mystery 'envelops the mechanism of migraine'.

J. R. FOZARD
AND J. A. GRAY*
Preclinical and 'Clinical Research, Sandoz Ltd., 4002 Basel, Switzerland.

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CURRENT TECHNIQUES

Identification of human serum cholinesterase variants using the polymerase chain reaction amplification technique

The polymerase chain reaction (PCR) is a newly developed technique for selectively amplifying RNA or DNA sequences (see Box). Variations of the technique are proving to be powerful tools in fields as diverse as cancer and population biology. In pharmacology, PCR will allow rapid identification of point mutations or deletions of DNA responsible for variant forms or functional deficiencies in receptors and other proteins of pharmacological interest which are present in low abundance. It has already been useful in the analysis of mutations responsible for variants of human serum cholinesterase.

Applications of PCR

PCR amplification is a remarkably powerful technique. If the human genome were 1000 km long, and contained about 100000 genes, the average gene of 50 kbp would occupy about 10 m. A 300 bp segment amplified by PCR would represent less than 10 cm. The repeated cycles of annealing, primer extension and heat denaturation (see Box) are an extremely efficient process. Millions of copies of the selected segment are generated, and the amplified DNA can easily be seen in agarose gels stained with ethidium bromide. Regions of interest can be sequenced directly using specific sequencing primers. Alternatively, amplification primers can be constructed with synthetic restriction sites at their 5' ends to allow the amplified segments to be inserted into suitable vectors, such as M13 phage, for single-strand sequencing. This is particularly useful for long amplified segments and may also be advantageous in searching for new point mutations within heterozygous samples, where very clean DNA is needed to reduce confusion with shadow bands.

The major limitation of the PCR technique is a direct consequence of its remarkable sensitivity: contamination of the sample by even minute amounts of any DNA recognized by the amplification primers can result in very efficient amplification of the unwanted material. Precautions must be taken to reduce the possibilities for contamination as much as possible, and controls (without template DNA) must be included routinely. PCR can be used for the analysis of DNA mutations that cannot readily be detected in any other way (for example, mutations producing an alternative codon for the same amino acid, so that the enzyme is unchanged). Quantitative structural variants may result from mutations affecting the half-life of the enzyme, without producing any changes in the quality of its catalytic activity. In fact, it may be impossible to identify these variants by any test based upon kinetic properties, such as a response to competitive inhibitors. PCR also provides a simple means of diagnosing such alleles in heterozygous individuals. Presumably, a structural change confers greater stability,
or faster turnover, on the protein, and the steady-state level of the variant enzyme is reduced. Careful studies of kinetic properties, even after denaturation experiments, may fail to reveal phenotypic differences between the variant and usual enzymes.

New methods are being developed using PCR-amplified DNA to diagnose mutations without sequencing the DNA each time, and simplify the detection of carriers. These technical advances offer unique opportunities for study of hereditary traits associated with abnormal responses to drugs and environmental chemicals (pharmacogenetics) non-invasively (except to obtain a small sample of blood DNA). This applies to all pharmacogenetic conditions—those due to differences in enzymes directly involved in pharmacokinetics and drug metabolism, and those caused by modified drug receptors and specialized proteins regulating drug effects at the pharmacodynamic level. Our studies on human serum cholinesterase variants provide an example of the use of this approach.

**Human serum cholinesterase**

Analysis of cholinesterase variants by the PCR reaction followed many years of basic work on the purification and characterization of serum cholinesterase and its variants. The 'atypical' (dibucaine-resistant) form of human serum cholinesterase with reduced affinity for choline ester substrates, particularly succinylcholine, was discovered in 1957. Since then, several additional qualitative and quantitative variants have been identified, including
some 'silent' variants with no detectable enzymatic activity. Most of these variants, alone or in combination, cause a prolonged duration of action of the drug succinylcholine, which is still commonly used as an adjunct in surgical anesthesia to obtain greater muscular relaxation. The pharmacogenetic importance of cholinesterase variants has, thus, been recognized for many years, but no definitive physiological role for serum cholinesterase (EC 3.1.1.8, butyrylcholinesterase, pseudo-cholinesterase, nonspecific cholinesterase) has been established.

Human serum cholinesterase is a glycoprotein (~24% carbohydrate) present primarily as a tetramer of ~342 kDa. Each of the identical subunits contains 574 amino acids and one active site. The subunits are held together by two interchain disulfide bridges and by hydrophobic, non-covalent forces. The average amino acid sequence of the enzyme isolated from plasma pooled from many unrelated people was identical, with the sequence deduced from cDNA clones isolated from an infant brain cDNA library. Thus, we conclude that human brain cholinesterases and that of human liver (the probable source of serum cholinesterase) are products of the same gene.

A number of tests using selective inhibitors such as dibucaine and fluoride have been developed to identify the cholinesterase variants. The ease of obtaining serum samples from patients who experience a prolonged response to succinylcholine has facilitated the development of diagnostic tests to characterize the altered properties of variant cholinesterases. Sera from closely related family members permitted detailed pedigree analyses and deduction of the inheritance patterns of these genetic traits. As expected, a simple, autosomal inheritance of the variant cholinesterase was found. The E1 locus, which determines the structural quality of cholinesterase, is located in the q21-qter region of chromosome 3 (Refs 16 and 17); it is closely linked to transferrin.

### Analysis of variants at DNA level

For most of the past 20 years, the dibucaine inhibition number, fluoride inhibition number and level of serum cholinesterase activity have been sufficient measures to identify most of the known cholinesterase phenotypes associated with succinylcholine sensitivity. During the last decade, however, several additional rare and common variants of the enzyme have been discovered, and at least ten distinctive variants are now known (Table I). Many of these are quantitative variants, with normal kinetic properties, as far as can be told from the standard tests. The increased number of possible phenotypes (28 phenotypes from ten alleles) has complicated the old system used for identifying phenotypes. In some instances, phenotypes conferring sensitivity to succinylcholine are easily confused with phenotypes that give a normal drug response. Clearly, a DNA structural basis for cholinesterase phenotyping would be helpful, as well as being a logical frame of reference for developing future diagnostic methods to distinguish the important new phenotypes.

Cholinesterase is present in human serum in very low concentration (~5 mg l^-1). Thus, it has been impractical to purify the variant enzymes and directly identify the anticholinesterase variants. By use of cDNA libraries, we undertook cloning the gene for cholinesterase for three reasons: (1) to identify the structural alterations responsible for the variant forms of the enzyme; (2) to establish a structural basis for the classification of these variants, including the quantitative variants that cannot be identified by any of the standard inhibitor or kinetic tests; and (3) to learn more about the genetic relationships between cholinesterases and the acetylcholinesterases, and other 'serine-type' esterases.

The atypical (dibucaine-resistant) cholinesterase variant is the one most commonly associated with succinylcholine sensitivity. Our approach involved determining the amino acid sequence of the usual cholinesterase, and using this information to construct suitable oligonucleotide probes to screen for and subsequently cDNA clones encoding a new enzyme from individuals homozygous for the usual enzyme. These sequences were compared with nucleotide sequences from white blood cell genomic library clones from a homozygous atypical individual. The amino acid alteration(s) in the atypical protein could then be deduced.

We found just one nucleotide difference among the 1722 bases representing the coding region of the mature atypical protein: a point mutation at nucleotide 209 (GAT to GGT), which changes codon 70 from aspartate to glycine. The resulting change of a single acidic amino acid to a neutral amino acid seems to be a reasonable explanation for the reduced binding affinity of succinylcholine at the anionic site of the atypical enzyme. However, it was necessary to verify that the point mutation in nucleotide 209 segregated

### Table I. Genetic forms and variants of human serum cholinesterase

<table>
<thead>
<tr>
<th>Name and characteristics</th>
<th>Frequency</th>
<th>Ref.</th>
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<tbody>
<tr>
<td>Usual</td>
<td>0.854</td>
<td>a,b</td>
</tr>
<tr>
<td>K-variant (quantitative; activity reduced ~30%) (Ala539Thr)</td>
<td>0.125</td>
<td>c,d</td>
</tr>
<tr>
<td>Atypical (dibucaine-resistant) (Asp70Gly)</td>
<td>0.017</td>
<td>e</td>
</tr>
<tr>
<td>Silent (no, or very low activity) (several types: Silent-1 = Gly11, Silent-2 = Asp11)</td>
<td>1 x 10^-5</td>
<td>f</td>
</tr>
<tr>
<td>Fluoride-resistant</td>
<td>6.6 x 10^-5</td>
<td>g,h</td>
</tr>
<tr>
<td>J-variant (quantitative variant; activity reduced ~70%)</td>
<td>rare</td>
<td>i</td>
</tr>
<tr>
<td>H-variant (quantitative variant; activity reduced 90%)</td>
<td>rare</td>
<td>j</td>
</tr>
<tr>
<td>Newfoundland</td>
<td>rare</td>
<td>j,k</td>
</tr>
<tr>
<td>Cynthiana (high activity)</td>
<td>various</td>
<td>l,m,n</td>
</tr>
<tr>
<td>German family high activity variant</td>
<td>four families</td>
<td></td>
</tr>
<tr>
<td>South African variant (high activity)</td>
<td>two families</td>
<td></td>
</tr>
<tr>
<td></td>
<td>one family</td>
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and 1600), and the sequences of short segments of intron DNA adjacent to the intron-exon junctions were known, three pairs of amplification primers were designed which could amplify the entire coded region of cholinesterase as three segments (Fig. 2). The largest of these (~1500 bases), which includes exon 2, could be sequenced directly with a series of sequencing primers spaced about 250 bp apart. Thus, the entire coding region can be amplified and sequenced using the PCR amplification technique, from the DNA obtained from small amounts (a few ml) of blood. This has made it practical to search for structural mutations underlying the other variants of cholinesterase.

Polymerase chain reaction

The polymerase chain reaction, permitted the selective amplification of a small segment (187 bp) of genomic white blood cell DNA including the nucleotide 209 region (Fig. 1). Sequencing of the amplified DNA revealed complete concordance between the nucleotide base at position 209 and the serum cholinesterase phenotype (usual, usual-atypical or atypical) in every individual tested.

Another special advantage of the PCR amplification technique is that, in DNA from heterozygous individuals, the sequences representing the usual and atypical alleles are both amplified; sequencing gels show both the mutant and ‘wild-type’ nucleotide bases at the level of the point mutation. The speed and convenience of the PCR analysis permitted us to sequence the region of interest in many individuals within a few weeks, and verify that the DNA sequences accurately predicted the serum cholinesterase phenotypes.

Since the location of introns (nucleotide positions -93, 1433

![Fig. 1. DNA sequences of usual (UU), heterozygous (UA) and atypical (AA) cholinesterases showing the nucleotide substitution in the atypical cholinesterase sequence. Genomic DNA from three individuals was amplified by the PCR technique and sequenced. The U and A alleles were identical except for the nucleotide indicated by an asterisk. Codon 70 is GAT in UU, GAT and GGT in UA, and GGT in AA. (Reproduced from Ref. 2 with permission.)]

- A frame-shift mutation at Gly117 (GGT to GGAT) has been identified as the basis for one type of silent (i.e. no enzyme activity) mutation in two unrelated families.
- Another point mutation changing Thr243 to Met (ACG to ATG) segregates with the fluoride-resistance trait in two members of one family; however, this explanation of the fluoride variant will remain provisional until it has been observed in other fluoride-resistant families.
- A threonine-alanine polymorphism has been detected at position 539 which depends upon the nucleotide at position 1615 (GCA/ACA, Ala/Thr; allele frequency 0.875/0.125)\(^9\). We suspect that the less common allele coding for Thr represents the quantitative K-variant, which is characterized by a reduction of about one-third in cholinesterase activity. This hypothesis is supported by the observation that in individuals with the atypical allele (Gly at position 209), which tends to be inherited in tandem with the less common allele for Thr539 (Ref. 19), the average level of serum cholinesterase is about one-third that of normal individuals, when measured immunologically\(^10\).

Several additional potential polymorphic sites are being evaluated in larger numbers of DNA samples using PCR. This will permit detailed linkage studies within the cholinesterase gene, and allow
high resolution of these closely linked markers, to determine how various combinations of these structural alternatives influence the properties and concentration of serum cholinesterase.

Acknowledgement
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References

This and That: exotic foods – prophylaxis and peril

EVER NEW MODES of conspicuous consumption evolve to soak up disposable, or surplus, income. The Japanese habit of eating raw fish in the form of sushi or sashimi has become common enough in the United States to have achieved the status of an editorial in the New England Journal of Medicine. This was provoked by a report of a 4.2 cm long bright red worm crawling out of the surgical incision in a patient undergoing surgery for apparent acute appendicitis. The worm was identified as an Eustrongylides, a common parasite of fish-eating birds, and had presumably been swallowed by the patient during a sushi-eating session a few days earlier. The authors warn illiterately that ‘perforation of the stomach or intestine by the invading larva may present as an acute abdomen’.

This is but the latest in an increasing number of reports on the parasitic dangers of consuming raw fish. Brookes may eulogize the ‘ripples with dark ecstasies’ in its smooth curving world, but the fact is that fish lead a worm-ridden existence, and are more likely to be rippling with parasitoid-induced discomfort. Over 80% of Pacific salmon and red snapper are infested with Anisakis. Most Atlantic cod and plaece is infested. Indeed, as the parasitic principle enunciated by Jonathan Swift in the jingle quoted below reminds us, to be infested with parasites is the natural state of mammals. This is a reality we lose sight of in our urban existence of sanitized toilet seats, chlorinated water and pill-choked cabinets. One has only to recall ‘Queeney’ Thrale, daughter of Samuel Johnson. Her life bridged the 18th and 19th centuries. She was a lifelong sufferer from roundworms, treated unavailingly with tin pills and senna pods.

So, naturalists observe, a flea
Hath smaller fleas that on him prey;
And these have smaller fleas
to bite ’em
And so proceeding ad infinitum

A ‘natural’ existence is short and uncomfortable, and public health problems erupt whenever we forget this. And, in the increasing habit of eating raw fish in the form of sushi, sashimi, ceviche, lomi lomi, or sunomono, we are forgetting this. The biggest risk appears to be anisakiasis. Anisakiasis, commonly called roundworm, has a life history in which it passes through fish and/or crustaceans and squid and on through marine mammals to complete its cycle. Anisakiasis is common in Japan and is increasing in North America, over 70% of the reported cases occurring since 1980. In an interesting application of the law of ecology which states that everything is connected to everything else, one factor in the increase in the Pacific states appears to be the Marine Mammal Protection Act of 1972, which has led to increased populations of sea mammals and a resulting increase in the percentage of fish associated with them that become infested.

The roundworm has a boring tooth, and can tunnel into intestinal walls, with serious consequences. Anisakiasis is commonly misdiagnosed as appendicitis, Crohn’s disease, gastric ulcer or cancer – a situation helped by the absence of parasites or eggs in the feces. Thus, in one large Japanese series, 37% of cases ultimately found to be anisakiasis were initially diagnosed as gastric cancer, and another 16% as appendicitis.

The worms may be coughed up after ingestion, or produce the ‘tingling throat’ syndrome as they wriggle in the esophagus. Antihelminthics are without effect, and surgical resection is the only effective treatment, as in a recent case where there was penetration of...