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BIOCHEMICAL TESTING OF POTENTIALLY HAZARDOUS CHEMICALS FOR TOXICITY USING MAMMALIAN LIVER CELL CULTURES

FINAL REPORT

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13. ABSTRACT (Maximum 200 words)

This proposal dealt with the testing of selected chemicals for their possible toxic effects on humans through the use of established mouse (HEPA-2) and human (HEP-G2) liver cell lines. The objective of the work was to eliminate the use of long-term more costly animal models to assess chemical toxicity. These established liver cell lines were chosen for use in this project because they are well characterized biochemically, and continue to express many of the normal functions carried out by the liver. The human HEP-G2 cells are known to secrete 17 different major plasma proteins into the culture medium. The HEPA-2 mouse cells are known to synthesize and to secrete albumin, alpha-fetoprotein, transferrin, ceruloplasmin and haptoglobin while being cultured. Must of the proposed work dealt with the activation of specific detoxifying enzymes of the liver that are involved in the metabolism of xenobiotics. On the other hand, not all hepatotoxins induce these enzyme systems while causing various forms of alteration in the hepatocytes. Therefore, alterations in the expression of the liver-specific functions listed above

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served as excellent indicators of hepatotoxicity following chemical treatment. In addition, chemical effects on cell morphology, growth and macromolecular syntheses were also assessed. A summary of these results includes the following:

- Known chemical carcinogens significantly induced Phase I and Phase II enzymes including glutathione S-transferase, UDP-glucuronyltransferase and aryl hydrocarbon hydroxylase.
- There is good correlation between the effects seen in mouse and human liver cell systems.
- These chemicals produced alterations in cell morphology, and significantly reduced the level of total protein synthesis, as well as the expression of albumin.
- As expected, the chemicals act in a dose- and time-responsive manner.
- Chemicals such as azidothymidine and pentamidine that do not induce biotransforming enzymes, were shown to have inhibitory effects on normal cellular processes when used at various concentrations.

In conclusion, the results of these studies indicate that mammalian cell systems may be used as a rapid, cost efficient and accurate screening method for chemicals that humans are or may be exposed. It is important to also realize that for each chemical tested, a separate time and dose profile are important aspects to consider as our studies indicate. Finally, since not all hepatotoxins are inducers of detoxifying enzymes, the continued expression of normal cellular functions and other bioassays may and should be considered in assessing the effects of xenobiotics on the cells.
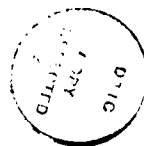
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Background:

It has been apparent for many years that chemicals, either natural or synthetic, are one of the major causes of many of the ills of man. It has been shown that some forms of cancer [1], various birth defects that are the consequence of genetic mutations [2,3] and cell death due to cytotoxic effects [4] are results of the body's response to an alteration in its biochemistry brought about by chemicals. The deleterious effects of chemicals have been intensely studied since Percivall Pott in 1775 showed that occupational exposure to soot and coal tar caused a significant increase in scrotal cancer in chimney sweeps. The active agent was found to be benzo(a)pyrene, an almost ubiquitous environmental pollutant that is present now in automobile and industrial exhausts, cigarette smoke and many other combustion products [5,6]. Benzo(a)pyrene is one of a class of compounds generally termed polycyclic hydrocarbons (PCH). The PCHs, along with aromatic amines, nitrosamines and aflatoxins, are among the important classes of chemical carcinogens that become bound to tissue macromolecules (e.g., proteins, DNA). It is believed that this binding (especially to DNA) is the event that initiates the mutagenic, carcinogenic, and/or teratogenic effects of these compounds. In an attempt to further investigate this macromolecular binding, in vitro experiments were done by Grover and Sims [7] and by Gelboin [8] in which radiolabeled chemicals and DNA were mixed in a test tube. It was found that the parent molecule could not bind to DNA. If, however, extracts from rat liver were also placed in the test tube, the binding of the chemical to DNA did occur. These experiments showed that for most of the chemicals found to be carcinogenic or mutagenic, enzymatic conversion (biotransformation) to an active form was necessary. It was soon learned that the enzyme system chiefly involved in carcinogen activation was the mixed function oxidase

(MFO) system (the system generally regarded, until then, as the drug detoxification system). The PCHs, and many other compounds with known deleterious effects in man, are very lipid-soluble. They would be stored in the body indefinitely were it not for their metabolism, which results in more water-soluble derivatives. However, along the metabolic pathway, electrophilic intermediates (such as epoxides) are produced which can react readily with nucleophilic groups on cellular macromolecules. Thus, in an attempt to detoxify these compounds, the MFO system actually aids in their activation. These biotransformation enzymes are located principally in the liver, though they are present to a lesser degree in all tissues tested to date. Traditionally, these enzymes are divided into two groups: Phase I and Phase II. The Phase I enzymes (which include the mixed function oxidases and various hydroxylases, dealkylases and epoxidases) introduce one or more water-soluble groups (e.g., hydroxyl, amino or carboxylic acid) into the parent molecule. This polar group is then used as a handle onto which the Phase II enzymes (such as glutathione S-transferase, UDP-glucuronyltransferase, sulfotransferase) conjugate an even more polar compound (e.g., glutathione, glucuronic acid) which leads to the increased water-solubility of the compound and its excretion from the body. The enzymes involved in both phases of biotransformation have been studied extensively [9,10]. It has been shown that one of the principal MFO enzymes, aryl hydrocarbon hydroxylase (AHH), has as its terminal oxidase cytochrome P-450 which is responsible for incorporating an atom of oxygen into the structure of PCHs and other chemicals. It has also been shown that the biotransformation enzymes are present in the cell at low levels, but the cellular concentration of these enzymes can be rapidly increased by exposure to one or more of a variety of xenobiotics [11-14]. The "inducibility" of the biotransformation enzymes (especially AHH) has been correlated with the enhanced

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carcinogenicity/mutagenicity/cytotoxicity of certain chemicals. Kouri et al. [15] studied the hepatic inducibility of AHH by the PCH, 3-methylcholanthrene (MC), in fourteen strains of mice and the oncogenesis produced by subcutaneous injection of MC, and found a good correlation between the two processes. Kellerman et al. [16] measured the levels of AHH present in cultured lymphocytes of normal subjects and subjects suffering from bronchogenic carcinoma and found that cultures from the cancer patients had significantly higher levels of this enzyme. Diamond et al. [17] found a direct correlation between the conversion of tritiated 7,12-dimethylbenz(a)anthracene into water-soluble metabolites and the cytotoxicity it exerted against a number of cultured cell lines. In 1971, Diamond [18] showed that only cells containing a high level of biotransformation enzymes were subject to the cytotoxic effects of benzo(a)pyrene and 7,12-dimethylbenz(a)anthracene, presumably caused by phenol intermediates produced during the conversion of these compounds to water-soluble forms. The conjugation (Phase II) enzymes are also induced by xenobiotics and, in some instances, have been found to play a role in the harmful effects of certain chemicals. The potent liver carcinogen 2-acetylaminofluorene is activated by microsomal N-hydroxylation to a proximal carcinogenic form, which in rat liver is converted by a sulfotransferase into a highly reactive, electrophilic sulfate ester, which is considered to be the ultimate carcinogenic form [19]. Glucuronidation has been implicated in bladder carcinogenesis. Arylamines, such as 2-napthylamine and 4-aminobiphenyl, are metabolized by the mixed function oxidase system and some of their metabolites appear as N-glucuronides. These glucuronides are transported to the bladder where they are hydrolyzed by B-glucuronidase, which yields a reactive metabolite that is capable of initiating the carcinogenic process in that organ [20]. Other studies have shown that glucuronide formation may also be

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a factor in the carcinogenic and mutagenic potential of PCHs. In in vitro experiments, Fahl et al. [21] found that covalent modification of DNA was increased after the addition of UDP-glucuronic acid, while Bock [22] showed that MFO activity was increased after glucuronic acid addition. These studies suggest that coupling of xenobiotic metabolism to the glucuronidation pathway may enhance carcinogenicity, possibly by removal of metabolites such as quinones that would have retarded the action of the MFO system of enzymes. Thus, the biotransformation enzymes (in both Phase I and Phase II) are important in both the activation and deactivation of carcinogens and, therefore, control to a certain extent the potential damage xenobiotics can do in mammals.

Other chemical agents (such as the nitrosamides nitrosomethylurea and N-methylmethanesulfonate) are direct-acting carcinogens and mutagens [23]. They do not require biotransformation to express their deleterious effects. As alkylating agents, these compounds are thought to exert their effect through direct reaction with macromolecular nucleophiles.

Hypothesis:

The cell undergoes a variety of biochemical changes when its normal environment is altered (e.g., the introduction of chemicals). Some of these changes include an induction of specific enzymes which causes an increase in protein synthesis, an alteration in the rate of cell growth and DNA synthesis and a change in specific cell functions, such as the secretion of albumin and cell morphology. In the work carried out in this proposal, we attempted to correlate these changes with the potential harmful effects of certain chemicals. Using mouse and human liver cells in culture, we first tested known carcinogens and cytotoxic compounds and monitored the induction of several key biotransformation enzymes, levels of protein and DNA synthesis,

as well as cell growth and morphology to determine whether specific changes in these parameters could serve as early warning signs of the deleterious effects of these chemicals. If a positive correlation could be made, it was thought that this in vitro system may serve as a reliable, time- and cost-efficient means to test other potentially harmful compounds to which man is or might be exposed.

Military Significance:

The significance of the proposed work to the military is that our approach would eliminate long-term more costly animal models to screen potentially hazardous chemicals for their toxicity to humans. The established liver cell lines used in the present studies have been shown to respond to known environmental pollutants and served as excellent model systems for rapid testing of potentially toxic substances. The studies support the mission of USAMRDC and proved to be quick, cost efficient and an accurate method of screening selected test chemicals.

Methodology:

Preparation and Treatment of Cell Lines:

The mouse hepatoma cells used in the proposed studies were a subclone of the BW7756 cell line isolated from a solid tumor which spontaneously arose in a female C57 leaden mouse at Jackson Laboratories at Bar Harbor, Maine [24]. This subclone is designated (HEPA-2) and has been maintained in the laboratory for well over 15 years. The cells are commonly kept in the log phase of growth by subculturing weekly and feeding approximately every other day. The cells are grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 4.5 gm of glucose per liter and supplemented with 10% fetal bovine serum, sodium bicarbonate and

penicillin/streptomycin. This particular cell line has been well characterized biochemically and continues to express certain tissue-specific differentiated functions, i.e., the synthesis and secretion of albumin, transferrin and alpha-fetoprotein [24-27]. We have previously shown that these cells respond to known enzyme-inducing chemicals. Like the mouse hepatoma cells, the human HEP-G2 cell line has been maintained in culture for several years and it, too, continues to express seventeen tissue-specific functions (e.g., albumin, haptoglobin, transferrin, plasminogen and ceruloplasmin) which serve as excellent markers for determining whether test chemicals play a hepatotoxic role in altering the expression of these particular proteins [28,29]. The HEP-G2 cells grow best in Eagle's or Dulbecco's Modified Eagle's Medium (EMEM & DMEM) respectively, supplemented with 10% fetal bovine serum and antibiotics (personal communication with Dr. Barbara Knowles, Wistar Institute, Philadelphia, PA).

Both log phase and stationary phase cells were tested initially. The various cell lines were treated with 10 μg of test chemical in a 100 μl volume for 24 hr as reported in our preliminary studies, since good induction of the enzymes was seen at this time and drug concentration. At the end of the treatment period, the cells were washed with ice-cold physiological saline to remove the medium and any unattached cells. The cell layer was scraped from the surface of the culture flasks and homogenized in 0.5M, 4°C Tris-HCl buffer, pH 7.2, containing 1.15% KCl and 10% glycerol with a Potter-Elvehjem homogenizer fitted with a teflon pestle. The homogenates were centrifuged at 9,000 x g for 1 hr at 4°C to obtain a supernatant fraction. In those cases where a cytosolic and a microsomal fraction were to be obtained, a 100,000 xg spin for 1 hr followed the initial 9,000 x g centrifugation step. The microsomes were collected and washed in the KCl buffer by recentrifuging for 30 min at 100,000 xg at 4°C.

Protein concentrations of the various fractions were determined by the method of Bradford [30]. When necessary, the fractions were aliquoted and quick-frozen in liquid nitrogen and stored at -80°C until needed.

Enzyme Assays Used:

1. Aryl hydrocarbon hydroxylase (AHH) assay:

   The method developed by Nebert and Gelboin [31] was used. The reaction mixture contained 50μmole Tris-HCl buffer (pH 7.5), 0.36μmole NADPH, 3μmole MgCl$_2$, 0.8nmole benzo(a)pyrene (added in 0.04 ml of methanol just prior to incubation) and supernatant in a total volume of 1 ml. The mixture was incubated in a gyratory water bath shaker set at 37°C for 30 min. The reaction was stopped by the addition of 1 ml of cold acetone. Hexane (3.25 ml) was then added and the mixture incubated with shaking for 10 min at 37°C. The organic phase was extracted with 3.0 ml of 1N NaOH and the amount of 3-hydroxy-benzo(a)pyrene in the alkaline phase was determined spectrofluorometrically using an Aminco Bowman Ratio II spectrofluorometer (excitation wavelength 396 nm; emission wavelength 522 nm).

2. UDP-glucuronyltransferase (UDPGT) assay:

   UDPGT activity was assayed by modifications of the procedures described by Burchell and Weatherhill [32]. The reaction mixture contained (in a volume of 0.7 ml) 5.8mM Tris-HCl, pH 7.3, 2.0mM p-nitrophenol (the substrate), 0.3mM MgCl$_2$, supernatant, an aliquot of 0.5% Lubrol PX (a detergent) and 2.5mM UDP-glucuronic acid. Aliquots (0.1 ml) of the reaction mixture were pipetted

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into separate test tubes and incubated at 37°C for 20 min. The reaction was terminated by the addition of 2.0 ml of 1.8% trichloroacetic acid. The protein precipitate was pelleted by 4°C centrifugation at 2500 x g for 10 min. An aliquot (0.2 ml) of the supernatant was removed and diluted with 2.0 ml of distilled water. Potassium hydroxide (0.4 ml of a 10N solution) was added and the mixture incubated at room temperature for 15 min. The amount of remaining free p-nitrophenol was determined spectrophotometrically at 400 nm.

3. Glutathione S-transferase (GST) assay:

GST activity was measured by the spectrophotometric method of Habig et al. [33]. The second substrate used was 1-chloro-2,4-dinitrobenzene (CDNB). Each reaction mixture contained 1mM CDNB, 1mM reduced glutathione, 1mM potassium phosphate buffer, pH 6.5 and tissue supernatant equivalent to 0.2 mg protein in a final volume of 3.0 ml. A Beckman DU-8 spectrophotometer equipped with a kinetics compuset was used to monitor production of product at 340 nm.

Hepatotoxicity Studies:

Not all hepatotoxins are inducers of metabolizing enzymes. Therefore, other assessments were made to determine whether the test chemicals were toxic to the cells. The following parameters were determined after the cells were treated.

1. Cytotoxicity - the HEPA-2 cells are generally very hardy. It was of interest to determine and compare these cells with the human cell line for viability via the trypan blue dye exclusion test. Significant cell death where present, was also

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readily apparent in assessing cell growth curves in (2) below.

2.   Cell growth - to determine the effect of selected chemicals on cell growth, low density cultures were established with known numbers of cells. Each day the number cf cells was determined following trypsinization of the cells from the surface of the flasks. A single-cell suspension was obtained and duplicate or triplicate cell counts made and averaged for plotting each day. Growth curves were constructed and assessed against control cultures. From these results we were able to determine whether the chemicals were effective towards the overall growth or cell cycle of liver cells.

3.   Cell morphology - numerous agents are known to cause changes in the typical shape of various cell types. Liver cells are commonly referred to as being epithelioid in culture. To maintain this anchorage-dependent shape requires energy, and as the cells become compromised they tend to round up and often detach from the substratum. Daily observations and photographs of the cells, using phase-contrast optics on an inverted tissue culture microscope, readily indicated the morphological status of the cells when compared with their paired controls.

4.   Expression of cell-specific functions - As cells become compromised, they often dedifferentiate or lose their ability to continue to synthesize their so-called luxury proteins. The specific function in question was the ability of the cells to continue to synthesize and secrete albumin into the culture medium, a function retained by both the mouse and human cell lines utilized in the studies. Tritiated leucine (^{3}H-

Leu) at 5-10μCi/ml of culture medium was added to the cells after it had been established that a particular chemical either induced certain enzymes or produced certain hepatotoxic effects on the cells. The cells were allowed to incorporate the isotope into newly synthesized proteins for various periods of time. Following the labeling phase, the cells were washed with ice-cold physiological saline to stop macromolecular synthesis after the medium containing the secreted proteins had been carefully and completely removed and stored for immunoprecipitation of the labeled product (albumin). The washed cell layer was scraped from the surface of the flasks using 10% ice-cold trichloroacetic acid (TCA) to precipitate the cellular protein. The TCA precipitates were stored at 4°C overnight and pelleted by centrifugation at 1,000 x g for 10 min. The pellets were then washed and recentrifuged 2X in ice-cold 5% TCA and the final washed pellets allowed to air dry. The dried pellets were solubilized in 1 ml of 1N NaOH and aliquots used to determine the total protein content [30] and total leucine incorporated via liquid scintillation spectroscopy using a Beckman II liquid scintillation counter.

In order to determine the level of radiolabeled albumin secreted by the cells, antibodies to human and mouse albumin were purchased commercially. The titer of the anti-albumin antibodies was determined by constructing immunoprecipitation curves to determine the zone of equivalence (highest concentration of albumin that will totally precipitate all of the antibody in a constant amount of IgG); 1:1, antibody:antigen [26]. This procedure was important, since the cells do not normally secrete enough albumin, over a period of hours, to obtain a good workable precipitate. Therefore, by adding non-labeled (carrier) albumin in quantities sufficient to form

precipitates, while working in the antibody excess range, the total albumin present (carrier and labeled secretory product) could be precipitated, pelleted, washed, solubilized and ultimately counted for radioactivity in a scintillation counter. For example, once the quantitative immunoprecipitation curves were done, aliquots of secretory product (0.3 ml) were added to small disposable glass tubes along with 0.5 ml of antibody and 0.2 ml of carrier albumin. The contents of each tube were mixed well, and allowed to form precipitates at 37°C for 1 hr. The tubes were stored at 4°C overnight and the antigen-antibody precipitates pelleted by centrifuging for 10 min at 1,000 x g. The supernatants were carefully decanted and the pellets washed 2X in cold 0.9% saline and recentrifuged. Following the final wash, the pellets were solubilized with 1N NaOH and 0.1 ml aliquots counted in Scinti-Verse scintillation cocktail in a Beckman II liquid spectrometer. The radioactivity was expressed in CPM or DPM per mg of protein for the cell cultures. The results of these studies indicated whether the test chemicals had an affect on total protein synthesis as well as the continued secretion of albumin by the liver cells.

RESULTS

Our initial studies involved the use of two proposed test compounds (2-acetylaminofluorene and benzo(a)pyrene) on the activity of several xenobiotic-metabolizing enzymes. Cultures of mouse HEPA-2 cells were treated with 10 μg of the test compound per ml of medium (total of 100 μg of each compound) for 24 h. Simultaneously, an untreated control culture was established as well as a culture that was treated with 100 μl of DMSO. The DMSO-control was used because the test compounds were dissolved in DMSO and 100 μl was the volume used to dispense the compound. At the end of the treatment period the medium was decanted, the cells were rinsed with cold saline and scraped into 3 ml of the cold

homogenization buffer (0.05 M Tris-HCl, pH 7.2, containing 1.15% KCl and 10% glycerol).

After homogenization, the sample was centrifuged at 10,000 x g for 30 min. The supernatant

was decanted and divided into 0.5 ml aliquots. The following assays were then performed:

protein content, UDP-glucuronyltransferase (UDPGT), glutathione S-transferase (GST), and aryl

hydrocarbon hydrolase (AHH), and the following results were obtained (Table I).

TABLE I.   INDUCTION OF BIOTRANSFORMING ENZYMES IN MOUSE HEPATOMA CELLS BY KNOWN
CHEMICAL CARCINOGENS

| | Protein | Assays Performed | | |
Treatments	Content (µg/ml)	Glutathione* S-transferase	UDP-Glucuronyl-* Transferase	Aryl Hydrocarbon* Hydroxylase x 10^5
Untreated Control	535	4.71	5.94	4.34
DMSO Control	540	4.42	4.47	3.84
2 Aminoacetyl fluorene	462	9.30	15.80	3.82
Benzo(a)pyrene	240	21.89	15.68	9.31

*Results reported as specific activity (nmole product/min/µg protein)
*Results reported as ▲ F (fluorescence) /min/µg protein)
The number of replicates for each assay was two.

Both experimental controls (untreated and DMSO-treated) gave approximately the same

results in all assays, including protein content. 2-Acetylaminofluorene caused a slight decrease

in protein content and no increase in Aryl hydrocarbon hydroxylase activity, when compared

to the controls.   However, treatment of cells with this compound did cause a two-fold

stimulation of glutathione S-transferase activity and an approximate three-fold increase in the

activity of UDP-glucuronyltransferase. BAP had a large effect on protein content (probably due

to significant cell death). It also appeared to significantly increase the activity of all enzymes

assayed: 4.65-fold increase of GST; 3-fold increase of UDPGT and 2-fold increase of AHH. The results suggest that the two compounds used to treat the cells cause distinct changes in the activity of specific enzymes. While both 2AAF and BAP cause similar increases in the activity of UDP-glucuronyltransferase, BAP appears to be a more potent inducer of glutathione S-transferase than 2AAF, and it appears to significantly increase the activity of aryl hydrocarbon hydroxylase while 2AAF has no effect on this enzyme.

In addition to the enzyme assays, the quantitation of the liver specific protein, albumin, was also initiated to assess whether the cells continue to express this particular product. Immunoprecipitation curves were first carried out to determine the titer of the commercially obtained antibodies to mouse albumin. The results indicated that the equivalence point (the maximum amount of albumin precipitated by a constant amount of antibody) was approximately 45 μg of albumin when the lyophilized antibody was reconstituted with 2 ml of distilled water and 0.1 ml used to form the specific precipitates. This was an important determination, since it was thought that the cultured cells would not produce amounts of albumin that could be readily precipitated. For this reason, 10-20μg of albumin (carrier) were added to each tube when the amount of newly synthesized radioactive albumin was being determined. By working in the "antibody excess" range we would be assured of forming precipitates that contained all of the secreted radioactive albumin made and contained in the aliquots of medium taken. Therefore, we would be able to accurately determine the level of albumin produced (secreted) per mg of cellular protein as well as determine whether there were any changes in the level produced following chemical treatment by counting the solubilized pellets in a liquid scintillation counter. In order to substantiate and build on our earlier findings, a series of studies was carried out during which the cells were labeled with either [^{3}H]-thymidine to assess DNA synthesis or

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[³H]-leucine to assess protein synthesis during or subsequent to BAP treatment. The results shown in Table II indicate that there is a decrease in leucine incorporation during BAP treatment. This difference was significant when compared with the control cell value.

TABLE II.   INCORPORATION OF [³H]-LEUCINE BY MOUSE HEPATOMA CELLS DURING INITIAL EXPOSURE TO BENZO(A)PYRENE

Control (cpm/mg protein)	Treated (cpm/mg protein)
571,282	422,163[+]
543,390	411,047[+]
	331,387[+]

Each number represents a different cell culture. [+]Significantly different from either of the two control values at $P < 0.01$ level, Student's t-test.

A slight decrease was also seen for the treated cells when assessing the incorporation of thymidine (Table III). This decrease was not statistically significant, however the morphological appearance of the cells following a 24-hr treatment clearly indicated an effect on the cells. This effect manifests itself at the microscopic level as more highly distended cell processes and an increase in the detachment of cells from the substratum. The latter point is also borne out by an overall decrease in total cellular protein in the treated cell cultures as determined by protein analysis.

TABLE III.   INCORPORATION OF [³H]-THYMIDINE DURING INITIAL EXPOSURE OF MOUSE HEPATOMA CELLS (HEPA-2) TO BENZO(A)PYRENE

Control (cpm/mg protein)	Treated (cpm/mg protein)
180,831	177,662[+]
215,935	187,968[+]
	167,071[+]

Each number represents a separate culture. [+]Not significantly different from either control at $P > 0.05$ level, Student's t-test.

Following our assessment of the level of leucine and thymidine incorporation during BAP treatment, we then determined the effect of BAP on albumin synthesis and secretion. The culture medium from the [³H]-leucine cultures was used for this determination, since the cells normally synthesis and secrete this tissue-specific protein directly into the culture medium. The results of our initial studies are found in Table IV. Unexpectedly, there was an approximately 2-fold increase in albumin synthesis in the treated cells when compared to the controls (Roman numeral I). Upon repeating the entire experiment in triplicate a second time, the same pattern persisted (Roman numeral II). This suggests a selective stimulation of the synthesis of at least one tissue-specific product.

TABLE IV.   SECRETION OF [³H]-ALBUMIN DURING 24 HR BENZO(A)PYRENE TREATMENT OF MOUSE HEPATOMA CELLS

Control (cpm/mg protein)		Treated (cpm/mg protein)	
I	II	I	II
8,700	739	15,650$^+$	2,861$^+$
8,200	852	24,000$^+$	2,467$^+$
	644	14,900$^+$	2,842$^+$

Each Roman number (I & II) represents a separate experiment, and each figure represents a separate culture.
$^+$*Significantly different from its experiment I or experiment II controls at P < 0.05 level, Student's t-test.*

To better assess this finding, we took aliquots of the culture medium from all the cultures receiving leucine to determine whether there was an overall 2-fold or better increase in total secreted products by the treated cells. The results in Table V suggest that there is a significant increase in the total secreted radioactive products.

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TABLE V.   TOTAL [³H]-LEUCINE LABELED PROTEIN SECRETED BY MOUSE HEPATOMA CELLS DURING
24-HR BENZO(A)PYRENE TREATMENT

Control (cpm/mg protein)	Treated (cpm/mg protein)
5,514	11,020[+]
6,336	12,282[+]
5,410	13,148[+]

Each number represents a separate culture. [+]Significantly different from the average of the three controls at
P < 0.05 level, Student's t-test.

In addition to determining the effect of BAP on total protein synthesis and secretion and albumin synthesis during the initial treatment phase, we treated the cells with BAP for 24 hr and then labeled with [³H]-leucine during the second 24-hr period. The results on total protein synthesis are found in Table VI. As seen when the cells are treated and labeled at the same time, there is a significant difference between the control and treated cells.

TABLE VI.   INCORPORATION OF [³H]-LEUCINE INTO CELLULAR PROTEIN FOLLOWING A 24-HR
TREATMENT OF MOUSE HEPATOMA CELLS WITH BENZO(A)PYRENE

Control (cpm/mg protein)	Treated (cpm/mg protein)
432,562	285,662[+]
447,288	293,862[+]
440,982	306,414[+]

[+]Significantly different from the average of the three controls at P < 0.01, Student's t-test.

What is even more striking, however, is the total secreted protein by these same cell cultures 24 hr post treatment (Table VII). There is a 4-fold increase in the secretory product for the treated cells. This suggests an even greater stimulation of secretion by BAP with time.

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TABLE VII.   *TOTAL [³H]-LEUCINE LABELED PROTEIN SECRETED DURING 24-HR POST BENZO(A)PYRENE TREATMENT OF MOUSE HEPATOMA CELLS*

Control (cpm/mg protein)	Treated (cpm/mg protein)
19,585	81,185[+]
15,215	69,540[+]
21,210	83,205[+]

[+]*Significantly different from the average of the controls at* $P < 0.01$ *level, Student's t-test.*

Finally, the medium from the above cell cultures was also used to precipitate the radiolabeled albumin secreted by the cells (Table VIII). The results here again clearly indicate a stimulation in the synthesis (secretion) of this particular protein.

TABLE VIII.   *SECRETION OF [³H]-ALBUMIN BY MOUSE HEPATOMA CELLS FOLLOWING A 24-HR TREATMENT WITH BENZO(A)PYRENE*

Control (cpm/mg protein)	Treated (cpm/mg protein)
2,870	6,060[+]
2,150	6,450[+]
2,680	6,110[+]

[+]*Significantly different from each of the controls at* $P < 0.01$ *level, Student's t-test.*

There were some concerns as to whether the use of cultured human and/or rodent cell lines (normal or transformed) would serve as a model for asssessing the responses that are or would be seen if humans were exposed to these same chemicals. In order to address this concern, a thorough literature search was undertaken in an attempt to correlate what is known to date.

Correlation of Biochemical Toxicology Studies in Man, Rodents and Cultured Cell Systems

One aspect of toxicological testing is to determine the effect of the agent tested on the enzymes responsible for the biotransformation of that agent. Some of the key enzymes involved in biotransformation are cytochrome P-450-dependent enzymes [e.g., aryl hydrocarbon hydroxylase (AHH), epoxide hydrase, glutathione S-transferase (GST) and UDP-glucuronyltransferase (UDPGT)]. Each of these enzymes exists in mammalian systems in many isozymic forms, which are inducible by a wide variety of xenobiotics.

Using enzyme activity in vitro or rodent systems as an indication of toxicologic response in humans, makes it necessary to determine whether the response seen in rodents correlates to those seen in humans. Since the liver has the greatest amount of xenobiotic metabolizing enzymes, the brief literature review below deals with the presence and inducibility of these enzymes in either rodent or human liver and in primary or transformed cell cultures of human or rodent hepatocytes.

Cytochrome P-450

Of the twelve forms of mammalian P-450 discussed by Geuengerich [34], eleven of them have been isolated in human liver and shown to be immunologically similar to P-450s from rat, rabbit and/or mouse liver. Many P-450-dependent catalytic activities in human liver extracts were also found to be correlated with the amount of the P-450 detected with antibodies raised against rodent forms of this protein. Peters and Kremers [35] also showed that P-450s of human liver react with the antibody raised against rat liver ß-naphthoflavone-inducible P-450. These data indicate that there are structural similarities between the rodent and human forms of P-450.

Studies on the inducibility of P-450 dependent enzymes showed that there is fair correlation between the results found in human and those from rodent and culture systems.

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Experiments with human liver extracts [36] showed that some P-450-dependent enzyme activities are increased five-fold by phenobarbital, whereas these activities were increased ten-fold in human hepatoma cells (HEP-G2) treated with 3-methylcholanthrene. Muakkassah-Kelly et al. [37] measured a two-fold increase in the P-450 of primary cultures of rat hepatocytes treated with phenobarbital and a three-fold increase in this portion when 3-methylcholanthrene was used as the inducer.

It should be noted here that clear correlations between the activity of biotransformation enzymes found in humans versus those in rodent or culture systems are difficult to establish since "untreated" human samples may be impossible to obtain for control studies and the inducers frequently used in rodent and culture systems are often carcinogenic (e.g., 3-methylcholanthrene). Another problem is the use of different substrates by investigators. It has been shown that the measured activity of the biotransformation enzymes is significantly affected by the substrate used in the assay. This reflects the fact that different forms of these enzymes (induced by different xenobiotics) preferentially use specific substrates.

Glutathione S-transferase (GST)

Ketterer [38] stated that data obtained for rat GST is relevant to man. He further stated that the same multigene families are seen in both species and there is considerable identity in primary structure across the two species. Tu et al. [39] and Hayes et al. [40] have demonstrated that there is sequence homology as well as immunological cross-reactivity between GST subunits from several rat tissues (including liver) and human liver. Studies by Mannervik [41] showed that the major isoenzymes of cytosolic GST from rat, mouse and human liver share structural and catalytic properties.

In comparative studies of the amount of GST in primary rat hepatocytes and whole liver

extracts, Vandenberghe et al. [42] found that when measuring the quantity of GST per milligram cytosolic protein there was no significant difference. However, the amount of some GST subunits decreased by as much as 50% after four days in culture.

Though numerous investigations have measured GST activity in human liver [e.g., 39, 43-45] we have uncovered no studies in the literature on the expressed activity of human hepatic GST after treatment with xenobiotics. However, studies by Tahir [45] showed that GST activity was increased two- to three-fold in normal rat liver and two-fold in rat hepatoma cells when both were treated with trans-stilbene oxide. Kulkarni et al. [46] found that phenobarbital increased GST activity two- to four-fold in mouse liver. This indicates a close correlation between the activity obtained from tissue extracts and transformed cells in culture.

UDP-glucuronyltransferase (UDPGT)

At least five forms of UDPGT have been detected in human liver by immunoblot analyses [47,48] which used antibodies prepared against purified rat UDPGTs. There is also immunologic cross-reactivity between mouse and rat hepatic UDPGT (Anaba and Stewart, unpublished observation). This indicates structural similarities between the rodent and human enzymes.

The activity of the human hepatic enzyme toward bilirubin can be stimulated three-fold by phenobarbital [36]; whereas activity toward the substrates paracetamol, benzopyrene-3,6-quinol, 4-methylumbelliferone and 1-naphthol is increased two-fold by 3-methylcholanthrene [36]. When the activity of this enzyme was studied in rat liver extracts, its activity was increased three- to forty-fold depending on the substrate used [4-methylumbelliferone, three-fold; 1-naphthol, four-fold; benzopyrene-3,6-quinol, eleven-fold; benzopyrene-3,6-quinol monoglucuronide, forty-fold] [49].

' <u>Summary</u>

Analyses of the data presented in these studies reveal that though there is not a perfect correlation between the activity of biotransformation enzymes in human liver and that in either rodent liver or transformed cells, enough of a relationship exists that the enzyme activities in these non-human or non-normal systems can be used as one phase of a study to assess (with a fairly high degree of certainty) the potential toxicologic effect of xenobiotics on humans.

A comparative analysis of the effects of one of our test chemicals on human vs. mouse hepatoma cells was performed. This was done to determine if the use of mouse cells for toxicity studies was valid for extrapolation to humans, since the initial studies were done with mouse cells prior to our obtainning the human cell line. Obviously, humans can not and have not been used to assess the effects of potentially toxic substances, so that a clear correlation can not be made in this regard. However, from the literature, it is apparent that studies carried out with intact animals or cell cultures indicate that the enzyme systems are present and respond in a similar manner and to similar induction levels. From this we conclude that the data that we have generated through the use of transformed cell lines is in keeping with earlier published reports, and indicate that our proposed model system is a valid method for assessing the potential toxicological effects of xenobiotics on humans.

The following table (Table IX) shows the biochemical results obtained when human (HEP-G2) cells and mouse (HEPA-2) cells were treated with benzo(a)pyrene. These data reveal that BAP has a drastic effect on protein concentration in both cell lines; causing a 2.0 to 2.5-fold decrease in both. GST and UDPGT activities were stimulated significantly by BAP in the two different cell lines. Though AHH activity was not increased in HEPA-2 cells by BAP, it was increased almost two-fold in the HEP-G2 cells. Earlier studies with HEPA-2 cells had shown

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that BAP caused a significant reduction in protein concentration (attributed to cell death) when compared to untreated cultures.

TABLE IX.   COMPARISON OF HUMAN (HEP-G2) AND MOUSE (HEPA-2) LIVER CELLS FOLLOWING BENZO(A)PYRENE TREATMENT

Cell Type	Protein (μg/ml)	GST*	UDPGT*	AHH*
HEPA-2:				
Control	1158 ± 65	11.25 ± 2.59	114.43 ± 5.89	22.43 ± 3.85
BP	566 ± 45	35.85 ± 5.28[+]	278.80 ± 14.93[+]	24.34 ± 4.ɔ3
HEP-G2:				
Control	2365 ± 81	8.14 ± 1.53	114.70 ± 6.39	16.94 ± 2.19
BP	965 ± 52	18.75 ± 3.16[+]	190.38 ± 8.26[+]	29.34 ± 2.70[+]

All values represent the average of two sets of experiments, each done in triplicate.

*Enzyme activity expressed as nmole product per minute per microgram of total protein.
*Enzyme activity expressed as the change in fluorescence (ΔF) per minute per microgram of protein.
[+]Significantly different from control value, $P < 0.05$, Student's t-test.

It appears that the human and mouse hepatoma cells respond similarly to treatment with BAP. With the acquisition of human cells, and results that were comparable, from this point onward the HEP-G2 human cell line was used in our assessments.

Time- and dose-response studies in human hepatoma cells (HEP-G2) with the xenobiotic benzo(a)pyrene

During the next phase of the project, variations in the time of treatment and concentrations of benzo(a)pyrene (BAP) were assessed in the human hepatoma cell line HEP-G2. These experiments were done to ascertain the minimum concentration of BAP and treatment time that would yield significant responses in the parameters measured (e.g., protein concentration, enzyme activity and albumin secretion). All data reported are the results of at least three

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separate experiments, each done in triplicate (Table X).

TABLE X.   *TIME RESPONSE STUDIES OF HUMAN HEP-G2 CELLS TREATED WITH BENZO(A)PYRENE (10µG/ML)*

	TIME (HR)						
	1	2	4	8	12	24	48
Protein Concentration	±	*l*	*l*	±	±	±	*J*
GST Activity	*l*	*l*	±	±	±	±	*l*
AHH Activity	±	±	±	±	±	±	*l*
UDPGT Activity	*l*	±	±	±	±	*l*	±

± = *Not significantly different from the control cultures, P≥0.05, Student's t-test.*
l = *Significantly higher than the controls, P≤0.05, Student's t-test.*
J = *Significantly lower than the controls, P≤0.05, Student's t-test.*

In the time-response studies, cells were treated for either 1,2,4,8,12,24 or 48 h with 10µg/ml BAP dissolved in 100% ethanol (10µl). Protein concentrations and enzyme activities were measured in the supernatant resulting from a 9,000 x g centrifugation of the cell homogenate. When protein concentration was determined, it rose sharply between 2 and 4h, was essentially unchanged between 4 and 24h and dropped drastically at 48h (to 27% of the value obtained for the 48h ethanol control). This pattern mirrors growth curves of these cells in the presence of BAP and reflects a sharp increase in cell death at 48h post-treatment.

These studies also showed that there are differences in the times needed to get peak activity of the various enzymes monitored. Glutathione S-transferase (GST) activity was increased significantly at 1,2 and 48h post-treatment, with the highest activity (a 2-fold increase over the control value) at 2h. A significant increase in aryl hydrocarbon hydroxylase (AHH) activity was seen only at 48h. At this point, there was a greater than 8-fold increase in this activity over the control value. Large increases (5-fold and 10-fold) were seen in UDP-

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glucuronyltransferase (UDPGT) activity at 1h and 24h, respectively.

In the dose-response studies, the concentrations of BAP used were 2.5 and 5.0 µg/ml. The treatment times used (2,24 and 48h) corresponded with the times at which peak enzyme activities were observed in the previous study (Table X).

Supernatant protein concentrations did not show a significant dose response at any of the times used. GST activity was stimulated 2-fold in cells treated with either 2.5 or 5.0 µg/ml BAP at 2 and 24h. However, 10- to 11-fold increases were seen in this activity at 48h with both doses. A BAP concentration of 2.5 µg/ml stimulated AHH activity more than 3-fold at 2h but seemingly suppressed it at 24 and 48h (20-51% of the control value was obtained at these time points). A higher BAP concentration (5.0 µg/ml) stimulated AHH activity slightly, but significantly, at 24h (1.5-fold), but caused no significant increase at 2h and a suppression at 48h (57% of the control value). The only significant change in UDPGT activity occurred at 2h with 5.0 µg/ml BAP (3-fold increase) (Table XI).

Table XI.   *DOSE RESPONSE STUDIES OF HUMAN HEP-G2 CELLS TREATED WITH BENZO(A)PYRENE*
(BAP)

	TIME (HR)		
	2	*24*	*48*
GST Activity			
(2.5µg BAP/ml)	*(2)*	*(2)*	*(10+)*
(5.0µg BAP/ml)	*(2)*	*(2)*	*(10+)*
AHH Activity			
(2.5µg BAP/ml)	*(3+)*	*[>20]*	*[>20]*
(5.0µg BAP/ml)	*±*	*(1.5)*	*[>50]*
UDPGT Activity			
(2.5µg BAP/ml)	*±*	*±*	*±*
(5.0µg BAP/ml)	*(3)*	*±*	*±*

Number in parenthesis ( ) = fold increase in activity, significantly different from control, P≤0.05, Student's t-test.
Number in brackets [ ] = fold suppression below control, significantly different at P≤0.05, Student's t-test.
± = Not significantly different from controls, P≥0.05, Student's t-test.

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In summary, the time-response and dose-response studies indicate that significant increases in the activities of the enzymes can be attained in as little as 2h using either 2.5 or 5.0 μg/ml BAP. These findings show that a significant response to a potentially hazardous xenobiotic can be demonstrated in human cells in a very short time span and at reasonably low concentrations. However, one should take into account the fact that each chemical has to be assessed for its own time and dose profile.

Following our demonstration that cultured human liver cells respond to drugs such as 2-acetylaminofluorene and benzo(a)pyrene, known inducers of drug detoxifying enzymes, we moved to assess the effects of other drugs that are presently widely used in humans. The two drugs examined, azidothymidine (AZT) and pentamidine, were chosen for the following reasons. First, AZT is a thymidine base analog that is approved for treatment of individuals suffering from AIDS, and AZT was being used to test its effects on other cell lines, T-lymphocytes in particular, presently in use in the laboratory. In addition, it was suspected that enzyme activation may not occur with a substance so closely related to thymidine, yet we were aware that AZT produces some very toxic side effects in humans.

The effects of AZT on cell growth and the induction of glutathione S-transferase (GST), UDP-glucuronyltransferase (UDPGT) and arylhydrocarbon hydroxylase (AHH) were tested. Following a 24-hr treatment with 500 μm AZT, the HEP-G2 cells showed little change in cell growth and viability. There was a two-fold increase in AHH activity and a three-fold increase in UDPGT activity over control values with this concentration of AZT. There was no increase in GST activity by this treatment regimen; however, GST activity was stimulated two-fold at shorter time periods (1-8 hr). A lower dose (125-250 μm) of AZT as well as shorter exposure

times (2-12 hr), also led to a significant increase in certain key drug metabolizing enzymes when compared to untreated control cells. These results indicate that AZT has significant effects on the activity of some Phase I and Phase II biotransformation enzymes which could in this case affect the bioavailability and therapeutic effectiveness of this anti-AIDS drug.

It should also be pointed out that the doses of AZT that we used were much higher than are commonly used to treat patients at any one time. On the other hand, these doses are commonly administered over a 24-h+ period. Conversely, when lower doses (10-50μm) are given there is no significant induction of detoxifying enzymes. These findings were reported at the First Annual NIH RCMI International AIDS Conference in Atlanta in September of 1990.

Finally, we studied the biochemical effects of pentamidine isethionate, an aromatic diamidine. Pentamidine is an anti-protozoan agent proven effective in the treatment of *Pneumocystis carinii* pneumonia in immunodeficient adults, such as those with AIDS. Since it is often given to AIDS sufferers, we wanted to determine its effects on human tissues, since it reportedly does not undergo biotransformation in the body, but leads to highly toxic side effects. Our assessment of physiological doses of pentamidine indicates that it does not stimulate detoxifying enzyme activity, but does lead to significant inhibition of DNA, total protein and albumin synthesis as well as altered cell morphology. This is a clear case of a drug expressing hepatotoxicity without activating detoxifying enzyme systems. Thus, these findings emphasize the importance of the use of various procedures, including bioassays in assessing drug toxicity (see Appendix I for further details).

Publications and Presentations Resulting from Work

Publications:

Stewart, J., Wright, B.G. and Parker, C.L., 1991. Effect of AZT on glutathione S-transferase and UDP-glucuronyltransferase activities in human cell lines. FASEB Journal Pt. 1, A824, Vol. 5, No. 4.

Parker, C.L., Finley, R.B., Wright, B.G., Nzeribe, R. and Stewart, J. 1991. Biochemical effects of pentamidine on cultured human liver cells. J. Cell Biol. 115:73a.

Parker, C.L., Finley, R.B., Wright, B.G., Nzeribe, R. and Stewart, J. 1992. Biochemical effects of pentamidine on cultured human liver cells. Toxicology In Vitro (submitted for publication).

Presentations:

Stewart, J., Parker, C.L., Wright, B.G. and Finley, R.B., 1990. The effects of AZT on growth and key drug-metabolizing enzymes in human cell lines. First NIH/RCMI International AIDS Symposium, Atlanta, GA, Sept. 27-29.

Parker, C.L., Finley, R.B., Wright, B.G., Nzeribe, R. and Stewart, J., 1991. The inhibitory effects of pentamidine on biochemical events in liver cells. Second NIH/RCMI International AIDS Symposium, Atlanta, GA, Sept. 5-7.

Stewart, J., Wright, B.G. and Parker, C.L., 1991. Effect of AZT on glutathione S-transferase and UDP-glucuronyltransferase activities in human cell lines. FASEB Meetings, April 1991.

Parker, C.L., Finley, R.B., Wright, B.G., Nzeribe, R. and Stewart, J. 1991. Biochemical effects of pentamidine on cultured human liver cells. Thirty-first Annual Meeting of the American Society for Cell Biology, Boston, MA, Dec. 8-12.

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APPENDIX I

MANUSCRIPT

THE INHIBITORY EFFECTS OF PENTAMIDINE ON BIOCHEMICAL EVENTS IN

HUMAN LIVER CELLS

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Running Title: Biochemical Effects of Pentamidine

ABSTRACT

The present study was undertaken to determine the biochemical effects of pentamidine on an established human liver cell line (HEP-G2) and its drug-resistant subclone (HEP-G2DR). The HEP-G2DR cells were included in the study to determine whether drug-resistance played a role in any of the observed effects seen during and/or following pentamidine treatment. The results indicate a $5\mu M$ concentration of the drug significantly reduced the level of protein synthesis, as well as the continued expression of albumin, a tissue specific product of both cell lines. Pentamidine also significantly inhibited DNA synthesis in HEP-G2, but not HEP-G2DR cells during the first 24h. Conversely, only the resistant cells showed a significant recovery in both protein and albumin synthesis after the drug was removed (reversal). The significant recovery in these biochemical parameters was observed during the initial 24-h reversal period, while a recovery in albumin secretion was not obtained in the HEP-G2 cells even after 72h. These findings suggest that drug resistance plays a role in the mechanism of action of pentamidine, since biochemical modulation of both cell lines is brought about by the drug, but only the resistant cells appear to recover fully following the removal of pentamidine from the culture medium.

INTRODUCTION

Due to the complex nature of the AIDS disease, many patients often seek the help of a physician for other prevalent ailments prior to the proper diagnosis and drug treatment regimen commonly associated with AIDS infections. Two of the illnesses commonly associated with susceptible AIDS victims are upper lobe or *Pneumocystis carinii* pneumonia and *Mycobacterium avium*-intracellulare complex. These diseases are referred to as secondary infections and are usually treated aggressively with a combination of drugs, i.e., co-trimoxazole (trimethoprim-sulfamethoxazole) (Fischi et al. 1988). Pentamidine isethionate, an aromatic diamidine, is another important drug used in the treatment of secondary infections brought about by protozoan organisms in AIDS patients (Drake et al. 1985). This anti-protozoal agent was developed in the 1940s for use in the treatment of illnesses such as African sleeping sickness and Indian kala-azar caused by trypanosomes. It was shown to be very effective in significantly reducing infant mortality caused by *P. carinii*, but even so, frequent and severe side effects were noted and the use of pentamidine became limited with the introduction of the less toxic antimicrobial combination agent (Goa & Campoli-Richards, 1987).

While pentamidine produces severe adverse reactions in general, it has been shown to be less toxic to AIDS patients than is co-trimoxazole. The present dramatic increase in *P. carinii* pneumonia cases among AIDS patients is what stimulated our interest in this drug. Most of the published accounts on the mechanism of action of pentamidine deal with its effects on protozoa, while little has been done to assess its effect on host tissues, even though drug treatment usually involves a two to three week course. Therefore, the goal of the present study was to gain a better understanding of the mechanism of action of pentamidine on the cells of the host. Two human liver cell lines were chosen to carry out this study; HEP-G2 the parental strain, and HEP-G2DR a drug resistant strain derived from the G2 cell line. These two cell lines continue to express tissue-specific functions including the synthesis and secretion of albumin (Knowles et al. 1980). We used the cells to assess the continued expression of this differentiated tissue-specific function via immunoprecipitation of the newly synthesized radioactive product, as well as other macromolecular processes following drug treatment.

MATERIALS and METHODS

Cell Culture

The human liver cell line (HEP-G2) was obtained from Dr. Edwin Ades at the Centers for Disease Control, Atlanta, GA. The drug resistant subclone (HEP-G2DR) was also developed in Dr. Ades' laboratory by growing G2 cells over a period of time in increasing amounts of adriamycin (Sigma Chemical Co.), and subdividing the cells that survived once they reached a maintenance dose of the drug. Both cell lines were grown in Dulbecco's Modified Eagle's Medium (DMEM) (J.R.B. Biosciences) supplemented with 10% fetal bovine serum (Hyclone), and an antibiotic/antimycotic solution consisting of penicillin, streptomycin and amphotericin B (Sigma Chemical Co.). Resistant cell cultures were routinely maintained in adriamycin (1.25×10^{-8}M). The cells were grown in a humidified atmosphere of 5% CO_2 at 37°C. For most of the kinetic studies, the cells were seeded in 60-mm^2 dishes and generally allowed to acclimatize overnight prior to experimental manipulations. Experimental cultures were treated with 1μM or 5μM pentamidine for various periods of time. The pentamidine stock solution (1000X) was made up in distilled water and filter sterilized just prior to its use. At the end of the treatment phase, the pentamidine-containing medium was removed and the attached cells washed with DMEM before the addition of fresh DMEM containing the radioactive isotopes.

Measurement of Albumin Secretion,
Total Protein and DNA Synthesis

To measure albumin secretion, [^{3}H]-leucine (5μCi/ml) was added to fresh culture medium and the cells allowed to grow for the appropriate times. At the end of the labeling period, the medium was removed and aliquots used to immunoprecipitate the radioactive albumin as previously described (Rosebrock et al. 1981). In brief, human albumin and rabbit anti-human albumin (ICN Immunobiologicals) were used to establish a quantitative immunoprecipitation curve. From this, we were able to determine that 0.1ml antibody and 20μg of albumin gave an antibody excess that was sufficient to precipitate all of the labeled albumin contained in the 0.5ml aliquots of culture medium tested, and to produce antigen/antibody pellets large enough to wash and analyze accurately. To determine the total cellular protein present on the culture dishes, as well as the amount of protein synthesized during the labeling period, the cell layers were washed (3X) with ice-cold physiological saline and scraped from the surface of the dishes into 10% trichloroacetic acid (TCA). The precipitates were incubated overnight at 4°C. The TCA pellets were collected by centrifugation (1000x g, 10min), and washed three times with 5% TCA by centrifugation and resuspension. After the last wash, the pellets were solubilized in 1.0ml of 1N NaOH and aliquots used for both protein determinations (Bio-Rad method) with bovine serum albumin used as a standard and liquid scintillation spectrometry.

For analysis of DNA synthesis, treated and control cells were given 1μCi [^{3}H]-thymidine/ml DMEM for 24h. At the end of the labeling period the medium was discarded and the cell layers carefully washed three times to remove any unincorporated isotope. The cells were then precipitated with TCA, solubilized in NaOH and used to assess the incorporation of thymidine and total protein as described above.

Radioactive isotopes were purchased from ICN Radiochemicals (^{3}H-thymidine, sp. act. 68 Ci/mmol) and NEN Research Products (^{3}H-leucine, sp. act. 156 Ci/mmol). All chemicals and reagents were of research grade. The pentamidine (Sigma Chemical Co.) was obtained as an isethionate salt. Statistical analysis of the data employed a two-tailed t test. Each experiment was done in triplicate and repeated at least twice.

RESULTS

PENTAMIDINE STUDIES following 48-h TREATMENT

In our initial studies, log phase HEP-G2 and HEP-G2DR cells were treated with 5μM pentamidine for 48h, washed free of the drug and then labeled with ^{3}H-leucine (5μCi/ml) for 24h along with their companion control cultures. The result was a highly significant reduction in total protein synthesis in the two treated cell series (Fig. 1a). The drug resistant cells have a longer doubling time than do the sensitive cells, but the overall rate of protein synthesis and inhibition following drug treatment appears to be comparable.

The level of albumin secretion was then assessed using the medium from these same cell cultures. Albumin is generally constitutively synthesized and secreted into the culture medium from a stable message. Therefore, it was of interest to determine whether the rate of albumin secretion was also affected following pentamidine treatment. The result was a significant reduction in the level of albumin secretion, parallel to that seen for total protein synthesis (Fig. 1b).
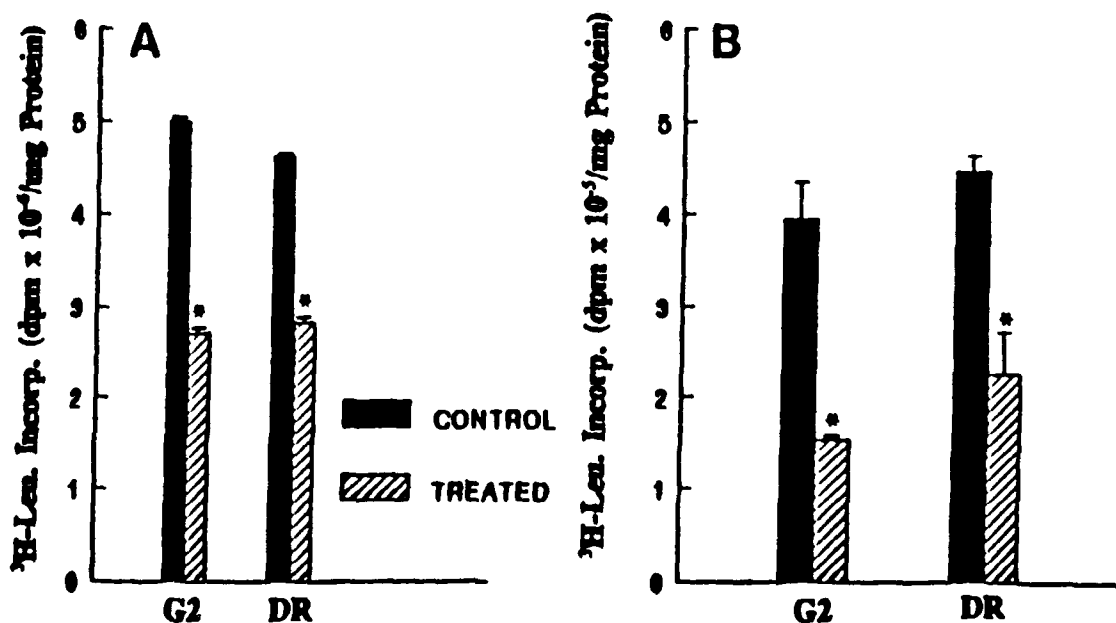

Fig.1. Incorporation of [^{3}H]-leucine (5μCi/ml) into total protein (A) and albumin (B) following treatment with 5μM pentamidine. The drug sensitive (G2) and drug resistant (DR) cells were treated for 48h, washed free of pentamidine and radiolabeled for 24h. In the case of both total protein synthesis (A) and albumin secretion (B), the treated cells were significantly reduced in their synthetic capacity (*). For protein, there was a significant affect at $p < 0.003$ and for albumin, $p < 0.05$.

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The cells were also observed with an inverted phase contrast microscope for morphological changes, and growth in general during the 48-h pentamidine treatment. There was an indication with time, that the number of cells present lagged behind the number on the untreated control dishes. It was possible that this might be due to either a lag in cell division or to the absence of clustering that is commonly seen as the cells continue to grow towards confluency. To address this, cells were then set up for a 24- and 48h treatment with pentamidine that was followed by the addition of ^{3}H-thymidine to assess the level of DNA synthesis (Fig. 2). The HEP-G2 cells showed a significant reduction in the rate of thymidine incorporation at both time intervals, while there was essentially no change in the HEP-G2DR cells even after treatment for 48h, when compared with their controls. To further corroborate this finding, both cell lines were grown in the presence or absence of pentamidine for a period of six days to determine if the treated cells would reach confluency as would be expected. The results clearly indicate that pentamidine has an effect on both growth and morphology of the cells (Fig. 3). In addition to the lack of clustering and a lag in the increase in cell density, the cells became more fibroblastic. Further, while the drug showed no significant effect on DNA synthesis by HEP-G2DR cells even at 48h of treatment, there was an indication of an effect on cell number at this time and this became more apparent over the next 2-3 days. In a series of studies using a 1μM concentration of pentamidine, the drug was found to have no significant effect on protein or albumin synthesis unless the treatment period was extended to 4-5 days. At this point, the results were similar to those obtained using the 5μM concentration.
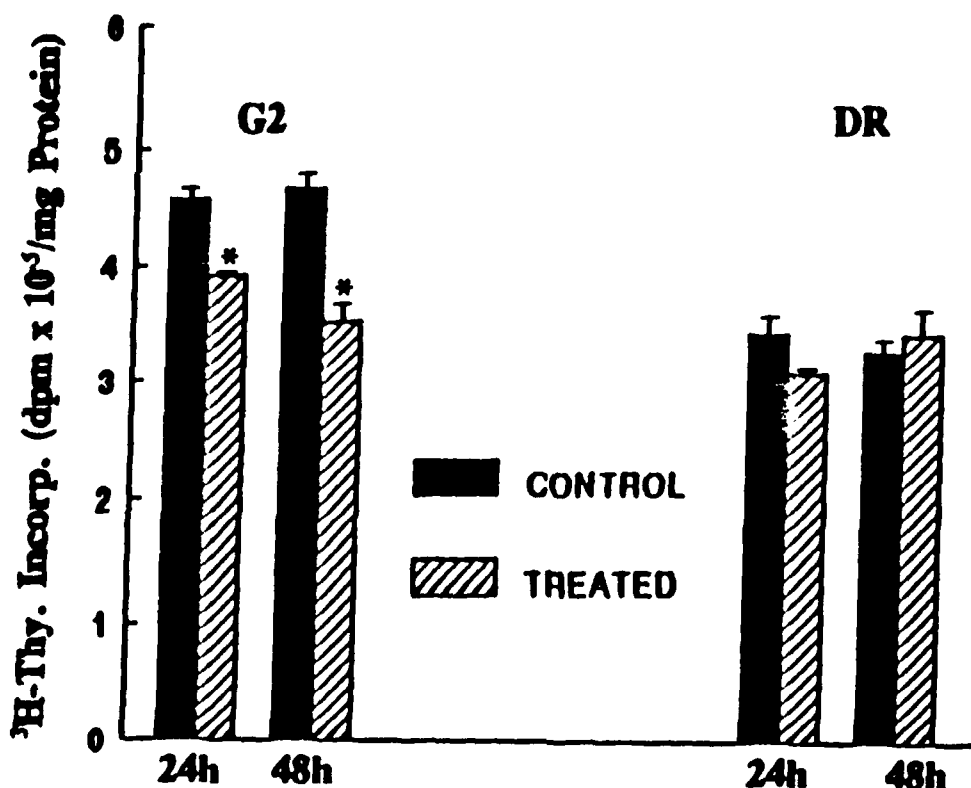

Fig 2. Tritiated thymidine incorporation into DNA by human liver cells. Both sensitive G2 and resistant DR cells were treated with 5μM pentamidine for 24 or 48h prior to the addition of [^{3}H]-thymidine for a 24-h labeling period. Note that the pentamidine-treated G2 cells are significantly reduced (*) in their rate of thymidine incorporation when compared to their controls. This was not seen in the pentamidine-treated drug resistant (DR) cells. * The decrease in thymidine incorporation is significant at p<0.05 level, Student's t-test.
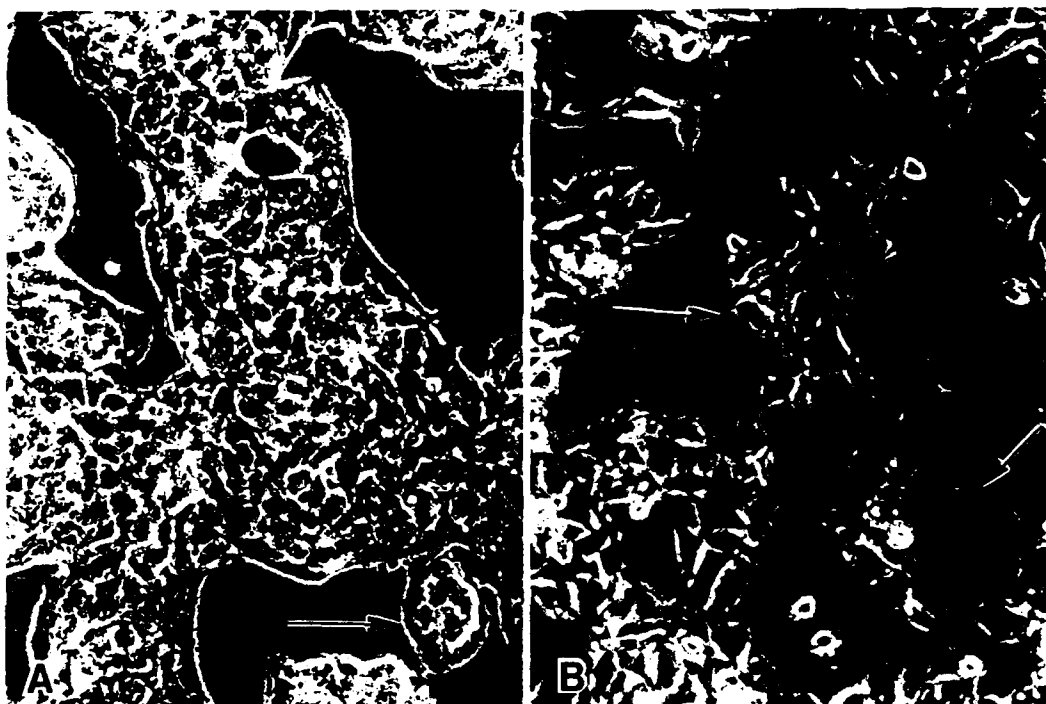
Fig. 3. Phase contrast micrograph of the growth and morphology of human liver cells. HEP-G2 cells were established in culture at a low cell density. Following overnight attachment and acclimation, half of the cultures were treated with $5\mu M$ pentamidine and the control (A) and treated (B) cells were allowed to grow for six days while being assessed for growth and morphology. In panel A, the cells are seen to form clusters (arrow) as they grow and produce a continuous monolayer. The treated cells in B lag behind in cell number, lack the typical cell clustering seen in the control, and become more fibroblast-like (arrows) than the more normally appearing epithelioid control cells in A.

In other mammalian cell systems, it has been shown that the rate of total and/or specific protein synthesis may differ greatly in log phase of growth from that seen when the cells reach stationary phase (Blume et al. 1970; Layman & Ross, 1973; Papaconstantinou et al. 1978). Since it was apparent that pentamidine had an effect on cell growth, it was important to know whether the long-term treatment, which led to a decrease in cell number while the control cells grew into confluency, might account for the reduction in protein and albumin synthesis observed in the treated cells. This was first addressed by seeding cells from both cell lines at log phase and stationary phase densities. After allowing the cells to attach and acclimatize overnight, the medium was changed and the cells were labeled with ^{3}H-leucine (5μCi/ml) for 24h. The results in Figure 4 indicate that the rate of total protein synthesis between log and stationary phase cells is not significantly different within each cell line. The same result is found in comparing the rate of total protein synthesis within or between the phases of the different cell lines. It is however, interesting to note that the rate of albumin secretion is significantly lower in log phase cells than in those at stationary phase. A similar finding was reported earlier by Kelly and Darlington (1988). This suggests that cells not having to direct their energies toward division, etc., are free to devote their energy towards tissue-specific functions. These results suggest also that the inhibitory effect on albumin secretion following pentamidine treatment may well have been related to cell density and not directly to the drug.
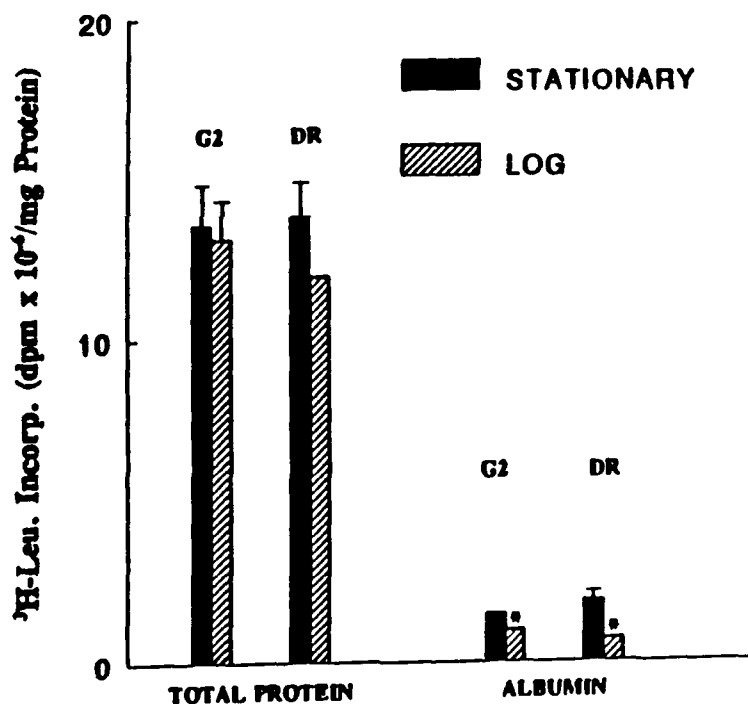

Fig.4. Rate of total protein and albumin synthesis in log vs stationary phase cells. The cells were set up and allowed to acclimatize overnight. They were then labeled for 24h with 5μCi ['H]-leucine/ml. There is essentially no difference in the rate of total protein synthesis between cells from stationary and log phase of the same cell line or between the two cell lines. There is, however, a significant difference in the rate of albumin secretion between log and stationary phase cells (*). This difference is seen in both the G2 and DR cell lines. * Significantly different from stationary phase cells at $p < 0.05$ level, Student's t-test.

In order to determine that the differences seen were due to pentamidine and not to cell density, the following experiment was carried out. The two cell lines were treated with ·pentamidine for 4 days prior to setting up the control cultures at a comparable cell density. The control and treated cells were allowed to grow for an additional 24h and then used to assess total protein and albumin levels (Fig. 5). The results again show that there is a significant reduction in total protein and albumin in both pentamidine treated series, indicating that it is pentamidine that modulates macromolecular processes in these studies.
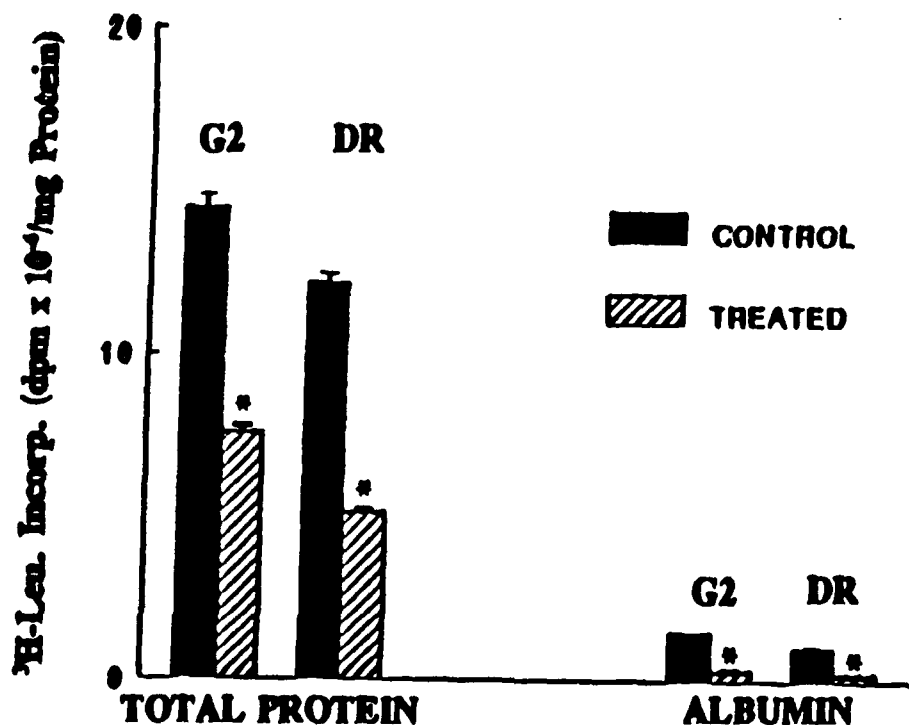
Fig. 5. Effects of pentamidine on liver cells cultured at comparable cell densities. The pentamidine-treated cells (1μM/ml) were set up 4 days prior to the controls, in order to allow the drug time to express its effect before establishing the control cultures at a comparable cell density. The cells were then maintained for an additional 24h to allow the controls to acclimatize prior to labeling with [³H]-leucine (5μCi/ml) to assess the level of total protein synthesis and albumin secretion following a 24-h labeling period. In each instance, the treated cell series is significantly reduced biochemically, * p<0.0003, Student's t-test.

REVERSAL of the EFFECTS of PENTAMIDINE

After demonstrating that pentamidine modulates various biochemical processes by the cells, it was of interest to determine if any of these could be reversed following the removal of the drug and maintenance of the cells in DMEM. In this series of studies, cells were treated with a 5μM concentration of pentamidine for 48h, and half of the cultures were reversed with fresh medium alone, while the other half received fresh medium containing pentamidine. A third of each series was then labeled with ³H-leucine each day over a 3-day period. Figure 6a clearly indicates that both HEP-G2 and HEP-G2DR reversed cells significantly increase their rate of protein synthesis over that seen in the cultures remaining in the drug. This increase was noted

within the initial reversal phase, and persisted over the 3-day study (Fig. 6a). On the other hand, when albumin secretion was assessed, it was striking to note that the reversed drug sensitive cells did not recover from pentamidine treatment even after 72h in the absence of the drug (Fig. 6b). Conversely, the drug resistant cells significantly recovered during the first 24-h period, and continued to increase their rate of secretion over that seen in the continuously treated series throughout the 72-h study. Again, 1μM of pentamidine leads to a similar set of results if the cells are first treated for 4-5 days and then allowed to recover.
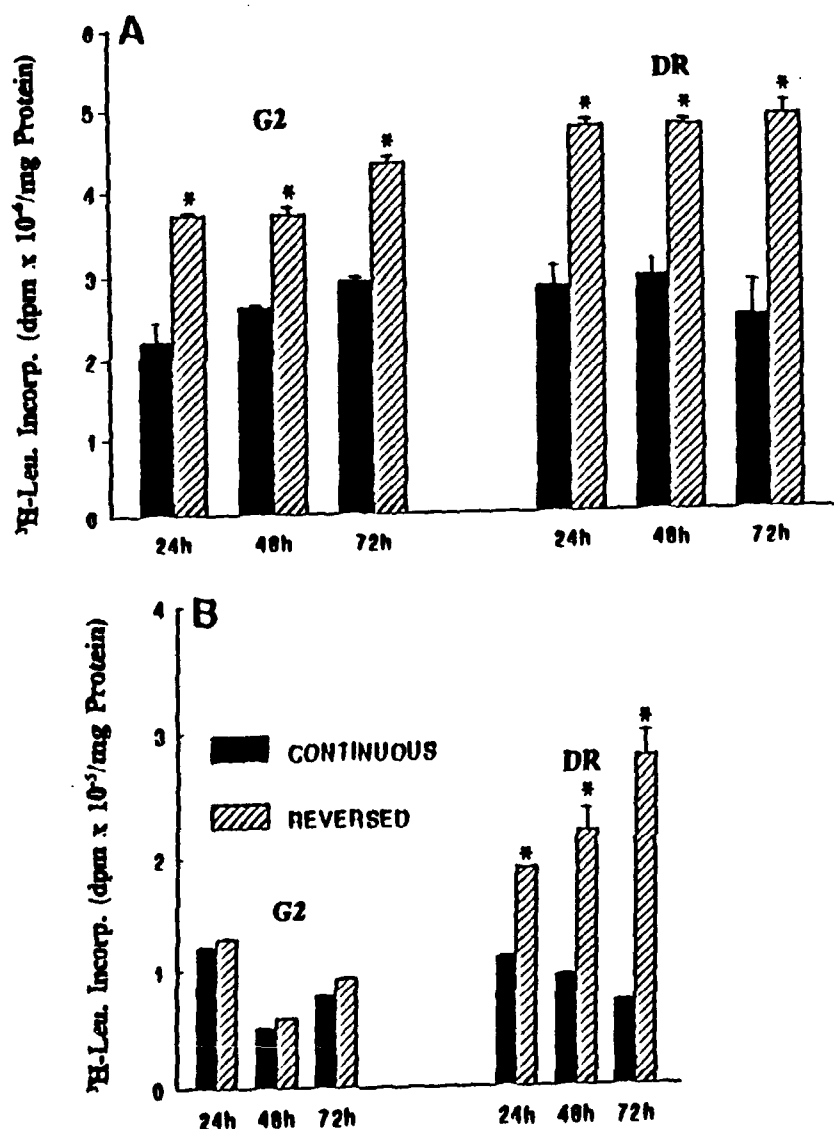

Fig. 6. Recovery of biochemical processes following the removal of pentamidine from the culture medium. Following an initial 48-h pentamidine treatment, the cells were washed and maintained in DMEM alone (reversed) or continued in DMEM containing fresh pentamidine. A series of each was then labeled with [³H]-leucine over a 3-day period to determine whether the reversed cells are capable of recovering from the effects of pentamidine. In panel A, it is clear that both G2 and DR reversed cells significantly increase their rate of total protein synthesis within the initial reversal phase. This pattern persists throughout the 72-h recovery period, $p < 0.003$. In panel B, only the reversed drug-resistant (DR) cells recover their ability to secrete albumin at levels comparable to that seen prior to pentamidine treatment. As noted for total protein synthesis, this is seen during the initial reversal phase and remains throughout (compare panels A and B). * Significantly stimulated over the continuously treated cells at $p < 0.002$ level, Student's t-test.

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DISCUSSION

Pentamidine has been shown to have a "cidal" role against protozoa, possibly via inhibition of nucleic acid and protein synthesis (Bornstein & Yarbro, 1970) or glucose metabolism (Pesanti & Cox, 1981). While it is used widely in the treatment of protozoan infections in AIDS patients, little is known concerning its effects on host tissues. The results of our studies corroborate those reported for protozoa, but also suggest that there may be a different mechanism of action or of metabolizing pentamidine by sensitive and resistant cell lines derived from the same tissue. The concentrations of pentamidine that we used (1 & 5μM) are in the range found in blood plasma following its administration to patients (Waalkes & DeVita, 1970). Specifically, our results indicate that protein synthesis in general, as well as tissue-specific functions such as albumin synthesis and secretion, are significantly affected by the drug. This is important to note, since these changes occur within the first 48-h use of a 5μM concentration of pentamidine, while most drug treatment protocols call for a 14-day or longer treatment course. There was also an affect on the rate of cell division, as measured by thymidine incorporation by drug sensitive HEP-G2 cells. This alone may not be of major concern in a patient, since the liver is not an organ in which the parenchyma cells readily divide. On the other hand, pentamidine is reported not to undergo hepatic biotransformation in humans, and is only slowly eliminated from the body due to its extensive binding to mammalian tissues (Waalkes et al. 1970). Thus, the drug has the potential of affecting most tissues of the body over this extended time frame. While the use of pentamidine is targeted to lung infections, the manner in which it is eliminated, as well as its route of administration, also lend credence to the possible involvement of other organs. Further, reports are now beginning to appear in the literature which document extrapulmonary *Pneumocystis carinii* infections in AIDS patients. These extrapulmonary sites include the liver, kidneys and spleen (Cote et al. 1990; Witt et al. 1991). The liver was also a primary choice in which to study the effects of pentamidine, because of its overall role in drug detoxification and numerous other essential functions.

The slow release of pentamidine along with its initial effects, prompted us to assess the reversibility of these altered processes once the drug was removed from the culture medium. In medium lacking pentamidine, the cells regain both their normal growth rate and morphology. The two cell lines significantly increase their rate of total protein synthesis and the HEP-G2DR subclone regains its ability to express the tissue-specific function of albumin synthesis and secretion. Surprisingly, this was not the case for the parental HEP-G2 cells. These cells did not significantly increase their rate of albumin secretion even after three days of growth. This suggests that drug sensitivity may play a role in the way these cells metabolize and/or eliminate pentamidine. This would not be surprising, since the HEP-G2DR cells were selected for their drug-resistant ability to grow in a low maintenance dose of adriamycin. It has been shown that prolonged treatment with one drug can cause resistance to be established to that as well as other structurally unrelated drugs (Cowan et al. 1986; Beck, 1987). This process is known as multidrug resistance, and it is not known whether these cells express this particular phenotype. It is known, however, that pentamidine increases the survival rate of immunodeficient individuals with *Pneumocystis carinii* pneumonia. Therefore, because this drug has been shown to be efficacious, it is important to know as much as possible about its mode of action especially since it has been documented to produce severe side effects. A better understanding of these secondary toxic effects should lead to improved drug treatment regimens in attempting to deal with repeated episodes involving protozoan infections in immunosuppressed individuals.

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