ogy as with DSPE–PEG 2000. Since the concentrations of PEG 5000 containing proteins could also be measured by Skoog's method (13), we believe that this modified method would also work.

In conclusion, a simple and direct colorimetric method, modified from Skoog's PEG measurement method (13), for determining the amount of DSPE–PEG 2000 in buffer solutions containing blood or albumin was reported. The concentrations of DSPE–PEG 2000 in those solutions could be determined at 435 nm as low as 25 μg/ml with simple sample preparations. It may facilitate the application of PEG–liposomes in drug delivery or blood substitutes.

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REFERENCES

1. Allen, T. M., Hansen, C., Martin, F., Redemann, C., and Young, A. Y. (1991) Liposome containing synthetic lipid derivatives of poly(ethylene glycol) show prolonged circulation half-lives in vivo. *Biochim. Biophys. Acta* **1066,** 29–36.
2. Allen, T. M. (1994) Long-circulating (sterically stabilized) liposome for targeted drug delivery. *Trends Pharmacol. Sci.* **15,** 215–220.
3. Woodle, M. C., Newman, M. S., and Martin, F. J. (1992) Liposome leakage and blood circulation: Comparison of adsorbed block copolymers with covalent attachment of PEG. *Int. J. Pharm.* **88,** 327–334.
4. Unezaki, S., Maruyama, K., Hosoda, J. I., Nagae, I., *et al.* (1996) Direct measurement of the extravasation of polyethylene glycol-coated liposomes into solid tumor tissue by in vivo fluorescence microscopy. *Int. J. Pharm.* **144,** 11–17.
5. Yoshioka, H. (1991) Surface modification of hemoglobin-containing liposomes with polyethylene glycol prevents liposome aggregation in blood plasma. *Biomaterials* **12,** 861–864.
6. Chung, T. W., Huang, Y. Y., and Wu, C. I. (1999) PE-PEG/LEH can reduce its interactions with plasma expanders indexed through viscosity measurements. *Art. Cells Blood Substrates Immobil. Biotech.* **27,** 215–227.
7. Phillips, W. T., Klipper, R. W., Awasthi, V. D., Rudolph, A. S., Cliff, R., Kwasiborski, V., and Goins, B. A. (1999) Polyethylene glycol-modified liposome-encapsulated hemoglobin: A long circulating red cell substitute. *J. Pharmacol. Exp. Ther.* **288,** 665–670.
8. Ishiwata, H., Vertut-Doï, A., Hirose, T., and Miyajima, K. (1995) Physical chemistry characteristics and bio-distribution of poly-(ethylene glycol)-coated liposome using poly(oxyethylene) cholesteryl ether. *Chem. Pharm. Bull.* **43,** 1055–1001.
9. Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal. Biochem.* **72,** 248–254.
10. Sedmak, J. J., and Grossberg, S. E. (1977) A rapid, sensitive, and versatile assay for protein using coomassie brilliant blue G250. *Anal. Biochem.* **76,** 544–552.
11. Parr, M. J., Ansell, S. M., Choi, L. S., and Cullis, P. R. (1994) Factors influencing the retention and chemical stability of poly-(ethylene glycol)–lipid conjugates incorporated into large unilamellar vesicles. *Biochim. Biophys. Acta* **1195,** 21–30.
12. Boada, J., Montserrat, G., and Estelrich, J. (1997) Determination of polyethylene glycol activated with cyanuric chloride in liposomes. *Anal. Biochem.* **253,** 33–36.
13. Skoog, B. (1979) Determination of polyethylene glycols 4000 and 6000 in plasma protein preparation. *Vox Sang.* **37,** 345–349.
14. Bio-Rad Protein assay, Bulletin 1069, 1123 US/EG, Bio-Rad Laboratories, CA.
15. Torchilin, V. P., Omelyanenko, V. G., Papisor, M. I., Bogdannov, A. A., Trubetskoy, V. S., Herron, J. N., and Gentry, C. A. (1994) Poly(ethylene glycol) on the liposome surface: On the mechanism of polymer-coated liposome longevity. *Biochim. Biophys. Acta* **1195,** 11–20.

Quantitative Polymerase Chain Reaction to Estimate the Number of Amplified Esterase Genes in Insecticide-Resistant Mosquitoes

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Amplification of genes coding for detoxifying esterases is the most common mechanism involved in resistance to organophosphate insecticides in the mosquito *Culex pipiens.* Previous attempts to estimate amplification levels were rather imprecise. The reliability of quantitative PCR is presently tested using a nonamplified gene (*Ace.2*) as an internal control.

Esterase overproduction involves alleles, at either *Est-3* or *Est-2* loci, expressing a higher amount of esterase and providing a substantial level of OP resistance. The number of those alleles involved in resistance is limited (contrarily to alleles expressing a basal amount of esterase and providing a normal level of susceptibility to OP), and a name has been given for each overproduced esterase: A*i* or B*i* where the letter corresponds to the *Est-3* or *Est-2* locus, respectively, the *i* indice indicating the discovery order (for a review, see Ref. 1). Esterase overproduction is due to a constitutive gene amplification in all cases but one, the overproduction of esterase A1 resulting from a change in gene regulation (2, 3). Due to their close proximity (2 to 6 kb of DNA between *Est-3* and *Est-2*), both loci are often coamplified as a single unit leading to a complete association of overproduced A*i* and B*i* esterases (2, 3). There is evidence that the number of copies varies between amplified alleles (2, 4) and for the same amplified allele between strains of different geographical origins (5). Variation in amplification levels might even have occurred within a single strain. In TEM-R strain,

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the *Est-2* allele coding for esterase B1 was found to be amplified 150 times (6) or at least 250 times (7) when estimated by the intensity of an autoradiography spot from a ^{32}P hybridization after a restriction fragment length polymorphism gel and a Southern transfer on a nylon membrane, but an estimate of only 20 copies has been found 10 years later using a beta imager to count the radioactive signal of a dot blot hybridization after serial dilutions (2). These differences either are artifacts due to the different techniques used or actually reflect a ca. 10-fold decrease in the amplification level in the TEM-R strain during a 10-year period.

In order to have a reliable technique to estimate the amplification level of *Est-2* in a single mosquito, a quantitative PCR approach was undertaken. Two PCR were performed on genomic DNA of each mosquito: one was specific for the *Est-2* locus and the other was specific for the *Ace-2* locus (coding a noncholinergic acetylcholinesterase) which is not involved in insecticide resistance and is present in a single copy per haploid genome. The quantitative comparison of these two PCR gave the mean copy number of the *Est-2* locus per haploid genome. This method was calibrated and assessed on various susceptible and resistant mosquitoes and was compared to a previous quantitative PCR attempt (8) which did not displayed the required conditions for a reliable estimation of gene copy number.

Specific amplification of Est-2 and Ace-2 loci. We designed primers in the exon 4 of *Est-2* (9) (Bquantidir 5′CCGACGAGCTGTCCTATCTG3′ and Bquantirev 5′CGTCGTTGGCAATGTTCAG3′) and in the exon 3 of *Ace-2* (10) (Acequantidir 5′GCAGCACCAGTCCAAGG3′ and Acequantirev 5′CTTCACGGCCGTTCAAGTAG3′) to amplify a 216- and a 208-bp fragment, respectively. The DNA quantity was estimated after each PCR cycle (performed with a Roche light cycler) by measuring the fluorescent dye incorporation (SYBR Green) in the PCR product. To avoid unspecific products that prevent quantitative measurements, we used a high annealing temperature and a hot start PCR using anti-*Taq* antibody (Clontech). Two standards curves were obtained for *Est-2* and *Ace-2* by dilution between 2×10^4 pg and 2 pg of genomic DNA of susceptible mosquitoes in salmon sperm DNA (5 μg/ml in water) as follows. The fluorescence profiles obtained for each dilution intercept an arbitrary noise band at distinct cycle numbers (Fig. 1, top). Regression of the cycle number of these crossing points to the known dilution factor defines then each standard curve (Fig. 1, bottom). The three criteria required for an accurate estimate of gene copy number were fulfilled: first no unspecific products were detected by melting curve analysis, second the log of fluorescence increased linearly along PCR cycles for a wide dilution range, and third all the linear parts of the log(fluorescence) curves

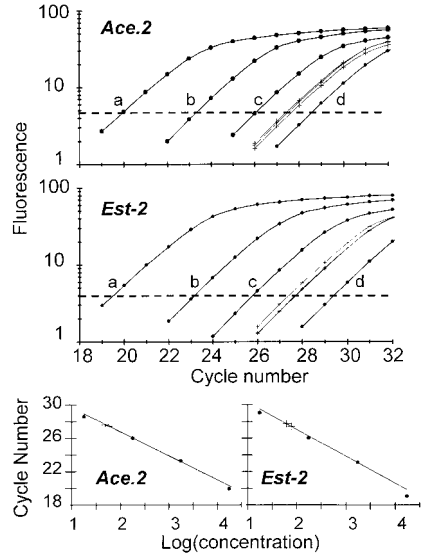

FIG. 1. Testing the quantitative PCR accuracy for measuring *Est-2* copy numbers relatively to *Ace.2* ones. 2 μl of genomic DNA (between 200 and 2000 pg) was mixed with 0.5μM each primer and 2.16 μl of anti-*Taq*-containing master mix and completed to 20 μl with water (master mix and anti-*Taq* antibody are used according to Roche light cycler instructions for SYBR technology (13)). PCR was run with 45 cycles defined by 94°C for 0 s, 65°C for 10 s, and 72°C for 15 s, followed by a continuous denaturation step between 70 and 94°C in order to generate the melting curve for T_m analysis. (Top) Fluorescence across cycle numbers for serial DNA dilutions (dots and a-to-d indexed lines; DNA quantities are 20,000, 2000, 200, and 20 pg in a–d, respectively) and for an experimental triplicate (crosses); dotted lines indicate the noise bands used. (Bottom) Standard curves corresponding to regression analyses of the number of cycles required for each dilution (dots) to intercept the same noise band and estimates (triplicate) using these regressions (crosses). Observed slopes for *Ace.2* (−2.86) and *Est-2* (−3.23) standard curves are close to the expected value (−3.22).

were parallel (Fig. 1). PCR products were sequenced (using the PCR quanti primers with a Big Dye Terminator on a ABI Prism 310 sequencer, Perkin–Elmer) and confirmed that the amplified genes were the expected ones.

Quantification of Est-2 copy numbers. Genomic DNA of each mosquito was diluted between 100 pg and 1000 pg/μl. Standard curves were performed for each light cycler run, such that the cycle number to which the log(fluorescence) profile of a given sample intercept the noise band used to establish these standard curves (see above) can be directly converted to a concentration. Each DNA template was analyzed in triplicate for *Est-2* and *Ace-2* quantification. The ratio between *Est-2* and *Ace-2* arbitrary concentrations provides the copy number of *Est-2* relatively to *Ace-2*, i.e., per haploid genome. The triplicates were used to compute 95% confidence intervals.

Variation in Est-2 copy numbers. Results for 14 mosquitoes lacking overproduced esterase are dis-

played in Fig. 2. Average *Est-2* copy number(s) among individuals (1.26 ± 0.42) is not different from the expected value of 1 (extreme values were found to be 0.88 ± 0.06 and 2.33 ± 0.15). These slight variations are best explained by pipeting errors during the loading of the capillary tubes. This precision level seems acceptable to confidently detect *Est-2* amplification, as shown by comparing this group of mosquitoes to the natural population JASMIN (Fig. 3A): in these mosquitoes, the range of *Est-2* amplification varied from 0.6 to 74, with a mean of 18.6 copies ($N = 28$). Estimates above 2.3 copies (i.e., the maximum found in absence of amplification) are likely to represent amplified alleles and are at the frequency of 0.82, which is not different (Fisher exact test, $P > 0.75$) from the frequency of A2–B2 detected by protein overproduction (i.e., 0.83, Ref. 11). The large variability of *Est-2* copy numbers in this field sample indicates that alleles with very distinct amplified level of A2–B2 are segregating in natural populations. This is not in contradiction with a recent estimate of a 80-fold amplification in an A2–B2 laboratory resistant strain (8), although this value is to be taken with caution, as data showing the good quality of the quantitative PCR (e.g., the linear and parallel relationship between the log(fluorescence) and the cycle number for a wide range of concentrations) were not apparent. As A2–B2 amplification results from one recent molecular event (12), this variation in amplification levels within an insular population (Tahiti) has been probably generated within few generations. This suggests that the molecular events generating this variation (possibly unequal recombination) are not uncommon and could be studied under laboratory conditions.

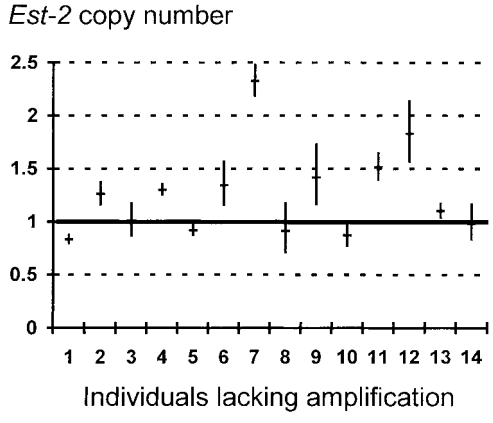

FIG. 2. Ratio (mean and 95% confidence intervals) of *Est-2* copy numbers (relatively to *Ace.2*) among mosquitoes lacking overproduced esterase, thus supposed to possess only a single copy of *Est-2* per haploid genome (from Belgium and The Netherlands, strains BRUGES-B (1 and 2) and HETEREN (5 and 6) (14); southern France, strain BLEUET (10 and 11) (15); California, strains S-LAB (12–14), TRANS (7–9), and PRO-R (3 and 4) (16, 17)).

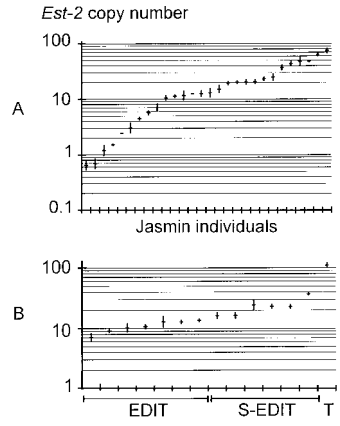

FIG. 3. Mean and 95% confidence intervals of the copy number estimates for two *Est-2* amplified alleles. (A) In population JASMIN (collected in Tahiti in 1992) where mosquitoes overproducing A2–B2 esterases are at a frequency of 0.83 (11). (B) In EDIT, S-EDIT (two strains differing by the insecticide treatment (18) and TEM-R strains (7), where each mosquito overproduces B1.

This was confirmed with EDIT and S-EDIT mosquitoes carrying the amplified allele coding esterase B1. These two strains were separated in April 1992 from the same family homozygous for a particular B1 amplification level and have been reared since then either in absence of insecticide (EDIT) or under continuous OP selection (S-EDIT). After less than 75 generations the amplification level differs between strains, with an average of 23 ± 7 copies in S-EDIT and 11 ± 2 in EDIT (Fig. 3B). Both amplification levels are lower than in the TEM-R reference strain (123 ± 13), indicating that a large potential increase in copy numbers still exists in S-EDIT.

Quantitative PCR appears as a convenient technique to estimate gene amplification level: it requires single individuals DNA amount, allows analysis of several DNA templates simultaneously, and offers the possibility to compute error estimates. The present results on the mosquito *C. pipiens* show that there is a substantial variation in *Est-2* copy numbers in field populations and that the increase in the amplification level in response to insecticide selection can be now accurately measured to better understand its rapid evolution.

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REFERENCES

1. Raymond, M., Chevillon, C., Guillemaud, T., Lenormand, T., and Pasteur, N. (1998) *Phil. Trans. R. Soc. Lond. B* **353,** 1–5.
2. Guillemaud, T., Makate, N., Raymond, M., Hirst, B., and Callaghan, A. (1997) *Insect Mol. Biol.* **6,** 319–327.

3. Rooker, S., Guillemaud, T., Bergé, J., Pasteur, N., and Raymond, M. (1996) *Heredity* **77,** 555–561.
4. Poirié, M., Raymond, M., and Pasteur, M. (1992) *Biochem. Genet.* **30,** 13–26.
5. Callaghan, A., Guillemaud, T., Makate, N., and Raymond, M. (1998) *Insect Mol. Biol.* **7,** 295–300.
6. Raymond, M., Beyssat-Arnaouty, V., Sivasubramanian, N., Mouchès, C., Georghiou, G. P., and Pasteur, N. (1989) *Biochem. Genet.* **27,** 417–423.
7. Mouchès, C., Pasteur, N., Bergé, J. B., Hyrien, O., Raymond, M., Robert de Saint Vincent, B., De Silvestri, M., and Georghiou, G. P. (1986) *Science* **233,** 778–780.
8. Paton, M. G., Karunaratne, S. H. P. P., Giakoumaki, E., Roberts, N., and Hemingway, J. (2000) *Biochem. J.* **346,** 17–24.
9. Vaughan, A., Hawkes, N., and Hemingway, J. (1997) *Biochem. J.* **325,** 359–365.
10. Malcolm, C. A., Bourguet, D., Ascolillo, A., Rooker, S. J., Garvey, C. F., Hall, L. M. C., Pasteur, N., and Raymond, M. (1998) *Insect Mol. Biol.* **7,** 107–120.
11. Pasteur, N., Marquine, M., Rousset, F., Failloux, A.-B., Chevillon, C., and Raymond, M. (1995) *Genet. Res.* **66,** 139–146.
12. Raymond, M., Callaghan, A., Fort, P., and Pasteur, N. (1991) *Nature* **350,** 151–153.
13. Wittner, C. T., Ririe, K. M., Andrew, R. V., David, D. A., Gundry, R. A., and Balis, U. J. (1997) *BioTecniques* **22,** 176–181
14. Raymond, M., Qiao, C. L., and Callaghan, A. (1996) *Genet. Res.* **67,** 19–26.
15. Rioux, J. A., and Puech, J. (1961) *C. R. Soc. Biol.* **155,** 343–344.
16. Georghiou, G. P., Metcalf, R. L., and Gidden, F. E. (1966) *Bull. World Health Org.* **35,** 691–708.
17. Priester, T. M., and Georghiou, G. P. (1978) *J. Econ. Entomol.* **71,** 197–200.
18. Guillemaud, T., Raymond, M., Tsagkarakou, A., Bernard, C., Rochard, P., and Pasteur, N. (1999) *Heredity* **83,** 87–99.

Quantification of Phosphatidylinositol 3,4,5-Trisphosphate by Liposome Lysis Assay with Specific Monoclonal Antibodies

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PI 3-kinase is an enzyme that catalyzes phosphorylation of PI 4,5-P_2 to yield PI 3,4,5-P_3. This enzyme is often activated after stimulation of animal cells with growth factors, elevating the level of PI 3,4,5-P_3 within a few minutes (1, 2). The roles of PI 3-kinase have been studied with selective inhibitors such as wortmannin or LY294002, dominant-negative or constitutively active mutant PI 3-kinases, and growth factor receptors defective in their interaction with PI 3-kinases, in a number of laboratories. PI 3,4,5-P_3 has been shown to be an important lipid second messenger that regulates cytoskeletal rearrangement, vesicle transport, and gene expression. A number of downstream target factors of PI 3,4,5-P_3 have been identified, such as atypical protein kinase C, Arf-guanine nucleotide exchange factor, or PI 3,4,5-P_3-dependent protein kinases (1, 2). PI 3,4,5-P_3 can then be dephosphorylated to yield PI 3,4-P_2, which is also an important second messenger capable of activating Akt/PKB, a serine threonine kinase that is involved in signal transduction for cell survival (1, 3). It is, therefore, important to quantify intracellular levels of PI 3,4,5-P_3.

To date, quantification of these phospholipids has been done mainly by labeling of the cells with appropriate isotopes and analysis of relevant deacylated products of lipids by high-performance liquid chromatography or by direct analysis of lipids by thin-layer chromatography (4, 5). However, these methods give only relative levels of PI 3,4,5-P_3. To establish a convenient method for quantification of PI 3,4,5-P_3, we produced monoclonal antibodies specific to PI 3,4,5-P_3.

Mice were immunized with D-(+)-1-palmitoyl, 2-arachidonyl- or D-(+)-1,2-dioctadecanoyl-PI 3,4,5-P_3 mixed with *Salmonella minnesota* which was fixed with acetone for 6 months at intervals of 2 to 4 weeks (6, 7). A booster injection was then given, and fusion with SP2/0 was performed 3 days later (7). Hybridoma supernatants were screened for antibodies against D-(+)-1-palmitoyl, 2-arachidonyl-PI 3,4,5-P_3 by liposome lysis assay. Liposomes were prepared as described by Miyazawa *et al.* (7). Briefly, 250 μl of chloroform solution containing 1.0 mM dimyristoylphosphatidylcholine, 1.2 mM cholesterol, 2.2 mM diethyl phosphate, and 0.022 mM PI 3,4,5-P_3 or other phospholipids was dried in a glass test tube by rotary evaporator at 40°C and further dried *in vacuo.* This dried film of lipids was dispersed in 150 μl of marker solution containing 75 mM calcein by vortexing. The resulting liposomes were spun down at 8000*g* and resuspended in HBS[3] solution containing 10 mM Hepes–NaOH, pH 7.4, and 150 mM NaCl. After repeating this procedure four times, the liposomes

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[3] Abbreviations used: HBS, Hepes-buffered solution; CHO-IR, Chinese hamster ovary cells overexpressing insulin receptor.

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