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THE CHARACTER OF 1,1,1-TRICHLORO-2,2-BIS-(*p*-CHLOROPHENYL)ETHANE
RESISTANCE IN MOUSE L 5178 Y LYMPHOMA CELLS

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Abstract—A population of DDT-resistant mouse L 5178 Y leukemic cells was selected by chronic treatment with sublethal concentrations of DDT. The DDT-resistant cells also exhibited resistance to the DDT pesticide analogues, Kelthane, DDD and methoxychlor, and to DDE, a DDT metabolite characteristic of mammalian detoxification. Compared with susceptible (control) L 5178 Y cells, the relative resistance to Kelthane was 5-fold greater than that to DDT, while resistance to methoxychlor was one-half that to DDT. A clone of cells selected from the resistant cell population and carried in continuous culture through 300 generations in the absence of DDT treatment retained the resistant characteristics of the parent culture. Though DDA was the least toxic DDT analogue tested, it was toxic to the same degree in both susceptible (control) and resistant leukemic cells. Treatment of resistant clones with ^{3}H-DDT indicated that resistance was not being expressed by detoxification or by structural alteration of the parent DDT molecule. It is concluded that the resistance to DDT expressed by resistant L 5178 Y cells must be due to a mechanism different from the established degradative systems described for insects and higher organisms.

THE TOXICITY of some organophosphorus and organochlorine pesticides to several tissue culture lines has been demonstrated, along with concomitant effects by several of the compounds on protein and nucleic acid synthesis.[1–4] At the same time, Gabliks[5] has shown that Hela and mouse L-929 cells become resistant upon chronic treatment with Di-syston,* chlordane or Dipterex. The mechanism of resistance was not determined, but was postulated to occur through selection of resistant cell types, induced mutations followed by selection or by induction of detoxicating enzymes. The latter mechanism is supported by several recent studies. It has been shown, for example, that aryl hydrocarbon hydroxylases are induced by benz[a]anthracene in mouse and hamster embryo cells.[6–8] More recently, DDE was found in ^{14}C-DDT-treated Hela cells,[9] which suggests the presence of a microsomal dehydrochlorinase.[10–13]

Recent studies in our laboratory on the mechanisms of toxicity and resistance of cultured mammalian cells to chlorinated hydrocarbons have revealed that cells can

* Abbreviations: *p,p'*-DDT, 1,1,1-trichloro-2,2-bis-(*p*-chlorophenyl) ethane; *p,p'*-DDE, 1,1-dichloro-2,2-bis (*p*-chlorophenyl)ethylene; *p,p'*-DDD, 1,1-dichloro-2,2-bis-(*p*-chlorophenyl)ethane; DDA, bis-(*p*-chlorophenyl) acetic acid; methoxychlor, 1,1,1-trichloro-2,2-bis(*p*-methoxyphenyl) ethane; Kelthane, 4,4'-dichloro-*α*-(trichloromethyl) benzhydrol; Di-syston, *o,o*-diethyl-S-(2-ethylthio) ethyl phosphorodithioate; Chlordane, 1,2,4,5,6,7,8,8-octachloro-3a,4,7,7a-tetrahydro-4,7-methanoindan; Dipterex, *o,o*,-dimethyl-(1-hydroxy-2,2,2-trichloroethyl) phosphonate; DMSO, dimethylsulfoxide.

develop resistance to several pesticides via mechanisms which apparently do not involve enzymatic or other types of modifications of the original pesticide molecule. This would appear to represent a heretofore little studied and novel defense exhibited at the cellular level against potentially harmful xenobiotic compounds. Accordingly, we have examined a number of aspects of tolerance of mouse L 5178 Y lymphoma cells to *p,p*-DDT and several other chlorinated hydrocarbons. The results of these experiments are reported here.

MATERIALS AND METHODS

Mouse L 5178 Y leukemic cells were grown in suspension as shake cultures in a model G25 Brunswick Gyrotory incubator-shaker at 37°. Fischer's medium, pH 7·0, was supplemented with 10% horse serum, 50 units of penicillin/l., 50 mg streptomycin/l., 2·2 g $NaHCO_3$/l., and 0·1% pluronic F-68 (Wyandotte Chemicals Corp., Wyandotte, Mich.). The cells were tested routinely for PPLO and were proven to be PPLO-free throughout the course of experiments described. Cell numbers were monitored in a model B Coulter counter. When pesticide toxicity was compared in control and resistant cell lines, cells were subcultured at an initial concentration of 0·125 $\pm$ 0·015 $\times$ 10^6 cells/ml in fresh media. *p,p'*-DDT and the various analogues dissolved readily in DMSO, at 75 mg/ml, and the final concentration of DMSO in the culture was held below 0·1%. In each culture series, one control culture was treated with the maximum volume of DMSO used. Inhibition of cell replication was measured after 24–27 hr of incubation at 37°. Toxicity is expressed in terms of the ID_{25} or ID_{50} concentration in micrograms per milliliter. Relative toxicity is expressed as an ID_{25} or ID_{50} ratio, e.g. ID_{50} resistant culture/ID_{50} control culture.

Cloning procedure

The cloning method was a modification of that reported by Chu and Fischer.[14] Fifty and 100 cells were suspended in 10-ml volumes of cloning media in 16 $\times$ 125 mm No. 3033 Falcon plastic tissue-culture tubes. Final concentrations of Noble agar and horse serum in the cloning medium were 0·15% and 20% respectively. In addition, the cloning medium was supplemented with 20% spent medium. The cultures were flushed with 5% CO_2 and air prior to setting the gel by cooling in an ice bath.

Cytochrome P-450 determination

Rat liver. Rats were sacrificed by decapitation. The liver was removed and washed in 0·25 M sucrose and homogenized in twice the volume (w/v) of 0·05 M tris–HCl buffer, pH 7·4. The homogenate was diluted to 10% (w/v) with buffer, and cell debris and mitochondria were removed by centrifugation at 11,000 *g* for 20 min. The supernatant was centrifuged at 105,000 *g* for 60 min. The microsomal pellet was washed in one-half of the previous volume of 0·15 M KCl at 105,000 *g* for 30 min, and then resuspended in tris–HCl buffer at the equivalent of 0·5 g liver/ml.

L 5178 Y mouse lymphoma cells. Approximately 2 $\times$ 10^9 cells of the RU-1 resistant culture were collected by centrifugation and washed in cold saline. All operations from this point on were carried out at 4°. The cell pellet was suspended in twice the volume of 0·05 M tris–HCl buffer, pH 7·4, and homogenized with a close-fitting

Dounce homogenizer. The supernatant from a 20-min centrifugation at 11,000 *g* was recentrifuged at 105,000 *g* for 60 min. The microsomal pellet was suspended in tris–HCl buffer at a concentration equivalent to $0{\cdot}5 \times 10^9$ cells/ml. The microsomal suspensions were further diluted either 1:3 or 1:6 with buffer prior to the cytochrome P-450 determinations, which were carried out according to methods described by Omura *et al.*[15,16] For detection of cytochrome P-450, the microsomal suspension was placed in the sample cuvette and treated with a few crystals of dithionite to reduce the cytochrome P-450. After gassing the suspension with CO, the absorption spectrum was measured between 550 and 340 mμ. An absorption peak at 450 mμ indicated the presence of a CO–P-450 complex. The difference spectrum was measured in a Beckman DK-2A dual-beam spectrophotometer.

Thin-layer chromatography

Cell culture suspensions and media were extracted with twice the medium volume of acetone–ether (1:1). After filtration through No. 1 Whatman paper, the solvent phase was taken to dryness under an air stream. The residue was solubilized in a convenient volume of chloroform, and aliquots were spotted on Silica gel GF plates (Analtech, Inc.). Chromatograms were developed in one dimension either in the mixed solvent system hexane–chloroform (1:1) or *n*-heptane as indicated. In those experiments where ^{3}H-DDT or its possible metabolites were to be recovered, the area of Silica gel between the origin and the solvent front was divided into $\frac{1}{8}$ in. sections. These sections were scraped into scintillation vials to which were added 10 ml of scintillator solution made up from 1330 ml of Triton X-100, 2670 ml toluene, 22g 2,5-diphenyloxazole (PPO) and 400 mg 1,4-bis[2-(5-phenyloxazolyl)]-benzene (POPOP). Radioactivity was determined with a Packard Tri-Carb liquid scintillation spectrometer (model 3380–544).

Chemicals

Analytical grade *p,p'*-DDT, 99·9% pure, was a gift from Geigy Chemical Corp. *p,p'*-DDE, *p,p'*-DDD, *p,p'*-DDA, Kelthane and methoxychlor, all of analytical grade, were generously supplied by Dr. Lawrence Fishbein, of the Chemistry Branch of this Institute.

^{3}H-DDT, specific activity 5 mc/34·7 mg, was obtained from New England Nuclear Corp.

RESULTS

It was proposed that if a tissue culture line could be selected that was resistant to a specific pesticide or group of pesticides, that resistant line would not only offer an opportunity to study the mechanism of resistance, but by comparison would offer insight into the mechanism of cytotoxicity in susceptible cells as well.

Mouse L 5178 Y lymphoma cells have a relatively short generation time, $10{\cdot}5 \pm 0{\cdot}5$ hr, and are easily cultured as a single cell suspension at high density, for they do not attach to a surface. These characteristics prompted the selection of the L 5178 Y cell line from among several other established lines for an attempt to establish resistance to a class of organochlorine pesticides. Since the metabolic pathway of *p,p'*-DDT

detoxification has been partially defined in mammalian tissues,[12,17,18] it was chosen as a model pesticide.

Establishment of DDT-resistant cultures

L 5178 Y cells were subcultured at concentrations of 0·1 × 10^6 cells/ml in 150-ml shake cultures and treated with DDT at various concentrations in DMSO. L 5178 Y cells can tolerate either DMSO or ethanol in concentrations up to 0·1% without any apparent ill effect. A typical concentration curve is given in Fig. 1. DDT in concentrations of 1 × 10^{-5}, 2 × 10^{-5} and 3 × 10^{-5} M inhibits cell replication 27, 59 and 100 per cent, respectively, after 24 hr of treatment, a period slightly in excess of twice the doubling time of the culture. To adapt cells, 150-ml cultures were treated daily with 1 × 10^{-5} M of the insecticide in the following manner. Cells from a 24-hr culture were sedimented and suspended in fresh media at a concentration of 0·2 × 10^6 cells/ml.

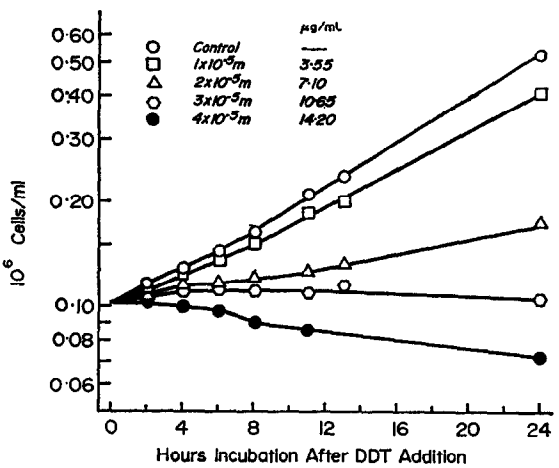

FIG. 1. *p,p'*-DDT toxicity in L 5178 Y cells.

DDT in DMSO was added in a volume of 0·05 ml. After 3 weeks of such treatment, a culture was adapted to the compound as indicated by the fact that all of the cells remained viable. At this time, the culture was divided. In one culture, the concentration of daily administered DDT was raised to 2 × 10^{-5} M. The other culture was treated as before. In another 3 weeks, the culture treated with the higher DDT concentration had stabilized and its resistance to DDT was compared to that of control (untreated) cells. Both control and resistant cells were exposed to DDT at various concentrations for 24 hr. The relative degrees of inhibition are given in Table 1. It is apparent that the resistant cells (DDT-R) tolerated amounts of DDT that result in 100 per cent inhibition of cell replication and cell death in control cultures.

The resistant character of the DDT-R culture was confirmed in a more sensitive system by comparing the cloning efficiency with that of the control culture over a range of DDT concentrations. The 50 per cent cloning efficiency for control cells in DDT occurred at a concentration of 9 μg/ml (Fig. 2a). In earlier cloning experiments, it was determined that 5 × 10^{-5} M DDT (17·8 μg/ml) was 100 per cent lethal for

TABLE 1. COMPARISON OF *p,p'*-DDT TOXICITY IN CONTROL AND RESISTANT L 5178 Y CELLS*

DDT concn		Per cent inhibition†	
(M)	(μg/ml)	Control culture	DDT-R culture
		0	0
1×10^{-5}	3·55	48	10
2×10^{-5}	7·10	82	20
3×10^{-5}	10·65	87	33
4×10^{-5}	14·20	100 CD‡	37
5×10^{-5}	17·75	100 CD‡	41

* Both control and DDT-R cells were subcultured at $0{\cdot}100 \times 10^6$ cells/ml in 150-ml suspension cultures.

† Per cent inhibition refers to inhibition of cell replication.

‡ There was a decrease in cell population from the initial concentration due to cell death (CD) at this concentration of DDT.

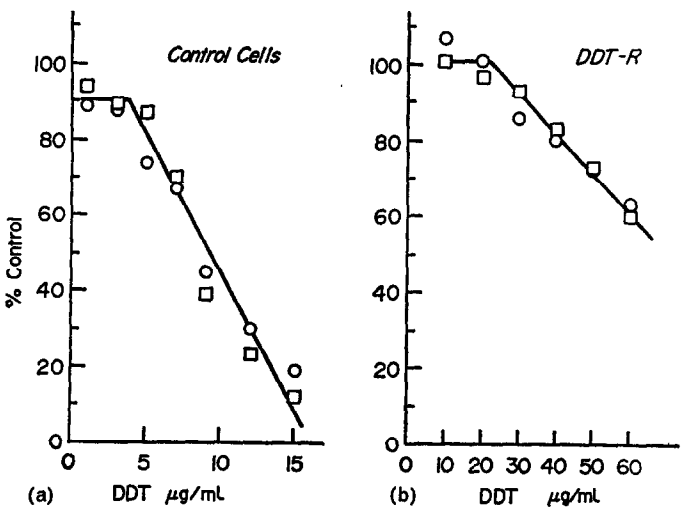

FIG. 2. Comparison of the effect of *p,p'*-DDT on the cloning efficiency in control (a) and resistant DDT-R (b) L 5178 Y cells. The open circles and open squares designate different experiments.

control cells. However, the cloning efficiency of cells from the DDT-R culture was higher than 50 per cent for all concentrations of DDT tested, up to 60 μg/ml (Fig. 2b). There was no attempt to further increase the concentrations of DDT, for the compound could not be kept in uniform suspension at higher levels. Thus the resistant character of the DDT-R culture was confirmed, for at a concentration of DDT (20 μg/ml) which was 100 per cent lethal to control cells, DDT-R cells had a cloning efficiency of 100 per cent.

Cross-resistance to DDT metabolites and analogues

Once the DDT-resistant culture was established, it was of interest to determine

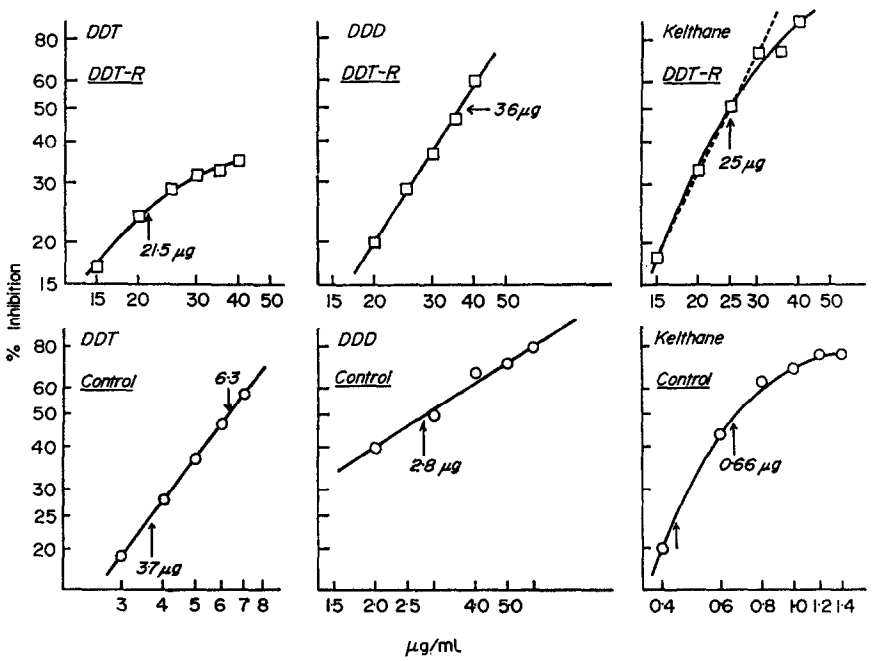

FIG. 3. Dose–response curves and comparison of ID_{50} concentrations in control and DDT-R cultures Per cent inhibition refers to inhibition of cell replication.

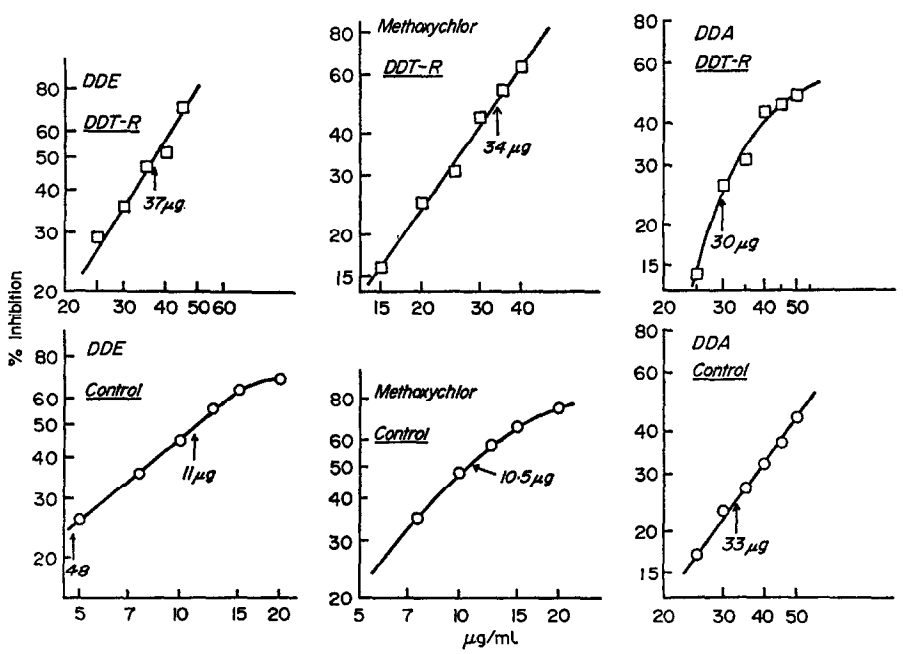

FIG. 4. Dose–response curves and comparison of ID_{50} concentrations in control and DDT-R cultures. Per cent inhibition refers to inhibition of cell replication.

whether the resistance was specific for *p,p'*-DDT or whether it was exhibited also toward the DDT analogues, Kelthane, methoxychlor and *p,p'*-DDD (which are also pesticides), and metabolites of DDT, e.g. DDE and DDA.[13,17]

The relative toxicity of these compounds to control and DDT-R cultures was compared by determining the ID_{50} dose (50 per cent inhibitory dose with respect to cell replication) from a dose–response curve. The relative resistance of the DDT-R culture to the control culture was expressed as the ratio of the ID_{50} or ID_{25} concentrations, e.g. DDT-R ID_{50}/control ID_{50}. Typical dose–response curves are given in Figs. 3 and 4. The ID_{50} and ID_{25} ratios are shown in Table 2. The DDT-R culture was resistant to all of the pesticide analogues. The DDT-R culture exhibited highest resistance to Kelthane, where the ID_{50} ratio was 33. The ID_{50} ratios for *p,p'*-DDD and methoxychlor were 12 and 3·5 respectively. The ID_{50} ratio for DDT was 6·6.

TABLE 2. COMPARISON OF TOXICITY BY *p,p'*-DDT ANALOGUES IN CONTROL AND RESISTANT L 5178 Y CELLS*

Compound	ID_{50} Concn (μg/ml)		
	Control culture	DDT-R culture	ID_{50} Ratio
Kelthane	0·8 ± 0·1	26 ± 1	33·3 ± 5·5
p,p'-DDD	2·8 ± 0·2	33 ± 3	11·9 ± 1·9
p,p'-DDT	4·7 ± 0·1	31 ± 4	6·6 ± 1·0
Methoxychlor	9·5 ± 1	33 ± 3	3·5 ± 0·7
p,p'-DDE	12 ± 1	42 ± 5	3·6 ± 0·7
	ID_{25} Concn (μg/ml)		
	Control culture	DDT-R culture	ID_{25} Ratio
p,p'-DDT	3·5 ± 0·3	22 ± 2	6·4 ± 1·1
p,p'-DDA	32 ± 1	31 ± 1	1·0 ± 0·1

* Except for DDA, these ID_{50} values are the average of three or more experiments. Over a 6-month period, the DDT-R culture became more resistant, so that later comparison to DDT toxicity had to be made on the basis of ID_{25} values. The ID_{25} and ID_{50} refer to that concentration which inhibits cell replication by 25 and 50 per cent respectively.

When control and DDT-R cultures were treated with *p,p'*-DDE and DDA, both compounds were less toxic to control and DDT-R cultures than any of the compounds previously tested (Table 2). The greater resistance of the DDT-R culture to DDE was reflected in the ID_{50} ratio of 3·6. This is very similar to the ID_{50} ratio for methoxychlor. The most striking effect was noted with DDA, which was only slightly toxic to control cells at the usual range of concentrations selected (Fig. 4), and it was not possible to dissolve or suspend a sufficient amount of DDA in the medium to achieve an ID_{50} concentration in the DDT-R culture. Therefore, the relative toxicity of DDA to control and DDT-R cells was compared at the ID_{25} dose level. Tolerance to DDA was high and essentially equal in both the control and resistant cultures; the ID_{25} ratio was 1·0. The decreasing order of toxicity of DDT, DDE and DDA observed in both cultures follows the order expected by the proposed pathway of DDT detoxification.[12,13,17]

Isolation of resistant clones

Further experiments were carried out to determine if single-cell clones derived from the DDT-R culture would retain their resistance if the daily exposure to DDT were terminated. Several of the clones surviving from the exposure of DDT-R cells to 60 μg/ml of DDT (Fig. 2b) were isolated and subcultured for several weeks while being treated daily with DDT.

In order to obtain sister clones that were of similar genotype, cells from one of the treated clones were recloned in media containing DDT over a range of concentrations as before. Sister clones which survived at 60 μg/ml of DDT were isolated; one clone was subcultured continuously in DDT-free medium, while another was treated daily with DDT in a manner similar to the DDT-R culture. The sister clones were designated RU-1 (resistant-untreated) and RT-1 (resistant-treated) respectively. These cultures were subcultured daily as described above for a period equivalent to 30 generation times (approximately 2 weeks) and then compared for resistance to DDT and DDT analogues and metabolites at intervals ensuing over the next few weeks. Throughout these experiments, it was noted that the degree of resistance to DDT exhibited by the clones was significantly greater than that of the parent DDT-R culture, which made it difficult or impossible to dissolve and suspend enough DDT to obtain an ID_{50} value. Thus resistance was compared on the basis of the ID_{25} rather than the ID_{50} dose. The RT-1 clone proved to be 25 per cent more resistant to DDT than the RU-1 clone (Table 3), and both were much more resistant than the original resistant (DDT-R) culture. The increase in resistance of the RT-1 clone apparently

TABLE 3. COMPARISON OF RESISTANCE TO *p,p'*-DDT AND DDT-ANALOGUES BY TWO RESISTANT CLONES OF L 5178 Y CELLS*

Compound	ID_{25} Concn (μg/ml)			
	RU-1	RT-1	DDT-R†	Control†
p,p'-DDT	32	40	22 ± 2	3·5 ± 0·3
p,p'-DDD	25	25	23	0·9
Kelthane	20	19	17	0·44
p,p'-DDE	35	32	24	4·8
Methoxychlor	29	22	21	5·7
DDA	33	31	31 ± 1‡	32 ± 1‡

* The RU-1 culture was carried in the absence of chronic DDT treatment from the day of isolation. The RT-1 culture was treated daily with 2×10^{-5} M DDT (7·1 μg/ml). The ID_{25} refers to that concentration which inhibits cell replication by 25 per cent.

† ID_{25} Values for DDT-R and control cultures from representative experiments have been included for comparison.

‡ These ID_{25} values are from Table 2.

resulted from selection of the more resistant cells of the clone. However, this favored resistance to DDT by the RT-1 clone was not extended to the DDT analogues and metabolites (Table 3), since resistance to DDD, Kelthane, DDE and DDA was the same in both clones. The RU-1 clone was slightly more resistant to methoxychlor than

the RT-1 clone. These data clearly indicate that the resistance of the RU-1 clone* could be maintained in the absence of DDT. The greater resistance of the two clones to DDT may have been due to the fact that they were selected from cells which survived 60 μg/ml of DDT in two sequential cloning experiences. The parent (DDT-R) resistant culture represents a continuing selection of cells from a heterogeneous population exposed to chronic treatment with a much lesser concentration of DDT, i.e. 7·1 μg/ml.

Nature of resistance to DDT

Ernster and Orrenius[19] have shown that increases in the level of microsomal hydroxylating activity in liver were accompanied by similar increases in the activity of cytochrome P-450. Thus cytochrome P-450 in the resistant cultures was studied as an indicator of the status of the microsomal hydroxylases in the cells. Cells from the RU-1 culture were grown in quantity, lysed, and the microsomal fraction examined for the presence of cytochrome P-450. A microsomal fraction from rat liver was prepared at the same time as a control. No trace of P-450 cytochrome in the RU-1 cell preparation was found. This is in accord with the observation of Estabrook,[20] who was unable to detect cytochrome P-450 in several established tissue culture lines.

In order to explore the possibility of DDT metabolism by the resistant cultures, ^{3}H-DDT was employed. Both the RU-1 and RT-1 cultures were grown for approximately 72 hr in the presence of ^{3}H-*p,p'*-DDT, specific activity 5 mc/34·7 mg, at a concentration of 10 μg/ml. The cultures were expanded in volume every 24 hr by addition of fresh medium. As a control, 300 ml media was incubated with the same concentration of ^{3}H-DDT for a similar time period. At the end of the incubation period, the cell suspensions were extracted as described under Methods, and an appropriate amount of the extract was examined by thin-layer chromatography, using hexane–chloroform (1:1) as the solvent system. In this system, DDT and DDE run very close together, with the DDE moving just ahead of the DDT. Since DDE was a possible metabolite,[9] both unlabeled DDT and DDE were added as carrier to the ^{3}H-DDT standard and the medium control extract to enhance resolution and to insure detection of both compounds by quenching of fluorescence. DDT and DDE were run separately as controls. Under these conditions, the only detectable spots from cell suspension and medium control extracts were those corresponding to DDT, or to DDE when the latter had been added as carrier.

In order to test for very small amounts of metabolites below the limits detectable by lack of fluorescence, the area of Silica gel between the solvent front and the origin was arbitrarily divided into $\frac{1}{8}$ in. sections, and the Silica gel from each section was recovered for radioisotope analysis. In every instance, 99·0–99·5 per cent of the total radioactivity on the plate was recovered in the spot identified as DDT (Table 4). However, the ^{3}H-DDT standard contained three spots with low activity and with mobility characteristics identical to similar spots detectable in the culture and medium extracts. One spot, C_1, was detected at the origin. Another, C_2, with an R_f of 0·10, moved only a few millimeters from the origin. A third, C_3, had an intermediate R_f of 0·40. The three radioactive contaminants were present in approximately the same

* The RU-1 (untreated) culture still exhibited the same degree of resistance after being carried for 6 months, or through approximately 360 replications.

TABLE 4. SEPARATION OF ^{3}H-*p,p'*-DDT FROM CONTAMINANTS BY THIN-LAYER CHROMATOGRAPHY AFTER INCUBATION WITH DDT RESISTANT L 5178 Y CELLS*

	^{3}H-DDT standard		^{3}H-DDT + medium		RU-1 culture		RT-1 culture	
	R_f	% dis./min	R_f	% dis./min	R_f	% dis./min	R_f	% dis./min
DDT	0·80	99·5	0·80	99·5	0·80	99·1	0·80	99·0
C_1	0·40	0·41	0·40	0·12	0·40	0·18	0·40	0·13
C_2	0·10	0·06	0·10	0·14	0·10	0·13	0·10	0·17
C_3 (origin)		0·13		0·25		0·35		0·69

* The solvent system was hexane–chloroform (1:1).

amount in the culture extracts as in the ^{3}H-DDT standard (Table 4). Thus, the experiment indicates that DDT was not metabolized even upon prolonged incubation with the resistant L 5178 Y cells.

However, since DDE had been reported as the major metabolite of DDT by Hela S cells,[9] and since DDT and DDE were nearly indistinguishable in the solvent system just described, the extracts were chromatographed in a second system with *n*-heptane as the solvent. In this system, there was excellent resolution of DDE and DDT. Unlabeled DDE was added as carrier to the extracts from the RU-1 and RT-1 cultures to facilitate detection of trace amounts of radioactive DDE that might be present. DDE and DDT were easily detectable as distinct nonfluorescing spots with R_f values of 0·43 and 0·33 respectively. The areas of resolution on the plates were partitioned as before, and the $\frac{1}{8}$ in. sections scraped into scintillation vials for radioactivity determinations. The results clearly showed that the radioactivity was associated with DDT and not DDE.

It is concluded, therefore, that within the limits of detection of the procedures employed, no DDT metabolites were formed by either of the two resistant cultures. Therefore, the resistance of the RU-1 and RT-1 cultures to DDT must be explained by some mechanism other than the established degradative systems found in some cultured cells[6–8] and in many organisms.[10–13] Further efforts are in progress to elucidate the nature of the tolerance mechanism.

DISCUSSION

When resistance to DDT and its analogues was first demonstrated in L 5178 Y cells (Table 2), consideration was given to the possibility that resistance to this class of organochlorine compounds was conferred by induced microsomal drug detoxification enzymes similar to those described in liver[17,18,21] or by the induction of a dehydrochlorinase.[10,11] The recent report by Huang *et al.*[9] encouraged this interpretation of the resistance phenomenon, for these investigators had demonstrated that in Hela cells treated with ^{14}C-DDT for 48 hr, 13 per cent of the parent compound was converted to DDE; DDA was not detected as a metabolite in these experiments.

When the presence of cytochrome P-450 could not be demonstrated in the DDT-resistant RU-1 culture, metabolism of DDT by induced microsomal oxidase enzymes

was ruled out. On the other hand, the absence of P-450 activity did not exclude the possibility that DDT was metabolized by another mechanism.

However, the failure to detect any evidence that ^{3}H-DDT was metabolized by the resistant mouse L 5178 Y cultures indicated that resistance was being expressed by some mechanism that did not depend on a structural or metabolic modification of the DDT molecule. This type of resistance to DDT and other insecticides is not unknown. In a recent review[22] concerning the genetic basis for insect resistance to insecticides, a little understood kind of resistance is discussed. Among the major known genes conferring resistance in the housefly, *Musca domestica L.*, are the recessive genes known as knockdown resistance (kdr) and knockdown resistance-Orlando (kdr-O). Not only does the gene kdr-O confer resistance to DDT and its analogues, but flies possessing this gene are resistant to all other organochlorine insecticides and the pyrethroid insecticides as well. In addition, it has been reported that flies possessing the kdr-O resistant gene have a decreased nerve sensitivity to DDT. A similar form of resistance to the cyclodiene insecticides also occurs in houseflies and apparently involves a mechanism independent of insecticide detoxication.[22]

The prominent signs of acute DDT toxicity in insects, birds and mammals, e.g. muscle tremor, incoordination and convulsions, indicate that the central nervous system is the target of insecticide activity. Although the actual poisoning mechanism of DDT remains unknown, there is agreement that the end result of DDT activity is disruption of the ion-transport mechanism in the nerve cell membrane.[23,24] There is increasing evidence that this potent effect of DDT as well as other organochlorine insecticides depends on a physical complex rather than a chemical reaction with certain protein components of the nerve membrane.[25–27] Recently these insecticides have been shown to inhibit membrane adenosine triphosphatases derived from brain tissues.[28,29,*]

The lack of evidence for DDT metabolism in L 5178 Y cells suggests that the compound may manifest its toxicity by binding with protein or some other component at specific sites on the cell membrane. Since the uptake and transport of essential nutrients from the medium are dependent on an energy-requiring process, interference with an ATP receptor protein or alteration of ion-exchange processes in the membrane could result in depressed cell growth and, if severe enough, ultimately cause cell death.

Current theories on the mode of action of DDT[27] emphasize the steric specificity required by receptor sites for forming a tight complex with DDT. Thus the resistance of L 5178 Y cells may be due to steric hindrance with respect to DDT and its analogues at specific sites on the cell membrane. This is indicated by the limited range of toxicity among these compounds in resistant cells compared to that in susceptible cultures (Table 3). Currently the characteristics of cell membranes in resistant and susceptible L 5178 Y cells are being compared to determine whether resistance is being expressed at the cell membrane. These investigations may provide insight into the mechanism of DDT toxicity as well.

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* Private communication from Dr. William Wilson of the Chemistry Branch of this Institute.

REFERENCES

1. J. GABLIKS and L. FRIEDMAN, *Proc. Soc. exp. Biol. Med.* **120,** 163 (1965).
2. J. GABLIKS and L. FRIEDMAN, *Proc. Soc. exp. Biol. Med.* **125,** 1002 (1967).
3. C. L. LITTERST, E. P. LICHTENSTEIN and K. KAJIWARA, *J. agric. Fd Chem.* **17,** 1199 (1969).
4. W. J. JOHNSON and S. A. WEISS, *Proc. Soc. exp. Biol. Med.* **124,** 1005 (1967).
5. J. GABLIKS, *Proc. Soc. exp. Biol. Med.* **120,** 168 (1965).
6. L. J. ALFRED and H. V. GELBOIN, *Science, N.Y.* **157,** 75 (1967).
7. D. W. NEBERT and H. V. GELBOIN, *J. biol. Chem.* **243,** 6242 (1968).
8. D. W. NEBERT and H. V. GELBOIN, *J. biol. Chem.* **243,** 6250 (1968).
9. E. A. HUANG, J. Y. LU and R. A. CHUNG, *Biochem. Pharmac.* **19,** 637 (1970).
10. H. LIPKE and C. W. KEARNS, *Adv. Pest Control Res.* **3,** 253 (1960).
11. C. F. WILKINSON in *Enzymatic Oxidations of Toxicants* (Ed. E. HODGSON), p. 118. North Carolina State University, North Carolina (1968).
12. W. J. HAYES, *A. Rev. Pharmac.* **5,** 27 (1965).
13. D. A. VESSEY, L. S. MAYNARD, W. H. BROWN and J. W. STULL, *Biochem. Pharmac.* **17,** 171 (1968).
14. M. Y. CHU and G. A. FISCHER, *Biochem. Pharmac.* **17,** 753 (1968).
15. T. OMURA and R. SATO, *J. biol. Chem.* **239,** 2370 (1964).
16. T. OMURA, R. SATO, D. Y. COOPER, O. ROSENTHAL and R. W. ESTABROOK, *Fedn Proc.* **24,** 1181 (1965).
17. J. E. PETERSON and W. H. ROBISON, *Toxic. appl. Pharmac.* **6,** 321 (1964).
18. A. MORELLO, *Can. J. Biochem. Physiol.* **43,** 1289 (1965).
19. L. ERNSTER and S. ORRENIUS, *Fedn Proc.* **24,** 1190 (1965).
20. R. W. ESTABROOK, in *Microsomes and Drug Oxidations*, p. 429. Academic Press, New York (1969).
21. L. J. HART and J. R. FOUTS, *Proc. Soc. exp. Biol. Med.* **144,** 388 (1963).
22. F. W. PLAPP, JR., in *Biochemical Toxicology of Insecticides* (Eds. R. D. O'BRIEN and I. YAMAMOTO), p. 179. Academic Press, New York (1970).
23. T. NARAHASHI and H. G. HAAS, *J. gen. Physiol.* **51,** 177 (1968).
24. B. HILLE, *J. gen. Physiol.* **51,** 199 (1968).
25. F. MATSUMURA and R. D. O'BRIEN, *J. agric. Fd Chem.* **14,** 36 (1966).
26. F. MATSUMURA and R. D. O'BRIEN, *J. agric. Fd Chem.* **14,** 39 (1966).
27. G. HOLAN, *Nature, Lond.* **221,** 1025 (1969).
28. R. B. KOCH, *J. Neurochem.* **16,** 269 (1969).
29. F. MATSUMURA, T. A. BRATKOWSKI and K. C. PATIL, *Bull. envir. Contamin. Toxic.* **4,** 262 (1969).