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Overexpression of Cytochrome P450 CYP6D1 Is Associated with Monooxygenase-Mediated Pyrethroid Resistance in House Flies from Georgia

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CYP6D1 is a house fly cytochrome P450 that catalyzes metabolism of pyrethroid insecticides. Overexpression of CYP6D1 is responsible for monooxygenase-mediated pyrethroid resistance in the LPR (Learn pyrethroid resistant) house fly strain, which was originally collected in New York State. To determine whether CYP6D1 is involved in monooxygenase-mediated pyrethroid resistance in other populations, we obtained two pyrethroid-resistant strains from Georgia and investigated the relationship between piperonyl butoxide suppressible permethrin resistance and *CYP6D1* gene expression. The Georgia strain, collected from a poultry facility in about 1983, showed no PBO suppressible permethrin resistance and CYP6D1 was not overexpressed. The NG98-u strain, collected from a poultry facility in Georgia State in 1998, showed PBO suppressible resistance and CYP6D1 mRNA was overexpressed. Furthermore, the level of resistance and CYP6D1 mRNA increased after only two generations of permethrin selection. Overall, the synergistic ratios were well correlated with the expression of *CYP6D1*. CYP6D1 protein detected by immunoblotting was well correlated with CYP6D1 mRNA expression. These results suggest that increased expression of CYP6D1 in the resistant flies collected from Georgia in 1998 is due to increased transcription of the gene (as has been identified in LPR) and that CYP6D1-mediated pyrethroid resistance is found in house flies from both New York and Georgia.

INTRODUCTION

The microsomal cytochrome P450 monooxygenases are an essential metabolic system found in all organisms. These important enzymes oxidize a wide variety of endogenous compounds and xenobiotics. In insects, they are involved in synthesis and degradation of hormones and pheromones and detoxification of pesticides and plant toxins (1). Insects commonly develop insecticide resistance by increasing monooxygenase activity leading to enhanced detoxification of insecticides (2).

Insecticide resistance is a major problem in controlling medically and agriculturally important insect pests. Insects can develop resistance to insecticides by different mechanisms, and one of the most important mechanisms of resistance to many classes of insecticides is enhanced detoxification mediated by cytochrome P450 monooxygenases (3, 4). To develop ways to overcome, or at least delay, the evolution of resistance, it is important to understand the mechanisms of resistance and develop assays to monitor resistance (5). In terms of monooxygenase-mediated resistance, one unanswered question is whether the P450 isoform(s) responsible for resistance to a given insecticide varies from one geographical region to another.

The Learn pyrethroid resistant (LPR) strain of house fly was originally collected in 1982 from a dairy in New York. After pyrethroid selection for 22 generations (6), this strain became homozygous for the major mechanisms of resistance and showed extremely high levels of resistance to pyrethroid insecticides (7). Synergism and *in vitro* metabolism studies showed that a major mechanism of the pyrethroid resistance in this strain is cytochrome P450 monooxygenase-mediated detoxification (7). A single cytochrome P450, CYP6D1, was purified from the LPR strain (8) followed by sequencing of the *CYP6D1* gene (9). Both CYP6D1 protein and mRNA are expressed at about 10-fold higher

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levels in LPR compared to susceptible strains (10). Using monospecific antisera it was shown that CYP6D1 is responsible for monooxygenase-mediated pyrethroid resistance because CYP6D1-specific antisera (11) blocked the enhanced pyrethroid metabolism by LPR microsomes (12, 13). The molecular basis for this resistance is due to increased transcription of CYP6D1 mRNA (14), and it is not due to gene amplification or RNA stabilization (10, 14). Although CYP6D1 has been well studied in the LPR strain, it is unclear what role this P450 has in pyrethroid resistance in other populations from different locations.

In this study, we investigated the relationship between PBO suppressible permethrin resistance and expression of CYP6D1 mRNA and protein in four house fly strains collected from the United States. Our goal was to determine whether CYP6D1-mediated pyrethroid resistance occurred in field populations.

MATERIALS AND METHODS

House Fly Strains

Five strains of house flies were used in this study: Cornell susceptible (CS) is an insecticide susceptible strain reared in our laboratory without exposure to insecticides (15). LPR is a multiresistant strain having high levels of resistance to pyrethroid insecticides due to increased oxidative metabolism mediated by cytochrome P450 6D1 (7, 12, 16). Two other mechanisms of resistance to pyrethroid insecticides in the LPR strain are insensitivity of the nervous system (*kdr*) and decreased cuticular penetration (*pen*) (7). Georgia is a pyrethroid-resistant strain collected from a poultry house in about 1983 (17), provided by Dr. F. W. Plapp Jr., and selected with permethrin for four generations using a dose that caused 71–87% mortality. NG98-u (unselected) was collected at a poultry farm in Georgia (obtained from Dr. C. Sheppard, University of Georgia) in 1998, and NG98 strain was established from NG98-u strain by two consecutive permethrin selections (by topical applications) using a dose that gave 56 and 72% mortality. House flies were reared as described previously (18).

Bioassay

Permethrin (94.7%, cis:trans ratio = 25:75) was obtained from AgrEvo (Wilmington, DE). Bioassays were carried out by the topical application of a 0.5-μl drop (up to 1.5 μl for extremely high doses) of insecticides in acetone solution to the thoracic notum of 3- to 5-day-old female flies. Each replicate consisted of 20 flies per dose and at least five doses, causing >0 and $<100\%$ mortality. All bioassays were run at 25°C and at least three replicates were completed for each insecticide dose. Controls were treated with acetone only. The mortality was assessed 24 h after treatment. LD_{50} values for each strain were calculated using log-probit mortality regression analysis (19). To determine the synergistic effects of piperonyl butoxide (PBO, a cytochrome P450 inhibitor), the house flies were tested with permethrin in combination with PBO. The synergist solution, PBO (90.0%, Aldrich, Milwaukee, WI) in acetone, was applied at a dose of 10 μg (0.5 μl) per fly 1 h prior to dosing with permethrin (20).

Preparation of Microsomes and Quantification of Cytochrome P450s and b_5

Microsomes were prepared from the abdomens of 200 female house flies as described previously (8, 21). Microsomal pellets were resuspended in 2 ml of resuspension buffer (21) and stored at −80°C. Protein was determined in triplicate for each sample using a Bio-Rad protein reagent (Bio-Rad, Hercules, CA) with bovine serum albumin as the standard. Cytochrome P450 and b_5 were quantitatively analyzed by the method of Omura and Sato (22) using a Beckman DU-640 spectrophotometer (1 mg/ml microsomal protein). Microsomes were prepared at least three times from each strain and each preparation was assayed three times for P450 and b_5 content.

Northern Blotting

Three to 5-day-old house flies of the different strains were used for isolating poly(A)$^+$ RNA. Poly(A)$^+$ RNA was isolated directly from house fly abdomens using the Quick prep micro mRNA

purification kit (Amersham Pharmacia Biotech, Piscataway, NJ) as described by the manufacturer and quantified with a Beckman DU-640 spectrophotometer. Three micrograms of poly(A)$^+$ RNA from each sample were separated by formaldehyde denaturing gel electrophoresis and transferred to a nylon membrane (23). A 1.4-kb CYP6D1v1 cDNA sequence was amplified by reverse transcription-mediated polymerase chain reaction (RT-PCR), labeled with [α-^{32}P]dCTP using a RadPrime DNA labeling system (Life Technologies, Gaithersburg, MD) and used as a hybridization probe. RNA blots were prehybridized at 68°C in QuickHyb solution (Stratagene, La Jolla, CA) for 3 h and then hybridized with the probe in the presence of sheared salmon DNA for 16 h. RNA blots were then washed twice for 15 min in 2× SSC + 0.1% SDS at room temperature followed by a 30-min wash with 0.2× SSC + 0.1% SDS at 65°C. Using these techniques our detection of CYP6D1 is highly specific (9, 10). Radioactive signal intensity from different samples was quantified using a phosphorimager (Molecular Dynamics, Sunnyvale, CA) and compared to the signals from serial dilutions (2-, 4-, 8-, and 16-fold) of CYP6D1 mRNA from LPR. After quantification, the blots were stripped by washing twice for 1 h in a boiling solution of 0.1× SSC + 0.1% SDS (23). To determine the amount of poly(A)$^+$ RNA loaded into each lane the blots were air dried and rehybridized at 68°C with a fragment of the ribosomal protein S3 cDNA (*RPS3*) from *Musca domestica.* All assays were replicated three times using RNA from different batches of house flies. A paired *t* test (24) was used to compare the levels of CYP6D1 mRNA between CS and the other strains.

RPS3 Isolation from House Flies

The following degenerate primers, designated based on the homologous region of *RPS3* genes from *Drosophila* (25), *Manduca* (26), human (27), and rat (28), were synthesized for PCR: 5′-CG(C/G)GA(A/G)CT(C/G)GC(C/T)GA(G/A)GATGGC-3′ (forward primer) and 5′-GGC(T/A)G(A/G/C)GGCTTCTT(A/G)GG(A/G)CC-3′ (reverse primer). The PCR products (537 bp) were purified, subcloned into TA-cloning vector (Invitrogen, Carlsbad, CA), and sequenced. The resultant sequence (Accession Number: AF207603) showed 82% identity to *Drosophila* ribosomal protein S3 (29). Signal intensity was quantified as described above and used to correct for any differences in sample loading (i.e., samples were normalized based on *RPS3* levels).

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS–PAGE) and Immunoblotting

SDS–PAGE was performed according to the method described by Laemmli (30). The stacking and the separating gel contained 4 and 10% acrylamide, respectively. Microsomal protein (15 μg) was combined with sample buffer containing β-mercaptoethanol, boiled for 2 min, cooled, loaded onto a 0.75-mm-thick gel, and separated using a 30-mA constant current. The separated proteins were transferred from the gel to a nitrocellulose membrane and immunoblotting was processed as previously described (18) using anti-CYP6D1-specific antisera (11).

RESULTS

Bioassay

The toxicity of permethrin to six house fly strains is shown in Table 1. The resistance ratios were calculated based on the LD_{50} of the susceptible CS strain. To examine whether cytochrome P450 monooxygenases are involved in permethrin resistance we used PBO as a synergist. LPR showed high resistance to permethrin (6080-fold) and the resistance level was reduced 79-fold by treatment with PBO; this agrees with previous results (7). The field-collected strain, NG98-u, also had a high resistance ratio (816-fold) with an LD_{50} value of 13 μg/fly. Following selection of this strain with permethrin for two consecutive generations, the resultant NG98 strain was 3660-fold resistant to permethrin. Both NG98-u and NG98 also had high synergism ratios (344- and 387-fold, respectively)

TABLE 1
Toxicity of Permethrin with and without PBO against Five Strains of House Fly, *Musca domestica*

	Permethrin				Permethrin + PBO				
Strain	n^a	Slope (±SE)	LD_{50} (ng/fly) (95% CI)	RR[b]	n^a	Slope (±SE)	LD_{50} (ng/fly) (95% CI)	RR[b]	SR[c]
CS	480	3.5 (0.3)	16.3 (14.9–17.9)	—	600	6.3 (0.6)	1.03 (0.98–1.08)	—	16
LPR	360	3.0 (0.4)	99,100 (84,500–114,000)	6080	480	3.9 (0.4)	79.7 (71.0–88.3)	77	1240
Georgia	660	4.4 (0.4)	330 (309–353)	20	540	4.0 (0.4)	10.2 (9.36–11.1)	9.9	32
NG98-u	660	1.9 (0.1)	13,300 (11,400–15,400)	816	540	3.2 (0.4)	38.7 (34.4–42.5)	38	344
NG98[d]	480	2.6 (0.5)	59,600 (44,800–78,800)	3660	600	3.8 (0.3)	154 (142–167)	150	387

[a] Number of females tested.
[b] Resistance ratio = LD_{50} of each strain/LD_{50} CS.
[c] Synergism ratio = LD_{50} without PBO/LD_{50} with PBO.
[d] Established by permethrin selections of NG98-u.

suggesting that P450 monooxygenases are involved in the resistance. There was only a slight decrease in permethrin resistance in the Georgia strain following treatment with PBO, suggesting that monooxygenases are of limited importance as a resistance mechanism in this strain.

Quantification of Cytochrome P450 and b_5

Elevated levels of cytochrome P450 were found in all permethrin-resistant strains tested (Table 2). The highest levels of P450 and b_5 were found in the LPR strain (3.2- and 1.6-fold, respectively, Fig. 1), which agrees with earlier work by Scott and Georghiou (31). In the NG98-u strain, the level of cytochrome P450 was 0.407 ± 0.033 nmol/mg protein and it was increased by 11% ($P<0.05$) after permethrin selection (i.e., NG98 strain) to 0.453 ± 0.021 nmol/mg protein. No difference in b_5 content was observed between these two strains.

The absorption maximum (λ_{max}) in the CO-difference spectrum was slightly different among the six strains, ranging between 449.0

TABLE 2
Levels of Cytochrome P450s and b_5 in the Five Strains of House Fly, *Musca domestica*

	Cytochrome P450		Cytochrome b_5	
Strain	nmol/mg protein	γ_{max}	nmol/mg protein	γ_{max}
CS	0.290 (0.022)	451.8 (0.1)	0.324 (0.013)	427.1 (0.1)
LPR	0.923** (0.024)	449.0 (0.1)	0.524** (0.012)	426.3 (0.1)
Georgia	0.338 (0.013)	451.1 (0.1)	0.286 (0.014)	427.2 (0.1)
NG98-u	0.407* (0.033)	450.4 (0.2)	0.355 (0.023)	426.9 (0.2)
NG98	0.453** (0.021)	450.0 (0.1)	0.350* (0.012)	427.0 (0.1)

Note. All values are the mean (±SEM) of at least three replicates.
* Significantly greater than CS strain ($P<0.05$).
** Significantly greater than CS strain ($P<0.01$).

and 451.8 (Table 2). Interestingly, cytochrome P450 λ_{max} was inversely proportional to the levels of cytochrome P450 (i.e., CS had the lowest level of P450 and the largest λ_{max} value while the opposite was true for LPR). This is likely due to the elevated levels of CYP6D1 in the resistant strains (see below). CYP6D1 has a λ_{max} of 447 nm, therefore increased levels of this P450 would be expected to shift the λ_{max} closer to this wavelength.

Northern Blotting

To evaluate whether PBO suppressible permethrin resistance was associated with increased CYP6D1 mRNA, we analyzed mRNA from five house fly strains by Northern blotting with a *CYP6D1*-specific probe (Fig. 2). The cDNA probe specifically hybridized with a single band (2.0 kb) in each strain. Poly(A)$^+$ RNA loading was standardized by reprobing the stripped membrane with ribosomal protein S3 cDNA (*RPS3*). Expression of CYP6D1 mRNA was significantly ($P<0.05$) greater in LPR (8.3-fold), NG98-u (2.8-fold), and NG98 (4.1-fold) compared to the CS strain. It is noteworthy that *CYP6D1* expression was enhanced after only two permethrin selections, suggesting involvement of CYP6D1 in permethrin metabolism and resistance.

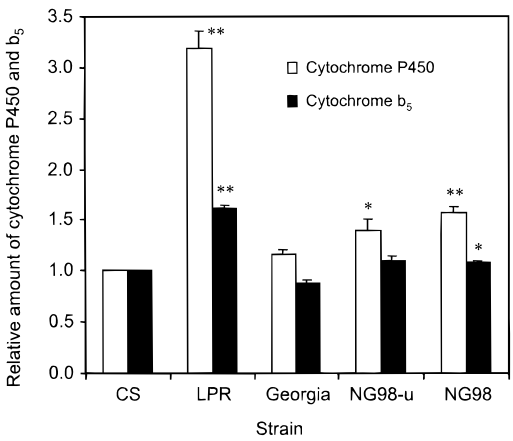

FIG. 1. The relative amount of cytochrome P450 and b_5 in five strains of house fly. Asterisks denote significantly greater than the CS strain (*$P < 0.05$, **$P < 0.01$).

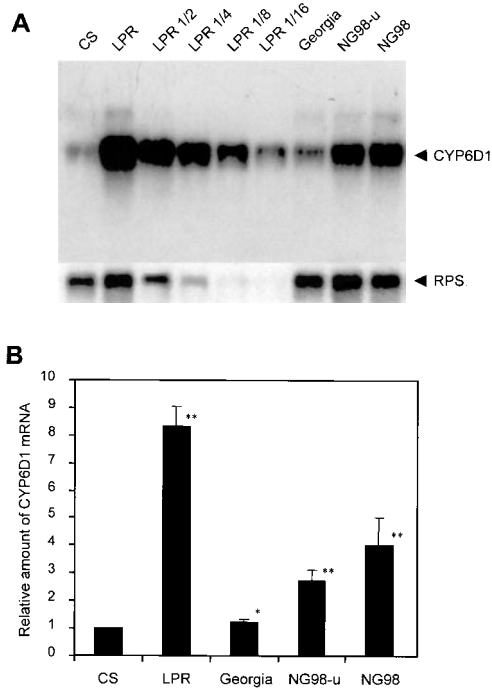

FIG. 2. Expression of CYP6D1 in five house fly strains. **A**. Northern blot analysis of CYP6D1 mRNA. A total of 3 μg of poly(A) RNA was loaded in each lane. The blot was hybridized with a 1.4-kb *CYP6D1* cDNA probe (top) and then stripped and rehybridized with ribosomal protein S3 (*RPS3*) cDNA (bottom). Serial dilutions (1-, 2-, 4-, 8-, 16-, and 32-fold) of mRNA from LPR were loaded as a standard. **B**. Relative CYP6D1 mRNA levels. Vertical bars indicate standard errors of the mean. Each value on the bar indicates the relative amount of CYP6D1 mRNA expression as CS = 1. Asterisks denote significantly greater than the CS strain (*$P < 0.05$, **$P < 0.01$).

Western Blotting

Microsomes were evaluated for the presence of CYP6D1 protein using SDS–PAGE/immunoblotting. Overproduction of CYP6D1 proteins was found from each resistant strain while the susceptible CS strain showed only a weak signal (Fig. 3). Additionally, the immunoreactive bands had the same mobility in CS, Georgia, NG98-u, and NG98 strains compared to that of LPR strain which strongly suggests that the band in each lane is CYP6D1. The amount of CYP6D1 (detected by immunoblotting) in each strain parallels the results of Northern blotting, suggesting

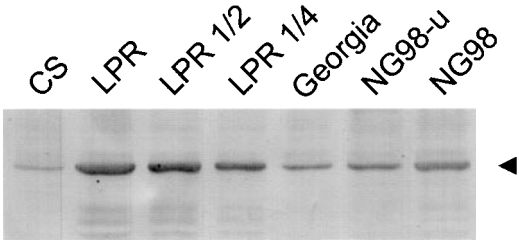

FIG. 3. Immunoblotting of microsomes from five house fly strains. A total of 30 μg of microsomal protein was loaded in each lane. The arrow indicates the position of CYP6D1. Serial dilutions (2- and 4-fold) of microsomes from LPR were loaded as a standard.

that overexpressed CYP6D1 mRNA is efficiently translated into protein.

DISCUSSION

NG98-u is a field-collected strain with no exposure to insecticides in the laboratory. Surprisingly, this population had already developed extremely high levels of resistance to permethrin for a field-collected strain (816-fold). The unusually high LD_{50} value, 13 μg/fly, suggests that permethrin is minimally effective where this population was collected. In addition, the relatively shallow slope of the log dose probit line (Table 1) indicated relatively high levels of heterogeneity in this population. Therefore, after just two selections of the NG98-u strain the resistance level increased to 3660-fold (NG98). NG98-u was collected from a poultry farm, an environment with somewhat limited immigration of house flies. Such facilities are excellent environments for rapid resistance development once a resistance gene is present in the population. In other words, for house flies it is possible to have high selection pressure not only in the laboratory, but also in field populations.

Overall, PBO suppressible permethrin resistance was well correlated with *CYP6D1* gene expression. In addition, the result of protein immunoblotting mirrored the expression of CYP6D1 mRNA. Since total P450s, expression of CYP6D1 mRNA, and production of CYP6D1 protein were enhanced by only two consecutive selections in NG98-u strain, CYP6D1 expression is clearly associated with permethrin resistance.

Comparison of the Georgia and NG98-u (or NG98) strains suggests that the first pyrethroid resistance mechanism present in house flies was *kdr* (32). This is likely due to previous use of DDT for fly control (32, 33). The appearance of CYP6D1-mediated monooxygenase resistance was more recent (since 1985) and correlated with the rapid increase in pyrethroid use occurring over the past 15 years. As such, some house fly populations in the United States will not have substantial CYP6D1-mediated resistance if the selection pressure has remained low. This appears to be the case in at least one population we tested from Nebraska (collected in 1996) which had a permethrin resistance ratio of about 25 which was not eliminated by PBO (i.e., similar to Georgia, data not shown). Unlike the case of organophosphate resistance in mosquitoes, where one resistance mechanism can be selected for and eventually replace the original resistance allele (34), *kdr* appears to be maintained in resistant populations of house fly (32). This is possibly due to neither *kdr* nor CYP6D1-mediated monooxygenase resistance being sufficient, without the other, for flies to survive field levels of pyrethroid exposure.

This study showed that CYP6D1 is not an "LPR-specific" resistance mechanism. We have now documented that CYP6D1 contributes to permethrin resistance in house flies from New York and Georgia, suggesting that it is likely that this is an important mechanism for other populations of house flies in North America. Previous studies found that overexpression of CYP6D1 mRNA is not due to gene amplification or high stability of mRNA but increased rate of transcription in the LPR strain (10, 14). Furthermore, enhanced gene transcription is regulated by at least two mechanisms *cis*- and *trans*-factors (14, 35, 36). Increased levels of CYP6D1 in the NG98 strain may also be regulated by these factor(s). The difference of *CYP6D1* expression between the LPR and NG98 strains may be due to the number of the factor(s) present in these strains. Although it is still unknown what precise

molecular factor(s) controls the enhanced transcription of *CYP6D1* in resistant strains, their identification will offer new methods for monitoring resistance, thus providing a critical resistance management tool. Recently, 5′ flanking sequences of the *CYP6D1* were determined from LPR and five pyrethroid susceptible strains, and some sequences unique to LPR were observed (37). Further comparison of the gene structure of other pyrethroid-resistant strains such as NG98, and analysis of the interaction between these sequences and transcriptional elements, will give us useful information for understanding increased transcription. It is also possible that a better understanding of CYP6D1-mediated resistance could lead to the discovery of novel target sites (e.g., factors controlling P450 transcription and/or inhibitors thereof) for the development of new insecticides and/or synergists.

Thus far it has been shown that CYP6D1-mediated pyrethroid resistance is present in house flies collected from the southern and northeastern United States. How common this is in other areas of North America or in other continents remains unknown. In the organophosphate resistant strain of *Culex* mosquito, it is known that overproduction of esterase B2, due to gene amplification, is a common worldwide resistance mechanism. Raymond *et al.* (38) proposed that the amplified esterase B2 gene originated from an initial event that has subsequently spread organophosphate insecticide resistance by migration. Since *CYP6D1* expression is regulated by multiple factors, the evolutionary history of *CYP6D1* expression may not be as simple and widespread as esterase B2 overexpression. Further studies aimed at investigating the extent of CYP6D1-mediated resistance throughout the world will help clarify the evolution of pyrethroid resistance.

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