Overproduction of detoxifying esterases in organophosphate-resistant Culex mosquitoes and their presence in other insects

(Immunology/insecticides/resistance/detoxification)

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ABSTRACT Anti sera raised against the denatured polypeptide of two organophosphate-detoxifying esterases (B1 and A1) of Culex mosquitoes were used in an immunoblot method to quantify esterase production in resistant versus susceptible strains and to detect the presence of immunologically related proteins in other insects. It was demonstrated that esterase B1 of Culex quinquefasciatus and esterase A1 of Culex pipiens are overproduced in resistant strains by factors of at least 500-fold and 70-fold, respectively, as compared with the corresponding susceptible strains. These factors approximate the levels of resistance to the organophosphate chlorpyrifos determined by bio assay—i.e., about 800-fold and 100-fold, respectively. Anti esterase B1 antiserum was found to react with other type B esterases (B2 of C. quinquefasciatus and B3 of Culex tarsalis) but not with type A esterases (A1 of C. pipiens, A2 of C. quinquefasciatus, or A3 of C. tarsalis); similarly, antiserum A1 antiserum was found to react with other type A esterases (A2 and A3) but not with type B esterases (B1, B2, and B3). Proteins immunologically related to esterase B1 were detected in Aedes aegypti L., Mycus persicae Sultzer, and Musca domestica L., although they were not overproduced in the organophosphate-resistant strains of these species. In none of these species were proteins immunologically related to esterase A1 found.

In many insect species, resistance to organophosphate insecticides is due to increased detoxification activity (1) resulting either from structural modification of the detoxifying enzymes involved or from enhancement of their synthesis. At present, there is no simple method for distinguishing between these two types of mechanisms, although it is clear that such knowledge would improve our understanding of the evolutionary dynamics of resistance and could have important implications in the formulation of strategies for resistance management.

In the present study we have developed immunological techniques for quantifying the synthesis of organophosphate-detoxifying esterases and have demonstrated that in organophosphate-resistant Culex mosquitoes these enzymes may be produced in markedly greater amounts, to 500-fold greater, than in susceptible strains.

In mosquitoes, as well as in many other insects (2–13), organophosphate-detoxifying esterases are able to degrade large quantities of naphthyl acetate substrates. In Culex, two types of such esterases, A and B, have been recognized (6). These esterases have different electrophoretic mobilities depending on the species and, in some cases, on the origin of the mosquito. To date, three "electromorphs," or variations of each esterase type have been recognized by direct electro-

trophoretic comparison (14)—namely, A1 in French Culex pipiens L.; A2, B1, and B2 in Californian Culex quinquefasciatus Say; and A3 and B3 in Californian Culex tarsalis Coq. Esterases A1 and B1 are encoded by different genetic loci (15) and differ considerably in their structure (16): esterase A1 is a 120-kDa dimer containing two identical 60-kDa subunits, whereas B1 is a 67-kDa monomer.

MATERIALS AND METHODS

Purification of Esterases and Preparation of Specific Antibodies. Esterases A1 and B1 were purified from strain S54 of C. pipiens and strain Tem-R of C. quinquefasciatus, respectively. These strains are homozygous for the presence of the respective highly active enzymes (15). Mass homogenates of adults were prepared in a 0.025 Mimidazole-saline buffer at pH 7.4 and subjected to two consecutive ultracentrifugations at 10,000 and 100,000 × g. Supernatant proteins were then submitted to chromatofocusing between pH 4.0 and pH 7.4 (16). The fractions containing esterase activity were pooled, and their proteins were precipitated by the addition of trichloroacetic acid to a final concentration of 10% (wt/vol). The precipitated proteins were resuspended in an electrophoresis sample buffer containing NaDodSO4 and β-mercaptoethanol (17) and denatured by heating for 10 min at 100°C. They were then separated by NaDodSO4/PAGE (17), and the bands corresponding to esterases A1 or B1 were excised from the gels following their identification by staining with Coomassie blue. This identification was facilitated by including on the same gel extracts in which the esterase polypeptides had been labeled with tritiated disopropyl fluorophosphate before heat denaturation (16, 18).

The excised gels containing the esterases were homogenized in sodium phosphate buffer, pH 7.2. After emulsification in complete Freund’s adjuvant, these homogenates were injected intradermally at multiple sites into rabbits. Rabbits were given booster injections of similar homogenates by using Freund’s incomplete adjuvant 3 and 6 weeks after the first injection and were bled 2 weeks later. A total of about 250 μg of antigen was used to raise each antisera. For control assays nonspecific sera were obtained from blood collected before immunization of the rabbits.

Detection and Quantification of Esterases in Various Culex and Other Insect Species Using the Antisera. Soluble proteins from mass crude homogenates of various insects (Table 1) were obtained by grinding insects in 0.025 Mimidazole buffer. The proteins were denatured by heating in the presence of NaDodSO4 and β-mercaptoethanol. After electrophoresis on 15% acrylamide/0.1% NaDodSO4 gels, proteins were transferred to nitrocellulose sheets by electrophot-
horseradish peroxidase-conjugated sheep anti-rabbit immunoglobulins as secondary antibody. The substrate for the peroxidase reaction was 4-chloro-1-naphthol (20).

RESULTS

Overproduction of Esterases B1 and A1 in Organophosphate-Resistant Strains Tem-R and S54. Rabbit antisera were produced (i) against the 67-kDa polypeptide contained in esterase B1 of the Tem-R strain of *C. quinquefasciatus* from California and (ii) against the 60-kDa polypeptide contained in esterase A1 of the S54 strain of *C. pipiens* from France. These antisera were tested against the resistant strains as well as the corresponding susceptible strains S-Lab and Bleuet (see Table 1 for list of strains).

Antiserum B1 reacted strongly with Tem-R extracts on immunoblots; after electrophoresis of the mosquito extracts and their transfer to a nitrocellulose sheet, a single polypeptide was evident (Fig. 1a, lane 2), corresponding to the 67-kDa polypeptide used to produce the antiserum. From the staining intensity of the peroxidase reaction, it was evident that this immunoreactive protein is present only at a very low level in extracts of the organophosphate-susceptible S-Lab mosquitoes—i.e., individuals lacking the highly active ester-

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Table 1. Species and strains of insects examined by immunoassay for the presence or absence of highly active esterases A and B

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain and origin</th>
<th>Status of organophosphate susceptibility*</th>
<th>Highly active esterase present</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. <em>Culex pipiens</em></td>
<td>Bleuet (France)</td>
<td>S</td>
<td>—</td>
</tr>
<tr>
<td>2. <em>Culex pipiens</em></td>
<td>MSE (France)</td>
<td>R</td>
<td>—</td>
</tr>
<tr>
<td>3. <em>Culex pipiens</em></td>
<td>S54 (France)</td>
<td>R</td>
<td>A1</td>
</tr>
<tr>
<td>4. <em>C. quinquefasciatus</em></td>
<td>Tem-R (California)</td>
<td>R</td>
<td>B1</td>
</tr>
<tr>
<td>5. <em>C. quinquefasciatus</em></td>
<td>Tem-S (California)</td>
<td>S</td>
<td>—</td>
</tr>
<tr>
<td>6. <em>C. quinquefasciatus</em></td>
<td>Coachella (California)</td>
<td>R</td>
<td>B1</td>
</tr>
<tr>
<td>7. <em>C. quinquefasciatus</em></td>
<td>Willow (California)</td>
<td>R</td>
<td>A2, B1, B2</td>
</tr>
<tr>
<td>8. <em>C. quinquefasciatus</em></td>
<td>Pat (California)</td>
<td>R</td>
<td>A2, B1, B2</td>
</tr>
<tr>
<td>9. <em>C. quinquefasciatus</em></td>
<td>S-Lab (California)</td>
<td>S</td>
<td>—</td>
</tr>
<tr>
<td>10. <em>C. tarsalis</em></td>
<td>Norco (California)</td>
<td>R</td>
<td>A3, B3</td>
</tr>
<tr>
<td>11. <em>Aedes aegypti</em></td>
<td>Tinker-R (Caribbean)</td>
<td>R</td>
<td>—</td>
</tr>
<tr>
<td>12. <em>Aedes aegypti</em></td>
<td>Rock (?)</td>
<td>S</td>
<td>—</td>
</tr>
<tr>
<td>13. <em>Musca domestica</em></td>
<td>Hirokawa (Japan)</td>
<td>R</td>
<td>—</td>
</tr>
<tr>
<td>14. <em>Musca domestica</em></td>
<td>WHO/SRS (Italy)</td>
<td>S</td>
<td>—</td>
</tr>
<tr>
<td>15. <em>Myzus persicae</em></td>
<td>R2 (U.K.)</td>
<td>R</td>
<td>E-4 (ref. 10)</td>
</tr>
<tr>
<td>16. <em>Myzus persicae</em></td>
<td>Avignon (France)</td>
<td>R</td>
<td>E-4</td>
</tr>
<tr>
<td>17. <em>Myzus persicae</em></td>
<td>Susc. (?)</td>
<td>S</td>
<td>—</td>
</tr>
<tr>
<td>18. <em>Apis mellifera</em></td>
<td>? (France)</td>
<td>S</td>
<td>—</td>
</tr>
<tr>
<td>19. <em>Apis mellifera</em></td>
<td>? (France)</td>
<td>S</td>
<td>—</td>
</tr>
<tr>
<td>20. <em>Drosophila melanogaster</em></td>
<td>MH19 (Canada)</td>
<td>S</td>
<td>—</td>
</tr>
</tbody>
</table>

* S, susceptible; R, resistant.
† Tem-S was derived from Tem-R by propagation of individuals lacking esterase B1, following the release of selection pressure.

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Fig. 1. Immunological evidence for an overproduction of esterases B1 and A1 in organophosphate-resistant mosquitoes. Two hundred micrograms of soluble proteins, extracted from adult mosquitoes of each strain, were submitted to electrophoresis and transferred to nitrocellulose sheets by electroblotting before (lanes 1 and 2) or after (lanes 3–9) serial dilution. The immunoblots were probed with the antisera raised against esterase B1 (a) or against esterase A1 (b): (a) proteins from the organophosphate-susceptible strain S-Lab (lane 1) and the organophosphate-resistant strain Tem-R (lanes 2–9); (b) proteins from the organophosphate-susceptible strain Bleuet (lane 1) and the organophosphate-resistant strain S54 (lanes 2–9). Positions of purified esterases A1 and B1 run on parallel lanes of the same gels are indicated. The apparent molecular masses of immunoreactive proteins were calculated from protein markers stained with Coomassie blue and run on a parallel lane of the same gels.
ase B1 (Fig. 1a, lane 1). As antiseras from preimmune rabbits gave no positive reaction, it can be concluded that the positive reaction observed is specific to the polypeptide injected into the rabbit and that this polypeptide is overproduced in Tem-R as compared with S-Lab mosquitoes.

Antiserum A1 reacted strongly with two immunoreactive proteins of S54 mosquito extracts (Fig. 1b, lane 2). One of these corresponds to the 60-kDa subunit of esterase A1 that had been injected into the rabbit. This protein showed a much stronger staining intensity (indicating higher quantity) in S54 mosquitoes—i.e., those possessing the highly active esterase A1, than in organophosphate-susceptible, Bleuet insects—i.e., those lacking A1 esterase with high activity (Fig. 1b, lane 1). The second protein had a size of 40-kDa and was present in equivalent quantities in both S54 and Bleuet mosquitoes. Neither the 60- nor the 40-kDa polypeptides were detected on immunoblots using a preimmune antiserum.

The quantities of esterases B1 and A1 contained in Tem-R and S54 mosquitoes were estimated in relation to those of the corresponding susceptible strains (S-Lab and Bleuet) using aliquots of serial dilutions. The immunoblots obtained with these aliquots (Fig. 1a, lanes 2–9, for Tem-R aliquots tested with antiserum-B1 and Fig. 1b, lanes 2–9, for S54 aliquots tested with antiserum-A1) indicate that esterase B1 is at least 500-fold more abundant in Tem-R than in S-Lab and esterase A1 is some 70-fold more abundant in S54 than in Bleuet. These quantities of esterases approximate the levels of resistance to the organophosphate chlorpyrifos determined by bioassay in Tem-R and S54—i.e., about 800-fold and 100-fold, respectively.

Detection of Esterases B and A in Various Culex Strains. The antibodies raised against esterases B1 and A1 from Tem-R and S54 mosquitoes were tested with whole-body extracts of mosquitoes belonging to various susceptible and organophosphate-resistant strains of Culex, some of which possess different electrophoretic forms of detoxifying esterases (Table 1).

With antiserum B1, a strong immunoreaction was observed in several organophosphate-resistant strains of Californian C. quinquefasciatus (Coachella Pat, and Willow, Fig. 2a, lanes 6–8) and in C. tarsalis (Norco, Fig. 2a, lane 9). In all cases, only one immunoreactive protein (57-kDa) was detected, as occurred with Tem-R extracts (Fig. 2a, lane 4). This result was expected with the Coachella strain that, like Tem-R, is homozygous for esterase B1 (14); it was surprising, however, with the Pat and Willow strains that, in addition to esterase B1, contain esterase B2 (14); and the result was totally unexpected with the C. tarsalis Norco strain that lacks esterase B1 but possesses esterase B3. Comparison of these serologically related polypeptides by V8 protease mapping (21) confirms without ambiguity that a protein identical or nearly identical to the Tem-R esterase B1 subunit was indeed overproduced in these mosquitoes. On the contrary, such a protein was not found, or found only at very low levels, in the organophosphate-susceptible strains of C. quinquefasciatus tested (S-Lab, Fig. 2a, lane 5, and Tem-S, data not shown). Such a protein was also absent from the various strains of C. pipiens tested—i.e., the organophosphate-susceptible Bleuet strain, the organophosphate-resistant MSE strain that possesses an insensitive acetylcholinesterase, and the S54 strain that contains an enhanced amount of esterase A1 (Fig. 2a, lanes 1, 2, and 3, respectively).

With antiserum A1, a strong immunoreaction was observed with a 60-kDa polypeptide from the two C. quinquefasciatus strains Pat and Willow (Fig. 2b, lanes 7 and 8, respectively). A polypeptide of slightly smaller size (higher electrophoretic mobility) was also detected in the C. tarsalis strain Norco (Fig. 2b, lane 9). Comparisons by V8 protease mapping of these serologically related polypeptides demonstrated that a protein nearly identical to the esterase A1 subunit that is contained in S54 was indeed overproduced in the C. quinquefasciatus Pat and Willow strains, as well as in the C. tarsalis Norco strain. By contrast, no immunoreaction, or only a very low immunoreaction, was detectable in the protein extracts from the C. pipiens Bleuet and MSE strains, or in the protein extracts from the C. quinquefasciatus S-Lab, Tem-R, and Coachella strains (Fig. 2b, lanes 1, 2, 4, 5, and 6, respectively).

Thus, antiserum B1 recognizes esterases B1, B2, and B3, whereas antiserum A1 recognizes esterases A1, A2, and A3 in the Culex genus. There is no cross reaction between antiserum B1 and the various type A esterases (A1, A2, and A3) or between antiserum A1 and the various type B esterases (B1, B2, and B3). This specificity of A and B esterase types was further confirmed by sequentially probing a nitrocellulose sheet, first with antiserum B1 and then with antiserum A1, after transfer of the proteins of mosquitoes containing both types of esterases from a NaDodSO4/polyacrylamide gel. After each antiserum addition, the antigen–antibody complexes were detected as described above. The two 67-kDa and 60-kDa polypeptides could thus be revealed sequentially (data not shown).

Search for Esterases B and A in Insects Other Than Culex. The finding of esterases B and A in C. tarsalis, a species distantly related to those of the C. pipiens complex, prompted us to survey immunologically various organophosphate-susceptible and resistant strains of other insect species (Table 1) for the presence of these esterases.

Antiserum B1 revealed the presence of proteins serologically related to the Tem-R esterase B1 subunit in strains of Aedes aegypti L., Myzus persicae Sultzer, and Musca domestica L. However, these proteins were present in equivalent quantities in the susceptible as well as in the
resistant strains of these species. No protein serologically related to esterase B1 was found in two colonies of *Apis mellifera* L., or in the single strain of *Drosophila melanogaster* Mg. tested.

No protein related to esterase A1 has been found in any of the above species other than in those of the genus *Culex*. It is also noteworthy that in *M. persicae* the highly active detoxifying esterase E4 responsible for organophosphate-resistance (10) is not recognized by any of our antisera.

**DISCUSSION**

The present study has provided direct evidence of abundant overproduction of detoxifying proteins in insecticide-resistant insects. In mosquitoes of the genus *Culex* studied here, these proteins are esterases of two types, A and B, that can be identified by specific antisera raised against a polypeptide that was purified from a particular electromorph of each esterase type (electromorphs A1 and B1). The immunological assays show that the two proteins, or proteins very similar to them, are present, at least at a low level, in most, if not all, of ten strains belonging to three species of *Culex*. In mosquitoes that are organophosphate-resistant due to increased detoxification by esterases, there is overproduction of either a single esterase type (esterase A in strain S54 of *C. pipiens* or esterase B in strains Tem-R and Coachella of *C. quinquesacius*) or of both types (esterases A and B in strains Willow and Pat of *C. quinquesacius* and in strain Norco of *C. tarsalis*). This overproduction varies with the strains examined: it was estimated by immunological assays to be 70-fold and 500-fold higher in the S54 and Tem-R organophosphate-resistant strains, respectively, than in the corresponding susceptible strains.

Surprisingly, no cross-serological relationship was detected between the two esterases. As their respective antibodies were raised against heat-denatured proteins and are directed against several epitopes of their antigens (C.M., unpublished data), it can be assumed that there are large differences in the amino acid sequences of esterases A and B. Thus, if the genes coding these enzymes have arisen from a duplication of an ancestral gene, this duplication has undoubtedly occurred a long time ago.

Both the absence of a serological relationship between the two esterases and the evidence that organophosphate-resistance in *Culex* results from an overproduction of these enzymes raise the prospect of developing immunological assays to survey for organophosphate-resistance in these mosquitoes. These assays can undoubtedly be developed for use on individual insects, and preliminary investigations indicate that they can be used on larvae as well as on adults. They may thus serve as a valuable supplement of traditional bioassays.

Moreover, as esterase B-type polypeptides have been identified in other insect genera in addition to *Culex*, immunological assays might eventually find wider application in resistance surveys.

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