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March 27, 1972

Dear Sir:

I am pleased to submit in duplicate an unsolicited proposal entitled "Studies on the Mechanism of Action of Compounds Affecting the Central Nervous System Using Tissue Culture Techniques."

This study would be under the direction of [redacted] Ph.D. Curricula vitae for Dr. [redacted] and others are appended as part of the technical proposal.

The cost to conduct this study is [redacted] The breakdown of costs appears in the cost proposal which is appended.

If there are any questions concerning this proposal, please do not hesitate to contact us. We look forward to working with you on this project.

Sincerely yours,

Enclosures

TECHNICAL PROPOSAL

STUDIES ON THE MECHANISM OF ACTION
OF COMPOUNDS AFFECTING THE CENTRAL NERVOUS SYSTEM
USING TISSUE CULTURE TECHNIQUES

Today there are many compounds known to have a pharmacological effect upon the central nervous system. However, the exact biochemical lesion(s) or effect(s) which triggers this response remains, for the most part, unknown. Until such time as the mechanism of action of these compounds is known, (i) accurate screening tests to measure the activity of these and other test compounds cannot be devised, (ii) a person who has been exposed to these materials cannot be identified, and (iii) the effects of these materials cannot be countered. The use of new analytical techniques in a tissue culture system provides a unique assay system for studying the mechanism of action of a compound with an effect upon the central nervous system. Tissue culture systems are much more readily controllable, more reproducible, more easily manipulated, cheaper, and can yield more data in a shorter period of time.

Because of the immense background data in man and animals on the pharmacologic effect of narcotics, these analgesic and addictive drugs will be studied as type-specific instances of compounds which affect the central nervous system. The same methodology and techniques are directly applicable to any other type of compound desired. There have been many theories developed as to what changes occur within the body following administration of a narcotic and/or development of the tolerant-dependent state (1,2). The best current explanation is that there is interference with several biochemical

- 2 -

reactions including a change in protein and nucleic acid synthesis as well as changes in the pathway of glucose metabolism.

There have been several reports in the literature demonstrating the feasibility of using tissue culture systems to study the action of central nervous system affecting compounds. These reports cover studies on the initial effect caused by administration of a narcotic (3) as well as the changes induced during development of the tolerant-dependent state. The following sections describe our experimental approach using narcotics as type-specific drugs.

EXPERIMENTAL APPROACH

A. Cell Lines and Growth Conditions

In a study such as this it is most important that several different cell lines be used so that common reactions can be distinguished from reactions specific for only one cell type. Also, there is the probability that certain cell lines may not show a reaction whereas others may. We propose to use human cell lines at first. If these are unsuccessful, we may have to switch to animal cell lines.

We will use human amnion (as a primary isolate), WI-38 (as a low passage diploid line), and HeLa (as a heterploid, established line) as the cell lines. The alternate animal choices would be chick embryo, LLC MK₂ monkey kidney, and L-929 mouse fibroblast, respectively. The primary isolates will, of course, be used immediately. The other cell lines

will be grown on Eagles Basal media plus 20% human or fetal bovine serum. For most of the experiments, where microscopic observation is involved and where the techniques are best known, the cultures will be grown and maintained as monolayers.

It has been reported that human serum albumin reacts with and binds with narcotics (5). Apparently this is not a serious problem as previous tissue culture studies have indicated an effect by morphine in serum-containing media.

B. Exposure to Narcotics

In addition to using several different cell lines it will be most important to use several different narcotics which differ in their analgesic and/or addictive properties. We propose to use _____ as a strong narcotic with addictive properties. We propose to use _____ as weak narcotic with limited additive capabilities and _____ as narcotic antagonists.

_____ have been shown to be toxic when added to HeLa cells in culture (3). Cell growth was inhibited and cytoplasmic vacuolization was induced. However, at certain levels the cells were made dependent upon _____. After 20 passages withdrawal of _____ from the culture

- 4 -

media lead to a decrease in the growth rate, cellular changes and death. If [redacted] was again added to duplicate cultures the cells regained their prior appearance and growth resumed.

C. Parameters to be Measured

(1) Growth Rate - The growth rate will be determined by the time required to form a confluent monolayer.

(2) Cell Size - Cell size distribution studies as a function of treatment will be performed using an ocular micrometer. Some idea of cell numbers will be obtained by the use

of an ocular grid and physical counting. It is possible that certain narcotics will influence cell size and cell numbers without causing a shift in the overall growth rate.

(3) Cytology - Careful attention will be paid to changes in cellular morphology as a result of treatment. Cytoplasmic vacuolization and the presence of irregular shapes have previously been noted as a result of exposure to narcotics (3,4). Any changes in cell morphology will be carefully documented and photomicrographs obtained as a permanent record.

(4) Karyotype - Although the literature on the mechanism of action of narcotics indicates that the effect is mediated by metabolic shifts rather than genetic injury, there is always a possibility that narcotics have some sort of a direct effect upon chromosomal structures. Chromosomal analysis, both as to number and morphology, will be made by the colchicine trapping method of Moorhead, et al (9). The results will be expressed as bar graphs of the percentage distribution of cells in the population as a function of chromosome number as well as photographs of the grouped chromosomes.

(5) Synthesis of Macromolecules - Narcotics are known to affect the synthesis of protein and RNA in the brain (1,10,11). If the synthesis of protein is blocked by the addition of actinomycin, puromycin or 8-azaguanine, there is a marked inhibition in the development of tolerance in vivo. Clouet and Ratner (10) studied the incorporation of ^{14}C -leucine into protein as a measure of protein synthesis after exposure to the narcotic.

The above specifically mentioned enzymes as well as certain other enzymes of general importance to the cell (e.g. lactic dehydrogenase, esterase, acid and alkaline phosphatase, etc.) will be assayed in the cell-free supernatants prepared from the various cell cultures after exposure to the various narcotics.

(7) Changes in Isoenzyme Distribution Pattern - Certain key metabolic reactions are catalyzed by more than one species of protein molecule. These families of different proteins all catalyzing the same reaction are called isoenzymes. When studied in sufficient detail they each appear to have slight different physical properties and therefore allow the cell to exercise fine control over its metabolism by stimulating one pathway and inhibiting another even though both pathways pass through a common intermediate. The literature is full of studies on isoenzyme forms of lactic, malic, isocitric, and glucose-6-phosphate dehydrogenase (6 and its references). There is some evidence for the presence of isoenzymes of phenylethanolamine-N-methyltransferase (20) and other N- and O-methyltransferases (19).

Most of the enzymes whose specific activity is determined will be subjected to isoenzyme distribution studies.

In this way an increase or decrease in specific activity can be attributed to a change either in one or more of its isoenzyme forms or across the board to all isoenzyme forms. This data will be quite important when it comes to deciding whether the effect of the narcotic was general (e.g., an effect on protein synthesis) or specific (e.g., an effect on one particular isoenzyme form).

The data from all of these different assays will then be summarized by cell line, narcotic used, and whether the cell was treated with the narcotic or was in the tolerant-dependent state. The simplicity of the tissue culture system coupled with its reproducibility will allow decisions to be made as to what functions and/or metabolic pathways within the cell are affected following narcotic administration versus development of the tolerant-dependent state. Knowing which pathways are affected by addictive versus non-addictive will allow definitive test systems to be developed and standardized for testing new compounds. The mechanism of action of a narcotic and indeed the mechanism of pain itself may be studied in much greater detail by these procedures. The establishment of a reliable tissue culture screening technique for the monitoring and assaying of compounds active upon the central nervous system will greatly facilitate future study. This methodology may then be readily applied to the study of any other pharmacologically active material.

PROPOSED BUDGET

DIRECT LABOR

<u>Name</u>	<u>Function</u>	<u>% of Time</u>	<u>Hours</u>	<u>Rate</u>	<u>Total</u>
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