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EFFECTS OF LEVAMISOLE ON PRIMARY CULTURES OF ADULT RAT HEPATOCYTES

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Abstract—Levamisole represents one of several new compounds that exhibit immunomodulating activity. Pharmacological data have documented a relationship between liver drug metabolism of levamisole and its subsequent immunomodulating activity. To directly investigate this relationship in a controlled manner, primary cultures of adult rat hepatocytes were treated with levamisole, and ultrastructural and biochemical effects were analyzed. Ultrastructurally, levamisole did not disrupt the cellular architecture of the hepatocytes. Biochemically, levamisole stimulated alkaline phosphatase activity and elevated microsomal cytochrome P-450 content after a 48-hr incubation. High pressure liquid chromatographic analysis of levamisole metabolites produced by cultured hepatocytes suggested the formation of a hepatocyte-specific metabolite(s) that may be associated with its immunological mode of action.

Levamisole is one of several new synthetic compounds possessing immunomodulating properties of medical importance to man [1–4]. Although therapeutic treatment with levamisole has been reported [5–7], the exact *in vivo* mechanism of action remains unresolved. Limited knowledge of the *in vivo* effects of this agent exists because the parent compound does not appear to directly mediate the immunomodulating functions [1–4].

Pharmacological studies have demonstrated that levamisole is absorbed rapidly after oral administration, is metabolized extensively by the liver, has a plasma half-life of only 4 hr, and has a non-linear dose–response effect [4, 8–10]. Graziani and DeMartin [8] and Benard *et al.* [11] have shown that radioactive levamisole is concentrated in the liver as soon as 30 min after oral administration. Although levamisole is rapidly metabolized by the liver, immunological effects persist 5–7 days after the administration of the drug [12]. Response to levamisole appears to be under polygenic control that is not associated with the major histocompatibility complex but is associated with the aromatic hydrocarbon hydroxylase locus (AHH), which suggests that the liver may play an important role in the immunological activity of levamisole [13, 14, ‡]. Based on this association, Renoux and Renoux [15] proposed that liver parenchymal cells (hepatocytes) may be the primary source of the immunomodulating activity of levamisole.

Although *in vivo* pharmacokinetics have documented the rapid metabolism/excretion of levamisole, the metabolite(s) associated with the immunomodulating activity of levamisole has not been characterized. Additionally, the probability of extrahepatic metabolism of levamisole *in vivo* greatly complicates the isolation and characterization of a hepatocyte-specific levamisole metabolite. Primary cultures of hepatocytes offer an excellent system for studying the metabolism of levamisole because they maintain many *in vivo* characteristics of the liver and can be manipulated easily. In this report, we describe the effects of levamisole treatment on the characteristics of adult rat hepatocytes in culture and show that levamisole is nonhepatotoxic at physiologically relevant concentrations and that a putative hepatocyte-specific levamisole metabolite is produced.

MATERIALS AND METHODS

Materials. Waymouth MB 752/1 medium was obtained from KC Biologicals, Lenexa, KS. Delta-amino-levulinic acid (ALA) was obtained from Porphyrin Products, Logan, UT. Collagenase was obtained from the Millipore Corp., Bedford, MA (100–125 units/mg). HEPES [4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid] was obtained from Research Organics, Inc., Cleveland, OH. Pyruvic acid (sodium salt) and bovine serum albumin (BSA) were obtained from the U.S. Biochemical Corp., Cleveland, OH. Levamisole, all amino acids, hormones, heme and acid soluble calf skin collagen were obtained from the Sigma Chemical Co., St. Louis, MO. Female, Fisher F344 retired breeders (approximately 12 months of age) were obtained from Harlan Sprague Dawley, Inc., Madison, WI, and weighed between 230 and 275 g.

Hepatocyte isolation and cell culture. Hepatocytes were isolated by collagenase perfusion as previously described in detail [16]. In brief, a modified Hanks' balanced salt solution (HBSS) containing 10 mM pyruvate, 5 mM glutamine, 6.2 milliunits of insulin/ml and reduced salt to maintain osmolarity near 300 milliosmoles was used. Cell yield using 0.05% collagenase digestion was 400–700 million par-

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enchymal cells (hepatocytes) per liver with viability (trypan blue dye exclusion) exceeding 90%. Hepatocytes were cultured at a density of 10 million cells/10 ml of medium in 100 mm diameter collagen-coated culture dishes (Corning) and incubated at 35° in a humidified atmosphere of air. The culture medium used was Waymouths MB 752/1 modified as previously described [17] to contain: 5 mM glutamine, 5 mM asparagine, 2.5 mM lysine, 2 mM tyrosine, 2 mM phenylalanine, 0.5 mM serine, 0.5 mM alanine, 0.4 mM ascorbate, 0.2 mM ALA, 20 mM pyruvate, 0.1 mg/ml BSA, 30 mM HEPES, 2.5 mM bicarbonate, 25 μg/ml gentimycin (Schering Corp.), 0.5 μg/ml amphotericin B (Sigma), 0.1 μM selenious acid (Aldrich Chemical Co.), and 1 ml of an ethanol/propylene glycol nutritional and hormonal supplement. A modified Decad *et al.* [18] hormone/nutrient supplement consisted of the following: 5 μl DL-tocopherol acetate, 5 μl oleic acid, 5 μl linoleic acid, 10^{-8} M glucagon, 10^{-7} M testosterone, 10^{-7} M beta-estradiol, 10^{-7} M dexamethasone and 10^{-7} M insulin. The resulting medium was brought to one liter, sterilized by membrane filtration (0.22 μm) with a final pH of 7.6 and osmolarity near 400 milliosmoles as described by Schwarze *et al.* [19]. Heme was dissolved in 0.1 M NaOH containing 1 mg/ml BSA at a concentration of 0.6 mM, and 16.5 μl was added to 10 ml of medium to yield a 1 μM concentration of heme [17]. Levamisole was dissolved in culture medium and added to each dish to yield a final concentration of 0.005 to 0.5 mM. Heme and levamisole were added to fresh medium after an initial 4 hr incubation period during which monolayer formation was completed. Fresh medium (± levamisole) was added each day thereafter.

Cellular enzyme activities. Using five to seven dishes, hepatocytes were resuspended in a homogenization buffer (50 mM Tris, 150 mM KCl, pH 7.3) and homogenized with a Polytron PT-10, an aliquot was then taken for DNA determination [20]. A second sample was diluted with 0.25% Triton X-100 containing homogenization buffer and used for enzyme analysis and protein determinations. The following enzyme activities were determined: lactate of dehydrogenase (LHD) [21], tyrosine amino transferase (TAT) [22], gamma glutamyl transpeptidase (GGTP) [23], alanine aminotransferase (ALT) [24], alkaline phosphatase (AP) [25], and 5'-nucleotidase (5'-ND) [26]. The remaining homogenate was used for microsomal isolation [27], and post-microsomal supernatant fraction was used for determination of tryptophan pyrrolase (TP) enzyme activity [28]. One unit of enzyme activity equals that amount of enzyme catalyzing the formation of a product or a decrease in a substrate of 1 μmole/min at the defined conditions for each assay. Statistical analysis was performed using a one-way analysis of variance (ANOVA) [29].

Cytochrome P-450, cytochrome b_5*, and protein determinations.* Microsomes were isolated by differential centrifugation of homogenized and sonicated cells in 0.1 M sodium pyrophosphate (pH 7.4) containing 50 μM butylated hydroxytoluene (BHT) [27]. The 106,000 *g* pellet was resuspended in 0.5 M Tris–HCl (pH 7.4), 1 mM EDTA and 20% glycerol [27]. The extinction coefficients of 91 mM^{-1} and 185 mM^{-1} were used for the quantification of cytochrome P-450 and cytochrome b_5 respectively [30]. Benzphetamine *N*-demethylase activity of the microsomal fraction was determined as described by Rikans and Notley [31]. Benzphetamine was obtained from Upjohn Pharmaceuticals, Kalamazoo, MI. Protein determinations were performed by the method of Bradford [32].

Electron microscopy. Ultrastructural analysis was performed using a JEOL JM-100C transmission electron microscope. Cultures were washed twice with HBSS, fixed in 2.5% glutaraldehyde, washed twice with HBSS, fixed with 1% osmium tetroxide, scraped from the plate, and processed for electron microscopy [33].

High pressure liquid chromatographic analysis of levamisole and its metabolites. Hepatocyte conditioned medium (CM) was collected at 24-hr intervals through 72 hr of culture. Hepatocyte CM was collected by centrifugation (1000 *g*, 10 min, 4°) to remove any cells or cellular debris, sterilized by filtration (0.2 μm) and prepared for high pressure liquid chromatographic (HPLC) analysis. Up to 1 ml of CM, containing 1.2 μg of levamisole and/or its metabolites, was placed on disposable extraction columns (Octadecyl C_{18} reverse phase) (J. T. Baker Chemical Co.), slowly drawn through the column under vacuum, and washed twice with 1 ml of distilled H_2O.

The volume of CM added to the disposable extraction column was 1 ml, 0.1 ml and 0.01 ml for the cultures treated with 0.005, 0.05 and 0.5 mM respectively. This was done to ensure that similar quantities (1.2 μg) of drug and/or metabolites were analyzed under identical chromatographic conditions. This step improved subsequent HPLC analysis and removed potential contaminants from the culture medium (i.e. phenol red). After washing with distilled H_2O, glass test tubes (12 × 75 mm) were placed under each column, two 150-μl volumes of HPLC grade chloroform (Fisher Scientific Co.) were drawn through the column under vacuum to elute levamisole and its metabolites, and the samples were evaporated to dryness under N_2 at 40°. The residue was resuspended in 50 μl of HPLC mobile phase (0.2% glacial acetic acid in water–absolute methanol–heptane sulfonic acid, 55:45:2) [34]. The entire sample was injected into a Waters 600A solvent delivery system, and reverse phase ion-paired HPLC [34] was performed using a radial compression separation system Z module with an Octadecyl C_{18} radial-pak liquid chromatography cartridge (Waters Associates, Inc.). Chromatographic standardization of levamisole was performed with levamisole dissolved in HPLC mobile phase and of levamisole extracted from culture medium after incubation for 24 hr at 35° in the absence of hepatocytes. Extraction efficiency of levamisole was found to exceed 90%. Levamisole metabolites are not available for standardization and were therefore identified based on their retention times.

RESULTS

Preliminary experiments were performed to determine the effect of levamisole on cellular viability

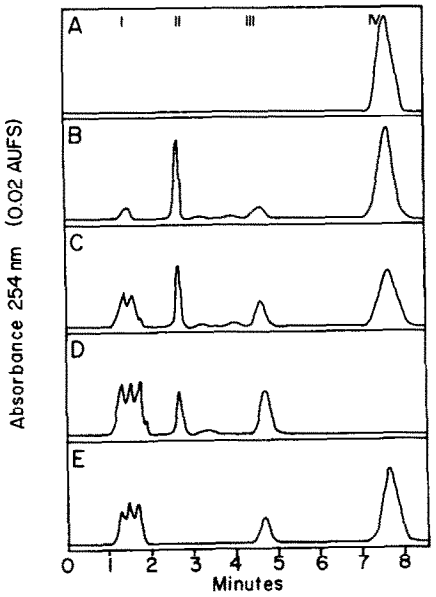

Fig. 1. High pressure liquid chromatographic analysis of levamisole metabolism by cultured rat hepatocytes. Cultured hepatocytes were incubated with 0.5 mM (B), 0.05 mM (C) or 0.005 mM (D) levamisole, and conditioned medium (CM) was collected after 24–48 hr of incubation. Cultured fibroblasts (E) were also incubated with 0.05 mM levamisole for 24 hr. The elution profile of the parent compound levamisole without any prior metabolism is shown in A. The volume of CM assayed was adjusted according to original levamisole concentration.

and morphology. When cultured hepatocytes were treated with levamisole concentrations greater than 1 mM, toxicity was detected by vital dye (trypan blue) staining and resulted in monolayer detachment (data not shown). It was subsequently found that concentrations of levamisole from 0.005 to 0.5 mM did not affect the viability of cultured hepatocytes through 1 week of treatment.

Metabolism of levamisole by cultured hepatocytes was studied using HPLC analysis of conditioned medium (CM). Ion-pair reverse phase HPLC was performed by mobile phase pH adjustment such that the ionic form(s) of levamisole and its metabolites were present, and a counter ion containing a strong lipophilic group (heptanesulfonic acid) was used for selective elution [34]. The detection of levamisole and its metabolites was based on the absorbance of its aromatic ring. Because many other biological compounds also have absorbance maxima at or near 254 nm, the possible contamination of CM extracts by other u.v absorbing compounds was determined. When either fresh culture medium or CM from untreated cultures was extracted and analyzed, in agreement with the data of Alvinerie *et al.* [34], no compounds exhibiting absorbance at 254 nm were detected (data not shown). The retention time of levamisole is shown in Fig. 1A. Because no other compounds eluted prior to the parent compound in untreated hepatocyte CM, the earlier elution of compounds absorbing at 254 nm from levamisole-treated hepatocyte CM were assumed to represent levamisole metabolites (Fig. 1). When cultured hepatocytes were treated with 0.005 mM levamisole for 24 hr, levamisole was completely metabolized to more hydrophilic components (Fig. 1D). Three groups of absorbing compounds were detected (Peaks I, II, and III). Peak I appeared to contain a group of hydrophilic compounds eluting prior to 2.5 min. Peaks II and III appeared to contain single hydrophilic compounds that eluted from the column at 2.8 and 4.5 min respectively. Although Peaks II and III appeared as single peaks, the presence of multiple compounds in each single peak cannot be discounted. Peak IV represents the parent compound levamisole which eluted from the column at 7.5 min. When cultured hepatocytes were treated with 0.005 mM levamisole, none of the parent compound remained after 24 hr. However, the metabolism of 0.05 and 0.5 mM levamisole by the cultured hepatocytes was saturated as shown by the presence of unmetabolized drug in the CM after 24 hr of incubation (Fig. 1C and 1B).

To detect metabolism occurring by mechanisms nonspecific to the hepatocyte, cultured fibroblasts were treated with 0.05 mM levamisole for 24 hr and the fibroblast CM was analyzed (Fig. 1E). Drug metabolism by fibroblasts is considered minimal, to the extent that V-79 cells (Chinese hamster lung fibroblasts) do not possess a full complement of drug-metabolizing enzymes [35]. As recently described by

Table 1. Percentage of levamisole metabolites formed

Levamisole concentration (mM)	Metabolites of levamisole (%)			Levamisole (%)
	Peak I	Peak II	Peak III	Peak IV
0.5	6	29	1	64
0.05	37	29	17	13
0.005	61	12	27	None
Fibroblast*	20	None	45	35

Cultured hepatocytes (N = 3–6) were treated with the concentration of levamisole indicated, and CM was collected after the 24–48 hr incubation period. The CM was extracted and analyzed by HPLC as described in Materials and Methods. The areas under each peak were used to determine the percentage of levamisole metabolized. Data represent the mean value obtained.

* Fibroblast cultures (N = 3) were treated with 0.05 mM levamisole for 24 hr.

Bend *et al.* [36], although the lung contains, at lower concentrations, virtually all of the hepatic pathways required for biotransformation, not all hepatic isozymes are present. Therefore, a deficiency in the P-450 isozyme mediating levamisole metabolism may exist in the human foreskin fibroblasts used in this study. However, several metabolites of levamisole were detected in fibroblast CM after treatment. Absent from the fibroblast CM was the metabolite(s) located in Peak II, which was always detected in hepatocyte CM. When the percentage of levamisole metabolites formed by cultured hepatocytes was determined, a dose response in metabolite formation was detected (Table 1). At the lowest concentration of levamisole examined (0.005 mM), levamisole was totally metabolized within 24 hr to hydrophilic compounds. When cultured hepatocytes were treated with 0.05 and 0.5 mM levamisole for 24 hr, 13 and 64% of the parent compound remained unmetabolized respectively. Although the percentage of levamisole metabolized decreased with an increasing dosage, the relative concentration of levamisole metabolized actually increased. Therefore, the maximal, non-toxic concentration of levamisole (0.5 mM) was used in all further experiments.

The ultrastructure of hepatocytes treated with 0.5 mM levamisole was unaltered with respect to subcellular architecture and cell-to-cell interactions (Fig. 2). Bile canaliculi and associated tight junctions, characteristic of hepatocytes *in vivo*, were reformed in culture and were not affected by levamisole treatment. No organelle, cell membrane or nuclear membrane damage was evident either ultrastructurally or morphologically.

To study the effect of levamisole treatment on hepatocyte physiology, a set of enzymes was chosen that is characteristic of the cellular physiology of cultured hepatocytes [37, 38]. Lactate dehydrogenase (LDH) was chosen as an enzyme marker of cellular viability because leakage of this enzyme into the culture medium indicates the loss of membrane integrity. Alkaline phosphatase (AP) was chosen because activity of this enzyme is related to bile formation. Alanine aminotransferase (ALT) was chosen because this enzyme is associated with the formation of pyruvate and energy from amino acid precursors. 5′-Nucleotidase (5′-ND) was chosen because this enzyme has important physiological functions in the maintenance of intracellular reducing equivalents. Gamma glutamyl transpeptidase (GGTP) was chosen because this enzyme is associated with fetal and neoplastic hepatocyte physiology.

The effect of 0.5 mM levamisole treatment on these five cellular enzymes is shown in Table 2. The cellular enzyme activities after culturing in the presence or absence of levamisole were statistically compared by one-way ANOVA to the enzyme activities detected in freshly-isolated hepatocytes (0 hr). LDH activity significantly increased ($P < 0.05$) above the 0-hr level with or without levamisole after 48 and 72 hr of incubation. ALT and GGTP enzyme activities decreased significantly ($P < 0.05$) below the 0-level with or without levamisole at 48 and 72 hr of incubation. The activity of 5′-ND did not change significantly during culturing with or without levamisole. The only enzyme system that exhibited any significant change after levamisole treatment was AP. Although the AP activity was elevated sig-

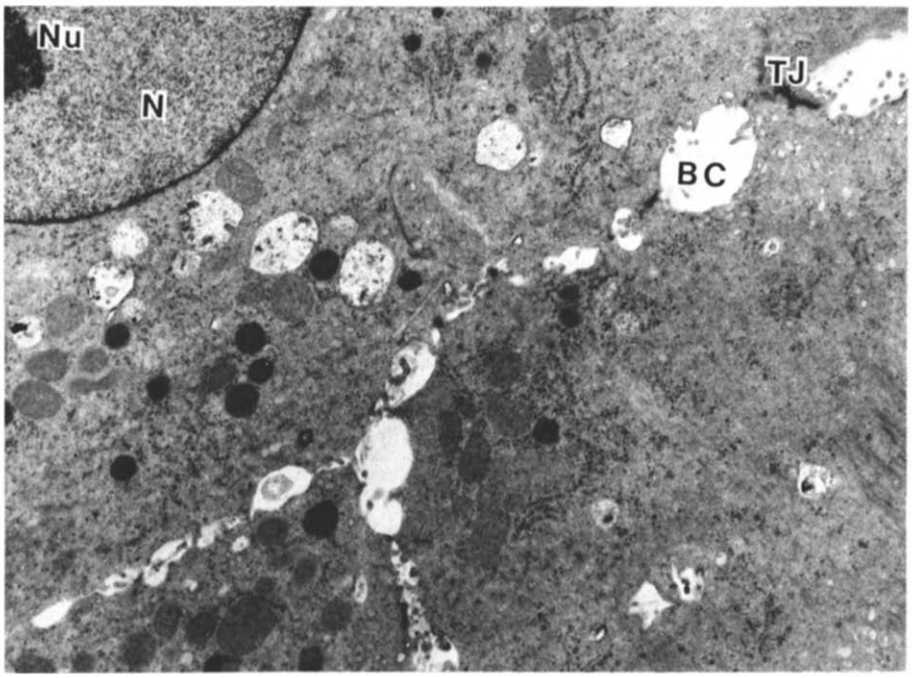

Fig. 2. Transmission electron micrograph of levamisole-treated cultured rat hepatocytes. Hepatocytes were cultured in the presence of 0.5 mM levamisole for 96 hr, fixed with glutaraldehyde, scraped off the dish, and processed for microscopy. The well developed bile canaliculus (BC) formation with associated membrane tight junctions (TJ) can be seen. N, nucleus, Nu, nucleolus. Magnification: 14,000×.

Table 2. Effect of levamisole on cellular enzyme levels in cultured rat hepatocytes

Culture time (hr)	Medium additions	Cellular enzyme activity (milliunits/mg protein)				
		LDH	ALT	5'-ND	AP	GGTP
0		3142 ± 217 (8)	257 ± 29 (11)	11.0 ± 0.4 (5)	5.9 ± 1.4 (11)	4.3 ± 0.6 (10)
24	None	3174 ± 114 (6)	175 ± 31 (9)	10.8 ± 1.0 (4)	65 ± 8* (5)	3.0 ± 0.5* (9)
	Levamisole	3104 ± 139 (4)	193 ± 29 (6)	9.9 ± 0.5 (3)	101 ± 16* (6)	3.9 ± 0.7 (5)
48	None	3919 ± 214* (8)	142 ± 21* (9)	9.1 ± 0.5 (5)	80 ± 6* (9)	2.2 ± 0.3* (8)
	Levamisole	3792 ± 111* (4)	152 ± 29* (6)	8.8 ± 0.5 (4)	141 ± 16*† (7)	2.7 ± 0.4* (5)
72	None	3767 ± 168* (8)	141 ± 28* (9)	9.5 ± 0.4 (6)	87 ± 9* (8)	2.4 ± 0.2* (9)
	levamisole	3911 ± 448* (4)	142 ± 33* (5)	10.0 ± 0.4 (4)	158 ± 24*† (6)	2.7 ± 0.4* (5)

Hepatocyte cultures were incubated with or without 500 μM levamisole. All values represent the mean ± S.E.M. of data obtained from hepatocytes isolated and cultured from the number of animals indicated in parentheses. Abbreviations: LDH, lactate dehydrogenase; ALT, alanine aminotransferase; 5'-ND, 5'-Nucleotidase; AP, aklaline phosphatase; and GGTP, gamma glutamyl transpeptidase.

* Significantly different ($P < 0.05$) from the 0-hr value of the respective enzyme activity.

† Significantly different ($P < 0.001$) from the corresponding enzyme activity of untreated cultures at the same culture period.

nificantly ($P < 0.05$) under both culture conditions, levamisole treatment resulted in a significantly higher ($P < 0.001$) activity than untreated cultures at 48 and 72 hr of incubation.

The responsiveness of levamisole-treated cultured hepatocytes to hormonal stimulation was studied by measuring the induction of TAT and TP by dexamethasone. As shown in Fig. 3, the dexamethasone induction of TAT and TP was not affected by levamisole treatment. The activities of TAT and TP were not significantly different from the values obtained in untreated cultures, as previously shown [17]. Therefore, hormonally responsive hepatocytes that retained many ultrastructural and enzymatic characteristics of hepatocytes *in vivo* were maintained in culture after levamisole treatment.

Levamisole has been reported to induce cytochrome P-450 (P-450) and cytochrome b_5 (b_5) content

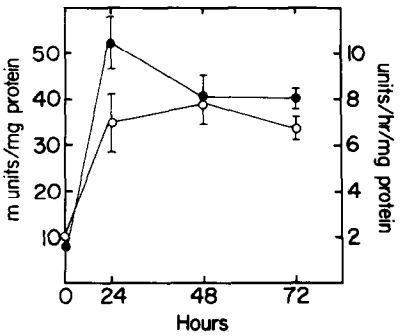

Fig. 3. Effect of levamisole on hormonal responsiveness of cultured rat hepatocytes. Induction of tyrosine aminotransferase (TAT) (●) and tryptophan pyrolase (TP) (○) in cultured hepatocytes above the basal values detected in freshly isolated hepatocytes by dexamethasone (10^{-7} M) in the presence of 0.5 mM levamisole. The basal units of TAT and TP were 8.36 ± 0.59 milliunits/mg protein and 2.02 ± 0.03 units/hr/mg protein respectively (means ± S.E.M., N = 3–10). All data points for both TAT and TP activities were significantly higher ($P < 0.01$) than the 0-hr values.

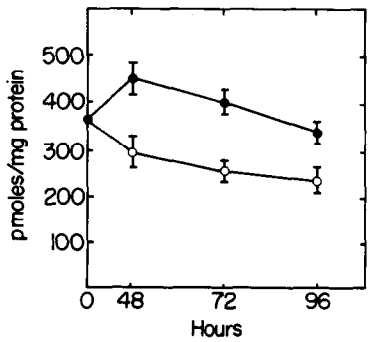

Fig. 4. Effect of levamisole treatment on cytochrome P-450 content of cultured rat hepatocytes. Hepatocytes were incubated in the presence (●) or absence (○) of 0.5 mM levamisole for 96 hr. The basal value of P-450 was 360 ± 53 pmoles/mg protein (mean ± S.E.M., N = 3–10). The microsomal P-450 contents of levamisole-treated and control cultures were not significantly different from 0-hr value through 72 hr. At 96 hr, control culture P-450 content was decreased significantly ($P < 0.01$). Throughout the culture period, P-450 content of levamisole-treated cultures was significantly higher ($P < 0.05$) than controls.

of the liver after *in vivo* administration [39]; therefore, the effect of levamisole on the microsomal cytochrome content of cultured hepatocytes was also determined (Figs. 4 and 5). Although a slight increase in P-450 content of levamisole-treated cultured hepatocytes above the 0-hr value was detected after 48 hr of incubation, the increase was not statistically significant ($P > 0.05$) (Fig. 4). However, the P-450 content of levamisole-treated hepatocytes was significantly higher ($P < 0.05$) than untreated cultures at 48 and 72 hr of incubation. A decline in b_5 content was seen in both levamisole-treated and untreated cultures (Fig. 5); the decline in b_5 content was significant ($P < 0.001$), but the difference between levamisole-treated and untreated cultures was not. The effect of levamisole on the mixed-function oxidase (MFO) activity of the cultured

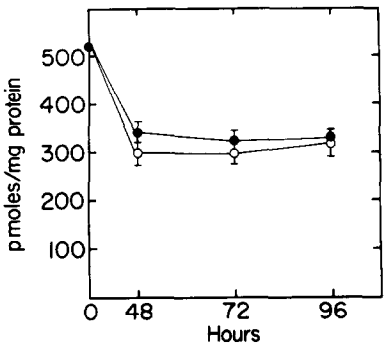

Fig. 5. Effect of levamisole treatment on cytochrome b_5 content of cultured rat hepatocytes. Hepatocytes were incubated in the presence (●) or absence (○) of 0.5 mM levamisole for 96 hr. The basal value of b_5 was 518 ± 17 pmoles/mg protein (mean ± S.E.M., N = 3–10). Microsomal b_5 content of all cultures was reduced significantly ($P < 0.001$) from the 0-hr value.

Table 3. Effect of levamisole on benzphetamine *N*-demethylase activity of microsomes

Culture time (hr)	Benzphetamine *N*-demethylase activity (nmoles/min/mg protein)	
	Untreated	0.5 mM Levamisole
0	5.48 ± 0.64	5.48 ± 0.64
24	5.35 ± 0.62	5.13 ± 0.49
48	4.71 ± 0.85	4.30 ± 0.46
72	4.76 ± 0.91	4.73 ± 0.89

Hepatocytes were cultured in the presence or absence (untreated) of 0.5 mM levamisole. The *N*-demethylation of benzphetamine by microsomes isolated from cultured hepatocytes was determined. Each value represents the mean ± S.E.M. of data obtained from hepatocytes isolated and cultured from three to six animals. There were no significant changes detected in benzphetamine *N*-demethylase activity in either levamisole-treated or untreated hepatocyte cultures.

hepatocytes was also investigated. The *N*-demethylation of benzphetamine was utilized as a measure of the MFO system (Table 3). The benzphetamine demethylase activity of cultured hepatocytes did not change significantly with culturing for up to 72 hr in either levamisole-treated or untreated cultures.

DISCUSSION

This study represents the first report of the effect of levamisole on cultured hepatocytes. By utilizing primary cultures of liver parenchymal cells, it was possible to study the effect of levamisole on a single cell type without interference from other organs and cell systems. We have shown that up to 0.5 mM levamisole was non-hepatotoxic to cultured rat hepatocytes. The concentration of levamisole in the liver after *in vivo* treatment has been shown to exceed 500 μg/g of tissue [40]. Schnieden [10] has reported that the peak blood level of levamisole in the rat after oral treatment is 0.04 mM. Therefore, the concentrations of levamisole used in our study (0.005 to 0.5 mM) are physiologically relevant.

When cultured hepatocytes were treated with 0.5 mM levamisole, no significant changes were observed in cellular ultrastructure or hormonal responsiveness. Because no change was observed in the activities of most of the enzymes studied, it appears that levamisole has very little effect on the cellular physiology of cultured hepatocytes. These data substantiate the *in vivo* observation of Symoens and Rosenthal [2] that levamisole is nonhepatotoxic. It has also been shown that levamisole, along with other immunomodulators, protects the liver against lipid peroxidation and membrane pertubations by free radical scavenging, thereby preserving liver function [41, 42]. However, the AP activity of levamisole-treated hepatocytes was significantly higher than those of untreated hepatocytes. Because AP activity is associated with bile formation, the increased activity of AP in levamisole-treated cultures may represent the formation of bile transported levamisole metabolites. These metabolites have been detected *in vivo* [8, 9].

Levamisole treatment significantly increased the P-450 content of the cultured hepatocytes above the level detected in untreated cultures. These data substantiate the observation of Reinke *et al.* [39] that *in vivo* levamisole treatment increases liver P-450 content. Although levamisole treatment *in vivo* induces P-450 content in female rats, the modest increase (22%) requires ten daily injections of 20 mg/kg [39]. Similarly, levamisole treatment was found to increase the P-450 level of cultured hepatocytes above the 0-hr value after 48 hr of incubation. In contrast, the induction of P-450 by phenobarbital using our culture system was found recently to be significantly above the 0-hr value [17]. Other immunomodulating agents used to treat rheumatic diseases decrease cytochrome P-450 content [43, 44], whereas levamisole has a slight stimulatory effect on the mixed-function oxidase (MFO) system of the liver. Although levamisole increased the P-450 content of cultured hepatocytes, levamisole had no effect on cytochrome b_5 or the ability of hepatocytes to *N*-demethylate benzphetamine (Fig. 5, Table 3). Reinke *et al.* [39] reported a modest increase in liver b_5 content and the N-demethylation of aminopyrine after chronic levamisole treatment *in vivo*. Because the benzphetamine demethylase activity did not correlate with the P-450 concentration of the cultured hepatocytes, the single substrate used to assess the MFO system may not accurately represent the complete MFO capabilities of the culture hepatocytes. Therefore, it appears that the subspecies of P-450 mediating benzphetamine demethylase is not rate limiting for this MFO activity. Guzelian *et al.* [45, 46] reported that various MFO activities of cultured hepatocytes do not correlate with P-450 levels maintained *in vitro*. This suggests that P-450 reductase, or a general decline in the MFO system *en toto*, may be rate limiting in our cultured hepatocyte system.

To analyze the metabolites of levamisole produced by cultured hepatocytes, a simplified system was developed to extract levamisole and its metabolites from potentially interfering substances found in tissue culture medium (e.g. phenol red, hormones,

etc.). Disposable C_{18} extraction columns allowed us to remove salts, phenol red, and other potentially interfering compounds from the CM. This extraction procedure may also be useful for analysis of levamisole and its metabolites in biological fluids. Using the HPLC system developed by Alvinerie *et al.* [34], we were able to study the metabolism of levamisole by cultured rat hepatocytes.

The HPLC analysis of levamisole metabolism by the cultured hepatocytes suggests the retention of phase I and phase II drug metabolism reactions (Fig. 1). The elution order of compounds from reverse phase HPLC columns are generally related to their increasing hydrophobic nature, i.e. the more water soluble the compound, the faster it will elute from the column. Because levamisole metabolites are not available for accurate standardization, we have speculated as to the identity and type of levamisole metabolite(s) located in each fraction. The compounds located in peak I could represent the products of synthetic reactions in which conjugates are formed between levamisole or metabolite(s) and endogenous compounds involved in phase II reactions (e.g. glucuronic acid, glutathione, etc.). The compound(s) located in peak II, since only obtained from hepatocyte CM, may represent the product(s) of a P-450 catalyzed phase I reaction (e.g. oxidation or reduction) that is not found in fibroblasts. Although fibroblasts contain little readily detectable P-450 [35], the absence of the peak II metabolite(s) after fibroblast biotransformation does not definitively define this(these) metabolite(s) as a phase I, hepatocyte P-450 mediated product. Structural analysis of this fraction and metabolic inhibition of its generation by a specific P-450 inhibitor are required before completely validating our hypothesis. Based on the retention time data reported by Alvinerie *et al.* [34], the compound located in peak II would appear to be para-hydroxylevamisole, a major urinary levamisole metabolite. Graziani and DeMartin [8] have also reported that para-hydroxylevamisole formation (phase I) and subsequent conjugation (phase II) represent the primary metabolite of levamisole. Adams [9] has similarly reported two major and six minor metabolites of levamisole with extensive conjugates detected in urinary metabolites. Another major levamisole metabolite is produced by thiazolidine ring hydrolysis and conjugation [8]. Although the opening of the thiol ring of levamisole yields OMPI [2-oxo-3-(2-mercaptoethyl)-5-phenylimidazolidine], detection of this water-soluble metabolite [10] in the hepatocyte CM would require additional oxidation/hydroxylation and/or conjugation. Although not directly identified with synthesized standards, the chromatographic detection of putative levamisole metabolites in hepatocyte CM suggests that *in vivo*-like metabolism had occurred.

Extensive experimental and clinical studies have shown levamisole to have unique immunological properties; however, the exact *in vivo* mode of action of levamisole remains unknown. Although levamisole is rapidly metabolized by the liver, an immunomodulating factor(s) has been found in the serum that may have originated from the liver [14, 15‡]. Because the liver is the major source of most serum proteins and drug metabolites, the effect of levamisole treatment on primary cultures on hepatocytes would provide an ideal system to attempt to isolate and characterize the levamisole-induced immunomodulating factor(s). We have shown that levamisole is non-hepatotoxic *in vitro*, and our cultured hepatocyte system retains many characteristics of the liver *in vivo*. A major functional characteristic of the cultured hepatocytes is the formation of many hydrophillic levamisole metabolites that may also be produced after *in vivo* administration of levamisole. Such levamisole metabolites may represent or stimulate the production of the reported immunomodulating factor(s) obtained *in vivo*. Therefore, if levamisole is able to stimulate a hepatocyte-mediated immunological factor, the conditioned media from levamisole-treated hepatocyte cultures should contain this(these) factor(s). Future studies will investigate the production of levamisole-induced factors by cultured hepatocytes using the system described in this study.

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‡ R. F. Van Ginkel, Unpublished Report: Jansen Pharmaceutical, Beerse, Belgium (1976).

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