Insecticide Resistance Genes in Mosquitoes: Their Mutations, Migration, and Selection in Field Populations

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Insecticides have been used intensively to control insect populations over the last 50 years and many species of insects have developed resistance to several families of insecticides. These resistances are mainly due to two mechanisms: mutation of the insecticide target protein (leading to a decrease in its affinity for the concerned insecticide family), and increased detoxification. Recent molecular studies suggest that the mutations conferring resistance are rare and sometimes unique events in any given species. The wide geographic distribution of some of these genes can then only be explained by the balance between migration and selection at the population level.

For the last 50 years, pesticides have been widely used to control insects. In 1989, among the 504 arthropod species that had become resistant to one or several insecticide families, 114 were mosquitoes, the most important vectors of human diseases (Georghiou and Lagunes-Tejeda 1991). Until recently the evolution of resistance could only be studied by bioassays, which measure the overall result of the resistance genes present in a population. With the development of research on the biochemical and molecular bases of resistance, techniques characterizing each resistance gene in each individual begin to be available, and investigations on the relative importance of the evolutionary forces controlling resistance emergence and expansion become possible.

In this article we will address two problems. The first concerns resistance genes and their mutations, and will attempt to answer the following questions: What are the nature of the genes involved in insecticide resistance? What are the mutations that transform a "susceptible" allele into a "resistant" one? How frequent are they? This will lead us to conclude that, for any given gene, mutations conferring high levels of resistance are probably rare and possibly unique events. How then can we explain that resistance is so widely spread in many species? This will be treated in the second part of this article, which will consider the interaction between migration and selection in the evolution of resistance in natural populations. Mosquitoes of the *Culex pipiens* complex for which we have at present one of the most comprehensive set of data will be taken as example.

Resistance Genes and Their Mutations

Insecticides act in impairing the function of molecular targets which are crucial for life. Any mutation that modifies the behavior or the physiology of the insect in such a way that the target remains at least partially functional will induce resistance. Thus, insecticide resistance may be due to one or several of the following mechanisms: target insensitivity, increased detoxification or excretion, decreased penetration, or behavioral avoidance of the insecticide.

Although resistance due to changes in behavior (Lockwood et al. 1984) and decreased penetration (Priester and Georghiou 1980) have been described in mosquitoes, no recent genetic studies have been undertaken. Thus, we will only consider the two most frequent resistance mechanisms, target modification and increased detoxification, which both may result in high levels of resistance.

The Target Proteins

The targets of synthetic insecticides are important molecules of the nervous system. Synapse acetylcholinesterase (AChE) is the target of organophosphorus (OP) and carbamate (CB) insecticides, Na^+ voltage-dependent (NaVdp) channel the target of DDT and pyrethroids (PY), and the

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GABA receptor of GABA-gated chloride channels ($GABA_A$) the target of cyclodienes (CYD) and avermectins (in house flies, CYD and avermectins work at distinct sites of the GABA receptor and have different effects: CYD block the chloride channel, whereas avermectins activate it; Clark et al. 1995). The targets of *Bacillus thuringiensis* (Bt) or *B. sphaericus* (Bs) toxins are proteins of the brush border membrane of the midgut epithelium (Tabashnik 1994), and in the Lepidopteran *Manduca sexta,* the Bt receptor was identified as an aminopeptidase N (Knight et al. 1994).

So far, insecticide resistances due to target proteins have been associated with a decrease of their binding affinity with the insecticide, and not to an overproduction of target molecules as sometimes recorded for herbicides or drugs (Caretto et al. 1994). This was shown for AChE and $GABA_A$ receptor, as will be seen below, as well for the targets of Bs in the mosquito *C. pipiens* (Nielsen-Leroux et al. 1995), of Bt in various Lepidoptera and Coleoptera (see Tabashnik 1994), and of pyrethroids and DDT (NaVdp) in *Drosophila melanogaster* (Pauron et al. 1989). There are at least two NaVdp channel genes in insects (*para* and *sch* genes), which have both been cloned in *D. melanogaster.* A mutation specific to a *Drosophila* strain resistant to DDT has been identified in the *sch* gene at an important position for the channel function (Amichot et al. 1992). However, it must be confirmed experimentally that this mutation is responsible for resistance.

Acetylcholinesterase (AChE). AChE terminates nerve impulses by catalyzing the hydrolysis of the neurotransmitter acetylcholine. OP and CB insecticides inactivate the enzyme by phosphorylating or carbamoylating the serine of the active site. Following intoxication, the termination of impulse transmission at cholinergic synapses no longer takes place, causing the death of the insect. AChEs that are less sensitive to insecticide inhibition have been selected in a number of arthropod species, including mosquitoes of the *C. pipiens* complex from various countries (see Bourguet et al., in press), *C. tritaeniorhynchus* (Hemingway et al. 1986b), *Anopheles albimanus* (Hemingway and Georghiou 1983), *An. nigerrimus* (Hemingway et al. 1986a), and in *An. sacharovi* (Hemingway et al. 1992). Inhibition by CB and OP insecticides is usually closely related to resistance ratio. Similar results have also been obtained in *D. melanogaster.* In this species, four sites of point mutations were identified in the AChE molecule (Mutéro et al. 1994). From comparisons with the three-dimensional structure of *Torpedo* AChE, it was determined that these mutations are positioned around the "mouth" of the active-site pocket. Thus, they modify the access of the active site and prevent the binding of OPs or CBs with the active-site serine while having little effect on acetylcholine hydrolysis. These mutations were introduced, alone or in combination, in a wild-type gene by in vitro mutagenesis, and expressed in *Xenopus* oocytes. The highest inhibitions in the presence of insecticides (which correspond to the highest resistance levels in vivo) were observed with enzymes with two or more mutations, and each combination of mutations gave a specific spectrum of resistance. These results, if confirmed in other species, suggest that the probability of occurrence of insensitive AChE giving high resistance levels is very low, since the accumulation of several mutations at specific sites is required in the same gene. Mutéro et al. (1994) think that this is achieved in nature by intracistronic recombinations. If so, identification of the mutations present in wild-type AChEs from a population could be used to predict the probability of occurrence of a gene conferring high resistance to insecticides.

GABA-gated chloride-ion channel ($GABA_A$). Mutations of the $GABA_A$ receptor are responsible for CYD resistance, which accounts for over 60% of insecticide resistance cases, including 73 mosquito species (Georghiou and Lagunes-Tejeda 1991). The gene was cloned in *D. melanogaster* and the sequences of dieldrin-resistant and susceptible insects were compared. Among the mutations detected only one (at position 302) was associated with dieldrin resistance as verified by patch-clamp electrophysiology of the receptor expressed in Xenopus oocytes following site-directed mutagenesis of a susceptible DNA (Ffrench-Constant et al. 1993). With one exception, CYD-resistant *D. melanogaster* possess a serine instead of an alanine at position 302. This same mutation is also present in dieldrin-resistant insects from three different orders: *D. simulans,* the yellow-fever mosquito (*Aedes aegypti*), the house fly (*Musca domestica*), the red flour beetle (*Tribolium castaneum*), the coffee berry borer (*Hypothenemus hampei*), and the American cockroach (*Periplaneta americana*) (see Ffrench-Constant 1994). Such a conservation of amino acid replacements between species infers that very few mutations can cause a sufficient resistance while an adequate function of the receptor is maintained. These mutations are most likely very infrequent events in any given species. Ffrench-Constant et al. (1994) report that preliminary results from sequencing a number of resistant alleles in *D. melanogaster* supports the unique origin of the mutation. However, this remains to be confirmed in this species as well as in others.

Detoxifying Enzymes

In susceptible insects, insecticides are metabolized (or detoxified) by three types of enzymes: mixed function oxidases (such as cytochrome P450 oxidases), glutathione S-transferases, or hydrolases (such as esterases). These enzymes degrade insecticides in non toxic or less toxic molecules or, sometimes, sequester them. Increased detoxification is a common resistance mechanism that prevents inhibition of the targets (Oppenoorth 1985). It is due to either a modification of the enzyme catalytic property or to an increased enzyme production, or to the association of both. Increased enzyme production is in some cases due to gene amplification.

Oxidase- and transferase-mediated resistances. Little is known of the nature of the mutations causing oxidase-mediated resistances to OPs, CBs, PYs, and DDT, except that overproduction of these enzymes is usually inducible in susceptible insects but constitutive in resistant ones. The ubiquitous P450 cytochrome-dependent (P450) oxidases are most often involved in such resistances. A P450 protein of the CYP6 family cloned in *M. domestica* was shown to be overproduced in resistant flies by increased transcription of the protein (Carino et al. 1994). In DDT- and PY-resistant *D. melanogaster* there is an increased production of several CYP6 and CYP4 P450 proteins that differ in their specific activities depending on the insecticide used for selection (Cuany et al., in press). However, this overproduction can be separated from resistance in crossing experiments. Thus, it seems that insecticide exposures select the constitutive overproduction of several P450, but that resistance develops only if one of these overproduced proteins have the capacity of hydrolyzing the insecticide (Bergé JB and Amichot A, personal communication).

Glutathione S-transferases (GST) confer resistance to OPs and DDT. Although GST-mediated resistance has not been reported in mosquitoes, genes of the two fami-

lies (*gst1* and *gst2*) have been cloned in *Ae. aegypti* (Grant and Matsumura 1988) and *An. gambiae* (Reiss and James 1993). In some resistant strains of *M. domestica*, the GST1 protein is clearly overproduced in the absence of gene amplification (Fournier et al. 1992), but in other strains it is likely that point mutations have modified the catalytic properties of the GST1 molecule or that GST2 proteins are also involved (Franciosa H and Bergé JB, personal communication).

Gene amplification of esterases. Increased esterase detoxification is a mechanism of resistance to OP and sometimes to PY in many insect species, including OP-resistant mosquitoes of the *C. pipiens* complex, *C. tarsalis, C. tritaeniorhynchus, An. albimanus, An. stephensi,* and *Ae. aegypti.* In *C. pipiens* and *C. tarsalis* mosquitoes and in *Myzus persicae* aphids, these esterases are overproduced, sometimes to very high levels (esterase B1 may represent up to 12% of the mosquito soluble proteins), and they confer resistance mainly by sequestering insecticides (Cuany et al. 1993; Devonshire and Moores 1982; Ketterman et al. 1992). Evidence that this overproduction is due to gene amplification is only documented for esterases A and B in *C. pipiens* and *C. tarsalis* (see below) and for esterases E4 and FE4 in *M. persicae* (Field et al. 1988).

The esterases involved in OP resistance were first identified on adult homogenates separated by starch gel electrophoresis in the presence of equal quantities of alpha- and beta-naphthyl acetates. This technique differentiates esterases A using preferentially the alpha isomer ("blue" electromorphs) and esterases B using preferentially the beta isomer ("red" electromorphs). Some esterases A are active only when EDTA is present in the electrophoresis buffers, and they were designated as esterases A' to distinguish them from those active in EDTA absence.

Esterases A, B, and A' are coded by distinct linked loci, *Est-1, Est-2,* and *Est-3,* respectively. At each locus, several electromorphs with either a "normal" or a "high" activity have been recorded in addition to null alleles. The high activity of *Est-2* and *Est-3* electromorphs is due to an overproduction of the enzymes which, in the case of *Est-2,* is due to gene amplification (Mouchès et al. 1990; Poirié et al. 1992; Raymond et al. 1991; Vaughan et al. 1995). Overproduction of *Est-3* electromorphs is also due to gene amplification (Vaughan and Hemingway 1995), but it concerns only some esterases A (Rooker et al., in press). At the *Est-2* locus, electromorphs with normal activity are very numerous: up to 8 electromorphs were recorded in southern France in the 1970s (Pasteur and Sinègre 1975) and 16 in Brittany (Raymond et al. 1996). Electromorphs with high activity have been named in order of their discovery: B1, B2, B4, etc. in the *C. pipiens* complex and B3 in *C. tarsalis.* As is often observed with "electrophoretic" alleles, each *Est-2* electromorph may correspond to different alleles that can be separated by sequencing the structural gene or by analyzing the RFLP pattern of the genomic region hybridizing with an esterase B probe. This was clearly demonstrated with an electromorph from strains collected in France and Cyprus, which corresponds to the amplification of two distinct alleles, namely B4 and B5 (Poirié et al. 1992), and for electromorph B1 from California and Cuba (Vaughan et al. 1995). In addition, the same highly active electromorph may correspond to an amplification with different numbers of gene copies (see below). *Est-3* electromorphs with high activity are always associated with specific *Est-2* electromorphs: A1 with the normally active $Est\text{-}2^{0.64}$ and A2, A3, A4, and A5 with B2, B3, B4, and B5, respectively. As for *Est-2, Est-3* electromorphs correspond to different alleles (Guillemaud T, personal communication).

In all amplifications, the sequences flanking the structural gene have been coamplified with the gene. The amplified region, or amplicon, was studied in detail by Mouchès et al. (1990) for the esterase B1 present in the Californian strain Tem-R. The amplicon covers at least 30 kbp and contains a constant and highly conserved "core" of 25 kbp, flanked with sequences of variable size and a lower degree of amplification than the core. In the amplicon, there is a single copy of the esterase B1 structural gene (2.8 kbp), and the gene is framed by two sequences (CE1 and CE2) which are repeated in other parts of the genome. The CE2 sequence contains a modified Juan element which is a truncated LINE-like retrotransposon (Mouchès et al. 1991). The nature of the CE1 sequence is not yet known; it is present in at least some of the variable sequences flanking the amplicon core on its 5' side and could thus be involved in the amplification process, but this is only speculative. Amplicons containing esterase B2, B4, and B5 alleles have widely different restriction maps (Poirié et al. 1992; Raymond et al. 1991), and Rooker et al. (in press) have recently shown that the esterase B2 amplicon also contains the esterase A2 structural gene. Little is known of the other amplified esterase B alleles, such as the Cuban esterase B1 (Vaughan et al. 1995) or the China esterases B6 and B7 (Xu et al. 1994), besides the size of a few restriction fragments hybridizing with the structural gene.

High activity of esterases B is inherited as a monofactorial character (at least in the strains studied, i.e., TEM-R for B1, SeLax for B2, VIM for B4). This is in agreement with the demonstration that, in the TEM-R strain, esterase B1 amplification is present in spermatozoa (Raymond and Pasteur 1989) and that all copies of the structural gene are grouped on the left arm of chromosome 2 as revealed by in situ hybridization on meiotic, mitotic, and polytene chromosomes (Heyse et al. 1996; Nancé et al. 1990). In strains homozygous for an amplified esterase B, interruption of insecticide selection is not accompanied by a rapid loss of esterase expression or resistance (Raymond et al. 1993; Pasteur N, unpublished) as is observed for the amplified esterase E4 in the aphid *M. persicae* (Field et al. 1989). Thus, in the Californian strains Tem-R and Edit with B1, and in SeLax with B2, resistance has remained stable for over 60 generations in absence of selection. However, we know that (1) various levels of esterase B1 amplification are associated with different levels of esterase activity and resistance in laboratory strains as well as in the field (Pasteur et al. 1984), and that (2) continuous selection of the homozygous Tem-R strain clearly increased resistance due to esterase B1 between 1974 and 1980 (Pasteur et al. 1980).

Although esterase B amplification is present in all cells, high quantities of the protein are observed only in some tissues (Nancé 1991). Thus, immunolocalization of esterase B revealed that B1 (in Tem-R strain), B2 (in SeLax strain), and B5 (in Cyprus strain) are overproduced in cells of the intestine and the Malpighian tubes. Esterase B1 is, in addition, overproduced in neurons of the brain, and thoracic and abdominal ganglia, as well as in cells lining the cuticle. These differences in tissue distribution may have important implications on the resistance levels, knowing that all overproduced esterases sequester insecticides. For example, a same 500-fold overproduction of esterase B (as compared to susceptible insects) is present in Tem-R and Cyprus mosquitoes, but Tem-R mosquitoes in which the enzyme is overproduced in the nervous system are 15 times

more resistant than Cyprus mosquitoes. At present, it is not known whether variations in tissue expression exist for nonamplified genes or whether they are a consequence of gene amplification.

Has amplification of the same esterase B allele occurred once or many times? To answer this much debated issue large-scale studies of natural populations have been undertaken. Electromorphs of overproduced esterases have been recorded in many countries around the world by our and other laboratories (see Raymond and Pasteur, in press). The most widely distributed overproduced esterase is B2 which is in high linkage disequilibrium with electromorph A2 in all samples investigated. Strains homozygous for the presence of B2 have been selected from populations collected in California, Texas, Martinique, Pakistan, Thailand, French Polynesia (Moorea), Egypt, Tunisia, Ivory Coast, Congo, and South Africa. Restriction maps of the amplified esterase B2 amplicon were constructed using 13 restriction enzymes. No variations were observed (Raymond et al. 1991, and unpublished data), indicating that the same esterase B2 allele is amplified in all these countries. Thus, the esterase B2 allele was either present in a nonamplified state in all these countries and has been recurrently amplified, or it was amplified once and has then acquired its present geographic distribution by migration. To answer this question an intensive study of nonamplified alleles has been undertaken (Raymond et al., 1996). The restriction maps of nonamplified esterase B alleles with 13 restriction enzymes are as different between themselves as they are from any of the amplified alleles. Surveys of single mosquitoes using a single endonuclease disclosed an extremely high polymorphism of the region flanking the structural gene. At present, it seems that the probability of occurrence of the amplification of a same sequence more than once is extremely low, as Raymond et al. (1991) concluded from the first such study.

Resistance Evolution in Field Populations

Analyses of the mutations of resistance genes suggest that these mutations are quite uncommon and possibly unique events. It follows that the spread of resistance genes is largely governed by migration and selection. At present, there are very few insect species in which all resistance genes can be identified in single individuals, as is the case of OP-resistance genes in the *C. pipiens* complex. The following will only consider this species that occupies temperate (*C. p. pipiens*) as well as tropical (*C. p. quinquefasciatus*) regions throughout the world, and has been submitted to heavy insecticide control in many countries since the 1960s. Control programs have used mainly OP insecticides, and many populations have developed resistance due to target (AChE) insensitivity or/and esterase overproduction.

Migration in *C. pipiens*

To appreciate the role of migration in resistance gene dispersal, gene flow between populations was studied by analyzing the electrophoretic polymorphism of putative neutral genes in different geographic situations.

Along the 1000 km of Mediterranean coast, between Valencia (Spain) and Nice (France) near the Italian border, *C. pipiens* populations are continuously distributed and display extensive genetic exchanges: N_m, the number of effective migrants between sites is unrelated to the geographic distance (Chevillon et al. 1995b). Similarly, genetic exchanges within islands of the Mediterranean (Corsica and Sardinia) or of French Polynesia (Tahiti, Moorea, etc.) are extremely high.

The situation is completely different when considering genetic differentiation between islands. Thus, a strong genetic differentiation was observed between populations from the south of Corsica and the north of Sardinia, separated by a 12 km stretch of water, indicating extremely low gene flow (Chevillon et al. 1995a), but in French Polynesia there was no genetic differentiation between Tahiti and Moorea separated by a 17 km stretch of water (Pasteur et al. 1995). One of the important differences between the two systems of islands is the intensity of commercial traffic: between Corsica and Sardinia, there are no organized commercial exchanges, whereas between Tahiti and Moorea more than 14,000 crosses by aircrafts or ships were recorded in 1992. Thus, gene flow over geographic barriers is strongly influenced by the intensity of commercial exchanges which must be a major component of introduction of resistance genes into new areas.

Selection Against Resistance Genes

Due to the large genetic exchanges between populations, resistance genes should be homogeneously distributed over large areas if there is no geographic barrier. This is not the case: for example, along the Mediterranean coast, Chevillon et al. (1995b) observed that the three common resistance genes (overproduced esterases A1 and A4–B4, and insensitive Ace^R) have high frequencies only where OP insecticides are used for mosquito control, suggesting that these resistance genes have a high fitness in the environment where OP insecticides are used, but not when insecticides are absent. Field investigations were conducted for measuring their fitness in field conditions (Chevillon 1994). In absence of OP, Ace^R was found to have a high cost in adults (1% of the Ace^R carriers die every day in overwintering females) as well as in preimaginal stages, whereas selection against A4–B4 and A1 is mainly expressed in preimaginal stages and is low as compared to that of Ace^R.

There are many examples of the evolution of resistance over time in field populations. Usually, resistance is first restricted to small areas and then expends geographically as insecticide use increases. This geographic expansion is often accompanied by an increase in resistance levels. In the south of France, *C. pipiens* control was started in 1960 and used the OP chlorpyrifos almost exclusively. The increase in OP resistance level with time was due to the successive selection of several genes: overproduced esterase A1 appeared in 1972, insensitive acetylcholinesterase (Ace^R) appeared around 1978, and overproduced esterases A4–B4 and A2–B2 appeared around 1986. In California, a single gene of OP resistance (the amplified esterase B1) was present between 1970 and 1980, and the increase in OP resistance observed during this period can only be attributed to the increase in the number of gene copies with time.

Conclusion

Evolution of resistance in field populations is due to the selection of resistance genes which appear by de novo mutation in the treated populations or are imported by active or passive migration from other areas. Present data seems to indicate that mutations transforming susceptible genes into resistance genes are extremely rare, indicating that the wide distribution of resistance in some species is due to migration. Studies of *C. pipiens* mosquitoes indicate that passive migration associated with commercial transport plays an im-

portant role in the dispersion of such mutations.

All resistance genes, whether conferring a low or high level of resistance are selected as long as the selection pressure is maintained because they confer a high fitness to their carriers. From the few studies done in field populations, it seems that in the absence of insecticide, these genes have a lower fitness than their susceptible counterpart. However, the intensity of selection against resistance genes varies widely with the nature of the genes. Evaluation of these fitnesses in natural conditions in the absence and presence of insecticides should allow us to devise control programs where both selection forces negate each other.

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