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THALIDOMIDE METABOLITE INHIBITS TUMOR CELL ATTACHMENT TO CONCANAVALIN A COATED SURFACES

by

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SUMMARY: The inhibitory effect of drug treatment on tumor cell attachment to plastic surfaces coated with concanavalin A correlates well with the in vivo teratogenicity of the drug. Using attachment as an assay, the effects of thalidomide and some of its metabolites have been examined for inhibitory activity. While thalidomide and its hydrolysis products did not alter attachment, metabolites of thalidomide produced by incubation of the drug with murine liver microsomes were inhibitory. Generation of inhibitory products required the presence of glucose-6-phosphate, NADP, glucose-6-phosphate dehydrogenase and magnesium chloride. The degree of inhibition was dependent on the duration of incubation at 37°C. These results suggest a model for the teratogenic action of thalidomide in which metabolites of the drug alter cell surface function leading to interference with normal morphogenic cell to cell interactions.

One of the unresolved problems in teratology is the mechanism by which the sedative thalidomide causes congenital malformations. This notorious drug was responsible for roughly 10,000 seriously malformed children in the early 1960's before its effects were discovered and the agent removed from the market (1-3). Although low doses of thalidomide cause severe limb, cardiac, gastrointestinal and renal malformations in embryos, it is a remarkably innocuous drug in adults. The oral LD_{50} in mice is greater than 10 gm/kg (4). Efforts to determine the mechanism of thalidomide's teratogenicity have been largely unsuccessful. The list of biochemical processes that are not inhibited by thalidomide includes energy, vitamin, nucleic acid and carbohydrate metabolism (5,6). In this paper we report that incubation of thalidomide with a microsomal activation system yields products that inhibit

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the attachment of tumor cells to concanavalin A coated plastic surfaces. Inhibition of attachment has been found to correlate with the teratogenic activity of many drugs (7). These results suggest that metabolites of thalidomide may interfere with normal embryonic cell to cell or cell to extracellular matrix interactions and thus produce abnormal morphogenesis.

In a revealing series of experiments, Lash and Saxen found that chondrogenesis in isolated, cultured, human limb bud was inhibited by thalidomide (7). Since chondrogenesis, in their system, was dependent on the presence of adjacent mesonephric tissue they suggested that thalidomide interfered with the interaction between mesonephros and limb bud. As ^{14}C labeled thalidomide accumulated primarily in the mesonephric mesenchyme rather than limb bud tissue they suggested that thalidomide acts indirectly on limb bud by altering some characteristic of mesonephric tissue.

Our interest in thalidomide stems from studies of ascites tumor cell attachment to surfaces coated with concanavalin A and other lectins. Tumor cell attachment to these surfaces requires metabolic energy, involves specific cell surface carbohydrate to lectin interactions and is sensitive to cytochalasins (9 and our unpublished observations). These features are similar to those involved in embryonic cell aggregation, in vitro (10-12). We have found that pretreatment of tumor cells with a wide variety of drugs inhibits attachment. A high correlation between the ability of a drug to inhibit attachment in vitro and the teratogenicity of the drug in vivo has been noted in a study of 55 drugs with known teratogenic potential (7). More than 80% of the drugs tested were correctly designated as teratogenic or non teratogenic according to their inhibitory properties. More recently we have found a good quantitative correlation between the in vitro inhibitory dose and the in vivo teratogenic dose for 53 inhibitory teratogens (13). We have speculated that since the interaction of tumor cells with lectin coated surfaces has features in common with morphogenic cell to cell or cell to ECM interactions, agents which interfere with lectin mediated attach-

ment in vitro might be expected to interfere with morphogenic interactions in vivo and lead to abberent development.

EXPERIMENTAL PROCEDURES

Ascites mouse ovarian tumor cells (20) were grown intraperitoneally in C3H/HeJ mice (Jackson Laboratory). The evening before use the mice were injected intraperitoneally with 0.2 mCi (^{3}H) thymidine (New England Nuclear). The following morning the cells were harvested, washed three times in phosphate buffered saline (PBS) and resuspended at 10^8/ml in PBS. Concanavalin A coated disks were made by floating 1.25 cm diameter polyethylene disks on an 0.1 mg/ml concanavalin A (Sigma), 2.5 % glutaraldehyde, PBS solution, waiting 30 mintues and inverting the disks. Incubation was continued at room temperature with gentle oscillation overnight, the disks washed in PBS and stored in 0.3M glycine. Bovine serum albumin coated disks were made similarly. To assay for attachment a suspension of cells treated as described in the figure legends was poured into a 35 mm bacterial petri dish (Falcon), and three coated disks introduced into the suspension. The cells were allowed to sedement onto the disks for 20 min, the disks removed with fine forcepts, washed in PBS and the attached radioactivity counted in a scintillation counter. Triplicate sample counts were averaged and background attachment to BSA coated disks subtracted. Background radioactivity never exceeded 5% of maximal attachment. Thalidomide was obtained from Chemie Grunenthal, Stolberg, West Germany. All other reagents were from Sigma.

RESULTS AND DISCUSSION

If, as suggested by Lash and Saxen, thalidomide interferes with an interactive process in vivo a parallel effect might be detectable in our in vitro system. Preliminary experiments, however, indicated that thalidomide did not alter attachment, even at saturating concentrations (1 mg/ml) of the drug.

We next examined the possibility that metabolites of thalidomide could inhibit attachment. Thalidomide undergoes spontaneous hydrolysis at pH 7.4 with a half life of 2.4 hours (14,15). Twelve different products are found in proportions dependent on incubation time. Aqueous solutions of the drug were hydrolyzed for 2,4,6,24 and 48 hours at 37°C and tested for their ability to inhibit attachment. Once again the results were negative, leading to the conclusion that spontaneous hydrolysis products of thalidomide did not alter attachment.

We then used a liver microsomal activation system, similar to that used in the analysis of carcinogens (16), in order to develop non-hydrolytic

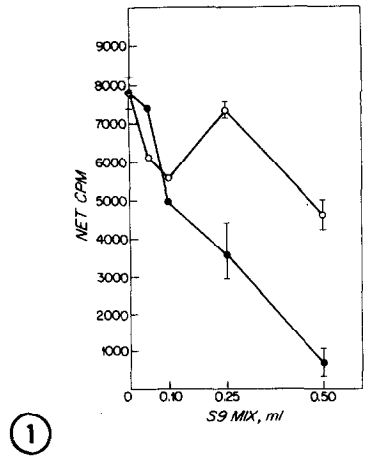
metabolites of thalidomide. This system yielded inhibitory metabolites as is shown in Figure 1. Ascites tumor cells were incubated with murine S9 mix, (16) with and without thalidomide. While S9 mix by itself inhibited attachment slightly, far greater inhibition was observed when thalidomide was added. No attachment inhibition was observed when NADP and glucose-6-phosphate were omitted from the incubation mixture.

To avoid the direct inhibitory effect of S9 on attachment we have subsequently used more purified microsomes obtained from the S9 fraction by high speed centrifugation (105,000 x G for 1 hr). The use of microsomes rather than S9 for thalidomide activation necessitated the addition of glucose-6-phosphate dehydrogenase to the incubation mixture.

The production of inhibitory thalidomide metabolites required incubation at 37°C. As shown in Figure 2, a 30 min incubation at room temperature did not yield inhibition. This figure also shows that the extent of inhibition was dependent on the duration of 37°C incubation.

Omission of NADP, glucose-6-phosphate dehydrogenase or magnesium from the incubation mixture prevented thalidomide dependent inhibition of attachment. The $MgCl_2$ concentration dependence was of particular interest. In the absence of Mg^{++} there was no inhibitory activity. At 1-2 mM $MgCl_2$ substantial inhibitory activity was observed. However, at concentrations in the 5 mM range no inhibitory products were obtained. This sharp magnesium optimum is in contrast to the wider optimum observed with hamster aryl hydroxylase (17) and suggests that another enzyme system may be involved.

These results are consistent with the suggestion put forward by Lash and Saxen, that thalidomide acts by interfering with cell to cell interactions during morphogenesis (8). These results are also consistent with an experiment of Klein et al (18) in which serum from rhesus monkeys dosed with thalidomide produced abberent growth of rat embryos in vitro. Thalidomide added directly to these cultures did not alter rat embryo morphogenesis. Thus, thalidomide



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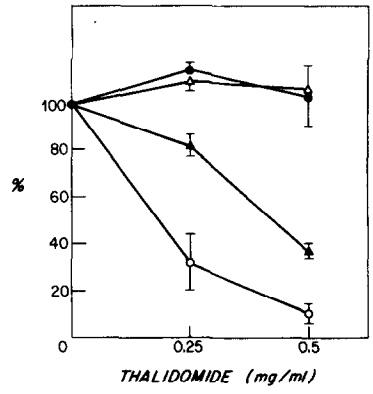

(2)

Fig. 1: Inhibition of tumor cell attachment by thalidomide incubated with murine liver S9 mix. One ml (final volume) of tritium labeled ascitic murine ovarian tumor cells at 10^7/ml in phosphate buffered saline (PBS) were incubated at 37°C with the indicated amount of S9 mix (from male C57/B6Kh mice) prepared according to Ames et al (16) with (●) or without (○) 0.5 mg thalidomide for 30 min. Thalidomide was routinely dissolved in dimethylsulfoxide (DMSO) at 50 mg/ml immediately before use. The DMSO concentration was held at 10 µl/ml in all samples. The treated cell suspension was poured into a 35 mm petri dish and attachment measured as described under experimental procedures. Standard errors are shown to indicate the variability of the data.

Fig. 2: Effect of incubation time on thalidomide mediated attachment inhibition. Tumor cells were incubated with the indicated concentration of thalidomide in a mixture including 0.32 mg (protein, Lowry) murine liver microsomes, 0.5 mM NADP, 10 mM glucose-6-phosphate, 0.8 units glucose-6-phosphate dehydrogenase, 1.5 mM $MgCl_2$, 20 mM sodium phosphate buffer pH 7.4. Following incubation the cells were assayed for their ability to attach to concanavalin A coated surface as described in the legend to Fig. 1. Attachment is expressed as a percentage of control (no thalidomide) value.

Symbols: no incubation (△), 30 min incubation at room temperature about 21°) (●), 30 min incubation at 37°C (▲), 60 min incubation at 37°C (○). Standard errors shown.

metabolites in monkey serum, rather than parent compound alter *in vitro* rat embryo development.

Non-hydrolytic metabolites of thalidomide have been found in the urine of treated rabbits. The detection of 3- and 4- hydroxyphtalic acids in urine by Williams et al (19) is an indication that 3- and 4- hydroxylation of thalidomide can occur *in vivo*. They point out that when hydroxylation is considered, over 100 different metabolic products of thalidomide can be postulated. Which of these products inhibit attachment and the question of whether these products are the ultimate teratogenic products of thalidomide

can only be resolved by the purification of inhibitory products. The attachment assay system can provide a simple and rapid method for monitoring the purification procedure.

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