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The determination of the uptake of arabinosyl-6-mercaptopurine by L1210 cells

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SUMMARY

1. The uptake of arabinosyl-6-mercaptopurine was studied *in vitro* in L1210 cells and in an arabinosyl-6-mercaptopurine-resistant subline. There was little or no difference in the rate of uptake, K_m and V values between the two cell lines. This strengthens the conclusion that the arabinosyl-6-mercaptopurine-resistant subline of L1210 cells is due to an insensitive ribonucleotide reductase and not due to an impairment in the capacity for arabinosyl-6-mercaptopurine accumulation.

2. A new technique is described for the rapid separation of cells from the reaction medium by centrifugation through silicone oil. This permitted the measurement of actual rates of antimetabolite or nucleoside movement in cells.

3. The water-permeable space was estimated to be 208 μl per 10^9 cells, since there was little or no detectable [*carboxyl*-^{14}C]inulin in the cell pellet.

It is well documented that arabinosyl-6-mercaptopurine, a nucleoside analog, is carcinostatic for a number of experimental tumors[1-3]. This purine nucleoside was reported to inhibit the CDP reductase and this was ascribed as its mechanism of action. In contrast to other purine and pyrimidine analogs which require conversion to nucleotides in order to be active[4,5], arabinosyl-6-mercaptopurine was neither phosphorylated *in vivo* and *in vitro* nor metabolized to any extent[1,6]. Recently, it was observed that arabinosyl-6-mercaptopurine was a competitive inhibitor of adenosine deaminase[7].

Nucleoside and nucleoside analog (antimetabolite) transport across mammalian cell membrane is generally accepted as a mediated process[8-13]. However, measurements of transport are complicated by the rapidity of this process as well as cellular nucleoside metabolism. By employing a murine (L1210) cell line which is resistant to arabinosylcytosine and low temperatures, Kessel and Shurin[10] were able to study the transport

kinetics of arabinosylcytosine and deoxycytidine. A rapid separation of erythrocytes from the medium by centrifugation of cells into a di-1-butylphthalate layer was reported by Oliver and Paterson[8] in their study of nucleoside transport. Unfortunately, the transport kinetics were followed by the disappearance of radio-activity in the medium.

This communication describes a technique with which actual rates of arabinosyl-6-mercaptopurine movement can be measured. The question of whether the resistance of L1210 cells to arabinosyl-6-mercaptopurine was due to impairment of membrane permeability to arabinosyl-6-mercaptopurine was investigated. The results obtained indicated that there was little or no difference in arabinosyl-6-mercaptopurine permeability between arabinosyl-6-mercaptopurine-sensitive and -resistant L1210 cells.

The technique used consisted of a modification of procedures described by Danon and Marikovsky[14] and Oliver and Paterson[8]. In our procedure the di-1-butyl-phthalate was substituted by silicone oil (Dow Corning 560 fluid, density 1.04) and a layer of 15% $HClO_4$ (density 1.09) was introduced under the silicone oil layer. The plastic centrifuge tube (Nalgene, 15 ml size) was layered from bottom to top as follows: 1.0 ml 15% $HClO_4$, 1.0 ml silicone oil and 2.0 ml medium containing cells. For the initial rate of uptake, the layered tube was placed in a bench-top centrifuge and the incubation was initiated by injecting the labelled compound into the medium. The incubation was terminated by top speed (1700 × *g*) centrifugation for 2 min.

After centrifugation, the samples were taken from the medium and acid layer (after removal of medium to avoid contamination) to determine the radioactivity and, thereby, the amount of labelled compound taken up by the cells. In parallel experiments, the water-permeable and "extracellular" space of the cells was determined by using ^3H_2O and [*carboxyl*-^{14}C]inulin[11], respectively. The intracellular concentration of nucleoside analog was then calculated.

The L1210 ascites tumor was grown in female BDF_1 mice by intraperitoneal inoculation of 10^6 cells per mouse. After 7 days, the mice were sacrificed, and the ascites tumor cells were collected by flushing the peritoneal cavity with cold saline (0.9% NaCl; in the case of the L1210-sensitive cell line, 2 mM EDTA was included in cold saline), followed by centrifugation at 1500 × *g* for 2 min (1.0 ml packed cell volume = 10^9 cells). Red blood cells, if present, were eliminated by a 30-s cold water lyse. The tonicity was quickly restored by the addition of an equal volume of 1.8% NaCl. The cells were suspended in a medium containing in mM: NaCl, 154; $MgCl_2$, 1.4; glucose, 5; potassium phosphate buffer (pH 7.4), 20.

As shown in Fig.1, the amount of tritiated water in the cell pellet was proportional to the number of cells in the incubation medium. The calculated water-permeable space[11] was 208 µl per 10^9 cells. This represents about 25% of the intracellular water-permeable space if one considers that 20% of the packed cell volume is extracellular fluid under our experimental condition[15]. This is in good agreement with Kessel's[11] estimation for L1210 cells. On the other hand the amount of inulin space was not proportional to the number of cells present. In a time-course study (not shown) up to 30 min the radioactivity in the cell pellet accounted for less than 0.1% of the total radio-

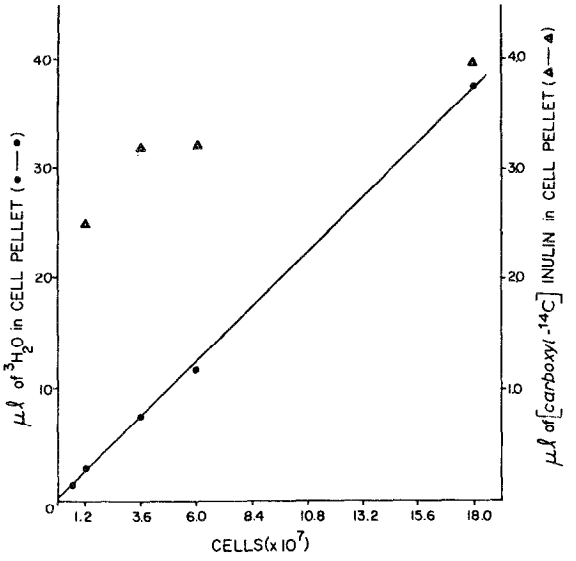

Fig.1. Determination of water and [*carboxyl*-^{14}C] inulin space in L1210 cells. $^{3}H_2O$ (Packard, $5.3 \cdot 10^5$ dpm) or [*carboxyl*-^{14}C] inulin (New England Nuclear, $3.6 \cdot 10^5$ dpm) was incubated with various amounts of arabinosyl-6-mercaptopurine-resistant L1210 cells for 10 min at room temperature. Then the cells were spun down through a silicone layer as described in the text. Samples were taken from the acid layer after removal of incubation medium. The neutralized samples were mixed with Aquasol (New England Nuclear, 10 ml per vial) and counted in a Packard Tri-Carb Scintillation Spectrometer, Model 3380. Quenching correction was made by the addition of [^{14}C] toluene or $^{3}H_2O$. The time-course study (not shown) ($6.0 \cdot 10^7$ cells incubated with $^{3}H_2O$ or [*carboxyl*-^{14}C] inulin from 30 s up to 30 min) showed constant radioactivity in the cell pellet.

activity added. This radioactivity observed may be due to association of [*carboxyl*-^{14}C]-inulin with damaged cells. So there was little or no [*carboxyl*-^{14}C]inulin associated with the cells while the silicone layer was traversed.

The time course of uptake of arabinosyl-6-mercaptopurine in arabinosyl-6-mercaptopurine-resistant and -sensitive L1210 cells are shown in Figs 2A and 2B, respectively. The initial rate of arabinosyl-6-mercaptopurine uptake was estimated to be 230 and 250 nmoles of arabinosyl-6-mercaptopurine accumulated per min per 10^9 cells for arabinosyl-6-mercaptopurine-resistant and -sensitive L1210 cells, respectively. Therefore, little difference was observed in initial rates of arabinosyl-6-mercaptopurine uptake between these two cell lines even though the concentration of arabinosyl-6-mercaptopurine used in the incubation medium was different. However, the concentrations of arabinosyl-6-mercaptopurine were in the saturation range with respect to arabinosyl-6-mercaptopurine uptake. After incubation for 30 min, the intracellular concentration of arabinosyl-6-mercaptopurine reached 3.4 mM (compare to medium 1.8 mM) and 3.2 mM (compare to medium 2.8 mM) for arabinosyl-6-mercaptopurine-resistant and -sensitive L1210 cells, respectively. This indicated that L1210 cells can concentrate arabinosyl-6-mercaptopurine like other nucleoside analogs[9] even though arabinosyl-6-mercaptopurine is known not to be phosphorylated *in vitro*[6]. From an extrapolation of the initial rate of

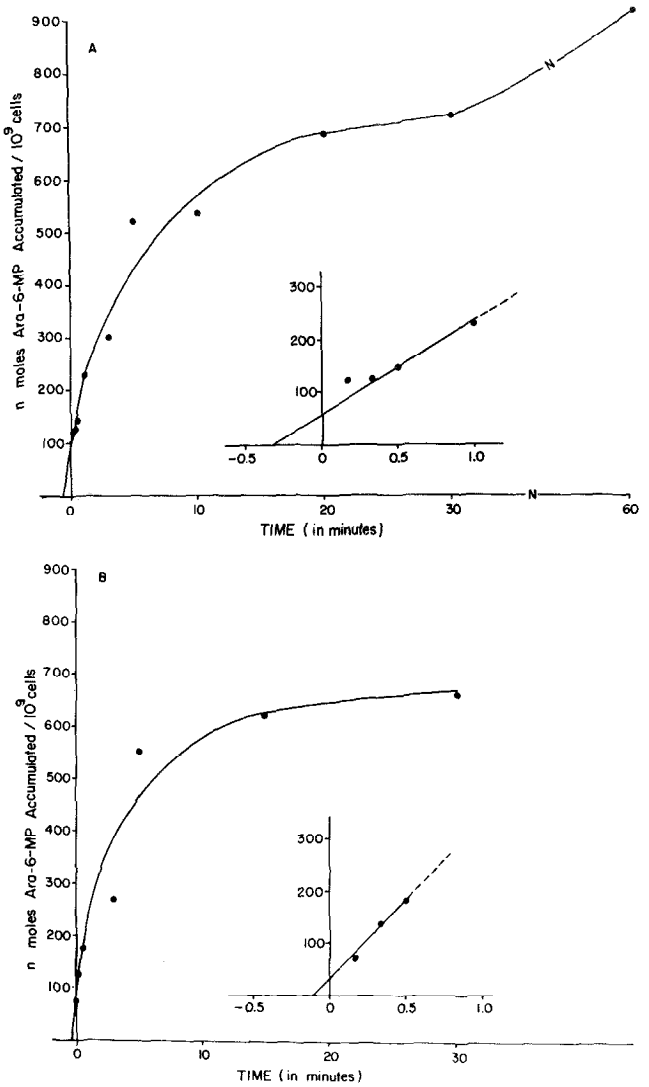

Fig.2. (A) Kinetics of arabinosyl-6-mercaptopurine (Ara-6-MP) uptake in the arabinosyl-6-mercaptopurine-resistant L1210 cells. Cells ($1.25 \cdot 10^8$) were incubated in the medium as described in the text, at room temperature. The incubation was started by the addition of arabinosyl-6-mercaptopurine to a final concentration of 1.8 mM. Uptake of arabinosyl-6-mercaptopurine in cells was followed spectrophotometrically (using an ϵ_{mM} (329 nm) = 22.9 at pH 1.0). (B) Kinetics of arabinosyl-6-mercaptopurine uptake in L1210 cells. Cells ($8.1 \cdot 10^7$) were incubated in the medium as described in the text, at room temperature. The incubation was started by the addition of arabinosyl-6-mercaptopurine to a final concentration of 2.8 mM.

arabinosyl-6-mercaptopurine uptake (Fig.2, insert), the time (dead time) required for the cells to travel through the silicone layer was estimated to be 10–30 s. This dead time can be shortened considerably if one uses the method described by Kraaijenhof *et al.*[16]. However, we found that the uptake of arabinosyl-6-mercaptopurine was linear for at least

30 s. Therefore, the technique described is more than adequate to measure the initial rate of transport of arabinosyl-6-mercaptopurine. The K_m and V values for the uptake of arabinosyl-6-mercaptopurine were found to be 1.3 mM and 313 nmoles/30 s per 10^9 cells, respectively, for arabinosyl-6-mercaptopurine-resistant L1210 cells and 1 mM and 323 nmoles/30 s per 10^9 cells, respectively, for arabinosyl-6-mercaptopurine-sensitive L1210 cells. From these kinetic parameters it is reasonable to exclude the possibility that resistance to arabinosyl-6-mercaptopurine in L1210 cells may reflect a permeability barrier.

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