THE EFFECTS OF LOW CONCENTRATIONS OF 2-DEOXY-D-GLUCOSE ON THE GROWTH, SURFACE ARCHITECTURE, AND GLYCOPROTEIN METABOLISM OF P388 LYMPHOMA CELLS

MARJORIE W. MYERS AND ALAN C. SARTORELLI

Department of Pharmacology, Yale University School of Medicine,

New Haven, Connecticut 06510

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SUMMARY

Exposure of P388 lymphoma cells to a concentration of 50 μM 2-deoxy-D-glucose (dglc) resulted in inhibition of growth only after exposure for several generations. Prior to inhibition of growth, radioactive dglc was maximally incorporated into cellular glycoprotein, surface architecture was altered as measured by plant lectin induced agglutination, and cellular fucose content was significantly increased. These phenomena were obtained in the absence of changes in the cellular content of ATP which decreased at levels of dglc greater than 200 μM. Following removal of dglc from cell cultures, radioactivity from dglc incorporated into glycoprotein was lost at a rate significantly greater than the loss of radioactivity from either glucosamine or fucose, cell growth resumed at a normal rate, and the susceptibility to plant lectin induced agglutination returned to the pattern present in untreated cells.

Dglc in sufficiently high concentrations is a potent inhibitor of cell growth, presumably by causing depletion of cellular ATP. At relatively lower levels, this carbohydrate antimetabolite has been shown to interfere with (a) synthesis of yeast cell wall glycoproteins and mannans (1,2), (b) viral replication (3), and (c) glycosylation of IgG_1 in neoplastic plasma cells (4). In addition, dglc is activated to a nucleotide-sugar complex and subsquently is incorporated into both mammalian and yeast cell glycoprotein, glycolipid, and mannan (5-7). This paper describes the relatively long term effects of dglc on the growth of murine P388 leukemic cells, particularly investigating the relationships between inhibition of growth rate, cellular glycoprotein metabolism and cell surface characteristics. A preliminary report of this work was described (8).

MATERIALS AND METHODS

P388 murine lymphoma cells were grown in suspension culture in Fischer's medium supplemented with 10% horse serum (9). Cultures were seeded at a level of 1 x 10^4

cells/ml. Cells were collected by centrifugation and numbers were determined with a Coulter Counter, Model B.

Protein was measured by the method of Lowry et al. (10) using bovine serum albumin as a standard. Fucose was determined by the method of Dische and Shettles (11) and was compared to that of appropriate standards (fucose or fucose plus hexoses)

Sialic acid was assayed by the method of Warren (12), using N-acetylneuraminic acid as a standard.

[^{3}H]-L-Fucose (13.7 mCi/umole), [^{14}C]-D-glucosamine-HCl (3.96 uCi/umole), or [^{14}C]-2-deoxy-D-glucose (6.83 uCi/umole) was added to cell growth media such that the final concentration of isotope per ml was: fucose, 1 uCi; glucosamine, 0.006 uCi; and deoxyglucose, 0.057 uCi. Cells were labeled with fucose and glucosamine for 12 hours and for the entire period of a growth experiment with dglc. Radioactivity was determined by removal of samples of 5 x 10^5 cells at selected intervals, washing as previously described (13), and counting the precipitate dissolved in NaOH in Aquasol with a Packard Tricarb Scintillation Spectrometer.

ATP was assayed by the luciferin-luciferase assay according to Weiner et al. (14). This was accomplished by washing 5 x 10^7 cells free of medium with sodium phosphate buffered 0.9% NaCl (pH 7.3) and homogenizing the cells in 10 ml of 0.8 M $KClO_4$. After centrifugation (5000 x g) for 10 min, the supernatant was neutralized with 0.8 M KOH and the precipitate was removed. The assay mixture contained 1 ml of 0.01 M potassium phosphate buffer (pH 7.4) $\pm$ ATP standards, 0.1 ml of cell extract, 0.1 ml of luciferin-luciferase extract (1 vial of Sigma FLE-50 to which 5 ml of H_2O had been added), and 2.8 ml of H_2O. Enzyme was added in a scintillation vial and counts were measured at a constant time interval. A standard curve of ATP versus initial counts observed in 0.1 min was obtained for ATP concentrations of 1 x 10^{-11} M to 5 x 10^{-10} M.

Cell agglutination studies were carried out by the method of Hwang et al. (15). Cells (1 x 10^7/ml) in Ca^{++}, Mg^{++}-free phosphate buffered 0.9% NaCl (pH 7.4) were placed in a 1 ml cuvette at 0°. Varied amounts of the plant lectins concanavalin

TABLE 1

Fucose and Sialic Acid Content of P388 Cells Grown in the Presence of 50 μM 2-Deoxy-D-glucose

	Control	2-Deoxyglucose
Cells/ml	4.6 x 10^5	2.8 x 10^{5*}
Cells/mg protein	3.5 x 10^7	3.7 x 10^7
Fucose (μg/mg protein)	11.7	17.1*
Sialic acid (μg/mg protein)	30.0	27.1**

The influence of 50 μM 2-deoxy-D-glucose on growth and protein, fucose, and sialic acid content of P388 lymphoma. Cells were grown in medium with or without dglc for 72 hours. Aliquots were taken for measurements as described in the Methods section. *$p < 0.01$; **$p < 0.05$.

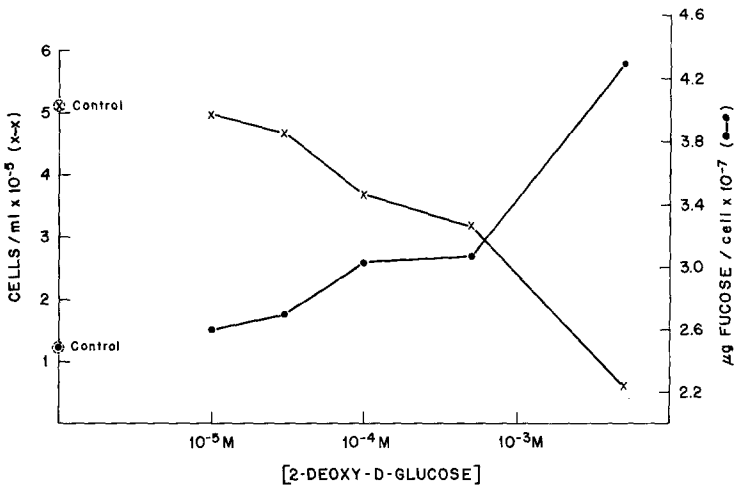

FIGURE 1. The effect of the concentration of 2-deoxy-D-glucose on the relationship between growth and fucose content of P388 lymphoma. Cells were grown in medium containing various concentrations of dglc for 64 hours. At this time, aliquots were taken for determinations of cell number (x) and fucose content (•). The results obtained with untreated cultures are presented as ⊗ and ⊙.

A and wheat germ agglutinin (Miles Laboratories) were added, mixed, and the cuvette placed in a recording Gilford spectrophotometer with the cuvette chamber maintained at 37°. Agglutination was monitored at 546 nm.

RESULTS AND DISCUSSION

The effects of exposing P388 cells to 50 uM dglc for 72 hours are given in Table 1. The rate of cell growth was inhibited as evidenced by fewer cells per ml of medium in drug treated cultures than in control medium. That inhibition of cellular growth at 50 uM dglc could not be explained by depletion of cellular ATP pools was supported by the finding that ATP levels (2.02×10^{-15} moles of ATP/cell) were identical in untreated and dglc treated cells. Cellular ATP concentrations were lowered only by concentrations of dglc greater than 200 μM.

The cellular contents of two components of glycoproteins and glycolipids (i.e., fucose and sialic acid) were significantly altered by 50 μM dglc. The fucose content of cells exposed to dglc was increased as demonstrated by a higher amount of fucose per mg of protein (Table 1). In contrast, sialic acid content was decreased in dglc treated cells. The quantity of protein per cell was unaltered by drug treatment.

The relationship of the concentration of dglc to the inhibition of growth and the fucose content of P388 cells is shown in Fig. 1. After 64 hours of exposure

to varying quantities of dglc, the cell number remained near control levels until the concentration of dglc exceeded 5 x 10^{-5}M. At concentrations of dglc above 5 x 10^{-4}, the cellular ATP concentration decreased and cell growth stopped completely. The fucose content per cell increased over the range of dglc concentrations employed, including dglc levels which produced growth inhibition but not ATP depletion. These findings suggest (a) a reciprocal relationship between cell growth inhibition by this agent and the composition of cellular fucosyl glycoproteins and glycolipids and (b) that it may be possible to differentiate between non-ATP depleting and ATP depleting lesions created by the carbohydrate antimetabolite.

The time course of cell growth in the presence of 50 μM dglc is shown in Fig. 2. The growth of P388 cells was not inhibited immediately following exposure to dglc; however, about 48 hours (4 cell generations) later the growth rate decreased to near zero. This effect was not reversed by the addition of glucose or additional

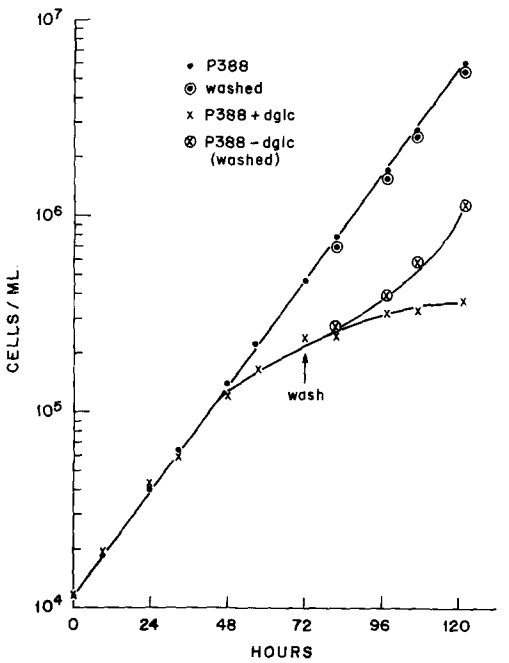

FIGURE 2. The time course of the effects of 50 μM 2-deoxy-D-glucose on the growth of P388 cells. Cell number was determined at various times after initiation of cultures in the presence and absence of dglc. At 72 hours some of the cells were collected by centrifugation, washed, and diluted in Fischer's medium in the absence of dglc and reincubated. Subsequent determinations of cell number were adjusted to reflect the dilution.

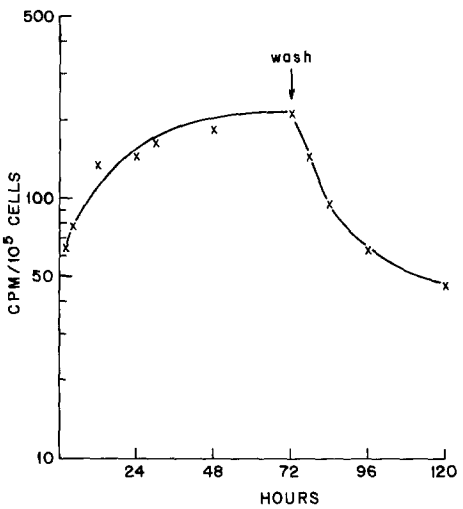

FIGURE 3. The rate of incorporation of [^{14}C]2-deoxy-D-glucose into the glycoprotein of P388 lymphoma. At 72 hours, cells were collected, washed, and diluted with fresh Fischer's medium in the absence of dglc to maintain a continuous culture.

medium to the culture. Cell death does not appear to occur since growth can resume after removal of dglc from the growth medium. Thus, following 12 to 24 hours after removal of dglc, cells initiated division and growth rate increased, becoming similar to that of control cells by 24-36 hours after washing. Untreated cells receiving similar treatment divided logarithmically after a short lag.

P388 cells incubated with [^{14}C]-labeled dglc over a similar time period incorporated radioactivity into acid- and ethanol-insoluble material, which is presumed to be glycoprotein. Fig. 3 presents the quantity of ^{14}C from dglc incorporated over a 120 hour time period. Cells were rapidly labeled in the presence of [^{14}C]dglc and approached maximum specific activity by 12-24 hours, which precedes the decrease in cellular growth rate produced by this agent (Fig. 2). Incorporated radioactivity remained constant during the period of inhibition of growth (48-72 hours); however, upon removal of dglc from the medium by washing, label from dglc was rapidly lost from the cellular population at a rate faster than cell turnover time. Thus, the t 1/2 for loss of dglc was 6 hours (Table 2). In similar experiments, cells were prelabeled with [^{3}H]fucose or [^{14}C]glucosamine. After removal of labeled material from the growth medium by collection of cells and washing, the rate of loss of

TABLE 2

Loss of Label from P388 Cells Grown in the Presence of 50 μM 2-Deoxy-D-glucose

Precursor	T 1/2 (hours)
[^{3}H]Fucose	7.2
[^{14}C]Glucosamine	11.3
[^{14}C]2-Deoxy-D-glucose	6.0

TABLE 3

The Effect of Dglc on Con A and WGA Induced Agglutination of P388 Cells

	Concanavalin A		Wheat Germ Agglutinin	
Treatment	Lag time (min)	Initial rate (A546/min)	Lag time (min)	Initial rate (A546/min)
None	1.6	0.004	4.6	0.026
Dglc for 24 h	0.9	0.037	5.6	0.014
Dglc for 72 h	0.85	0.40	6.0	0.016
24 h after removal of dglc	1.0	0.05	5.4	0.018

Agglutinability by Con A and WGA of P388 cells grown without dglc, with 50 μM dglc for either 24 or 72 hours, or for 72 hours in the presence of dglc and then for 24 hours in fresh, non-dglc containing medium. Results are expressed as lag times before onset of decreasing absorbance and the initial rate of decrease of absorbance at 546 nm.

acid-insoluble labeled material was determined in a manner analogous to that employed with dglc. The t 1/2 of the fucose label was 7.2 hours, while that of glucosamine was 11.3 hours; neither carbohydrate affected the cell generation time of about 10.5 hours. Additionally, the turnover-time of radioactivity from fucose and glucosamine was the same in both dglc treated and untreated cells; these values for the physiological carbohydrates were significantly greater than the turnover of the dglc label, suggesting the possibility of cellular recognition and removal of abnormal glycoproteins and/or glycolipids.

Further evidence for the presence of abnormal glycoproteins (and glycolipids) in cells grown in the presence of dglc was obtained through the use of the plant lectins Con A and WGA to probe the surface architecture of the drug treated cells. Table 3 presents the results of experiments which compare both the lag time prior to absorbance change and the rate of change of absorbance at 546 nm of untreated cells and those exposed to dglc for either 24 or 72 hours, as well as cells exposed to dglc for 72 hours and then washed free of dglc and grown for another 24 hours in dglc-free medium. Sensitivity to Con A induced agglutination was increased by treatment with dglc, as evidenced by both a decreased lag phase and an increased rate of agglutination, while susceptibility to WGA was decreased. These findings correspond to the increased agglutinability by Con A of normal hamster cells exposed to dglc which has been reported by Steiner et al. (16). The maximum change in cellular architecture as measured by agglutination with both Con A and WGA occurred at 72 hours, a period when growth is fully retarded. Prior to this time and following recovery of the capacity for growth after removal of dglc, agglutination rates were closer to those of untreated control cells. These findings indicate a relationship between the architecture of the cell surface and the growth characteristics of these cells.

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Abbreviations: dglc, 2-deoxy-D-glucose; Con A, concanavalin A; WGA, wheat germ agglutinin.

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