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Coamplification of human acetylcholinesterase and butyrylcholinesterase genes in blood cells: Correlation with various leukemias and abnormal megakaryocytopoiesis

(acute myeloid cell anomalies/cholinesterase gene family/thrombocytopoietic disorders/progenitor cell differentiation/leukemia-related gene amplification)

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ABSTRACT **To study the yet unknown role of the ubiquitous family of cholinesterases (ChoEases) in developing blood cells, the recently isolated cDNAs encoding human acetylcholinesterase (AcChoEase; acetylcholine acetylhydrolase, EC 3.1.1.7) and butyrylcholinesterase (BtChoEase; cholinesterase; acylcholine acylhydrolase, EC 3.1.1.8) were used in blot hybridization with peripheral blood DNA from various leukemic patients. Hybridization signals (10- to 200-fold intensified) and modified restriction patterns were observed with both cDNA probes in 4 of the 16 leukemia DNA preparations examined. These reflected the amplification of the corresponding AcChoEase and BtChoEase genes (ACHE and CHE) and alteration in their structure. Parallel analysis of 30 control samples revealed nonpolymorphic, much weaker hybridization signals for each of the probes. In view of previous reports on the effect of acetylcholine analogs and ChoEase inhibitors in the induction of megakaryocytopoiesis and production of platelets in the mouse, we further searched for such phenomena in nonleukemic patients with platelet production disorders. Amplifications of both ACHE and CHE genes were found in 2 of the 4 patients so far examined. Pronounced coamplification of these two related but distinct genes in correlation with pathological production of blood cells suggests a functional role for members of the ChoEase family in megakaryocytopoiesis and raises the question whether the coamplification of these genes could be causally involved in the etiology of hemocytopoietic disorders.**

Two types of cholinesterases (ChoEases) have been extensively studied, both capable of rapidly hydrolyzing the neurotransmitter acetylcholine. "True" acetylcholinesterase (AcChoEase; acetylcholine acetylhydrolase, EC 3.1.1.7) is involved in the termination of signal transmission in neuromuscular junctions (1) and is also intensely expressed in the human central nervous system (2) and the erythrocyte membrane (3). "Pseudo-" or butyrylcholinesterase (BtChoEase; cholinesterase; acylcholine acylhydrolase, EC 3.1.1.8) is a soluble plasma enzyme, presumed to be produced by the liver but also present in muscle and brain (4). Biochemical and histochemical analyses indicate that both enzymes are expressed at high levels in fetal tissues of various eukaryotic organisms (5) in which ChoEases are coordinately regulated with respect to cell proliferation and differentiation (6). However, no specific role could be attributed to ChoEases in embryonic development and their biological function(s) in these tissues remained essentially unknown (1, 4, 5, 7).

In addition to its presence in the membrane of mature erythrocytes, AcChoEase is also intensively produced in developing blood cells *in vivo* (8) and *in vitro* (9), and its activity serves as an accepted marker for developing mouse megakaryocytes (10). Furthermore, administration of acetylcholine analogs as well as ChoEase inhibitors has been shown to induce megakaryocytopoiesis and increased platelet counts in the mouse (11), implicating this enzyme in the commitment and development of these hematopoietic cells.

We have recently cloned the cDNAs encoding both human BtChoEase (12) and AcChoEase (13) and have localized BtChoEase cDNA hybridizing sequences to chromosome sites 3q21–26 and 16q12 (14). It is important to emphasize that the chromosome 3q21–26 region includes breakpoints that were repeatedly observed in peripheral blood chromosomes from patients with acute myelodysplastic leukemia (AML; refs. 15 and 16). These patients all featured enhanced megakaryocytopoiesis, high platelet count, and rapid progress of the disease (17). A growing flux of recent reports implicates chromosomal breakpoints with molecular changes in the structure of DNA and the induction of malignancies (18). Therefore, the connection between (*i*) abnormal control of megakaryocytopoiesis in AML as well as in mouse bone marrow cells subjected to ChoEase inhibition; (*ii*) ChoEase gene location on the long arm of chromosome 3; and (*iii*) chromosomal aberrations in that same region in AML has appeared to us as more than coincidental (see ref. 14 for discussion of this issue).

To examine the putative correlation between the human genes encoding ChoEases and the regulation of hematopoiesis, or, more specifically, megakaryocytopoiesis, we initiated a search for structural changes in the human AcChoEase and BtChoEase genes (ACHE and CHE) from peripheral blood DNA in patients with leukemia, platelet count abnormalities, or both. Our findings demonstrate a significant coamplification of both the ACHE and CHE genes encoding AcChoEase and BtChoEase, respectively, from peripheral blood cells in patients with leukemia and/or abnormalities in their platelet counts and strongly suggest an active role for these enzymes in the progress of human hemocytopoiesis.

METHODS

Blood samples were drawn in 13.3 mM EDTA (pH 7.5) from seven patients from our department (H.Z.) suffering from abnormal platelet counts and leukemia. Blood DNA from 30

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Abbreviations: AcChoEase, acetylcholinesterase; BtChoEase, butyrylcholinesterase; ChoEase, protein of the cholinesterase family; AML, acute myelodysplastic leukemia; CHE, gene encoding BtChoEase; ACHE, gene encoding AcChoEase.

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apparently healthy individuals served as controls. In addition, DNA from 14 patients with various types of leukemia was gratefully received from E. Canaani (The Weizmann Institute of Science). For hybridization experiments, 10-μg samples of purified DNA from peripheral blood were digested to completion with various restriction endonucleases (Boehringer Mannheim) and electrophoretically separated on 1.2% horizontal agarose gels (1.2 mA/cm; 18 hr). DNA was transferred onto GeneScreen membranes (NEN, DuPont) according to the company's instructions. Filters were subjected to hybridization with electrophoretically purified fragments from AcChoEase cDNA (13) and BtChoEase cDNA (12), 1500 and 2400 nucleotides long, respectively, labeled by a "multiprime" DNA polymerase reaction (Boehringer Mannheim) with [^{32}P]ATP to 5×10^9 dpm/μg. DNA preparation, hybridization, x-ray film autoradiography, and optical densitometry were performed as described (19) using the isolated cDNA fragments for quantitative analysis.

RESULTS

To search for putative structural changes within the human ACHE and CHE genes, we first examined their restriction fragment patterns in peripheral blood DNA from 16 patients with various leukemias as compared with DNA from 30 healthy individuals. For this purpose, DNA blot hybridization was performed with equal amounts of patients' DNA after complete digestion with the restriction endonucleases *Pvu* II and *Eco*RI and gel electrophoresis. Hybridization with ^{32}P-labeled AcChoEase cDNA and BtChoEase cDNA repeatedly revealed invariant restriction patterns and signal intensities for DNA from all of the healthy individuals. The same restriction pattern and signal intensities were observed in DNA from 12 of the leukemic patients. In contrast, the hybridization patterns in the 4 remaining samples displayed both qualitative alterations and a clear signal enhancement with both cDNA probes, which was unlikely to be due to incomplete digestion by the restriction endonucleases that were used. Fig. 1 presents the DNA blot hybridization results obtained with three of the latter leukemia DNA samples and with one of the controls. It reveals intensified labeling of bands that also existed in the control lane, as well as the appearance of various other labeled bands.

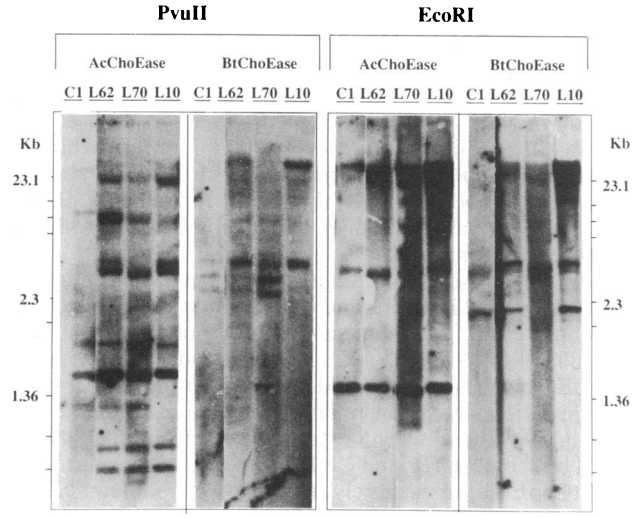

FIG. 1. DNA blot hybridization of leukemic DNA samples. Samples of peripheral blood DNA (10 μg) from three AML patients and one healthy control (L10, L62, L70, and C1; see Table 1 for details) were subjected to complete enzymatic digestion with the restriction endonucleases *Pvu* II and *Eco*RI, followed by agarose gel electrophoresis and DNA blot hybridization with ^{32}P-labeled AcChoEase cDNA (13) and BtChoEase cDNA (12) probes. The experimental conditions were as detailed in *Methods* and in previous publications (4, 12–14). Ethidium bromide staining of the agarose gels was used to ascertain that equal amounts of DNA were loaded and electrophoretically separated in each of the lanes. Note the appearance of intensified labeling signals in bands that are also present in the control lanes and the occurrence in leukemic DNAs of labeled bands that are absent in the control lanes. Exposure was for 10 days at −70°C with an intensifying screen. *Hin*dIII-digested DNA from λ and ϕX174 phages served as molecular weight markers. Kb, kilobases.

In view of these first promising results and the previous reports correlating ChoEases with megakaryocytopoiesis and platelet production (8–11), we examined DNA from additional patients with platelet disorders, whether or not defined as leukemic. Significantly enhanced hybridization signals with both cDNA probes were found in 3 of 5 such patients examined, one of them leukemic. Interestingly, the intensity of hybridization in 2 of these samples was much higher than it was in any of the previously tested leukemic DNA samples. Furthermore, the amplification events in these two samples appeared to involve many additional *Pvu* II-cut DNA fragments, due to either nucleotide changes producing additional *Pvu* II restriction sites or different regions of DNA having been amplified. Figs. 2 and 3*A* present these hybridization results.

To further compare the restriction fragment patterns of the amplified genes, we subjected the relevant lanes from these autoradiograms to optical densitometry. This analysis, presented in Fig. 3*B*, clearly demonstrates the appearance of slightly enhanced hybridization signals at equal migration positions to those observed in control DNA for a representative leukemic DNA sample, termed L70, with moderate amplification. In another leukemic DNA sample, termed L04, and taken from a patient with reduced platelet counts, the densitometry signals were higher by an order of magnitude and presented several additional short *Pvu* II-cut fragments. Yet much higher signals and more bands of various sizes were observed with the P03 sample, derived from a

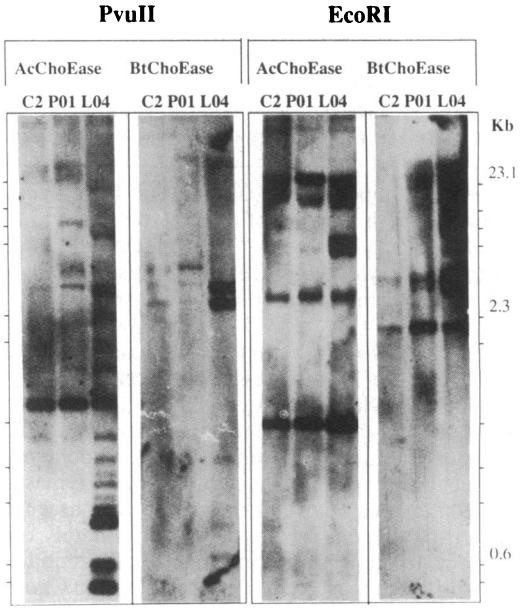

FIG. 2. Amplification of ACHE and CHE genes in DNA from patients with hematocytopoietic disorders. Peripheral blood DNA was prepared from one patient with highly increased platelet counts (P01), from a leukemic patient with decreased platelet counts (L04), and from a healthy donor (C2). Experimental details were similar to those in Fig. 1. Note the pronounced enhancement of hybridization signals with both probes and the appearance of *Pvu* II and *Eco*RI restriction fragments in the case of L04. Kb, kilobases.

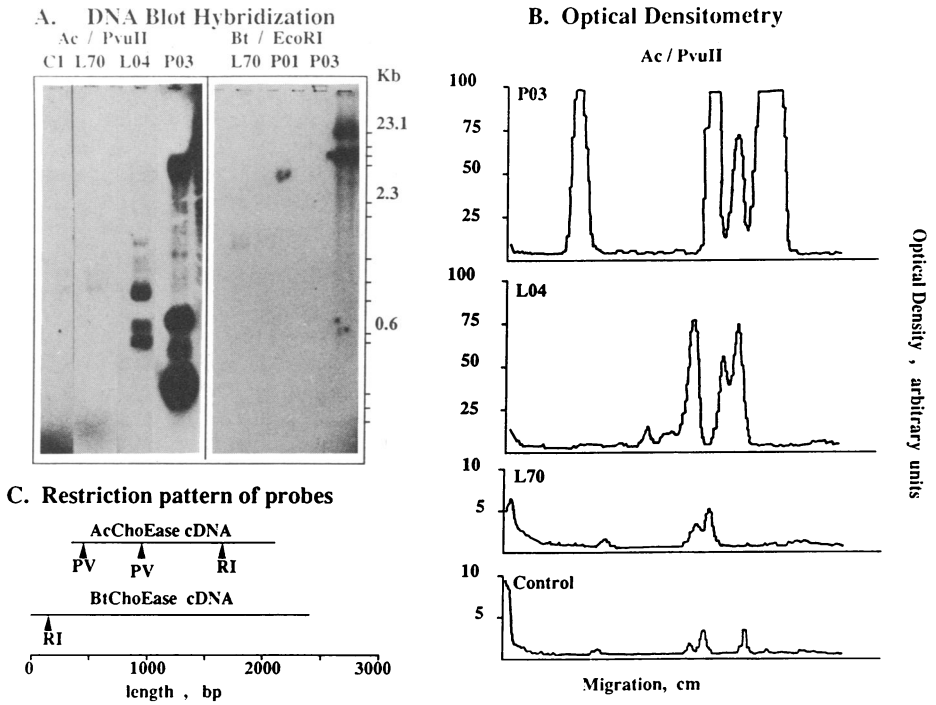

FIG. 3. Intensified amplification is accompanied by structural differences between the amplified DNA regions. Comparative analysis of representative DNA samples from a healthy control (C1), a leukemic AML patient with moderate amplification (L04), and a nonleukemic patient with a pronounced decrease in platelet counts (P03) was performed by DNA blot hybridization using ^{32}P-labeled probes. (*A*) Blot hybridization with *Pvu* II-cut genomic DNA and AcChoEase cDNA probe (Ac) and with *Eco*RI-cut genomic DNA and BtChoEase cDNA probe (Bt). (*B*) Optical densitometry of individual lanes from the *Pvu* II-treated AcChoEase cDNA-hybridized blot was performed at 545 nm as detailed elsewhere (19). Note the increased intensity of the densitometric measurements and the appearance of additional labeled restriction fragments in P03 and L04 lanes as compared with L70 and C1. (*C*) Restriction sites for *Pvu* II and *Eco*RI on the cDNA probes. Note that the number of *Pvu* II-cut DNA fragments in P03 that were labeled with AcChoEase cDNA exceeds the expected number of three fragments based on the *Pvu* II restriction pattern of AcChoEase cDNA, reflecting structural changes and appearance of *Pvu* II restriction sites within the amplified DNA sequence. Exposure was for 6 days in conditions otherwise identical to those in Fig. 1. Kb, kilobases; bp, base pairs.

nonleukemic patient with a pronounced decrease in platelet counts (thrombocytopenia).

The variable degrees of amplification occurring in the ACHE and CHE genes in these individuals were quantified by slot-blot DNA hybridization, using a 1:5 dilution pattern. Cross-hybridization between the AcChoEase and BtChoEase cDNA probes was exceedingly low (<0.01), demonstrating that the observed amplification events indeed occurred in each of these genes and did not merely reflect similarity in their sequences. A demonstration of the slot-blot experiments is presented in Fig. 4, clearly showing that 1 μg of P03 DNA included genomic sequences equivalent to $\approx$0.1 and $\approx$0.01 ng of the purified BtChoEase cDNA and AcChoEase cDNA inserts, respectively. Parallel analysis using similar quantities of C2 control DNA revealed considerably lower signals with both probes. Taking the total complexity of human genomic DNA as 4×10^9 base pairs, this implies that at least 40–100 copies of both these sequences are present in the DNA of P03. L04 and P01 DNAs featured $\approx$10 times lower signals with BtChoEase cDNA than those determined for P03 DNA, reflecting a more modest amplification in the order of up to 20 copies per genome. Repeated hybridization of the same blots with a cDNA probe, detecting a nonrelated gene encoding a ribosomal protein (20), demonstrated no amplification in all of the examined samples and similar labeling intensities for both patient and control DNAs.

Altogether, 6 cases of coamplification within the ACHE and CHE genes were observed in DNA samples from 20 patients with abnormal hematocytopoiesis, while DNA from 30 healthy individuals showed neither amplification nor polymorphism with respect to the restriction patterns obtained with these probes. The DNA samples presenting these amplifications were derived from four cases of AML with 10–50

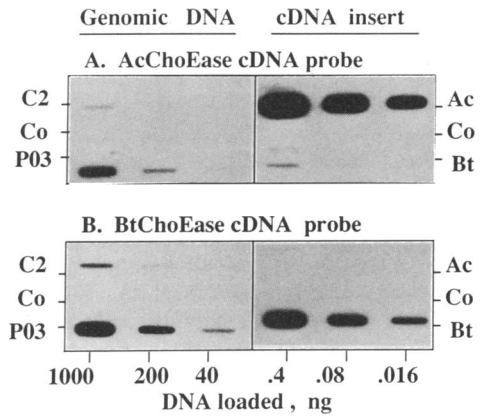

FIG. 4. Quantification of the amplification levels in diseased DNA samples by slot-blot hybridization. Denatured genomic P03 DNA, analyzed in Fig. 3, was spotted onto a GeneScreen filter using a slot-blot applicator (Bio-Rad). Electroeluted AcChoEase cDNA (Ac) and BtChoEase cDNA (Bt) inserts (see Fig. 3*C*) were spotted in parallel for calibration. Genomic DNA from an apparently healthy individual (C2) and herring testes DNA (Co) served as controls. All samples contained the noted quantities of genomic or insert DNAs supplemented with denatured herring testes DNA to yield a total of 2 μg of DNA per slot. Hybridization, wash, and exposure were done as detailed elsewhere (19) with ^{32}P-labeled AcChoEase cDNA or BtChoEase cDNA as noted. Note the minimal levels of cross-labeling between the corresponding cDNAs and the intense labeling of P03 DNA as compared with controls.

copies of both ACHE and CHE genes, and three cases of platelet count abnormalities. One expressed excess platelet count and 10–20 copies of the ACHE and CHE genes, and the two others showed reduction of platelet count and featured 10–200 copies of the same genes. These striking concomitant multiplications, summarized in Table 1, present a highly significant correlation ($P < 0.001$) between amplification of ChoEase-encoding genes and the occurrence of abnormal myeloid progenitor cells or promegakaryocytes in the examined individuals.

Table 1. Appearance of amplified ACHE and CHE genes in hematocytopoietic disorders

No.	Type	Defective progenitors	Approximate amplification	
			AcChoEase	BtChoEase
		Leukemias*		
1 (L23)	AML	Myeloid	N	N
2 (L38)	AMegL	Promegakaryocytes	N	N
3 (L26)	AMOL	Monocytes	N	N
4 (L10)	AML	Myeloid	5–15	5–15
5 (L41)	AMML	Myeloid/monocytes	N	N
6 (L42)	AML	Myeloid	N	N
7 (L79)	AML	Myeloid	N	N
8 (L70)	AML	Myeloid	10–20	10–20
9 (L20)	AML	Myeloid	N	N
10 (L96)	AML	Myeloid	N	N
11 (L62)	AMML	Myeloid/monocytes	10–20	5–15
12 (L59)	AMML	Myeloid/monocytes	N	N
13 (L15)	AML	Myeloid	N	N
14 (L12)	AML	Myeloid	N	N
15 (L03)	AML_2	Myeloid	N	N
16 (L04)	AML_2	Myeloid	40–60	10–20
		Megakaryocytopoietic disorders†		
	Platelet count			
16 (L04)	Low	Promegakaryocytes	40–60	10–20
17 (P01)	High	Promegakaryocytes	10–20	10–20
18 (P02)	Low	Promegakaryocytes	N	N
19 (P03)	Low	Promegakaryocytes	40–100	50–200
20 (P04)	Low	Promegakaryocytes	N	N
		Controls‡		
21 (C1)	Normal	None	N	N
22 (C2)	Normal	None	N	N
23–50 (C_x)	Normal	None	N	N

The characteristic types of hematopoietic progenitor cells, which appear to be defective in each class of the screened leukemias, are noted (17). The approximate extent of amplification was separately determined for ACHE and CHE genes by slot-blot DNA hybridization and optical densitometry. Numbers reflect the -fold increase in the calculated number of copies as compared with control DNA. N, normal.

*Peripheral blood DNA from 14 leukemic patients was received, together with clinical classification of the disease type, from E. Canaani (The Weizmann Institute of Science). Two other patients (L03 and L04) were diagnosed and classified in our department. (AMegL, acute megakaryocytic leukemia; AMOL, acute monocytic leukemia; AMML, acute monocytic/myeloid leukemia; AML_2, Fab subclassification of AML.)

†Peripheral blood DNA from five patients from our department (H.Z.), suffering from abnormal platelet counts, was analyzed as detailed above. Abnormalities in platelet counts are noted, where "low" implies <80,000 per mm^3 and "high" is >600,000 per mm^3 (normal counts are considered 150,000–400,000 platelets per mm^3). Note that L04 (no. 16) appears twice.

‡DNA samples from currently healthy individuals with normal platelet counts and blood ChoEase activities served as controls and were analyzed as detailed above. C1 and C2 correspond to representative control DNAs, shown in Figs. 1–4. Similar results were obtained in 28 more controls (C_x).

DISCUSSION

In this work, the postulated relationship between the family of ChoEases and hematopoietic commitment and differentiation was investigated by using cDNA probes. These probes detected the presence of multiple copies of the genes encoding ChoEases in 25% of the leukemic DNA samples examined. Amplification of DNA sequences occurring at specific chromosomal breakpoints has been increasingly found in various malignancies (18). In several cases, these changes were correlated with cellular growth and development effects (21). One region that is conspicuously altered in leukemias appears on the long arm of chromosome 3 (15–17), where we recently mapped the CHE genes (14). In the mouse, ChoEase inhibitors and acetylcholine analogs induce abnormal proliferation of megakaryocyte progenitor cells both *in vivo* (9) and *in vitro* (10–11). Taken together, this appeared to be sufficient to initiate a search for structural changes within the ACHE and CHE genes in leukemias. Our finding of 6 of 20 amplification events among both genes, in cases of hematocytopoietic abnormalities, suggests that these apparently unrelated pieces of evidence might be connected.

The occurrence of these gene amplification events could reflect a specific origin of replication within the amplified CHE genes or in an adjacent oncogene (18, 21). Yet another possibility is that of the insertion of a retroviral sequence, followed by the extension of its amplification into the chromosomal region of the ChoEase genes. The amplification of the CHE gene on chromosome 3 that we recently found in a family exposed to chronic doses of parathion, a potent ChoEase inhibitor (19), could be an example for the first option. It should be noted, however, that in that particular family the CHE gene was the only one to be amplified (H.S., C.A.P., and H.Z., unpublished observation). Other examples are the changes in the immunoglobulin genes close to the *MYC* oncogene in Burkitt lymphoma (22) and the amplification of cellular DNA sequences at the boundaries of the insertion site of polyoma DNA (23).

It is not known whether the ACHE and CHE genes are localized on the same chromosome and whether they are linked to each other. The coamplification of these genes in our patients may indicate that they were colocalized at the same chromosomal region prior to the amplification event and were amplified together. Alternatively, it can reflect the occurrence of recombination events between these two genes during the amplification process. Yet another possibility is that the ACHE and CHE genes can be independently subjected to the same selection pressure to be amplified. Amplification of various genes, including colocalized ones, has repeatedly been found in multidrug-resistant cell lines (24). Chromosomal rearrangement has also been proposed to facilitate gene amplification in drug-resistant cells by juxtaposing homologous segments (25). In *Drosophila*, a high frequency of recombinational events was noted for the *Ace* locus, carrying the structural ACHE gene (26). Precise mapping of the yet unlocalized gene(s) encoding human AcChoEase on chromosomes from normal and diseased individuals will be required to clarify this issue.

The appearance of restriction fragments in pronounced cases of ACHE and CHE gene amplifications could be due to overlapping, but nonequal, regions of DNA having been amplified in the various individuals, perhaps reflecting variable origins of replication resulting from retroviral transposition. Various insertion sites for amplifiable retroviral sequences have been observed in the human genome, including a chromosome 3q site for leukemia virus sequences (27) close to the location of the CHE genes (14). Alternatively, the different patterns obtained in the various analyzed DNAs could reflect genetic alterations in the amplified genes, such as those observed for the amplified *MYC* protooncogene in

primary breast carcinomas (28) or those occurring in the dihydrofolate reductase (*DHFR*) gene in methotrexate-treated leukemic cells (29).

The possibility should be considered that the amplification of ChoEase-encoding genes was induced by continuous exposure to ChoEase inhibitors (i.e., agricultural organophosphorus insecticides; see ref. 4). The amplification of the ACHE and CHE genes in leukemias is not a random process, as it does not involve irrelevant sequences such as those encoding ribosomal proteins (20). ChoEase gene amplification could be advantageous to blood cells to which ChoEase activities are essential by creating acquired resistance to ChoEase inhibitors, like the amplification and overexpression of multidrug-resistance genes (30) and the amplification of genes induced by arsenic (31). To further examine this possibility, the levels of expression of the amplified ACHE and CHE genes in hematocytopoietic disorders will have to be measured in various tissues from individuals under chronic exposure to organophosphorus insecticides as well as in additional members of their families.

The putative involvement of ChoEases in the etiology of hematocytopoietic disorders is of particular importance in view of the multiple reports implicating these enzymes with growth and development (1, 2, 6–9). Further correlations between the appearance of the amplified ChoEase genes, the stage of the diseases in the examined patients, and their treatment protocols should be pursued to investigate this issue. If ChoEases are indeed important for hematocytopoiesis, the amplification of ChoEase-encoding genes would be analogous to other amplifications in malignancies. Examples include that of the genes encoding the epidermal growth factor receptor in malignant gliomas (32–34), the amplification of the *NEU* oncogene in breast cancer, which is correlated with relapse and survival (35), and the amplification of *N-MYC* in neuroblastoma, associated with the rate of progress of the disease (36). Although ChoEases are not homologous to oncogenes, we have previously found altered modes of their expression in malignant gliomas (37) and, more recently, in the serum of patients with various carcinomas (38). At present we cannot exclude the possibility that the coamplification of ChoEase genes, altering their mode of expression, could result from drug treatment (38). It would be interesting to reveal whether modified expression of these genes in other tissues reflects parallel amplification phenomena, giving multiple types of tumor cells growth advantages.

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