MAY 14 1965

BNWL-SA-78
DNBSA-78

CONF-650113-1

MASTER

THE USE OF TISSUE CULTURE AND CYTOCHEMICAL
TECHNIQUE IN TOXICOLOGIC RESEARCH*

W. J. Clarke, D.V.M., Ph.D., J. L. Palotay, D.V.M., M.S.
and D. D. Mahlum, Ph.D.

Pacific Northwest Laboratory
Battelle Memorial Institute
Richland, Washington

The action of physical and chemical agents in vivo initiates complex reactions and interactions in various organs and tissues that are difficult to unravel. Tissue culture techniques, on the other hand, allow the effects of such agents to be studied independently of blood or nerve supply, under stringently controlled conditions. Complicated problems can thus be broken down into their elements and the question decided whether or not the action of a particular insult is due to a direct effect on the tissue, or whether it is mediated through in vivo metabolic pathways. The comparative ease with which many cultures can be kept under known, identical experimental conditions allows not only the detection and sequential observance of these effects, but also the opportunity to qualitate and quantitate these changes.

In the past, there have been many studies on the effects of toxic materials on cell, tissue, and organ cultures. Most of these studies have been morphologic in nature, designed to observe the gross behavior of tissue culture cells in response to different toxic agents. Although a great deal of information has been obtained by these observations, with the advent of new and improved analytical, microscopic, and cytochemical techniques, fresh definitive approaches to qualitative and quantitative

* Work performed under Contract No. AT(45-1)-1350 between the Atomic Energy Commission and the General Electric Company.

DISCLAIMER

This report was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government nor any agency Thereof, nor any of their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof.

DISCLAIMER

Portions of this document may be illegible in electronic image products. Images are produced from the best available original document.

toxicologic research have developed. I would like to discuss some of these techniques and briefly cite some of our work along these lines.

This past year we became interested in the early toxic action and carcinogenic properties of DMAB, p-dimethylaminoazobenzene, better known as butter yellow, on the liver. Rats fed this azo dye (Table I) for ninety days have a drop in the proportion of their hepatic parenchymal cells from an original value of 60% to one of about 25%. Similarly, the proportion of littoral cells goes down from 35% to about 10%. This is due primarily to an extensive proliferation of the bile duct cells, the proportion of which goes up from 2% to about 55%. Hence, the average cell, which meant $2/3$ a parenchymal cell and $1/3$ a littoral cell in normal liver has become under these conditions roughly $2/3$ a bile duct cell and $1/3$ a parenchymal cell. Biochemical determinations on liver samples from DMAB treated animals, therefore, reflect only variations in the composition of the cell population and not actual variations in the cells.

While biochemical methods can give precise figures on the composition of the "average cell" of a tissue, histochemical methods, in contrast, can add much information about the differences between individual cell types or individual cells of each type. This complimentary approach has been used in our laboratory in various toxicologic experiments with encouraging results.

Although with this experimental approach we were able to validate our quantitative biochemical results at the tissue level, we still could not answer the specific question of the loci and extent of toxic effects at the cellular level. It became apparent, especially in our DMAB studies, that the culturing of hepatic parenchymal cells, the insult of these cells,

and the qualitative and quantitative measure of their responses to this toxic stimulus could provide the answers. Consequently, we have initiated studies on the toxicity of DMAB in hepatic cell tissue cultures.

The Chang human liver cell strain was chosen rather than a primary culture of rat liver cells, not only for their established biochemical and morphological characteristics, but also because of the comparative ease with which these particular cultures can be kept under known, identical experimental conditions. The Chang liver line is one of four cell lines established from non-malignant, normal human tissue in 1954 by R. S. Chang. Subsequent serial subcultures from these cells now number approximately 255, thus firmly establishing their viability, growth characteristics, morphology, and metabolic patterns.

Chang human liver cells, set in 16 x 150 mm Leighton tubes with 11 x 22 mm coverslips, were obtained from a commercial source. The original inoculum consisted of 1.5 mls of Basal Eagle Medium, 2% calf serum, 100 units each of penicillin and streptomycin per ml., and 1.5×10^6 liver cells. Upon receipt of the cells, each tube was checked for cell viability and coverslip growth, and a growth media containing 5% calf serum was substituted for the maintenance media. The tubes were incubated at 37° C for 48-72 hours until logarithmic growth and a beginning confluent sheet of cells was observed on the coverslip. New growth media containing DMAB was then introduced into the tubes and random samples selected at various time intervals post-insult for morphological and cytochemical study. The dose levels utilized were 0.25, 2.5, 25, 250, and 2500 ug DMAB per tube. At least six experimental and three control tubes were sampled at each time interval and dose level.

All tubes containing 25 or more ug showed complete cellular death at 48 hours. The tentative LD₁₀₀ 48 hours for DMAB insulted Chang liver cells was, therefore, between 2.5 and 25 ug. Future studies will further define this dose level. Because we were primarily interested in the detection and sequential observance of toxic effects in these cultures, the dose levels of 25 ug or less during the critical 48-hour period were of special interest.

In a general oversight survey of the cells in these dose levels, using May-Grunwald-Geimsa staining, certain morphologic changes were noted. Four-hour control cells showed a basophilic granular to filamentous cytoplasm, and nuclei of rather uniform size. The nucleoplasm varied from a light, granular, eosinophilic coloration to a light to medium purple, coarse appearance. Nucleoli varied from one to four in number and showed variation in form and size. Mitoses appeared normal in numbers and appearance.

The 0.25 and 2.5 ug cultures at four hours did not vary significantly from the controls. The 25 ug level, however, showed a slight increase in mitotic figures and multinucleated cells, and a decrease in interphase cell nuclear diameters. In addition, many of the nucleoli were fused into elongated, bizzare forms.

At twelve hours post-insult, the 0.25 ug cells had a moderate decrease in mitoses and a slight decrease in multinucleates and nuclear diameters. In the 2.5 ug level, cells with bizzare nuclei began to appear. Irregular nuclear outlines in the form of "bean or cauliflower-like" shapes were prominent. Large, fused nucleoli and coarse hyperchromatic chromatin were common in these cells. In addition, the nuclear size of most

interphase cells was slightly decreased. The 25 ug cells also showed an increase in bizzare forms, but the most striking changes were in the increased numbers of degenerating cells, and the moderate to marked decrease in cell nuclear size.

By 24 hours, the 0.25 ug dose level changes were comparable to those seen for this level at 12 hours, except that mitotic activity did not appear to be as greatly depressed. The 2.5 and 25 ug cells were similar in change in that there was a moderate increase in bizzare forms and a marked decrease in mitoses and nuclear size in these cultures. However, impending lethality was apparent in the 25 ug level as evidenced by the marked decrease in cell numbers and in the numbers of degenerating cells.

At 48 hours, bizzare nuclei were observed for the first time in the 0.25 ug level, as were degenerating cells. Numbers of multinucleates were increased, while nuclear size continued to appear moderately depressed. Mitoses, however, were comparable in number to those of the control cells. The effects on the 2.5 ug cells were similar, except that there appeared to be more degenerating cells and a continuing marked depression of mitotic activity. In the 25 ug level, cellular destruction was complete. Only a few pyknotic cells and collections of cellular debris could be seen on the coverslips.

To substantiate the findings of this general oversight survey, tubes were randomly selected from the control and experimental groups and random areas from each coverslip were photographically enlarged. All cells in each photographic field were then analyzed for mitotic indices, multi-nucleated cells, bizzare nuclei, and cellular degeneration and necrosis. In addition, random samples of interphase cell diameters were measured

with a Zeiss particle size analyzer. A total of 10,000 to 20,000 cells were analyzed in this manner. In all instances, except for minor variations, results obtained by these methods were in agreement with those of the general oversight scanning method.

Cytochemical determinations were conducted on the control and 25 ug level cells at four-to-eight-hour intervals during a 24-hour post-insult period. This particular time period and dose level was chosen in an effort to delineate some of the early biochemical changes prior to cell death at 48 hours.

At four hours, only a few isolated interphase control cells showed PAS positive granules in their cytoplasm. Amylase digestion of these samples prior to staining blocked the magenta coloration of the PAS reaction, indicating that these granules were glycogen. In contrast, the 25 ug level cells showed increased numbers of PAS positive granules with a tendency toward clumping, especially in the multinucleated cells. Amylase digestion of these samples also blocked the PAS reaction. Control cultures at eight hours did not differ significantly from those at four hours. The 25 ug cells, however, showed a slight diffuse activity with concentrations of granules in their hypertrophic and multinucleated cells. By 16 hours, there was a slight increase in numbers of granules in the controls, especially in the interphase cells, while the 25 ug cells continued to show moderate glycogen content with apparent multinucleated cell concentration. At 24 hours, glycogen in the control culture was similar to that of the 16-hour samples. The 25 ug cells, however, showed less activity than the controls, although concentrations of granules were visible in the hypertrophic cells.

Lipid determinations of the cultures revealed increased concentrations of general lipids in the DMAB treated cells as compared to the controls that persisted throughout the experimental observation period. Parallel cultures stained for neutral and acidic lipids gave additional corroborative evidence of lipid increase, particularly in the neutral lipid fraction. Control interphase cells during the 24-hour sampling period showed consistent dark blue nucleoli and medium blue nuclear outlines indicative of acidic lipids. A few scattered blue globules were also evident in the perinuclear cytoplasm of some cells. Only a few red globules of neutral lipids could be discerned. The 25 ug cultures, on the other hand, had a consistent marked decrease in acidic lipid content, and a marked increase in neutral lipids in the cytoplasm of the interphase cells. This increase was particularly noticeable perinuclearly in some of the mitotic cells.

Succinic dehydrogenase staining was utilized as a general marker for mitochondrial activity. In general, the controls showed moderate amounts of formazan granules in their cytoplasm, with slightly increased amounts in the more active cells. The 25 ug cultures, in contrast, evidenced increasing amounts of formazan deposits during the sampling period, with concentration and clumping noticeable in the multinucleated cells.

The correlation of these morphologic and cytochemical studies, though not complete at this time, were very informative as to the nature and sequence of events following hepatic cell insult by DMAB. Mitotic indices (Table II) at four hours were depressed significantly only in the 2500 ug level. At 12 hours, mitotic activity was relatively the same for the control, 2.5, and 25 ug level cells, while that of the .25 ug cultures appeared to be one-half that of the control cells. By 24 hours, all dose

levels showed markedly lower indices except for the .25 ug cells. This effect carried over through 48 hours in the surviving .25 and 2.5 ug dose levels until 72 hours, when mitotic activity seemed to be at a standstill. At 96 to 120 hours post-insult, however, these cells showed a resurgence of mitoses that equalled and finally surpassed that of the controls. From these observations, it was apparent that though mitotic activity was initially depressed by DMAB, either recovery of the injured cells or the survival of resistant strains had taken place. Also to be considered as a probability is the fact that mitotic activity usually appears fairly early following hepatic insult, even preceding or occurring simultaneously with histologically visible necrosis. It is hoped that future studies with continuous flask cultures at these lower dose levels will provide the answers to this question.

Multinucleated cells (Table III) in cell cultures are considered to be evidence of either amitotic division, potentially neoplastic cells, or cellular coalescence and impending cell death. As can be seen from Table III, multinucleates are commonly found in Chang liver cell cultures. However, the increased numbers of these cells in the lower dose levels at 48 plus hours, coupled with the resurgence of mitotic activity seen in these levels, points more towards amitotic division as the source of these cells. The experimental observation period was not long enough to induce visible neoplastic transformations in these cells, and as for these multinucleates representing coalesced, dying cells, the cytochemical studies prove that these cells are, in most instances, more metabolically active than the non-multinucleated cells.

A certain number of cultured cells show irregular or bizzare nuclear and nucleolar forms (Table IV), and the Chang liver cells are no exception. The treated cells did not differ from the controls in this respect until 12 to 24 hours post-insult, when increasing numbers of apparently dose-dependent, bizzare forms began to appear. Of particular interest were the 2.5 ug level cells which evidenced more irregularity of nuclear and nucleolar form than the controls throughout the observation period. Hypertrophy of nucleoli are not unique in this instance, having been seen in numerous other toxicologic studies. Biochemical analysis of isolated nuclei with hypertrophic nucleoli in these circumstances has revealed an increase in RNA content and RNA: protein nitrogen ratio. However, the most interesting finding is the similarity in the increased size and number of nucleoli in both DMAB and other intoxications, which points to the fact that these compounds do not act uniquely by altering the permeability of the cellular membranes. They must have a particular and precocious action on the nucleus, the cause and pathogenic action of which is unknown at this time. These particular effects will also be of extreme interest in our future studies.

Tissue culture cells grown in monolayer form in Leighton tubes have a limited life span (Table V). Though media is routinely replenished at 72-hour intervals, population growth and metabolic end-product buildup outstrips the space environment to such an extent that these cultures cannot be maintained without subculturing much beyond 120 hours post-innoculation. This can be readily seen in the control values for 120 hours. Another important point to consider when observing initial cytotoxic effects in monolayer coverslip culture is that the more sensitive cells

have been destroyed and have dropped off the coverslip, leaving only the less severely affected or more resistant cells adhering to the surface. Time of sampling is also important when utilizing high dose levels, in that a shorter sampling interval is necessary in order to observe degenerative changes in the surviving cells. These aforementioned effects were all apparent in the values found for the 4, 12, and 24-hour samples. At 48 through 96 hours, however, cytotoxic changes were particularly prominent in the surviving experimental cells, a finding that would be expected in any dose-dependent effects study such as this.

The cytochemical studies on these cells, though incomplete at this time, were very informative as to the loci of cellular damage in these insulted cells (Table VI). Cellular glycogen is normally present in two fractions: one firmly bound to protein, and the other soluble in trichloroacetic acid and readily available for tissue requirements. Only the latter can be demonstrated cytochemically. It has been shown that the agranular or smooth endoplasmic reticulum is apparently associated with glycogenesis and glycogenolysis. Reduced glycogen content or its complete loss is a well-known happening in damaged cells, and is especially rapid in the presence of most of the carcinogenic dyes. This reduction or loss of cellular glycogen in the intact animal, however, was not corroborated during the 24-hour observation period in our cultured liver cells. Glycogen content of the DMAB treated cells was greater than in the controls through 16 hours post-insult, decreasing slightly at the 24-hour sampling period. This would seem to indicate that damage to the smooth endoplasmic reticulum is not an important factor in the early cytotoxicity of DMAB.

As for lipid metabolism and synthesis, the treated cells showed a marked increase in general lipids throughout the 24-hour observation period. This increase was entirely in the neutral lipid fraction, which includes triglycerides, cholesterol esters, steroids, some higher alcohols, and some of the carbohydrate-containing lipids. Acidic lipids, composed of fatty acids, chromolipids, and phospholipids, in contrast, were markedly reduced. These same lipid responses are seen in the toxic fatty livers of intact animals. In normal lipid metabolism and synthesis, fatty acids are oxidized in the mitochondria, synthesized in the soluble fraction of the cytoplasm, and incorporated into glycerides in the so-called microsomes or endoplasmic reticulum. Because fatty acid incorporation into glycerides was still taking place, as evidenced by the increased cellular content of neutral lipids, it was evident that endoplasmic reticulum damage, if present, was not severe. Instead, it would appear that there had been a breakdown in the biosynthesis of phospholipids with a resultant shunting of available α , β - diglycerides into the synthesis of triglycerides. The most probable site of breakdown would be in the enzymatic phosphorylation of choline or in the reaction of these phosphorylated bases with the mitochondrial nucleotide cytidine triphosphate. Further studies utilizing radioactive tracer techniques will elucidate these questions.

Succinic dehydrogenase staining reactions are localized exclusively in the mitochondria. This enzyme is concerned with cellular oxidation as part of the tricarboxylic acid or "Krebs" cycle. In the intact animal, inactivation of this enzyme is noted within 48 hours following DMAB administration. In our tissue culture system, however, this effect was

not noted during the 24-hour experimental period. In fact, a marked increase in concentration was noted during this time interval, indicating increased cellular oxidative activity. This finding appeared to correlate well with the increased glycogen content noted in these cells.

Though our experience to date with these new toxicologic research methods has been limited, their promise in the field of quantitative and qualitative toxicology can be readily recognized. Many new avenues of research in the study of the effects of DMAB, in this instance, were found in this preliminary study. This is not to say that tissue culture toxicologic research should supplant our classical methods of in vivo testing and biochemical study in this field, but rather that it should be used as a supplement to these methods. Cell cultures are rapid, quantitative, reproducible, and relatively inexpensive compared to in vivo screening. Also, they are extremely useful in testing materials available only in small quantities, thus allowing selection of which compounds should be prepared in larger quantities for in vivo testing.

We are presently planning future experiments to extend our quantitative and qualitative cytochemical measurements to include autoradiographic, radioisotopic, cytophotometric, fluorometric, and electron microscopic techniques. Also, selected organ cultures will be utilized to study toxic and carcinogenic substances at a controlled tissue level. It is hoped that by these methods our knowledge will be extended concerning the basic actions of certain physical and chemical agents on various organs and tissues.

TABLE I

Cellular Population of One Gram of Rat Liver

	Control	DMAB fed for 90 days
	<hr/> (x 10 ⁶) <hr/>	
Parenchymal cells	120	50
Littoral cells	70	20
Bile duct cells	4	110
Other cell types	6	20
	<hr/>	<hr/>
Total	200	200

Hanford - Biology
WJC 12-10-64

TABLE II

Mitotic Index of DMAB Insulted
Chang Liver Cell Cultures
(Mitoses/100 Cells)

	4 hr	12 hr	24 hr	48 hr	72 hr	96 hr	120 hr
Control	4 $\pm$.4*	11 $\pm$.8	6 $\pm$.4	3 $\pm$.3	1 $\pm$.1	1 $\pm$.1	0
.25 ug	-	5 $\pm$.3	4 $\pm$.4	3 $\pm$.3	0	2 $\pm$.1	2 $\pm$.1
2.5 ug	-	10 $\pm$.9	1 $\pm$.1	1 $\pm$.1	0	1 $\pm$.1	2 $\pm$.1
25 ug	5 $\pm$.4	10 $\pm$.4	2 $\pm$.1	-	-	-	-
250 ug	4 $\pm$.3	-	3 $\pm$.1	-	-	-	-
2500 ug	3 $\pm$.3	-	2 $\pm$.1	-	-	-	-

* 95% Confidence Limits

Hanford - Biology
WJC 12-10-64

TABLE III

Multinucleated Cells in DMAB Insulted
Chang Liver Cell Cultures
(Multinucleates/100 Cells)

	4 hr	12 hr	24 hr	48 hr	72 hr	96 hr	120 hr
Control	3 $\pm$.1*	2 $\pm$.1	2 $\pm$.3	1 $\pm$.1	1 $\pm$.1	0	0
.25 ug	-	1 $\pm$.1	2 $\pm$.1	2 $\pm$.1	2 $\pm$.1	2 $\pm$.1	2 $\pm$.3
2.5 ug	-	1 $\pm$.1	2 $\pm$.1	1 $\pm$.1	1 $\pm$.1	1 $\pm$.1	2 $\pm$.1
25 ug	4 $\pm$.4	3 $\pm$.1	2 $\pm$.1	-	-	-	-
250 ug	2 $\pm$.3	-	2 $\pm$.1	-	-	-	-
2500 ug	2 $\pm$.3	-	1 $\pm$.1	-	-	-	-

* 95% Confidence Limits

Hanford - Biology
WJC 12-10-64

TABLE IV

Bizzare Cells in DMAB Insulted
 Chang Liver Cell Cultures
 (Affected Cells/100 Cells)

	4 hr	12 hr	24 hr	48 hr	72 hr	96 hr	120 hr
Control	$3 \pm .3^*$	$2 \pm .1$	$1 \pm .1$	$1 \pm .1$	$2 \pm .1$	0	$1 \pm .1$
.25 ug	-	$2 \pm .1$	$1 \pm .1$	$2 \pm .3$	$2 \pm .1$	0	0
2.5 ug	-	$3 \pm .1$	$2 \pm .1$	$2 \pm .1$	$1 \pm .1$	$1 \pm .1$	$2 \pm .1$
25 ug	$2 \pm .1$	$3 \pm .1$	$3 \pm .1$	-	-	-	-
250 ug	$3 \pm .1$	-	$3 \pm .1$	-	-	-	-
2500 ug	$3 \pm .1$	-	$4 \pm .3$	-	-	-	-

* 95% Confidence Limits

Hanford - Biology
 WJC 12-10-64

TABLE V

Degeneration and Necrosis of Cells in DMAB
Insulted Chang Liver Cell Cultures
(Affected Cells/100 Cells)

	4 hr	12 hr	24 hr	48 hr	72 hr	96 hr	120 hr
Control	$6 \pm .3^*$	$5 \pm .4$	$6 \pm .4$	$4 \pm .3$	$6 \pm .4$	$8 \pm .3$	$16 \pm .8$
.25 ug	-	$4 \pm .3$	$4 \pm .3$	$6 \pm .4$	$13 \pm .7$	$16 \pm .5$	$15 \pm .8$
2.5 ug	-	$4 \pm .4$	$6 \pm .7$	$8 \pm .7$	$12 \pm .7$	$10 \pm .4$	$15 \pm .8$
25 ug	$5 \pm .5$	$13 \pm .3$	$3 \pm .3$	-	-	-	-
250 ug	$3 \pm .3$	-	$8 \pm .4$	-	-	-	-
2500 ug	$5 \pm .3$	-	$12 \pm .4$	-	-	-	-

* 95% Confidence Limits

Hanford - Biology
WJC 12-10-64

TABLE VI

Cytochemistry of Cells in DMAB Insulted
Chang Liver Cell Cultures

	4 hr		8 hr		16 hr		24 hr	
	Control	25 ug	Control	25 ug	Control	25 ug	Control	25 ug
Glycogen	+-	+	+-	+	+	++	+	+-
Lipids, General	+	+++	+	+++	+	+++	+	+++
Neutral Lipids	+	+++	+	+++	+	+++	+	+++
Acidic Lipids	+	-	+	-	+-	-	+-	-
Succ. Dehydrogenase	+	++	++	+++	++	+++	+	+++

Hanford - Biology
WJC 12-10-64