THE EFFECTS OF LECTIN INTERACTION WITH LD_{50} ULTRASOUND ON CULTURED HUMAN KIDNEY CELLS

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THE EFFECTS OF LECTIN INTERACTION WITH LD_{50} ULTRASOUND ON CULTURED HUMAN KIDNEY CELLS

A Research Paper

Presented to

the Graduate Council of

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In Partial Fulfillment

of the Requirements for the Degree

Master of Science

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by

Allen Harley West

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To the Graduate Council:

I am submitting herewith a Research Paper written by Allen Hanley Hunt entitled "The Effects of Lectin Interaction with ID_{50} Ultrasound on Cultured Human Kidney Cells." I recommend that it be accepted in partial fulfillment of the requirements for the degree Master of Science, with a major in biology.

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Major Professor

Accepted for the
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William V. Ellis
Dean of the Graduate School

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Chapter I

INTRODUCTION

The technology for producing ultrasound and the physical aspects of sonic wave propagation have been known for many years. The first major attempt to apply the knowledge practically was made in the unsuccessful search for the oceanliner Titanic in 1912. Early attempts to apply this technology to medical diagnosis were equally unsuccessful. The research effort that accompanied World War II developed sonar devices to detect submarines. Since this first important successful application of ultrasound technology, progress has been rapid. Because x-ray imaging in obstetrics has been condemned, the use of ultrasonic imaging has flourished. It has been estimated that by the mid-1980's virtually all infants born in the United States will have been exposed to ultrasound <u>in utero</u> (Neill, 1977). Apparently the vast majority of persons involved with obstetric ultrasonography are of the opinion that diagnostic doses of ultrasound are innocuous to the developing fetus. This opinion is well supported by most of the literature on the subject. A recent article in "Science" (Popp and Macovski, 1980) begins in accordance:

> Ultrasound is very attractive as a diagnostic method, from both the patient's and the physician's viewpoint. This non-ionizing form of radiation is applied at low levels, and no harmful effects have been found in humans over the 25 years of very active clinical application. The absence of patient discomfort, apparent safety, ease of performance,

> and measurement accuracy make medical ultrasonography nearly ideal for use in humans.

The subject is more controversial than this carefully worded statement leads the reader to believe. At present there is no known danger associated with clinical ultrasonography, but evidence has been presented to show that the procedure may not be entirely harmless.

It is clear that high intensity, focused ultrasound can kill most if not all biological materials. In the clinic, of course, the intensity levels encountered are neither high nor focused. The probe (transducer) is in constant motion and the dose is pulsed as the probe alternately sends and receives an ultrasonic signal. Yet because of the widespread use in obstetrics, involving the subtleties of genetics, and because there is no reason to assume that ultrasound has had any previous influence on our phylogenetic experience, some researchers are looking for more sensitive means of detecting biological damage. The immense difficulties involved in a multiple generation study have precluded any attempts to date (Fry, 1979). Consequently bioeffects data are based on first generation studies.

Most published works report damage only at exposure levels greatly in excess of the diagnostic range. Early reports of chromosomal damage in human leukocytes after diagnostic exposure (MacIntosh and Davey, 1970) were not confirmed in other laboratories. Subsequently MacIntosh

presented a paper entitled "Failure to Reproduce Chromosome Abberation Attributed to Ultrasound" (1974). It has since been suggested that the chromosome-breakage studies *in vitro* may not be the most sensitive indicator of genetic damage.

It has recently been shown that diagnostic doses of ultrasound may retard rapidly growing tissues (Pizzarello et al., 1978). Doreen Leibeskind and her associates at the Albert Einstein College of Medicine have reported evidence of DNA repair synthesis following exposure to clinical levels of ultrasound (1979a). A subsequent report (1979b) shows increased sister chromatid exchanges in lymphocytes following a thirty minute clinical dose. Since probe motion was not a factor considered, no extrapolation to the actual clinical situation is made.

The Radiation Biology Section of Vanderbilt University Medical Center is currently investigating the parameter of cellular attachment as an indicator of the effects of low level ultrasound on cultured human cells. These researchers have found that a significant number of cells become unattached from their plastic substratum following a pulsed dose well within clinical limits (Siegel et al., 1979a). In addition their results indicate a greater sensitivity in cells originating from human embryonic tissue. To insure that the cells were not merely shaken loose by mechanical vibrations, the culture dishes were coupled to the transducer with a jelly incapable of supporting a shear force. These results

suggest the possibility that the cell surface architecture submits to low levels of ultrasound.

Glycoproteins integral to the cell membrane have been associated with the phenomenon of cellular attachment (Roseman, 1975). They have also been related to intercellular adhesion and to interactions with lectins, unusual plant extracts that are known to agglutinate some cells and to stimulate mitosis in others.

The experiments reported in this research paper represent an attempt to detect cell surface changes that result from an LD_{50} dose of ultrasonic radiation (the dose required to destroy the reproductive capacity of fifty percent of the cell population under specific laboratory conditions). The lectins concanavalin-A (CON-A), phytohemagglutinin (PHA), and pokeweed mitogen (PWM) were used, as well as fucose (FUC) and mannose (MAN). The latter are two sugars that have been associated with glycoprotein cell surface receptors. A survey of the literature failed to find studies reporting the use of lectins or sugars with cultured human kidney cells (T-1 cells).

Chapter II

MATERIALS AND METHODS

In all experiments conventional methods of cell culture maintenance and propagation were used (Paul, 1975). Cells were grown as monolayers in glass bottles or plastic petri dishes in humidified 5% CO_2 incubators at 37°C, using Eagle's Minimal Essential Medium fortified with 10% fetal calf serum and penicillin/streptomycin. Routine subculturing and harvesting were accomplished with 0.05% trypsin and gentle agitation. All experiments were begun while the cells were in the exponential growth phase, synchronized by trypsinization 24 hours earlier. The T-1 cell line used was established in 1958 (Van der Veen et al., 1958) and has been used for radiobiological investigations (Broerse, 1969) and for studies of thyroid hormone metabolism (Siegel, 1979b). The insonation of the dishes was accomplished with a Mettler Electronics "Sonicator" therapeutic ultrasound device, using Unirad conductivity jelly to assure good acoustic contact.

Lectins were obtained from Sigma Chemical Company. According to the manufacturer, Type IV CON-A (an extract from Conavalia ensiformes) agglutinates 2% fresh human type O red blood cells in 0.01M phosphate buffered saline (PBS) at a pH of 6.8 and a concentration of 16 µg/ml. Phytohemagglutinin (from Phaseolus vulgaris) agglutinates 2% fresh type A red blood cells in 0.01M PBS (pH 6.7) at 8 µg/ml. At 2.5 µg/ml

this lectin induces mitosis in human lymphocytes. Pokeweed mitogen (from Phytolacca americana) agglutinates 2% fresh human type A red blood cells in 0.01M PBS (pH 6.8) at 4 µg/ml, and induces mitosis in human lymphocytes at 2.5 µg/ml.

Sigma was also the source of α-L(-)fucose (6-deoxy-L-galactose). Fucose is known to be a core sugar of the oligosaccharide side chains of some plasma membrane proteins which protrude from the cell surface (Lehninger, 1975). Reagent grade d(+)mannose was obtained from Fisher Scientific. At 156 µg/ml this sugar has been found (by Sigma) to inhibit the agglutinating effect of CON-A on type O red blood cells.

Plating efficiency experiments (PE-1 and PE-2) were conducted preliminarily. Replicate 100mm petri dishes were supplied with 10 ml of culture media, then with the cells. The number of cells per dish was estimated at 300 by counting on the hemocytometer. The dishes were incubated for two weeks, then retrieved and stained with methylene blue to visualize colony formations. Plating efficiency was then estimated:

$$\text{Plating Efficiency} = \frac{\#\text{ Colonies Observed}}{\#\text{ Cells Seeded } (= 300)} \times 100$$

The plating efficiency averaged for both experiments was 68%; results are tabulated in the appendix.

The LD_{50} experiments followed (LD-1, LD-2, and LD-3) using the same techniques but seeding 150 cells each into replicate 80mm dishes containing 5 ml of media. After a

minimum of one hour in the incubator to allow the cells to recover from the "insult" of trypsinization and to reattach to the substratum, the dishes were retrieved and insonated at doses varying from 0 to 0.6 W/cm^2 for one minute. They were then returned to the incubator for two weeks, after which the colonies were stained and counted. The results of the LD_{50} experiments are listed in the appendix as C-1 through C-5, the control dishes; 0.1-1 through 0.1-5, the dishes insonated at 0.1 W/cm^2 for one minute, etc. The mean, the standard deviation, and the percent of control for each five plate series are listed with the last entry of the group. After the first experiment it was apparent that 0.6 W/cm^2 totally inhibits the reproductive capacity of these cells. This level of insonation was omitted in the subsequent experiments.

The techniques used in the lectin and sugar experiments were similar to those of the LD_{50} experiments. Culture media containing the stated dilution of lectin or sugar was introduced into each dish. The number of cells then added to each dish was calculated to be 150, taking into account the plating efficiency error. Insonation was at 0.3 W/cm^2, the approximate LD_{50} value indicated by the previous LD_{50} experiments. The results of these experiments are tabulated in the appendix as the letter "S" (insonated) or "O" (not insonated) followed by two numbers. The first number represents the concentration of the lectin in micrograms per

Chapter III

RESULTS

The negative linear correlation between the number of reproductively surviving cells and increasing doses of ultrasound is graphically illustrated in Figure 1. The LD_{50} experiments, LD-1, LD-2, and LD-3, are displayed in A, B, and C, respectively. Each curve shows a shoulder of sublethal injury extending to 0.1 W/cm^2. A summary of these experiments is shown in Figure 1-D as the averaged percent of control as a function of ultrasonic dose. The dose that resulted in the reproductive death of fifty percent of the cells was 0.3 W/cm^2. This value represents the experimentally established LD_{50} dose of ultrasound to T-1 cells.

The percent of control values for the lectin and sugar experiments are compared in Figure 2. Statistical analysis of the data generated in these experiments, comparing each sample set to the controls, is displayed in Figure 3 employing Duncan's Multiple Range Test (Steel and Torrie, 1960).

Results obtained with CON-A at all concentrations tested are similar to control (Figure 2). This is supported by analysis with Duncan's test (Figure 3) which shows no significant difference between the mean of any experimental group and the control mean in both the insonated and the uninsonated series.

Phytohemagglutinin at 0.5 µg/ml and 1 µg/ml is not

significantly different from control. The reproductive survival of cells insonated in the presence of 5 µg/ml PHA, however, was significantly inhibited (Figure 3). This effect is not observed in the uninsonated correlate, thus these results suggest that 5 µg/ml PHA amplifies the lethal effect of ultrasound on T-1 cells.

Pokeweed mitogen at 5 µg/ml has a selective effect on uninsonated cells (Figure 3). The mean number of cells that survived incubation with 5 µg/ml PWM significantly differed from the control mean in the uninsonated group, but did not significantly differ from the control mean in the insonated group. At other concentrations tested, no significant differences were observed.

Mannose at 10 mM resulted in a zero percent reproductive survival of the cells, ultrasound notwithstanding. Thus, this data has been omitted from Figure 3. The presence of lesser concentrations of mannose in the culture media had no apparent effect on cell survival in the insonated groups, yet caused a significant decrease in survival in the uninsonated groups.

At all concentrations tested of fucose, survival was inhibited in the uninsonated cell cultures (Figure 3). A decrease in survival, significantly different from control, followed the insonation of cells in the presence of 0.1 mM fucose and 10 mM fucose. Although the survival depression with 10 mM fucose is acute, it may be an effect of the sugar

and not an amplification of the ultrasound effect since the depression is apparent in both the insonated and uninsonated cells.

A statistical cross-comparison of the cell survival means of all sample sets is shown in Figure 4, again using Duncan's Multiple Range Test. Cells insonated in the presence of 10 mM fucose and 5 µg/ml PHA are significantly different from cells exposed to any other experimental treatment in terms of survival. These results indicate that the amplification of ultrasonic effects may involve cell surface binding sites since PHA at 5 µg/ml has no effect on uninsonated cells but greatly depresses survival in insonated cells (Figure 4). Fucose at 10 mM depresses cell survival in both the insonated cells and the uninsonated cells, suggesting that the observed effect may result solely from the presence of this sugar in the culture media.

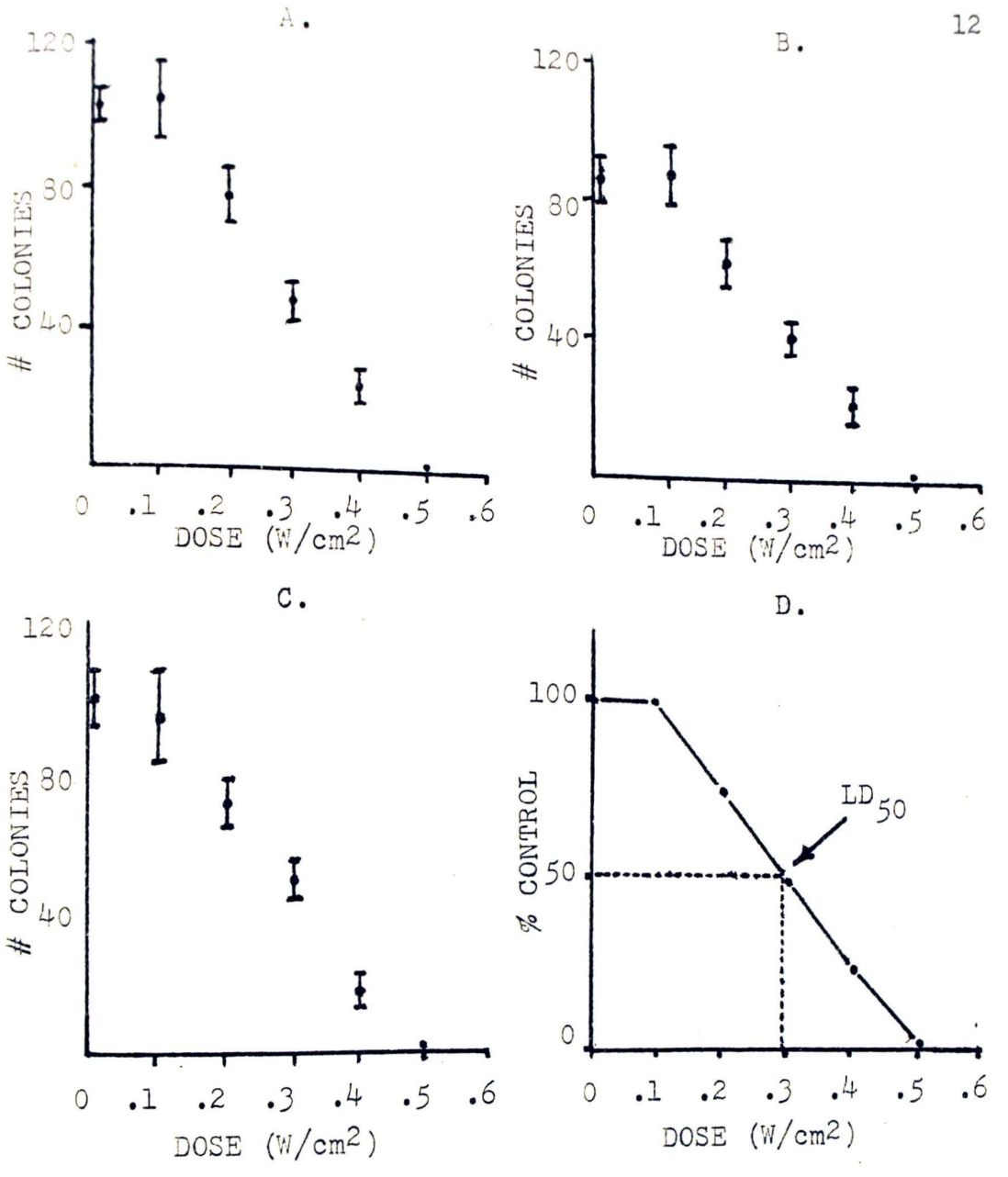

FIGURE 1. Number of surviving colonies as a function of increasing dose in A (LD-1), B (LD-2), and C (LD-3). The percent of control averaged for these three experiments is shown in D as a function of dose. The LD_{50} was determined to equal 0.3 W/cm^2.

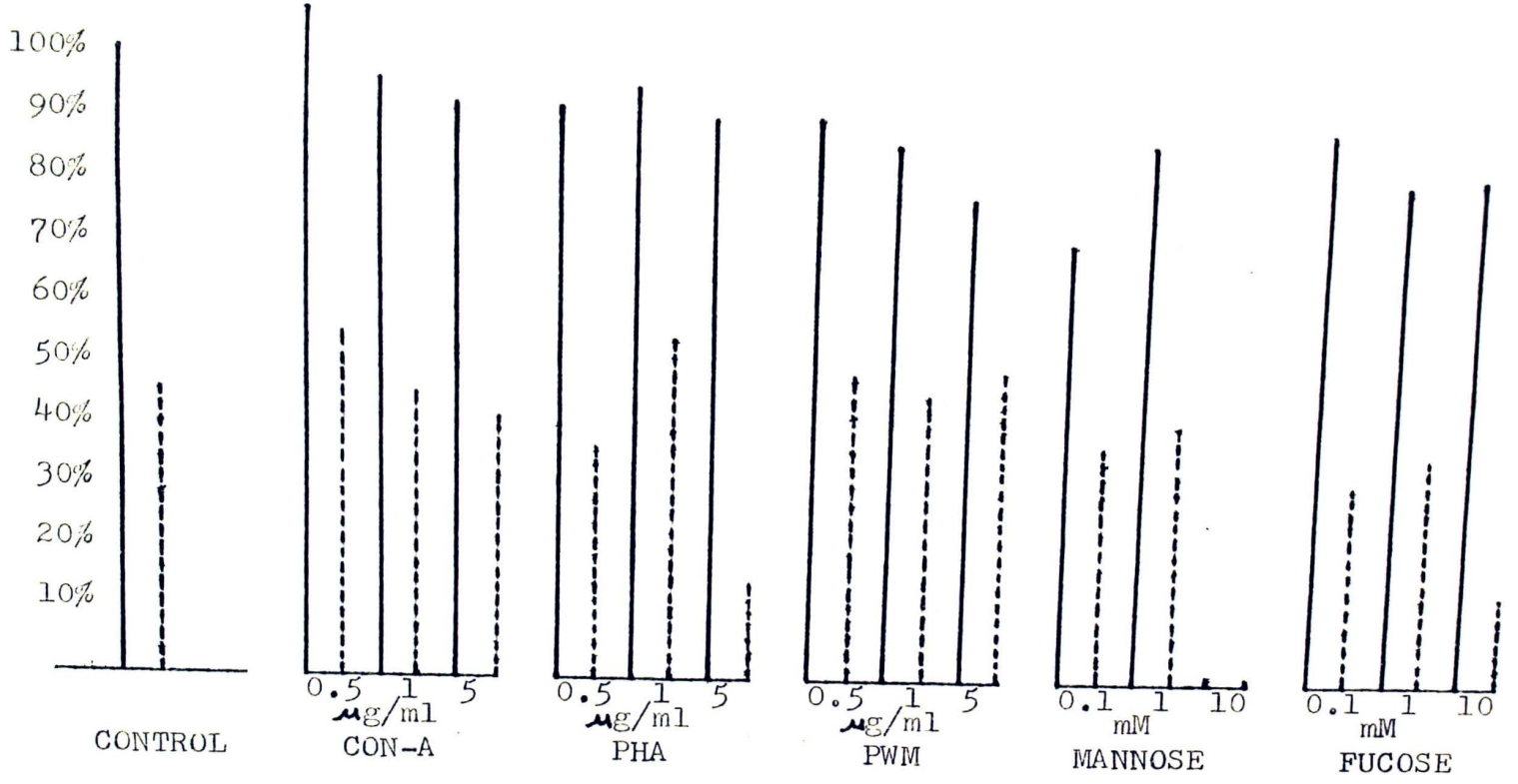

FIGURE 2. A comparison of percent of control values. Numbers above lectin names represent concentration in μg/ml. Numbers above sugar names represent concentration in millimoles. Continuous lines show average values for uninsonated sample sets. Dotted lines show average values for sample sets insonated at 0.3 W/cm^2.

CONCANAVALIN-A (µg/ml) and CONTROL

S-5	S-0	S-1	S-0.5	0-5	0-1	0-0	0-0.5
(58)	(62)	(62)	(79)	(132)	(137)	(145)	(154)

PHYTOHEMAGGLUTININ (µg/ml) and CONTROL

S-5	S-0.5	S-0	S-1	0-5	0-0.5	0-1	0-0
(21)	(50)	(62)	(76)	(130)	(132)	(135)	(145)

POKEWEED MITOGEN (µg/ml) and CONTROL

S-0	S-1	S-0.5	S-5	0-5	0-1	0-0.5	0-0
(62)	(64)	(68)	(69)	(112)	(123)	(130)	(145)

MANNOSE (mM) and CONTROL

S-0.1	S-1	S-0	0-0.1	0-1	0-0
(54)	(56)	(62)	(102)	(126)	(145)

FUCOSE (mM) and CONTROL

S-10	S-0.1	S-1	S-0	0-1	0-10	0-0.1	0-0
(19)	(44)	(51)	(62)	(116)	(119)	(128)	(145)

FIGURE 3. Duncan's Multiple Range Test is used to compare each sample set with the control. The group designations are as follows: "S" indicates insonation at the LD50, "0" indicates no insonation. Numbers after these leters indicate the concentration of the lectin in µg/ml, or of the sugar in millimoles. The average number of colonies in the group is given in parentheses under the designation. Underlying lines indicate no significant difference at $p = 0.05$.

FUCOSE	S-10	(19)
PHA	S-5	(21)
FUCOSE	S-0.1	(44)
PHA	S-0.5	(50)
FUCOSE	S-1	(51)
MANNOSE	S-0.1	(54)
MANNOSE	S-1	(56)
CON-A	S-5	(58)
CONTROL	S-0	(62)
CON-A	S-1	(62)
PWM	S-1	(64)
PWM	S-0.5	(68)
PWM	S-5	(69)
PHA	S-1	(76)
CON-A	S-0.5	(79)

MANNOSE	0-0.1	(102)
PWM	0-5	(112)
FUCOSE	0-1	(116)
FUCOSE	0-10	(119)
PWM	0-1	(123)
MANNOSE	0-1	(126)
FUCOSE	0-0.1	(128)
PHA	0-5	(130)
PWM	0-0.5	(130)
CON-A	0-5	(132)
PHA	0-0.5	(132)
PHA	0-1	(135)
CON-A	0-1	(137)
CONTROL	0-0	(145)
CON-A	0-0.5	(154)

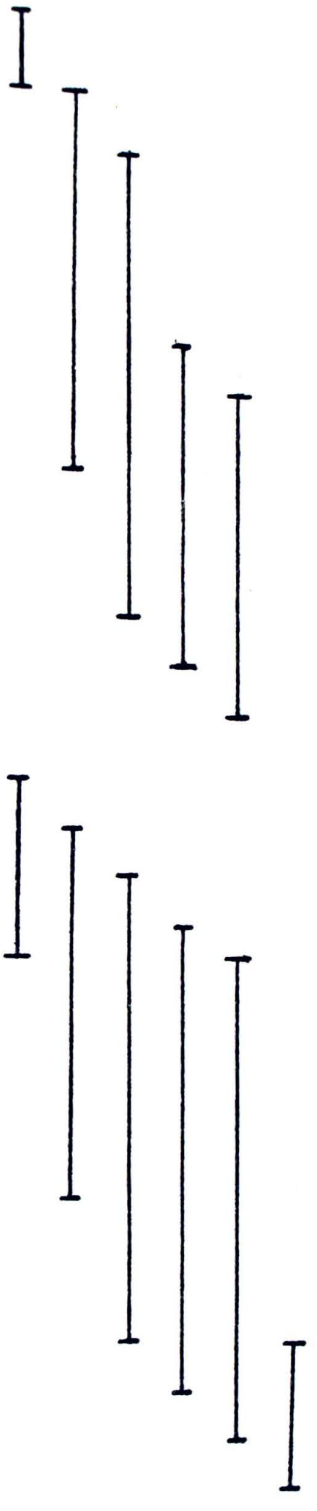

FIGURE 4. Duncan's Multiple Range Test to cross-compare all sample sets. An explanation is given in figure 3.

Chapter IV

DISCUSSION

Ultrasound is similar to x-rays in that both are waves transmitting energy. One important difference is that x-rays pass readily through a vacuum while sound requires a medium for its transmission. Another is that ultrasound usually does not cause ionization in matter; thus effects related to this phenomenon, such as free radical production and bond breakages, are not involved. The target theory (Lowry, 1974) used to explain interactions of ionizing radiations with biological materials is not applicable to ultrasonic radiations. Target theory postulates N targets (most likely DNA) in each cell, such that each target must be hit once (alternately, one target hit N times) for lethal damage to occur. The random nature of the hits (x-rays can also be described as massless bullets) results in an exponential cell survival curve when the surviving fraction is plotted as a function of dose. The survival curve following ultrasonic radiation, however, is approximately linear since all structural elements of the cell are hit (Figure 1).

Three general categories of interactions of ultrasound with living tissue are recognized (Baker and Dalrymple, 1978). "Thermal effects" result from the friction of mechanical vibrations. "Cavitational effects" occur in many liquids as a result of ultrasound. Most ordinary liquids contain gasses

dissolved in the form of stable microbubbles which have been found to increase in size during the negative pressure phase of a sound wave. At exposure levels far in excess of typical clinical studies (one thousand fold) the mechanical energy is sufficient to cause the bubbles to collapse completely during the compression phase of the vibration. In aqueous media one consequence of the collapse phenomenon is the production of free radicals, analogous to one of the most damaging aspects of ionizing radiation. At lower doses the cavitational effect can result in localized regions of stress in the liquid sufficient to break subcellular structures. This is a resonance phenomenon; thus its importance in the clinic is negligible because the administered doses are pulsed. "Direct effects" include everything other than the thermal and cavitational effects, implying a lack of knowledge of the events that occur between the application of the stimulus and the observation of the effect. Disruptions of macromolecules, chemical reaction rates and cell surface charges, among other reported phenomena, may ensue. The effect of the doses used in this experiment may involve all of the effects noted, with the probable exception of the collapse phenomenon.

Results observed during this study indicating an amplification of ultrasonic effects suggest that a direct effect may be occurring at the level of the cell membrane. The presumption made is that lectins are interacting with the glycoprotein cell surface receptors. The phenomenon of

detachment noted by Siegel is also likely to have been affected by a modification of these receptors. The "fluid-mosaic" model of the cell membrane (Singer and Nicholson, 1972) conceivably allows for membrane glycoproteins to shift in position following exposure to ultrasound. These ideas demand the attention of persons concerned about the possible long-range deleterious effects resulting from the widespread use of ultrasound in obstetrics. Siegel summarizes the problem (1979a):

> Of considerable clinical significance is the liklihood that attachment may be linked to "anchorage dependence", i.e., the inability of normal cells to grow and replicate unless attached to substrata. Particularly pertinent for assessing the biological consequences of ultrasonography is the presumption that alterations in attachment may affect implantation in situ, morphogenesis, and ontogenesis. In our view, these implications emerging from the present investigations urgently call for further studies.

Chapter V

SUMMARY

The lectins concanavalin-A, phytohemagglutinin, and pokeweed mitogen, and the sugars mannose and fucose were introduced into culture media in varying concentrations. Cultured human kidney cells (T-1 cells) were incubated in these media for one hour, then exposed to a predetermined LD_{50} ultrasonic dose for one minute. The surviving fraction was counted to measure the effects of the lectins and sugars. Concanavalin-A results were not significantly different from control. Phytohemagglutinin (5 µg/ml) significantly inhibited cell survival following insonation, but did not inhibit the survival of uninsonated cells. Pokeweed mitogen (5 µg/ml) showed a selective inhibition on the survival of uninsonated cells. Mannose had no apparent effect on cell survival in the insonated groups, yet caused a significant decrease in survival in the uninsonated groups. At all tested concentrations of fucose survival was inhibited in the uninsonated cell cultures, and at 0.1 mM and 10 mM the presence of this sugar in the medium significantly decreased survival in insonated cells. The most apparent interaction with ultrasound was the acute depression of cell survival following insonation with 5 µg/ml phytohemagglutinin. This result may indicate a potent amplification of the lethal effect of ultrasound and suggests that cell membrane changes may be involved in this phenomenon.

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APPENDIX

TABULATED RESULTS OF THE EXPERIMENTS

I. PLATING EFFICIENCY EXPERIMENTS:

EXPERIMENT	PLATE	COLONIES	% OF 300
PE-1	1	213	71
	2	209	69.7
	3	218	72.7
	4	208	69.3
PE-2	1	186	62
	2	194	64.7
	3	206	68.7
	4	197	65.7

II. LD_{50} EXPERIMENTS:

EXPERIMENT	PLATE	COLONIES	$\bar{x}$	SD	% CONTROL
LD-1	C-1	97			
	C-2	111			
	C-3	103			
	C-4	105			
	C-5	99	103	6	100
	0.1-1	92			
	0.1-2	105			
	0.1-3	109			
	0.1-4	120			
	0.1-5	95	104	11	101
	0.2-1	69			
	0.2-2	77			
	0.2-3	81			
	0.2-4	90			
	0.2-5	75	78	8	76
	0.3-1	45			

EXPERIMENT	PLATE	COLONIES	$\bar{x}$	SD	% CONTROL
	0.3-2	51			
	0.3-3	58			
	0.3-4	42			
	0.3-5	44	48	7	47
	0.4-1	23			
	0.4-2	29			
	0.4-3	31			
	0.4-4	21			
	0.4-5	21	25	5	24
	0.5-1	0			
	0.5-2	0			
	0.5-3	3			
	0.5-4	1			
	0.5-5	2	2		2
	0.6-1	0			
	0.6-2	0			
	0.6-3	0			
	0.6-4	0			
	0.6-5	0	0		0
LD-2	C-1	82			
	C-2	95			
	C-3	88			
	C-4	79			
	C-5	85	86	6	100
	0.1-1	90			
	0.1-2	101			
	0.1-3	82			
	0.1-4	83			
	0.1-5	79	87	4	101
	0.2-1	71			
	0.2-2	62			

EXPERIMENT	PLATE	COLONIES	$\bar{x}$	SD	% CONTROL
	0.2-3	58			
	0.2-4	70			
	0.2-5	55	63	7	74
	0.3-1	47			
	0.3-2	42			
	0.3-3	44			
	0.3-4	38			
	0.3-5	36	41	4	48
	0.4-1	19			
	0.4-2	17			
	0.4-3	28			
	0.4-4	21			
	0.4-5	25	22	5	26
	0.5-1	1			
	0.5-2	0			
	0.5-3	1			
	0.5-4	2			
	0.5-5	0	0.8		0.9
LD-3	C-1	110			
	C-2	95			
	C-3	92			
	C-4	102			
	C-5	98	99	7	100
	0.1-1	113			
	0.1-2	88			
	0.1-3	96			
	0.1-4	92			
	0.1-5	80	94	12	95
	0.2-1	58			
	0.2-2	71			
	0.2-3	78			

EXPERIMENT	PLATE	COLONIES	$\bar{x}$	SD	% CONTROL
	0.2-4	69			
	0.2-5	72	69	7	70
	0.3-1	55			
	0.3-2	48			
	0.3-3	51			
	0.3-4	50			
	0.3-5	42	45	5	49
	0.4-1	15			
	0.4-2	18			
	0.4-3	14			
	0.4-4	24			
	0.4-5	21	18	4	18
	0.5-1	0			
	0.5-2	3			
	0.5-3	0			
	0.5-4	0			
	0.5-5	0	0.6		0.6

III. LECTIN AND SUGAR EXPERIMENTS:

EXPERIMENT	PLATE	COLONIES	$\bar{x}$	SD	% CONTROL
CONTROL	0-0-1	152			
	0-0-2	—			
	0-0-3	158			
	0-0-4	127			
	0-0-5	142	144	14	100
	S-0-1	56			
	S-0-2	70			
	S-0-3	—			
	S-0-4	71			
	S-0-5	49	62	11	43

EXPERIMENT	PLATE	COLONIES	$\bar{x}$	SD	% CONTROL
CON-A	0-.5-1	–			
	0-.5-2	156			
	0-.5-3	145			
	0-.5-4	151			
	0-.5-5	165	154	8	106
	S-.5-1	65			
	S-.5-2	81			
	S-.5-3	77			
	S-.5-4	74			
	S-.5-5	96	79	11	54
	0-1-1	121			
	0-1-2	135			
	0-1-3	124			
	0-1-4	142			
	0-1-5	161	137	16	94
	S-1-1	62			
	S-1-2	–			
	S-1-3	47			
	S-1-4	59			
	S-1-5	80	62	14	43
	0-5-1	142			
	0-5-2	130			
	0-5-3	120			
	0-5-4	–			
	0-5-5	135	132	9	91
	S-5-1	–			
	S-5-2	74			
	S-5-3	47			
	S-5-4	55			
	S-5-5	54	58	12	40

EXPERIMENT	PLATE	COLONIES	$\bar{x}$	SD	% CONTROL
PHA	0-.5-1	112			
	0-.5-2	141			
	0-.5-3	125			
	0-.5-4	123			
	0-.5-5	157	132	18	91
	S-.5-1	–			
	S-.5-2	49			
	S-.5-3	50			
	S-.5-4	38			
	S-.5-5	65	50	11	35
	0-1-1	136			
	0-1-2	150			
	0-1-3	133			
	0-1-4	128			
	0-1-5	128	135	9	93
	S-1-1	97			
	S-1-2	82			
	S-1-3	94			
	S-1-4	51			
	S-1-5	56	76	21	53
	0-5-1	123			
	0-5-2	119			
	0-5-3	136			
	0-5-4	145			
	0-5-5	129	130	10	90
	S-5-1	12			
	S-5-2	–			
	S-5-3	14			
	S-5-4	31			
	S-5-5	26	20	9	14

EXPERIMENT	PLATE	COLONIES	$\bar{x}$	SD	% CONTROL
PWM	0-.5-1	128			
	0-.5-2	110			
	0-.5-3	135			
	0-.5-4	—			
	0-.5-5	146	130	15	90
	S-.5-1	63			
	S-.5-2	66			
	S-.5-3	49			
	S-.5-4	72			
	S-.5-5	88	68	14	47
	0-1-1	112			
	0-1-2	135			
	0-1-3	117			
	0-1-4	122			
	0-1-5	131	123	10	85
	S-1-1	79			
	S-1-2	62			
	S-1-3	24			
	S-1-4	65			
	S-1-5	92	64	25	44
	0-5-1	110			
	0-5-2	—			
	0-5-3	99			
	0-5-4	118			
	0-5-5	121	112	10	77
	S-5-1	—			
	S-5-2	72			
	S-5-3	—			
	S-5-4	61			
	S-5-5	74	69	7	48

EXPERIMENT	PLATE	COLONIES	$\bar{x}$	SD	% CONTROL
MANNOSE	0-.1-1	88			
	0-.1-2	100			
	0-.1-3	98			
	0-.1-4	114			
	0-.1-5	110	102	10	70
	S-.1-1	53			
	S-.1-2	44			
	S-.1-3	58			
	S-.1-4	51			
	S-.1-5	62	54	7	37
	0-1-1	—			
	0-1-2	142			
	0-1-3	113			
	0-1-4	121			
	0-1-5	130	126	12	87
	S-1-1	--			
	S-1-2	55			
	S-1-3	52			
	S-1-4	49			
	S-1-5	68	56	8	39
	0-10-1	0			
	0-10-2	0			
	0-10-3	0			
	0-10-4	0			
	0-10-5	0			
	S-10-1	0			
	S-10-2	0			
	S-10-3	0			
	S-10-4	0			
	S-10-5	0			

EXPERIMENT	PLATE	COLONIES	$\bar{x}$	SD	% CONTROL
FUCOSE	0-.1-1	123			
	0-.1-2	119			
	0-.1-3	125			
	0-.1-4	134			
	0-.1-5	140	128	9	89
	S-.1-1	40			
	S-.1-2	42			
	S-.1-3	47			
	S-.1-4	51			
	S-.1-5	40	44	5	30
	0-1-1	136			
	0-1-2	109			
	0-1-3	—			
	0-1-4	98			
	0-1-5	122	116	16	80
	S-1-1	58			
	S-1-2	52			
	S-1-3	57			
	S-1-4	47			
	S-1-5	42	51	7	35
	0-10-1	112			
	0-10-2	119			
	0-10-3	115			
	0-10-4	121			
	0-10-5	129	119	6	82
	S-10-1	11			
	S-10-2	22			
	S-10-3	19			
	S-10-4	17			
	S-10-5	27	19	10	13