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Transport of the Immunosuppressant 15-Deoxyspergualin in Human Peripheral Blood Lymphocytes

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SUMMARY: 15-deoxyspergualin (DSG) is a potent immunosuppressive compound currently in clinical trials. In this study, we have characterized the uptake and intracellular localization of DSG in human peripheral blood lymphocytes (PBL's). DSG is transported into human PBL's and reaches an estimated maximum concentration of approximately 500μM in 6 hours. The majority of the [^{3}H]-DSG remains in the cytoplasm of cells and that which is associated with the nucleus is only loosely associated. DSG was transported by HeLa cells, as well, suggesting uptake is not specific for hematopoietic cells. Positively charged amino acids and polyamines, which are structurally similar to DSG, were unable to compete for DSG transport suggesting that DSG is transported into cells via a pathway distinct from amino acids or polyamines.

The area of immunomodulation continues to develop rapidly and recent studies continue to elucidate the mechanism of action of drug-mediated immunosuppression (1,2,3,4). 15-deoxyspergualin (DSG), a derivative of spergualin which was originally isolated from *Bacillus laterosporus (5),* has been shown to have potent immunosuppressive activity in *in vivo* animal models (6,7,8) and is currently in phase I/II clinical trials. Recent studies indicate that DSG appears to act in a distinctly different manner than other current immunosuppressive agents, such as cyclosporin A and FK506 (9,10) and also that the intact DSG molecule itself is the active species and not a metabolic product (unpublished observations). The intracellular binding protein and putative target for DSG has recently been identified as the cognate form of heat shock protein 70 (Hsc70) (11,12).

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Abbreviations used: DSG, 15-deoxyspergualin, PBL, peripheral blood lymphocyte. Hsc70, cognate form of heat shock protein 70, PBS, phosphate buffered saline, HEPES, 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid, PMSF, phenylmethanesulfonyl fluoride.

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In order to characterize the internalization of DSG, radiolabelled DSG was synthesized (13) and its transport into human cells and localization inside cells was investigated. To confirm that the incorporation of DSG into cells was not cell type specific, uptake was determined on HeLa cells as well. As DSG appears structurally similar to both the polyamine spermidine and the amino acid arginine, we have also investigated the effect of these compounds on DSG transport.

MATERIALS AND METHODS

Isolation of Peripheral Blood Lymphocytes: 120cc of fresh human blood were drawn from healthy volunteer donors into heparinized syringes and peripheral blood lymphocytes were isolated as described previously (14). Briefly, whole blood was diluted into RPMI 1640 media 3:1 and underlayered with 10 mL ficoll hypaque (density 1.077, Organon Technika). After a 1500 x g., 30 minute centrifugation at room temperature, lymphocyte bands were harvested from the hypaque/media interface and washed 4-times with RPMI 1640 to deplete the suspension of platelets. Cells were then resuspended in 100 mL RPMI 1640 + 10% human serum (NABI) and allowed to sit in 2 T-175 flasks in a 37°C incubator for 1 hour to deplete some of the monocytes through plastic-adherance. The remaining cells were counted and used for further experimentation.

[^{3}H]Deoxyspergualin Uptake: Peripheral blood lymphocytes were suspended at 0.5-2 x 10^7 cells/sample (1 mL aliquots) in 37°C RPMI + 10% human serum and labeled DSG was added to 10 μM (approximate maximum plasma concentration reached in humans, our unpublished observations, (15)) and samples were incubated at 37°C with gentle mixing. At designated time points, aliquots were pelleted, washed extensively (5X) in ice cold PBS, solubilized in 2 mL counting fluid and counted in a liquid scintallation counter to determine uptake of label. Similarly, cultured HeLa cells were plated onto 6-well tissue culture plates at approximately 2.5 x 10^5 cells/well in DMEM media, 10% fetal calf serum + 2mM aminoguanidine (to inhibit polyamine oxidases which are found in high levels in calf serum and which also oxidize DSG). The following day, labeled DSG was added as described above. To harvest, wells were rinsed once with PBS and then scraped into 1 mL PBS. Cells were washed extensively, solubilized and counted as above.

Subcellular Fractionation: Cells were aliquotted, harvested and washed as described above. Samples were then resuspended in Lysis buffer (10 mM HEPES, 150mM NaCl, 10mM $MgCl_2$ + 0.1% Triton X-100 detergent and a protease inhibitor cocktail containing aprotinin, leupeptin, pepstatin, trypsin inhibitor and PMSF at recommended concentrations) and incubated on ice. After 10 minutes, nuclei were pelleted and washed 1X times with ice cold lysis buffer. Cytosol (removed after the first spin) and nuclei were counted as described above.

Uptake Competition Studies: Cells were prepared and aliquotted as described above. Labeled DSG and either 10-fold excess polyamine (Putrescine (PUT), Spermidine (SPD) or Spermine (SPM)) or 10-fold media excess of amino acid(lysine, arginine or glycine) was added at the same time as the label. Cells wereharvested after 4 hours of incubation, washed and counted as above.

RESULTS

Transport of [^{3}H]-DSG into Human Peripheral Blood Lymphocytes and HeLa Cells:

The uptake of DSG at 37°C into human peripheral blood lymphocytes is linear, reaching a maximum concentration in about 6 hours. As shown in Fig. 1a, the majority of DSG transport appears to be temperature dependent suggesting that it is taken up via an active transport process. The small amount of DSG

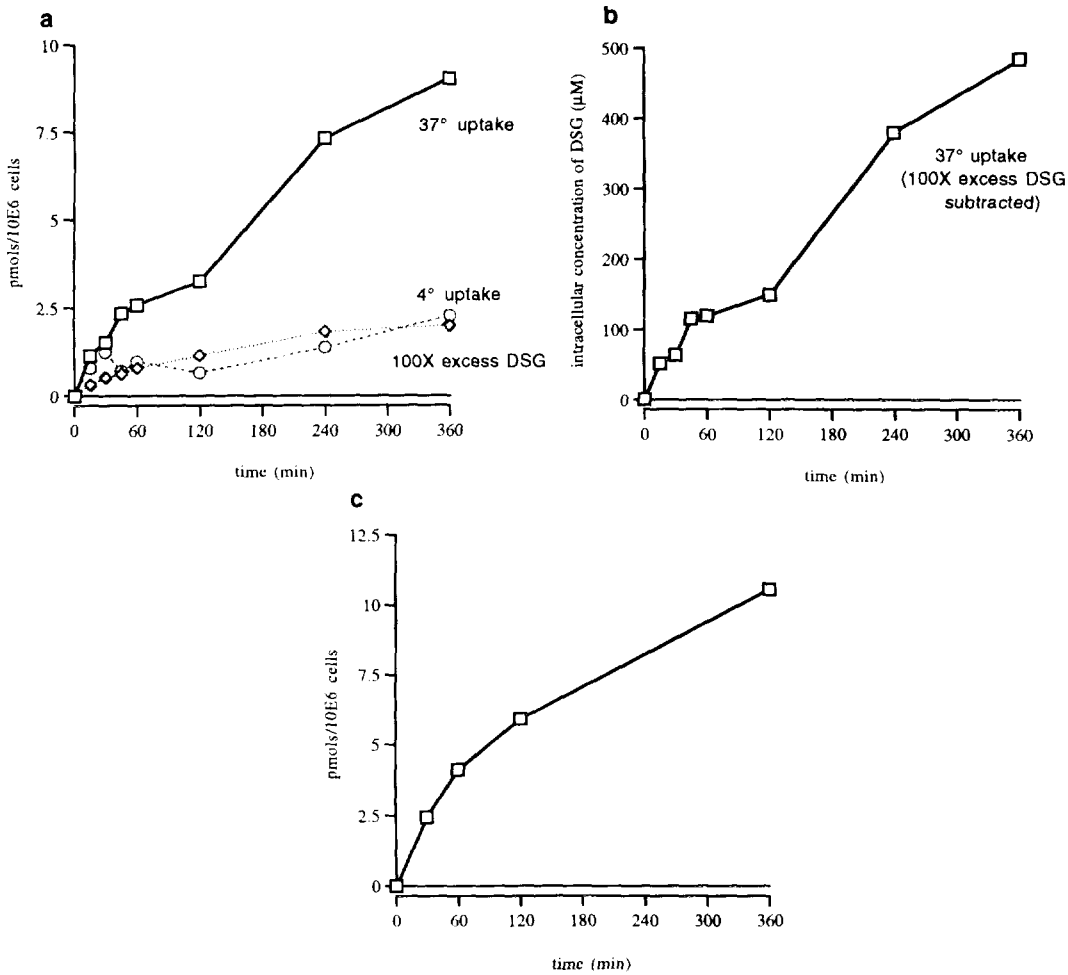

Figure 1a,b and c. Uptake of [^{3}H]-DSG in human peripheral blood lymphocytes and HeLa cells. (a) Human PBL's were incubated with 10μM labeled [^{3}H]-DSG and, at designated time points, samples were harvested, washed extensively and counted for radioactivity as described in Materials and Methods. (b) Transport of DSG into PBL's at 37°C minus uptake in the presence of a 100-fold excess of unlabeled DSG. The data is plotted as intracellular concentration using a volume of 175 femtoliters. (c) Transport of DSG into HeLa cells as described in Materials and Methods.

transported at 4°C is probably due to passive diffusion and subsequent binding to an intracellular target. Since a 100-fold excess of unlabeled DSG did not completely inhibit uptake, it is likely that the intracellular target(s) is(are) of high capacity. As seen in Figure 1b, assuming an intracellular volume of approximately 175 *f*liters (16,17,18,19,20), DSG reaches quite high concentrations of approximately 500μM after 6 hours. We have determined by HPLC analysis that, after 6 hours, approxiamtely 50% of the radiolabel is present as DSG. The remainder appear to be metabolic products (data not shown). To be sure that DSG transport into lymphocytes was not a lymphocyte-specific phenomenon, uptake was evaluated in HeLa cells (Fig. 1c). As shown, the rate and amount of uptake of DSG in both cell types is similar and not specific to cells of hematopoetic lineage.

Distribution of Label in Fractionated Cells:

After simple fractionation of cytosol and nuclei, the majority of labeled DSG was found in the cytosol (Fig. 2). Extensive washing of intact cell nuclei was attempted but much of the label dissociated upon washing indicating a loose association of label with nuclei (data not shown).

Competition of DSG Uptake by Amino Acids and Polyamines:

Since DSG structurally resembles both the amino acid arginine and the polyamine spermidine, uptake of labeled DSG in the presence of a 10-fold

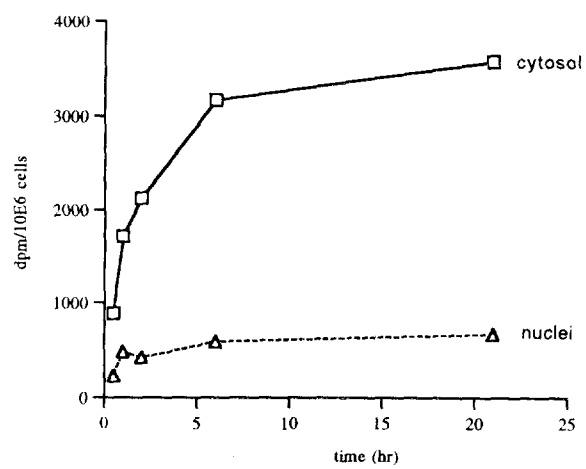

Figure 2. Cytosolic and nuclear localization of [^{3}H]-DSG in PBL's. Cells are prepared as described in Fig. 1 but after washing, cells were resuspended in lysis buffer for 10 minutes. Cell lysis was confirmed microscopically. After centrifugation, the supernate (cytosol) was removed and counted for activity. Nuclei were washed once and also counted. Subsequent washing of nuclei continued to remove substantial radioactivity, indicating that label is only weakly associated with nuclei (data not shown).

excess of 3 amino acids (two positively charged, arginine and lysine, and one neutral, glycine) and 10-fold excess polyamines (putrescine, spermidine and spermine) was performed. Results indicate (Fig. 3a) that neither positively charged ,nor neutral amino acids, had a marked inhibitory effect on DSG transport into human PBL's. In addition, excess polyamines had only a minimal effect on DSG transport. These data suggest that DSG is transported into human PBL's by a mechanism distinct from that of either polyamines or amino acids.

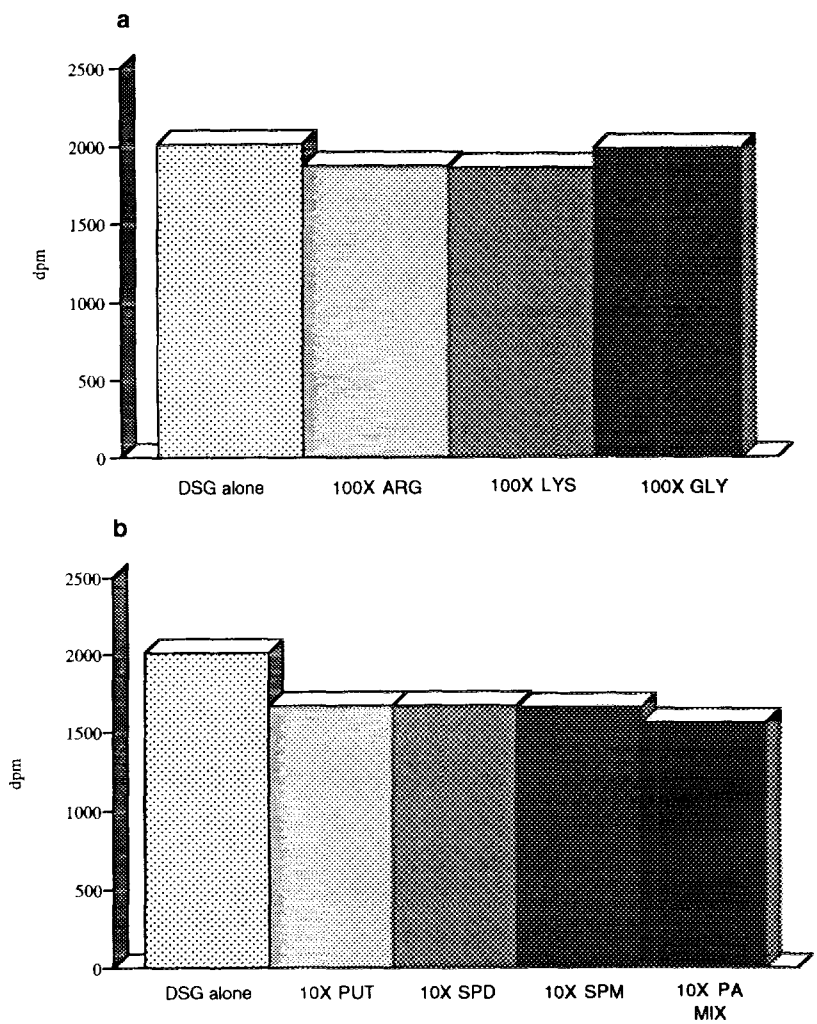

Figure 3a & b. Competition of DSG-Uptake with Amino Acids and Polyamines. Cells were prepared as described for previous experiments but samples were co-incubated with either (a) 10-fold media excess* amino acid (arginine, lysine or glycine) or (b) 10-fold excess polyamine (putrescine, spermidine or spermine) and harvested after 4 hours at 37°C. Polyamines were used at only 10-fold excess due to their known toxicity to cells at higher concentrations. *Because culture media had high levels of amino acids, some higher than 100-fold drug, a 10-fold excess of media levels of amino acid levels was used.

DISCUSSION

15-Deoxyspergualin is a potent immunosuppressive agent whose mechanism of action remains unknown. Recently, a member of the Hsp70 family of heat shock proteins was found to specifically bind to DSG. DSG appears to interact with Hsc70 with a K_d between 0.6 to 5.0μM (12). In order to determine whether DSG reaches concentrations in lymphocytes sufficient to saturate Hsc70, the intracellular concentration was determined. DSG is taken up in a slow process similar to that described for polyamine transport in lymphocytes (21). Although DSG is transported slowly, it reaches quite high intracellular levels in lymphocytes. These levels are well above the K_d for the interaction between DSG and Hsc70 suggesting that this protein may be an in vivo target for the binding and immunosuppressive action of DSG.

Interestingly, DSG appears to be transported into peripheral blood lymphocytes via a process distinct from that for polyamines and amino acids. This process, however, is not restricted to hematopoetic cells since we have shown that DSG is taken up by HeLa breast carcinoma cells equally as well. Further studies are in progress to characterize this unique mechanism of drug transport.

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