

## Effects of the Antibiotic Tunicamycin on Murine Lymphosarcoma Cells.

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### Introduction

Metastasis, the spread of tumor cells from the site of origin to other distant sites, is a complex biological phenomenon and depends on both host and tumor cell properties (1, 5, 10). Several experimental model systems have been developed to study some of the various aspects of cancer metastasis (4, 11). One such model system, a murine lymphosarcoma, was developed by Brunson and Nicolson (2) using the Abelson leukemia virus induced murine lymphosarcoma RAW 117 cell line. A highly malignant and liver metastatic variant line RAW 117-H10 was derived from the parental less malignant line RAW 117-P by using an *in vivo* selection method of cycles of intravenous inoculation of tumor cells and harvesting solid liver tumors in syngeneic BALB/C mice. The metastatic variant RAW 117-H10 line differed from the parental tumor cell line in that the RAW 117-H10 cells formed about 100-200 times more liver tumor nodules than the parental line RAW 117-P, expression of viral glycoprotein component gp70 was decreased on the RAW 117-H10 cells when compared to the parental line RAW 117-P and the highly malignant cells bound less concanavalin A lectin than their parental counterpart RAW 117-P (12).

It was also shown in our laboratory that there are two major antigens expressed on these cell lines as identified by xenoantisera and that expression of antigens on the two cell lines are different (3, 7). One antigen is expressed more on the parental line RAW 117-P and distributed uniformly over the cell surface. A second antigen is expressed more on the highly malignant RAW 117-H10 cells than the less malignant RAW 117-P cell line and the distribution of this second antigen is of a patchy nature (Joshi and Brunson, unpublished). These two antigens have been characterized as glycoproteins which are displayed on lymphosarcoma cell surfaces.

Although distinct changes in the cell surface properties associated with neoplastic transformation is well documented, the exact relationship of cell surface properties and cancer metastasis is poorly understood. In the present study the antibiotic tunicamycin, a known glycosylation inhibitor, was used to study glycosylation inhibition effects on growth, glycoprotein synthesis, and both *in vitro* and *in vivo* biological behavior of these tumor cells.

### Materials and Methods

#### *Cell Culture*

Both the RAW 117-P and RAW 117-H10 cells were maintained in 60mm x 10mm petri dishes (Falcon 1007, Falcon Plastics, Oxnard, California) containing Dulbecco's Modified Eagle Medium (DMEM) with 10% bovine calf serum (Flow Laboratories, McLean, Virginia) and no antibiotics.

#### *Growth Inhibition Assay*

5 x 10<sup>2</sup> cells in DMEM containing 2% bovine calf serum (BCS) were plated in each well (0.1ml/well) with or without different concentrations of tunicamycin

in microculture tissue culture plates (Linbrow, Flow Laboratories). Tunicamycin (Sigma Chemical Co., St. Louis, Missouri) stock solution was prepared by dissolving 2mg of the drug in 1 ml 10mM NaOH, diluted to the required concentration with DMEM and sterilized by filtering through 0.45 filters (Gelman, Ann Arbor, Michigan). Six hours before harvesting the cells 0.5  $\mu$ Ci of  $^3$ H Thymidine (ICN Chemicals, Irvine, California) was added to each well. Cells were harvested at 24 hour intervals using a "Mini Mash" cell harvester (MA Bioproducts, Walkersville, Maryland). The cells harvested in the Mini Mash device on glass fiber filter papers were counted in a liquid scintillation counter (Beckman model LS230) to determine tritiated thymidine incorporation.

#### *Measurement of Glycoprotein Synthesis*

Glycoprotein synthesis was measured by culturing  $5 \times 10^3$  cells/ml (5 ml total volume 60mm petri dish) in DMEM containing 2% BCS, and  $^3$ H-fucose (New England Nuclear, Boston, Massachusetts) was added 6 hours before harvesting the cells. Procedures for harvesting and counting the radioactivity were essentially as described above for tritiated thymidine incorporation.

#### *Measurement of Adherent Cell Populations*

$5 \times 10^3$  cells per ml (total 10 ml) were plated in 100mm x 15 mm (1001 Falcon) petri dishes with or without the appropriate amount of tunicamycin. Cells were then incubated for 36 hours at 37°C in an atmosphere containing 5%  $\text{CO}_2$  and 95% air. After the incubation period nonadherent (floating) cells were first carefully removed with a pipette, and any settled nonadherent cells were removed by immediate gentle washing of the dish with two volumes of 5 ml each DMEM. Five ml of 0.02 M EDTA (Sigma Chemical Co.) in calcium-free phosphate buffered saline, pH 7.2, was added to each dish and allowed to remain at 37°C for 5 min. Adherent cells were then harvested by vigorous pipetting. Cell suspension samples were counted and the percentage of adherent cells in each total population was determined.

#### *In Vivo Experiments*

Biological properties of the tunicamycin treated (36-hr incubation, 37°C in 5%  $\text{CO}_2$ ) and untreated cells were assayed by inoculating  $5 \times 10^3$  cells in 0.1 ml of serum-free DMEM intravenously (tail vein) into syngeneic BALB/C mice. Death of the animals were noted daily following injection of tumor cells.

### **Results and Discussion**

Figure 1 shows the growth inhibitory effects of tunicamycin at various concentrations on the less malignant parental line RAW 117-P. At least 1  $\mu$ g/ml concentration of tunicamycin was necessary for significant growth inhibition of these cells compared to control (no tunicamycin) cell cultures. A dose of 1  $\mu$ g or more tunicamycin per ml completely inhibited the growth of the RAW 117-P cells for as long as 72 hours after addition of the drug. Interestingly, the tunicamycin concentration (1  $\mu$ g/ml) which inhibited growth of RAW 117-P cells did not exhibit the same effect on highly malignant RAW 117-H10 cells. Even a tunicamycin concentration of as much as 2.5  $\mu$ g/ml did not have any significant growth inhibitory effect on the RAW 117-H10 cells (Fig 2). As shown in Fig 3, a dose of at least 5  $\mu$ g/ml tunicamycin concentration was necessary to exert a growth inhibitory effect on the highly metastatic variant cell line RAW 117-H10 similar to that seen with the RAW 117-P cells at 1  $\mu$ g/ml.

## EFFECTS OF TUNICAMYCIN ON RAW 117-P CELLS

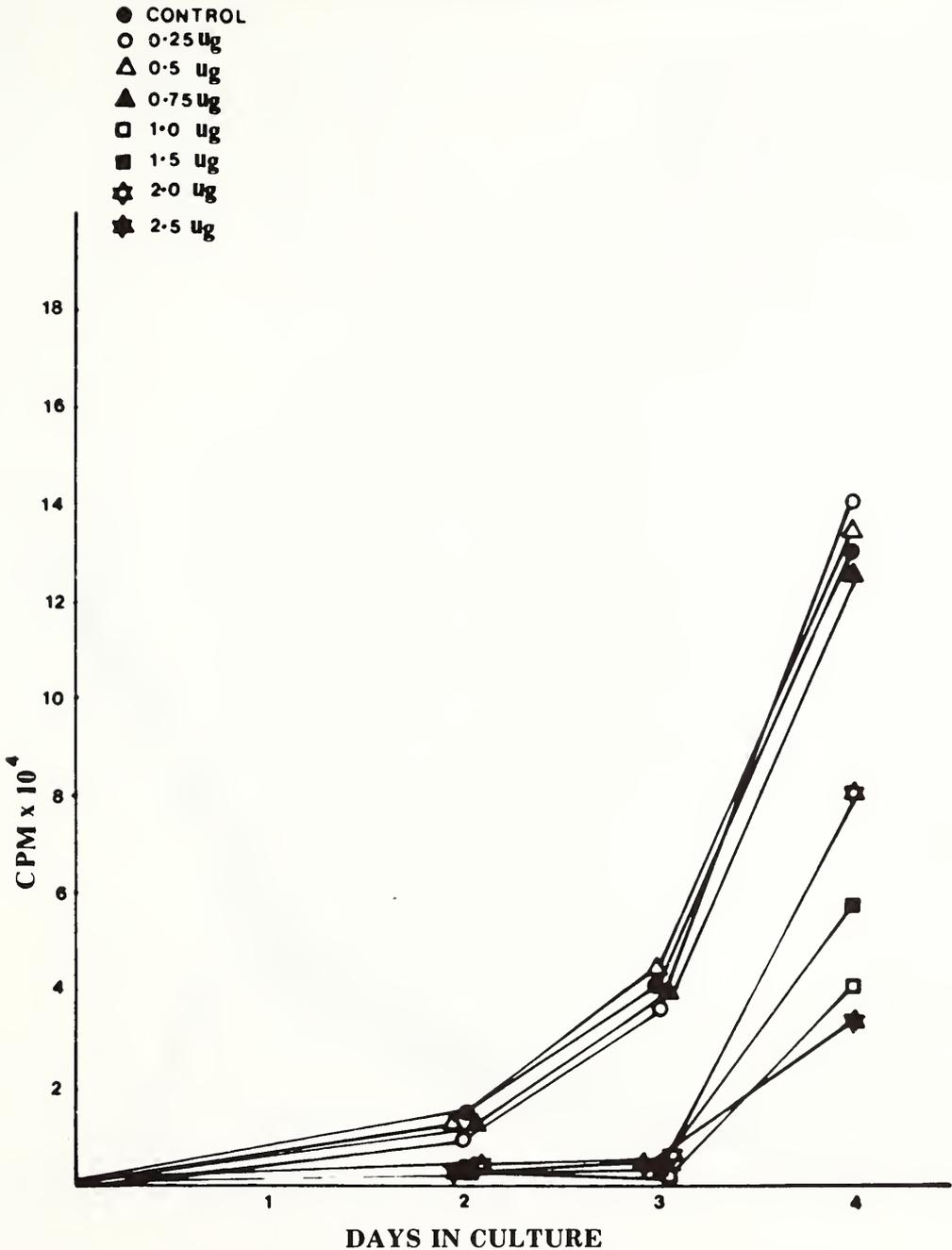


FIGURE 1:  $5 \times 10^2$  RAW 117-P cells were plated in 0.1 ml volume in each well in microculture plates and incubated at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$ , with or without an appropriate concentration of tunicamycin. Six hrs. before harvesting the cells,  $0.5 \mu\text{Ci}$  of  $^3\text{H}$ -thymidine was added. Each point represents the average of triplicate determinations (Cpm).

As expected, tunicamycin was shown to have an inhibitory effect on glycoprotein synthesis in these cells, as measured by  $^3\text{H}$ -fucose uptake. As shown in Figure 4, at a concentration of tunicamycin of 1 and  $2 \mu\text{g/ml}$  there is inhibition of glycopro-

## EFFECTS OF TUNICAMYCIN ON RAW 117-H10 CELLS

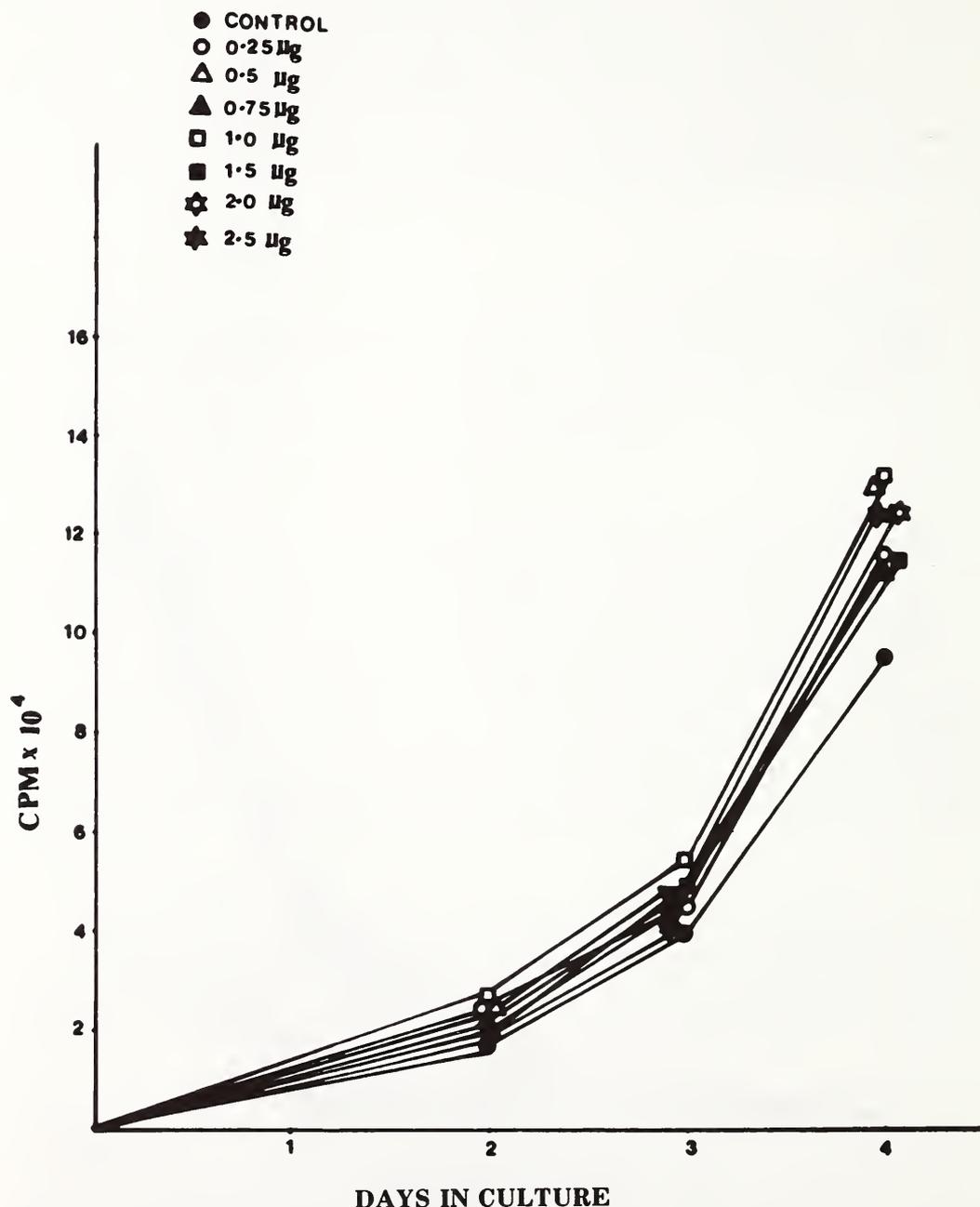


FIGURE 2:  $5 \times 10^2$  RAW117-H10 cells were plated in 0.1 ml volume in each well in microculture plates and incubated at 37°C in 5%  $\text{CO}_2$ , with or without an appropriate concentration of tunicamycin. Six hours before harvesting the cells, 0.5  $\mu\text{Ci}$  of  $^3\text{H}$  thymidine was added. Each point represents the average of triplicate determinations (Cpm).

tein synthesis of about 30% and 50%, respectively, in the less malignant RAW 117-P cells. The highly malignant RAW 117-H10 cells showed only a slight inhibition of glycoprotein synthesis with 1 and 2  $\mu\text{g/ml}$  concentration of tunicamycin (approximately

EFFECTS OF TUNICAMYCIN ON RAW 117-H10 CELLS

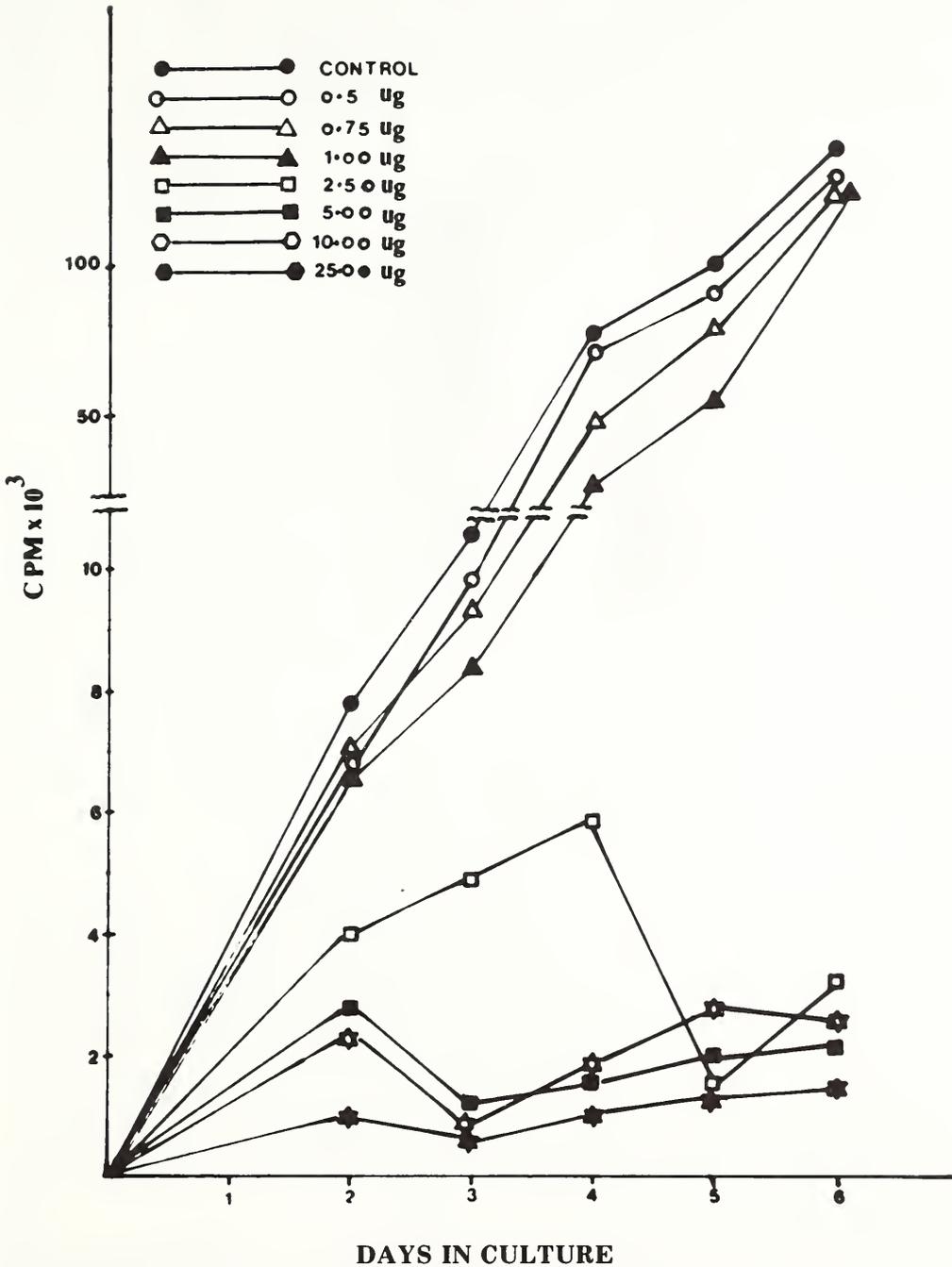


FIGURE 3:  $5 \times 10^2$  RAW117-H10 cells were plated in 0.1 ml volume in each well in microculture plates and incubated at 37°C in 5%  $CO_2$ , with or without an appropriate concentration of tunicamycin. Six hours before harvesting the cells, 0.5  $\mu$  Ci of  $^3H$  thymidine was added. Each point represents the average of triplicate determinations (Cpm).

10% inhibition at 2  $\mu$ g/ml) although higher concentrations did show similar inhibition of glycoprotein synthesis (data not shown).

Tunicamycin was also shown to affect the adherent properties of these lym-

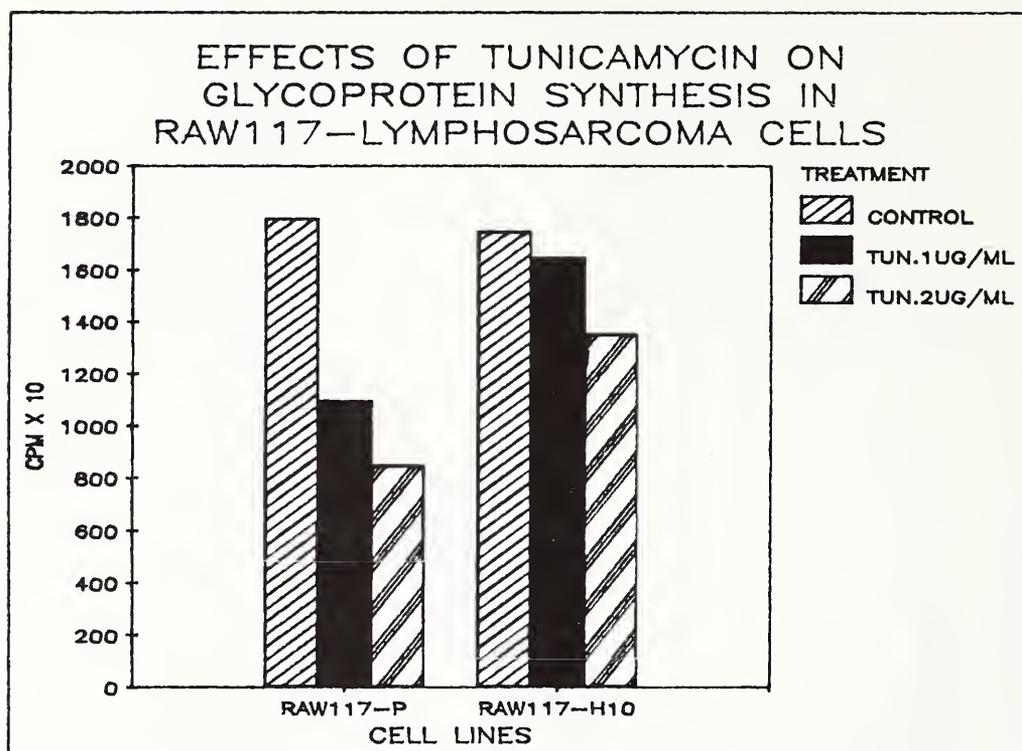


FIGURE 4:  $5 \times 10^3$  cells/ml (5 ml/dish) were plated in 60mm petri dishes and incubated with or without appropriate concentrations of tunicamycin. Six hours before harvesting the cells,  $0.5 \mu$  Ci of  $^3$ H-fucose was added. Each point represents the average of triplicate determinations of radioactivity (Cpm).

phosarcoma cells. The lymphosarcoma cells grow in suspension culture, although a certain percentage of cells adhere to the culture dish. The percentage of adherent cell populations varies somewhat in cultures of both cell lines, and does not appear to be a constant feature of either the RAW 117-P or RAW 117-H10 cell line. Tunicamycin decreased the adherent cell population in both these cell lines. As shown in Table 1, in RAW 117-P cell line cultures the percentage of adherent cell population was decreased from 11% to 3% of the total cell population at a concentration of  $1 \mu$ g/ml tunicamycin. A similar decrease in adherent cell populations in the RAW 117-H10 tumor cell cultures was noticed only at  $5 \mu$ g/ml concentration of tunicamycin. At a tunicamycin level of  $1 \mu$ g/ml, little or no decrease in adherence of RAW 117-H10 cells was seen.

TABLE 1. *Effect of Tunicamycin on Adherence Properties of Lymphosarcoma Cells*

CELL TYPE	% ADHERENT CELLS		
	Control	Tunicamycin $1 \mu$ g/ml	Concentration $5 \mu$ g/ml
RAW 117-P	11	3	N.D.
RAW 117-H10	19	17	7

N.D. = not done

Table I -  $5 \times 10^3$  cells per ml were plated in 100mm x 15mm (1001 Falcon) petri dishes with or without (control) the appropriate amount of tunicamycin ( $1 \mu$ g/ml or  $5 \mu$ g/ml). Cells were incubated for 36 hours at  $37^\circ$ C in an atmosphere containing 5%  $\text{CO}_2$  and 95% air. Adherent cells were calculated then as described in the text.

Figure 5 depicts the *in vivo* properties of tunicamycin treated cells. Preliminary studies on the biological properties of tunicamycin treated cells revealed that in the experimental protocol used, tunicamycin increased the malignant properties of RAW 117-P cells but did not alter the malignant properties of RAW 117-H10 cells which are already highly malignant in nature. About seventy percent of the animals inoculated (6 mice per group) with tunicamycin treated (2  $\mu\text{g/ml}$ ) parental cells (RAW 117-P) died by day 26 post-injection but most control animals injected with untreated parental cells were all alive at that time. The effect of tunicamycin in increasing the malignancy of these cells appeared to be a dose dependent phenomenon, since tunicamycin treatment of RAW 117-P cells at a lesser concentration (1  $\mu\text{g/ml}$ ) resulted in death of only 20% of the injected animals on day 26 and death of 50% of the injected animals on day 30.

Tunicamycin, a protein glycosylation inhibitor, is produced by *Streptomyces lysosuperficus* (12). It inhibits the growth of gram positive bacteria, fungi, yeast and viruses. Tunicamycin blocks the synthesis of N-acetylglucosaminyl pyrophosphoryl polyisoprenol by inhibiting the transfer of N-acetyl-glucosamine-1-phosphate to dolichol monophosphate (7). In most of the mammalian cells studied the amount of tunicamycin required for significant growth inhibition was at the sub-microgram level, usually 0.1 to 0.5  $\mu\text{g/ml}$  (6, 7). A much higher concentration of tunicamycin was found to be required for growth inhibition of RAW 117-P and RAW 117-H10 lymphosarcoma cells. We observed a marked difference in the amount of tunicamycin required for growth inhibition of these two cell lines. The highly metastatic variant cell line RAW 117-H10 required almost 5 times more tunicamycin than the parental cell line RAW 117-P for similar growth inhibition.

IN VIVO EXPERIMENT

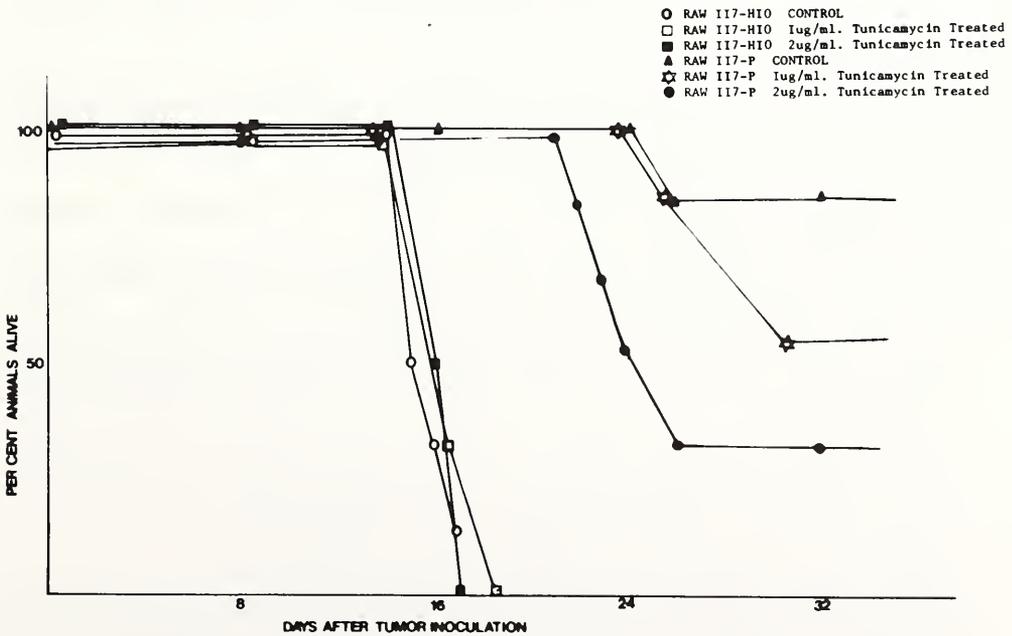


FIGURE 5:  $5 \times 10^3$  viable tunicamycin treated or untreated RAW117-P or RAW117-H10 cells in a volume of 0.1 ml were injected into each mouse via the tail vein. Each group consisted of 6 animals. Death of the animals were noted daily.

The reason for this increased resistance to tunicamycin by the highly malignant cell line is not known.

The expression of at least two major glycoprotein cell surface antigens (as recognized by xenoantiserum) "shifts" with altered malignant capacity of the RAW 117 lymphosarcoma cell lines. One of these antigens, a viral-coded glycoprotein (gp 70) is expressed much less on the highly malignant variant RAW 117-H10 cell line than on the less malignant parental RAW 117-P cells. It is likely that tunicamycin, through inhibition of glycosylation of cell surface components, prevents complete expression of major glycoprotein antigens such as gp 70, and may allow the less malignant RAW 117-P cells to masquerade as the more malignant RAW 117-H10 cells, perhaps at least long enough to establish experimental metastatic liver foci in the recipients. Quantitative assays of gp 70 and other cell surface glycoprotein expression will be necessary to see if the idea of molecular modulation of malignancy is correct. Further studies will also be required to determine the molecular basis for *in vitro* adherent difference in the tunicamycin treated and untreated cells. There appears to be no direct relationship between *in vitro* adherence and malignancy, since these adherence properties are similar in control (untreated) cultures of RAW 117-P and RAW 117-H10 cells.

#### Acknowledgments

This study was supported in part by funds from the Lake County Medical Center Development Agency. We thank Donya Warren for secretarial assistance.

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