Biochemical Effects and Therapeutic Potential of Tunicamycin in Murine L1210 Leukemia

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ABSTRACT

Tunicamycin, an antibiotic which specifically inhibits the dolichol-mediated synthesis of glycoproteins, significantly decreased the incorporation of [3H]-mannose and -glucosamine into L1210 ascites leukemia cell glycoproteins at concentrations which affected the biosynthesis of proteins minimally. Mice receiving inoculations of L1210 cells pretreated with 10 μM tunicamycin in vitro survived nearly twice as long as did mice receiving implants of untreated tumor cells. A nonlethal dose of X-irradiation (350 rads) to mice 24 hr prior to receiving their inoculation of tunicamycin-treated L1210 cells prevented this increase in life span. Thirty-eight percent of the long-term surviving mice which received 1 x 10^6 L1210 cells pretreated with 10 μM tunicamycin in vitro were then resistant to a subsequent challenge with 10^6 untreated L1210 ascites cells. Direct i.p. administration of tunicamycin to mice resulted in potent liver toxicity (50% lethal dose, 2.0 mg/kg) which obviated any therapeutic efficacy when administered to L1210 ascites-bearing mice. The administration of nontoxic levels of β-mannose prior to the administration of tunicamycin decreased the toxicity of the antibiotic in vivo and, when combined with β-mannose in vitro, exhibited cytotoxic additivity in terms of the inhibition of L1210 leukemic cell growth. A therapeutic regimen incorporating a 24-hr infusion of the sugar prior to multiple administrations of tunicamycin gave evidence of a small therapeutic response in terms of the survival of tumor-bearing mice. These results suggest that tunicamycin, an inhibitor of glycoprotein biosynthesis, might be able to alter tumor cell growth and immunogenicity provided that host liver toxicity is diminished.

INTRODUCTION

Earlier work from our laboratory has described the synthesis and evaluation of a large number of membrane sugar analogues as potential inhibitors or modifiers of tumor cell surface glycoconjugates (3-5, 27). This work led us to investigate the antitumor activity of tunicamycin, an amphipathic antibiotic which specifically inhibits the first committed step in the synthesis of glycoproteins containing asparagine-linked oligosaccharides (20, 21). Accordingly, tunicamycin has been widely used as a biochemical probe to study the role of the synthesis of these glycoconjugates in secretory processes (17, 33), plasma membrane turnover (11, 30), and other cell surface phenomena. The established antimicrobial and antitrypanosomal activity of the antibiotic (8, 28) and the demonstration of a preferential cytotoxicity of tunicamycin in several virally and chemically transformed versus nontransformed cells in vitro (22, 31, 34) have prompted investigation of the use of tunicamycin in L1210 leukemia (26).

Several different studies have demonstrated that increases in tumor cell immunogenicity have occurred as a result of modifications of the tumor cell surface by sulfhydryl- or hydroxyreactive agents (32), alkylating agents (15), neuraminidase (2, 6), or inhibitors of protein and glycoprotein synthesis (7). Indirect evidence, accumulated in investigations of the Thy-1-negative lymphocytic cell (23), suggested that a genetic aberration in the synthesis of some glycoproteins was responsible for modified antigenic determinants at the surface of these cells. Furthermore, recent studies have demonstrated that the synthesis or expression of tunicamycin-sensitive glycoproteins may affect the implantation and growth of B16 melanoma tumor cells in the lungs of mice (19). In this report, we describe the effects of the exposure of L1210 leukemic ascites cells to tunicamycin, both in terms of modifications in glycoprotein biosynthesis and tumor immunogenicity and in the expression of host toxicity.

MATERIALS AND METHODS

DBA/2 mice (Leo Goodwin Institute for Cancer Research, Fort Lauderdale, Fla.) were given i.p. inoculations of 1 x 10^6 L1210 ascites cells and were sacrificed 3 days later. The cells were removed under aseptic conditions by peritoneal lavage with cold Dulbecco's phosphate-buffered saline (Grand Island Biological Co., Grand Island, N. Y.) and were washed 3 times by repeated resuspension and centrifugation for 5 min at 1200 x g. After the final wash, the ascites cells were resuspended to a cell density of 1.0 x 10^6 cells/ml in serum-free RPMI 1640 supplemented with HEPES-MOPSA buffer (16 mw HEPES and 8 mw MOPSA; Sigma Chemical Co., St. Louis, Mo.), pH 7.35, containing penicillin (50 units/ml) and streptomycin (50 μg/ml). Two-mi aliquots of this cell suspension were added to culture tubes containing TM (provided by Dr. John Douros, National Cancer Institute, Bethesda, Md.) and 0.25 μCi of [3,4,5-3H]-leucine (0.66 mCi/mmol, 1.25 μCi of [2-3H]mannose (0.63 Ci/mmol) or 3.0 μCi of [6-3H]glucosamine (1.5 Ci/mol, New England Nuclear, Boston, Mass.). Cultures were incubated for 24 hr at 37° in a humidified atmosphere of 95% air/5% CO2. The incorporation period was terminated by the addition of 2 ml of cold RPMI 1640, the cells were pelleted by centrifugation, the medium was aspirated, and the cell pellets were precipitated with 1% phosphotungstic acid (1 g/100 ml of 0.5 N HCl). The resultant precipitates were repelleted, and the acid-insoluble material was washed twice more in 10% trichloroacetic acid (10 g/100 ml H2O). The final acid-insoluble pellet was extracted in 1 ml of absolute ethanol and 1 ml of ethyl ether, was recovered by centrifugation, and was solubilized in 200 μl of 1 N NaOH. An aliquot of the solubilized material was assayed for protein content by the method of Lowry et al. (24), and the remaining material was neutralized with 1 N HCl. The radioactivity of the neutralized samples was assessed in 3a70 complete counting cock-
Results of the incorporation of glycoprotein precursors into L1210 tumors

### Table 1

<table>
<thead>
<tr>
<th>Precursor</th>
<th>1 µM TM</th>
<th>3 µM TM</th>
<th>10 µM TM</th>
</tr>
</thead>
<tbody>
<tr>
<td>[3H]Leucine</td>
<td>92 ± 7</td>
<td>95 ± 6</td>
<td>89 ± 6</td>
</tr>
<tr>
<td>[3H]Mannose</td>
<td>12 ± 1</td>
<td>13 ± 3</td>
<td>13 ± 3</td>
</tr>
<tr>
<td>[3H]Glucosamine</td>
<td>39 ± 3</td>
<td>35 ± 4</td>
<td>36 ± 2</td>
</tr>
</tbody>
</table>

When given injections of ascites cells exposed to 5 µM tunicamycin, immunocompetent mice survived longer than did irradiated mice. Competent mice receiving cells pretreated with 10 µM tunicamycin in vitro survived nearly twice as long as did competent mice receiving untreated cells. The sole long-term (30-day) survivor among the 20 mice receiving 1 x 10⁶ viable tumor cells 30 days after receiving 1 x 10⁶ viable tumor cells was considered to be a long-term survivor and was rechallenged at that point with a lethal titer of 1 x 10⁹ viable, untreated L1210 ascites cells.

### Toxicity and Therapeutic Effects of Tunicamycin in the Presence or Absence of a Prior Administration of D-Mannose

Treatment of L1210 ascites tumor-bearing SPF DBA/2 mice with tunicamycin at concentrations from 1 to 10 µM significantly increased the length of survival of SPF DBA/2 mice given inoculations of 1 x 10³ treated viable cells over those mice which received an equal number of untreated viable cells (Chart 1). A nonlethal (350-rad) dose of X-irradiation 24 hr prior to inoculation revealed statistically significant increases in the survival of competent hosts (Chart 1, open bars) over that of immunologically compromised mice (Chart 1, hatched bars). When given injections of ascites cells exposed to 5 µM tunicamycin, immunocompetent mice survived longer than did irradiated mice. Competent mice receiving cells pretreated with 10 µM tunicamycin in vitro survived nearly twice as long as did competent mice receiving untreated cells. The sole long-term (30-day) survivor among the 20 mice receiving 1 x 10⁶ ascites cells treated with 10 µM tunicamycin did not survive a subsequent rechallenge with 1 x 10⁹ viable, untreated ascites cells.

### Parallel Studies in which 1 x 10⁹ viable, untreated, or tunicamycin-treated ascites cells were inoculated into competent and immunologically compromised SPF DBA/2 mice revealed analogous but more extensive increases in host survival. Both nonirradiated and irradiated mice succumbed to 1 x 10⁹ untreated L1210 ascites cells without significant differences in mean survival time (Chart 2). Competent mice given inoculations of tumor cells pretreated with 1 or 5 µM tunicamycin survived longer than did irradiated mice, and one long-term survivor was noted among the 16 competent mice given inoculations of ascites cells treated over the concentration range used. However, the incorporation of mannose and, to a lesser extent, glucosamine into ascites cells glycoproteins was inhibited significantly (12 to 13% and 35 to 39% of control values, respectively). These results suggested that, under the described conditions of cell culture exposure to tunicamycin, the synthesis of glycoproteins was significantly diminished in these cells. Corroborative evidence for the biochemical specificity of the antibiotic was obtained in other studies in which the activity of the tunicamycin-sensitive glycosyltransferase was measured in crude preparations from L1210 ascites cell microsomes. These studies confirmed that the formation of dolicholpyrophosphoryl-N-acetylglucosamine was completely inhibited at concentrations of tunicamycin (30 nM) which did not inhibit the formation of other lipid-linked intermediates such as dolicholpyrophosphorylmannose (data not shown). In intact cells, the modified expression of L1210 cell surface glycoconjugates can also be measured by the development of resistance to agglutination with wheat germ agglutinin after exposure to similar concentrations of tunicamycin (26).
Glycoprotein Synthesis as Target for Cancer Therapy

with 5 \( \mu M \) tunicamycin for 24 hr in vitro. With in vitro exposure to 10 \( \mu M \) tunicamycin, the mean survival periods of irradiated mice given inoculations of these cells was not significantly greater than that of irradiated mice given inoculations of untreated cells (Chart 2). However, the mean survival periods of immunocompetent mice given inoculations of treated cells was twice that of competent mice given injections of untreated cells. Five of 13 mice in this group were long-term survivors and, of these, 2 mice were fully resistant to a subsequent rechallenge with 1 \( \times 10^5 \) viable, untreated cells on Day 40. Bars, S.D.

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Tunicamycin Toxicity and the Effects of Prior Treatment with \( \alpha \)-Mannose. Examination of the lethal effects of tunicamycin, when administered as a single i.p. injection in mice, revealed a LD_{50} of 2.0 mg/kg and a LD_{100} of 3.5 mg/kg (Chart 3). Gross pathological evaluation of several organs suggested that the major pathological manifestations of tunicamycin toxicity occurred first and foremost in the liver. The acute toxicity of tunicamycin severely restricted the therapeutic efficacy of the antibiotic in the direct treatment of L1210 ascites tumor-bearing mice. The treatment of SPF DBA/2 mice, which had been given inoculations of 1 \( \times 10^5 \) ascites tumor cells 24 hr previously with a single i.p. dose of tunicamycin, resulted in no therapeutic effect at doses of 0.5 to 1.0 mg/kg (Chart 4). At higher doses, the toxicity of this agent was evident without any therapeutic effect, as demonstrated by the shorter survival periods of treated mice than those of tumor-bearing control mice.

The prior infusions of simple sugars which, theoretically, could alter the availability or synthesis of dolichol intermediates in situ were used in initial attempts to modify the toxicity of tunicamycin in mice. Preliminary investigations of the effects of subtoxic doses of \( \alpha \)-glycosamine prior to the administration of a range of doses of tunicamycin in vivo revealed no decrease in the expression of the toxicity of the antibiotic. However, infusion of \( \alpha \)-mannose at a maximally tolerated dose of 23 g/kg body weight,
infused over a 24-hr period prior to the administration of tunicamycin, decreased the lethality of the antibiotic significantly (Chart 3). With this pretreatment, a 2.0-mg/kg dose of tunicamycin, which represented an LD₅₀ value in noninfused mice, was found to be entirely nonlethal, and the apparent LD₅₀ for tunicamycin was increased by 50% (from 2.0 to 3.0 mg/kg; Chart 3).

Therapeutic Effects of Combinations of α-Mannose and Tunicamycin. Evaluation of the growth-inhibitory effects of the sequential addition of α-mannose followed by tunicamycin to L1210 leukemic cells grown in vitro demonstrated a cytotoxic additivity for the combination in terms of the inhibition of L1210 cell growth (Chart 5). This result, taken with the observation of a reduced host toxicity when α-mannose and tunicamycin were administered to mice sequentially, suggested that this protocol might offer significant therapeutic advantages in tumor-bearing mice.

Examinations of the effects of a single injection of tunicamycin (1.0, 1.5, or 2.0 mg/kg) following a 24-hr infusion of α-mannose revealed no appreciable therapeutic response in terms of host survival. The survival of ascites-bearing mice was also evaluated after multiple administrations of lower doses of the antibiotic with or without prior infusion with α-mannose. The results of these studies revealed that the treatment of mice with tunicamycin (0.1 mg/kg) on 3 successive days after the administration of α-mannose resulted in a modest (22%) increase in the life span of these mice (Table 2). An equal dose of tunicamycin in the absence of a prior infusion with the sugar resulted in no therapeutic effect, which suggested that the basis for the minor improvement with a pretreatment of α-mannose was due to a potentiation of the toxicity of the antibiotic similar to that seen in vitro or to the lessening of host toxicity. It was also apparent that a prior exposure to α-mannose had no effect on the expression of the toxicity of tunicamycin given in multiple doses as high as 0.5 mg/kg (Table 2).

DISCUSSION

Studies in which L1210 ascites cells were incubated at various concentrations of tunicamycin in vitro for 24 hr and then inoculated into immunocompetent and X-irradiated mice revealed that exposure to the antibiotic appeared to increase tumor cell immunogenicity without decreasing the tumorigenicity of these cells to a comparable extent. These effects were more pronounced at lower tumor cell inocula (1 × 10⁵ cells/mouse), at which 5 of 13 mice given inoculations of ascites cells exposed to 10 μM tunicamycin in vitro were long-term survivors and, of these, 2 were resistant to a subsequent rechallenge with 1 × 10⁶ viable, untreated L1210 ascites cells. The dose-dependent nature of the antibiotic-induced effect on tumor cell transplantability in vivo and the differences observed at the 2 tumor cell inocula suggested that the host response to tunicamycin-modified L1210 leukemic cells was dependent on both the extent of the biochemical alterations induced within the tumor cell population and on the initial tumor burden of the host. The observed increase in life span in immunocompetent mice resulted only after sufficient time had elapsed after the addition of tunicamycin to the L1210 cells in vitro. Simple admixture of tumor cells with tunicamycin prior to tumor cell implantation resulted in no subsequent increase in life span, thus ruling out any nonspecific cytotoxic effect of the antibiotic.

The basis for the apparent increase in the immunogenicity of the ascites cells following exposure to tunicamycin in vitro has not yet been directly determined, but it is likely that this modification is related to inhibition of plasma membrane glycoprotein biosynthesis. In an earlier study (26), we observed that tunicamycin altered L1210 cell plasma membrane glycoprotein biosynthesis and structure resulting in an inhibition of wheat germ-induced cellular agglutination. These alterations in plasma membrane structure and function may be the basis for changes in tumor cell membrane antigenicity. Alternatively, exposure to tunicamycin may result in the inhibition of the synthesis of a putative L1210 ascites cell surface, antigen-masking glycoprotein similar to those described for other ascites tumor cell lines (9, 18), or a specific block in the synthesis pathway. As asparagine-linked oligosaccharides of glycoproteins might result in a concomitant increase in the synthesis and expression of serine- or threonine-linked oligosaccharides which may function as tumor-associated antigens. Another level at which the biochemical effects of tunicamycin could result in alterations in the immunogenicity of L1210 ascites cells is a decreased capacity of tunicamycin-treated L1210 cells to undergo antigenic modulation. Old et al. (29) first described the loss of thymic leukemic antigens in the thymic leukemic antigen-positive ascites leukemia cell line when those cells were exposed to anti-thymic leukemic antigen antibody. The active process of release of antigens (10) into the extracellular space may result in the effective protection of tumor cells from the immunological defenses of the host (1).

In another recent study that we performed (8), therapeutic
effects of tunicamycin treatment were noted in trypanosome-infected mice. This response was probably due to inhibition of trypanosomal coat glycoprotein biosynthesis. In trypanosomes, the glycoprotein coat determines the antigenicity of these organisms in host animals, and throughout the course of the infection the antigenic character of the parasite population changes, thus allowing evasion of normal host immune defenses (35). Therefore, treatment of trypanosomiasis with tunicamycin may result in the inability of these parasites to modulate their cell surface coat, thereby becoming more susceptible to host immune defense mechanisms.

In our present studies with tunicamycin-treated L1210 cells inoculated into immunocompetent and X-irradiated mice, we have interpreted the increases in life span in immunocompetent animals to be due to tunicamycin-induced changes in tumor cell immunogenicity. Attempts to evaluate host immune effector mechanisms responsible for the observed effects have not been fruitful. Using both complement-dependent and complement-independent cellular cytotoxicity assays with splenic lymphocytes attained from syngeneic mice given inoculations of tunicamycin-treated L1210 cells, we observed no significant increases in the lysis of tunicamycin-treated or untreated $^{51}$Cr-labeled L1210 target cells. The lack of response in this in vitro syngeneic assay system does not, however, rule out that the observed increases in life span in mice receiving tunicamycin-treated L1210 cells were not due to changes in cellular immunogenicity. The negative results may indicate only that the in vitro system used did not have sufficient sensitivity to completely mimic the in vivo syngeneic situation. Most reports in the literature documenting activity for these in vitro systems use far more highly antigenic, allogeneic tumor systems in which responsiveness is easily ascertainable. The L1210 tumor system used in these studies is nonimmunogenic in syngeneic DBA/2 mice with the inoculation of low titers of cells (10 cells or less) sufficient to form lethal tumors.

The direct administration of tunicamycin to mice by i.p. injection of tunicamycin revealed a potent toxicity for this agent (LD$_{50}$ of 2.0 mg/kg for a single dose) which obviated any therapeutic efficacy when given to L1210 ascites tumor-bearing mice. Gross pathological examination of mice given injections of a toxic dose of the antibiotic indicated that the primary manifestation of toxicity occurred in the liver. Administration of nontoxic levels of $\alpha$-mannose by continuous infusion 24 hr prior to the administration of the antibiotic decreased this toxicity significantly. This, together with the apparent additivity of $\alpha$-mannose and tunicamycin in terms of the inhibition of L1210 leukemic cell growth in vitro, suggested that this regimen might offer therapeutic advantages in L1210 ascites tumor-bearing mice. Evaluation of a 24-hr infusion of $\alpha$-mannose prior to a single administration of tunicamycin in tumor-bearing mice revealed no therapeutic benefits in terms of host survival. However, additional studies in which the infusion of $\alpha$-mannose was followed by treatment with 3 successive administrations of lower doses of the antibiotic gave evidence of a small but significant increase in host survival.

Trapping of uridylates in the liver by hexosamines (12) may be indirectly related to the liver toxicity we observed following tunicamycin administration. Other studies recently completed in our laboratory (26) have demonstrated dramatic increases in intracellular UDP-sugar pools and ultrastructural modifications in L1210 cells following exposure to tunicamycin in vitro. These studies suggest that the inhibition of oligosaccharide biosynthesis and the concomitant perturbation in intracellular nucleotide or nucleotide sugar pools may together form the basis for the toxicity of the antibiotic in both liver and tumor cells. At this level, the sparing effects of $\alpha$-mannose may also be expressed in hepatocytes. A prior infusion of this sugar would be expected both to increase hepatic intracellular pools of GDP-mannose and to decrease GTP pools, effects which might counteract a tunicamycin-induced decrease in pyrimidine pools. Thus, the administration of $\alpha$-mannose before exposure to tunicamycin could be postulated to facilitate the maintenance of a metabolic balance in the hepatic tissues of treated mice. However, this general mechanism cannot readily account for the apparently additive cytotoxic activities of $\alpha$-mannose and tunicamycin when used sequentially against L1210 leukemic cells grown in vitro, nor can it account for the absence of a protective effect by $\alpha$-mannose under conditions of multiple administrations of tunicamycin in vivo. Moreover, the effects of a prior infusion with $\alpha$-mannose on both the pharmacokinetics of tunicamycin and on the immunological response of tumor-bearing mice to L1210 leukemic cells must be examined further before the actual basis for its therapeutically beneficial effects can be determined.

In conclusion, our studies have demonstrated increased survival due to apparent increases in tumor cell immunogenicity in vivo following exposure of L1210 leukemia cells to tunicamycin in vitro. Extrapolation of these results to an effective therapeutic course in tumor-bearing animals has been difficult, due to the toxicity of this antibiotic to the host. Attempts to increase the therapeutic efficacy of tunicamycin with a prior infusion with $\alpha$-mannose resulted in a small increase in survival in mice with L1210 leukemia and diminished toxicity to the host. This type of approach may provide a basis from which future studies can be designed and tested.

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