction of plasma membranes and other organelles is supported by the inhibitory effect of the antioxidant, vitamin E, on the killing of chronically infected cells by Li-GLA.

The back-titration data showed that the killing of the $H9_{RF}$ cells does not release infectious virus into the supernatant. This is a more sensitive and specific assay of virus infectivity than the determination of HIV-1 p24 or reverse transcriptase activity as the latter values may include the measurement of defective virions. This is an important finding for any future in vivo studies.

The cell-fusion assays indicated that pretreatment of cells at a sub-toxic concentration (5 µg/ml) of Li-GLA delays the onset of syncytium formation. This indicates that Li-GLA may have a transient effect on cell-to-cell transmission of virus. Syncytium formation was also markedly reduced when the H9, but not the $H9_{RF}$ cells had been exposed to Li-GLA. This latter observation may represent an additional activity of Li-GLA operating at lower concentrations and by a different mechanism to the selective toxicity seen in the other studies.

In conclusion, we have demonstrated that Li-GLA in vitro selectively kills cells chronically infected with HIV. Our data is consistent with a model in which HIV-chronically infected cells have an alteration in their oxidative metabolism of lipids, resulting in the production of cytotoxic metabolites of Li-GLA. Further work is in progress to investigate the properties of Li-GLA in HIV infections.

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