Survey of Atypical Pseudocholinesterasemia in Okayama District (Preliminary report) and a New Method for Its Detection

Iwao IUCHI, Shigeko AMENO and
Susumu SHIBATA
Kawasaki Hospital and Tumol Institute, Okayama, and Department of Medicine, Yamaguchi University School of Medicine, Ube
(Received January 25, 1969)

Atypical pseudocholinesterasemia is a congenital anomaly of serum enzyme inherited as Mendelian codominant character.

Although the carrier of pseudocholinesterasemia has no remarkable disabilities in daily life, he has a marked hypersensitivity to the choline derivatives, especially a muscle relaxant, succinyl dicholine (SCC) which is widely and frequently used as a muscle-relaxation-inducing drug in the field of anesthesiology and psychiatry.

When this drug is injected intravenously to the individuals with this anomaly, a dangerous long-lasting apnea (30 minutes～several hours) and asphyxia occur as the result of muscle paralysis, particularly in respiratory muscles.[1][2][3][4] This hypersensitivity is now clearly elucidated by qualitatively or quantitatively aberrant pseudocholinesterase (PsChE) in blood which is responsible for the decomposition of SCC. When a usual dose of SCC, for instance 100 mg, are given to the individuals with normal serum PsChE activity, almost all the SCC is decomposed into ineffective derivatives within a few minutes while staying in the blood stream and only a trace but sufficient amount of it for producing muscular paralysis arrive at neuromuscular endplate. On the other hand, the individuals with atypical cholinesterasemia split very slowly the SCC in the blood, and result in a large amount of SCC coming onto the endplates. This is why such individuals cause long lasting apenea.[3][5]

It, therefore, is reasonably thought that the best way for detection of an individual with SCC hypersensitivity is to measure directly serum SCC splitting activity by incubation together with SCC and serum. However, any clinically useful methods based on this idea has not yet been developed except semiquantitative method measuring the contraction of rectus abdominalis of a medium sized frog, rana pipiens.[6] At present, such a patient with hypersensitivity is detected and classified into their subtypes by measuring three parameters, namely, serum PsChE activity and dibucaine and fluoride inhibition effect upon the activities.[7]

Last year, the first instance of an atypical PsChE family with silent and fluoride resistant gene in Japan was reported by us from a group extracted from Okayama

district inhabitants.[8)9)] Since then, it has become evident that such abnormalities might be distributed in other parts of Japan, and a survey of larger scale has been conceived necessary in order to know how frequently this sort of anomalies are distributed among the Japanese.

During the studies of the first family of PsChE anomaly, it was felt that the standard method described in Kalow's[10)12)] and Harris and Whittaker's methods[11)] are accurate and reliable, but unsuitable for the screening test for dealing with many specimens simultaneousiy, since they have to trace optical density at wave length 240 mμ for several minutes for a determination from sample to sample. In fact, many simplified screening methods have been published in Europian countries.[13~20)] However, these methods are established chiefly for detection of dibucaine resistant type anomaly which is distributed in relatively high frequency in those countries. Therefore, application of those methods to the Japanese seems unsuitable since they are too eccentric for the use in the survey in this country where the types of PsChE anomaly are to be investigated. Accordingly, our first endeavours were concentrated for establishing the screening method which enables exact detection and classification of the aberrant PsChE.

The purpose of this paper is to describe the new screening method and the result of the survey carried out in Okayama district.

I. Determination of serum PsChE activity and its subclassification

A) Reagents:

1) Phosphate buffer solution (0.1 M, pH: 8.0)

Amounts of 0.34 g of KH_2PO_4 and 17.0 g of $Na_2HPO_4 \cdot 12H_2O$ are dissolved with water and made to 500 ml.

2) Buffered substrate solution (5×10^{-4}M)

An amount of 0.0317 g of butyrylthiocholine (Sigma) is dissolved in 200 ml of phosphate buffer solution (reagent 1).

3) DTNB solution (10^{-3}M)

An amount of 0.0398 g of 5, 5'-dithiobisnitrobenzoic acid (DTNB, Aldrich) is dissolved in 100 ml of phosphate buffer solution (reagent 2).

4) Sodium lauryl sulphate solution (0.6 per cent)

An amount of 0.6 g of sodium lauryl sulphate is dissolved in 100 ml water.

5) Dibucaine solution (3×10^{-4}M)

An amount of 0.0114 g of dibucaine (Ciba) is dissolved into 100 ml of water.

6) NaF solution (1.5×10^{-3}M)

The solution is prepared by dissolving 0.0063 g of NaF into 100 ml water.

7) Coloration and inhibition reagent

The reagent is prepared prior to use by mixing equal volumes of DTNB

solution and sodium lauryl sulphate solution.

8) Standard solution

a) Working DTNB solution (10^{-4}M)

DTNB solution (10^{-3}M, reagent 3) is diluted ten fold by phosphate buffer solution (pH: 8.0)

b) Glutathione solution (10^{-3}M)

An amount of 0.0031 g of reduced glutathione (GSH, Sigma) is dissolved with 10 ml of phosphate buffer solution (pH: 8.0)

Procedure:

1) An aliquot of 0.02 ml of serum is diluted with 0.5 ml of water by use of micropipette. This makes 26-fold diluted serum.

2) For one serum specimen, the following reagents are added into the test tubes, T, D, F, and B, in the order described in the table below.

Test tubes	T	D	F	B	
26-fold diluted serum	0.02	0.02	0.02		
Dibucaine solution		0.05			
NaF solution			0.05		
H_2O	0.05			0.07	(ml)

3) One and a half ml of buffered substrate prewarmed at 25 °C are added into each tube and incubated for 60 min. at 25°C.

4) At the end of incubation time, 1.5 ml of the coloration and inhibition reagent are added into individual tubes.

5) After setting a colorimeter at wave length 412 mμ, or with optical filter S = 43, the absorbances of t, d, and f corresponding to tube T, D, and F, respectively, are read out against B as colorimetry blank.

6) Making calibration curve:

Into the test tubes A′, B′, and C′, amounts of 0.75, 0.60 and 0.30 ml of DTNB standard solution are added, respectively, followed by addition of 0.75, 0.60 and 0.30 ml of glutathione solution, respectively, and then make final volumes to 3.07 ml with coloration and inhibition reagent. Calibration curve is constructed, regarding the absorbances of a, b, and c corresponding to tube A′, B′, and C′, as 200, 160 and 80 units (μM/ml/hr/25°C). The line is straight passing through the origin of the graphic chart.

7) Calculation:

a) Total activity: The total activity is obtained by collating the absorbance t on the calibration curve.

b) Dibucaine (DN) and fluoride (FN) numbers: The numbers are calculated by the following equation

$$DN = \frac{t-d}{t} \times 100\ \%$$

$$FN = \frac{t-f}{t} \times 100\ \%$$

8) Normal Values:

total activity	85 – 140	units
DN	67 – 97	%
FN	45 – 69	%

II. Methods of survey for the detection of atypical cholinesterase

The blood specimens sent to our laboratory from the hospital and out-patient clinics for routine chemistry tests were subjected to the screening test. And when abnormal PsChE were detected, they were examined by standard Kalow's,[10] and Harris and Whittaker's[11] methods scrupulously.

III. Result and Discussion

1) Methodologicals:

The optimal pH for serum PsChE activity was seen in a range of pH 8.0–9.5 as depicted in Fig. 1. The value of 8.0 was therefore chosen for the pH of the buffered substrate medium, because butyrylthiocholine became increasingly revealed unstable with rise in alkalinity.

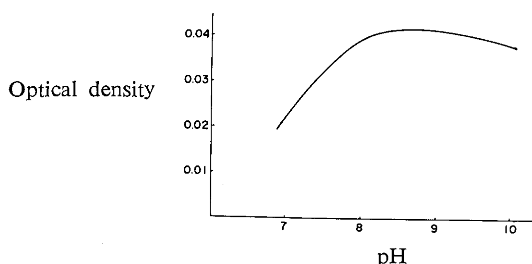


Fig. 1. Variation of usual PsChE activity as a function of pH

When substrate (BTC) concentration was changed from 5×10^{-6} to 5×10^{-2} M under fixed dibucaine and NaF concentration at 10^{-5} and 5×10^{-5} M, respectively, the three parameters, namely, the activity, DN and FN of the normal PsChE varied as illustrated in Fig. 2. It is evident that the three parameters vary depending upon the substrate concentration, a different attitude of the dependence of the DN and the FN lines on substrate concentration might indicate that they had different inhibitory mechanisms upon PsChE molecules. This figure also indicates the importance of the selection of the substrate concentration prior to establish the method. Accordingly, the final substrate concentration of 5×10^{-4} M where highest PsChE activity, and DN and FN value were observed was selected in this new method.

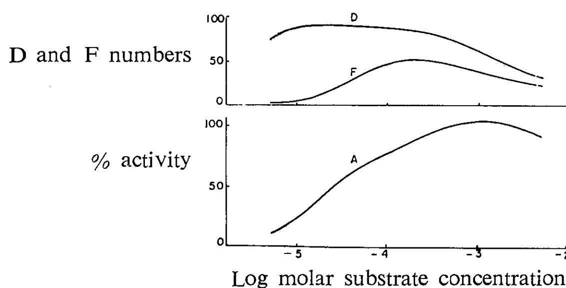


Fig. 2. Dependence of PsChE activity and dibucaine (D) and fluoride (F) numbers on substrate concentration. The concentration of dibucaine and fluoride was fixed at 1.0×10^{-5} M, and 5.0×10^{-5} M, respectively.
A: activity, D: dibucaine number, F: fluoride number

Inhibition curve with dibucaine and NaF of the normal PsChE by changing their concentration under fixed substrate strength of 5×10^{-4} M is shown in Fig. 3, together with another inhibition curve concerning the serum PsChE anomaly of fluoride-resistant character. From these experimental results, the concentration of NaF of 5×10^{-4} M, ten times as high concentration as that specified in the original method[11] was preferred for the sake of clearer differentiation of the two PsChE types (D and F).

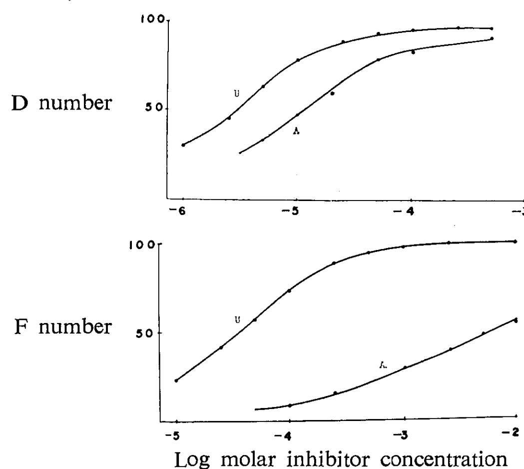


Fig. 3. PsChE inhibition by dibucaine and NaF.
U: PsChE in usual serum
A: Fluoride resistant PsChE in serum of heterozygote of silent/E_1^f gene.

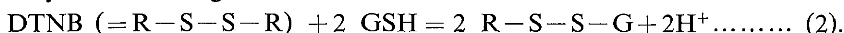
As for the dibucaine concentration of incubation mixture, a serum having slightly decreased PsChE activity on account of fluoride-resistant anomaly was used for the experiment, since unfortunately proper specimen of primary dibucaine-resistant anomaly was not available. The inhibition curve concerned with such

serum was sigmoid in shape similarly to and in close proximity to that of normal PsChE serum. However, the data accumulated from these instances were not satisfactory to appraise properly both dibucaine and NaF concentration. Therefore, the present method followed Kalow and Harris[10][11] in the adoption of optimum concentration of these PsChE inhibitors after contemplation of the fact that even in this concentration the abnormality of the PsChE can be differentiated successfully. This experiment provided another important evidence for the necessity of checking up the inhibition curve before establishing identification of PsChE anomaly of an unknown new specimen.

It seems reasonable to assume that reduced thiocholine (ChSH), a hydrolysed product of butyrylthiocholine by PsChE, should be used as standard for calibration, but it is a considerably labile substance which is not easily available commercially. For this reason, DTNB which is stable and reacts stoichiometrically with ChSH was chosen as the standard substance in this method. It was observed when DTNB was mixed with ChSH or with glutathione (GSH), they reacted stoichiometrically giving rise to the same symmetrical absorption peak with the same optical density at 412 mμ. Thus, no difference was seen between the two SH compounds in coloration. When equimolar DTNB reacted with GSH, the reaction proceeded as follows:

$$\text{DTNB } (=R-S-S-R) + \text{GSH} = R-S-S-G + \text{RSH} \quad \ldots\ldots\ldots\ldots\ldots (1)$$

The resultant mixture gave molar extinction coefficient $e^{412} = 13600$ at wave length 412 mμ. When GSH was added in more than five times as high molarity as that of DTNB in the mixture, a molar extinction coefficient of $e^{412} = 27200$ is obtained by the following reaction



$$\text{DTNB } (=R-S-S-R) + 2\ \text{GSH} = 2\ R-S-S-G + 2H^{+} \quad \ldots\ldots\ldots (2).$$

Therefore, molar extinction coefficient per one molar SH compound is calculated as 13600. In the medium of activity measurement of the present method, equation (1) takes place, because ChSH molarity produced by enzymatic decomposition of butyrylthiocholine is far below the level expected from the molarity of DTNB, while in the standard DTNB medium, the condition for equation (2) was chosen to complete the coloration of standard DTNB by adding about ten times higher molarity of GSH. (Fig. 4)

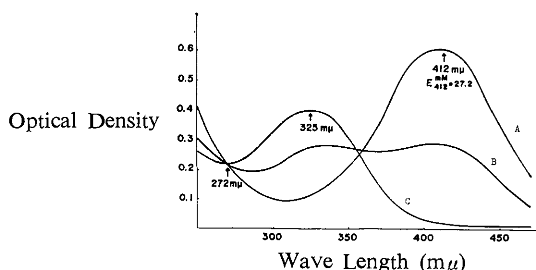


Fig. 4. Effect of glutathione upon DTNB coloration. The molar ratios of glutathione/DTNB of the absorption curve of A, B and C are 10/1, 1/1, and 1/10, respectively.

Colorized solution of this method was stable for many hours at room temperature. The within day and the day to day variations of the determinations were 4 and 4.5 per cent, respectively, throughout several months.

Buffered substrate solution is stable and useful for more than one month when it was kept in a refrigerator. Even when serum PsChE activity is low, it can be accurately determined by adjusting the dilution of serum adequately or by prolongation of incubation time up to two hours.

Lauryl sulphate was adopted as PsChE inhibitor in this method, for it does not affect the coloration.[14]

The normal range obtained by this method was established as 85－140 units (μM/ml/hr/25°C), for serum PsChE activity, 67－97 per cent for D number, and 45－95 per cent for F number on the basis of the examination of more than 200 individuals who were diagnosed as normal by blood chemistry tests including estimation of serum PsChE activity by Sasaki's routine laboratory method which uses acetyl choline as substrate.[21]

2) Populational survey:

The serum specimens totaling 2000 individuals were screened by this new method measuring their activities, D numbers and F numbers as shown in table 1 and the abnormal values were rechecked by the standard methods of Kalow's and Harris and Whittaker's.[10][11][12] The result of the survey demonstrated no detection of dibucaine resistant anomaly, namely homo- or hetero-zygotes of E_1^a gene.[22] However, three instances having fluoride anomaly were found. From the level of the decrease in fluoride numbers, they were considered to be heterozygotes. One of them was hospitalized by abdominal surgical wound and was given SCC, but no incidence of hypersensitivity was seen. The individuals with E_1^s gene[23][24] and silent type regulating gene[4][9][25] resulting in marked decrease in PsChE activity in homozygote were not encountered in this survey. These silent type PsChE anomalies are not detected by the simple screening test of the patients with ordinary diseases who come to the hospitals until the homozygotes are detected. However, it is certain that silent type anomaly is distributed in Okayama as we previously reported detection of two families of such anomaly.[8][9]

Table 1. Survey of atypical pseudocholinesterasemia in Okayama district

Sex	No. of Case	D number		F number	
		Median	95% Confidence limit	Median	95% Confidence limit
Male	1080	72.8	68.5—92.3	51.5	44.5—66.8
Female	970	72.9	67.8—91.6	52.1	43.0—66.5
Total	2050	73.1	68.1—92.0	51.5	44.0—66.4

The activities of PsChE are not tabulated since almost all the specimens screened are pathological, being of the patients who came to the hospitals.

Few survey on PsChE anomaly in Japan has been attempted for these ten years. Omoto sent blood specimens totaling 390, devided on several occasions to foreign countries, for the check of this anomaly and found two cases of fluoride type heterozygous anomaly, but could not discover any of the silent, E_1^s and E_1^a genes.[26) 27)] Motulsky et al, did not encounter any individuals with dibucaine resistant PsChE among the 140 people of Japanese-Canadian extraction living in Seattle.[28)] These observations and our survey are in good agreement in that E_1^a gene is rare in Japan if it is existing at all. However, E_1^s (silent) and E_1^f (fluoride-resistant) genes are certainly distributed with significant frequency, making a contrast to a high frequency of E_1^a (dibucaine-resistant) in Americans and Europeans. However, the presence of E_1^a gene among Japanese is not refutable since Oriental-Seattlians who may be consanguinously mixed with Japanese has its anomaly in frequency of 4/426 and the Taiwanese who may have close relations with Japanese have E_1^a with frequency of 1/340. These population problems would be gradually clarified by the future progress of serum PsChE anomaly in Japan.

CONCLUSION

A new method employing butyrylthiocholine as substrate was devised. This is recommended for the screening of pseudocholinesterase anomaly as well as for the routine laboratory test since it is very specific to PsChE, easily processed, and reliable.

In our experience, it is thought important to examine the inhibition curves of pseudocholinesterase by dibucaine as well as by sodium fluoride in addition to the measurement of D and F numbers for differentiation of aberrant PsChE types.

In our survey, 2000 individuals living in Okayama district were screened for PsChE anomaly by our new method. It disclosed the absence of dibucaine resistant type E_1^a gene, and presence of three heterozygotes of fluoride-resistant type, E_1^f gene, in three separate families. Silent and E_1^s type gene was not encountered in this survey although its existence was presumed from the result of our study on a consanguineous family inhabiting Okayama Prefenture which was reported previously. The populational distribution of atypical pseudocholinesterase gene in Japan may be denser for E_1^f, silent and E_1^s gene types than for E_1^a type.

REFERENCES

1) Lehmann, H. and Liddel, J.: Genetical variants of human serum pseudocholinesterase. In Steinberg, A.G.: *Progress in Medical Genetics*, **3**: 75-105, Grune and Stratton (N.Y. and London), 1964.

2) Kalow, W.: Cholinesterase types. In Wolstenholme, G.E. and O'Conner, C.M.: *Ciba foundation symposium on biochemistry of human genetics*. pp. 39-59, Churchill (Lond.), 1959.

3) Kalow, W.: The distribution, destruction and elimination of muscle relaxant. *Anesthesiology*, **20**: 505-518, 1959.
4) Lehmann, H.. Liddel, J., Blackwell, B., O'Conner, D.C., Daws, A.V.: Two further serum pseudocholinesterase types as causes of suxamethonium apnea. *Brit. Med. J.*, April 27: 1116-1118, 1963.
5) Foldes, F.F., Foldes, V.M., Smith, J.C. and Zsigmond, E.K.: The relation between plasma cholinesterase and prolonged apnea caused by succinylcholine. *Anesthesiology*, **24**: 208-216, 1963.
6) Kvisselgaard, N. and Moya, F.: Estimation of succinylcholine blood levels. *Acta Anaeth. Scand.*, **5**: 1-11, 1961.
7) Lehmann, H.: Pseudocholinesterase deficiency and some other pharmacogenetic disorders. In Stanbury, J.B., Wyngaarden, J.B. and Fredrickson, D.S.: *Metabolic Basis of Inherited Disease*, pp. 1356-1369, McGraw Hill (N.Y.), 1966.
8) Iuchi, I., Hashimoto, Y., Shoda, T., and Matsunaga, N.: The first family of serum Pseudocholinesterase anomaly found in Japan (in Japanese). *Med. J. Kawasaki Hosp.*, **1** (2): 41-74, 1968.
9) Shibata, S., Iuchi, I., Hashimoto, Y. and Shoda, T.: A consanguinous kindred of serum pseudocholinesterase anomaly found in Japan. *Proc. Jap. Acad.*, **43**: 907-912, 1967.
10) Kalow, W. and Genest, K.: A method for the detection of atypical forms of human serum cholinesterase. Determination of dibucaine numbers. *Canad. J. Biochem. Physiol.*, **35**: 339-346, 1957.
11) Harris, H. and Whittaker, M.: Differential inhibition of human serum cholinesterase with fluoride: Recognition of two new phenotypes. *Nature*, **191**: 496-498, 1961.
12) Kalow, W. and Lindsay, H.A.: A comparison of optical and manometric methods for the assay of human serum cholinesterase. *Canad. J. Biochem. and Physiol.*, **33**: 568-574, 1955.
13) Szasz, G.: Comparison between thiocholinester and O-nitrophenyl butyrate as substrates in the assay of serum cholinesterase. *Clin. Chem.*, **14**: 646-659, 1968.
14) Morrow, A.G. and Motulsky, A.G.: Rapid screening method for the common atypical pseudocholinesterase variant. *J. Lab. & Clin. Med.*, **71**: 350-356, 1967.
15) Karahasanoglu, A.M. and Özand, P.: Rapid screening test for serum cholinesterase. *J. Lab. & Clin. Med.*, **70**: 343-351, 1967.
16) Ernst, E.A. and Smith, J.C.: A pharmacogenetic study of a family exibiting aypical and "Silent" genes for plasma cholinesterase. *Anesthesiology*, **28**: 1085-1089, 1967.
17) Ellman, G.L., Courtney, K.B., Andres, V., and Featherstone, R.N.: A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmcol.*, **7**: 88-95, 1961.
18) Swift, M.R. and La Du, B.N.: A rapid screening test for atypical serum cholinesterase. *Lancet*, March 5: 513-514, 1966.
19) Harris, H. and Robson, E.B: Screening tests for the "atypical" and "intermediate" serum cholinesteterase types. *Lancet*, August 3: 218-221, 1963.
20) Radam, G.: Zur Bedeutung und Bestimmungstechnik erblicher pseudocholinesterase-Varianten. *Deut, Gerichtl. Med.*, **57**: 222-233, 1966.
21) Sasaki, M.: Ultramicro determination of serum cholinesterase with m-nitrophenol as indicator. *Jap. J. Clin. Path.*, **12**: 555-558, 1964.
22) Motulsky, A.G.: pharmacogenetics. In Steinberg, A.G.: *Progress in Medical Genetics*, **3**: 49-74, 1964. Grune and Stratton, (N.Y. and Lond.)
23) Kattamis, C.H., Davis, D. and Lehmann, H.: The silent serum cholinesterase gene. *Acta Genet. Basel*, **17**: 299-303, 1967.
24) Simpson, N.E. and Kalow, W.: The "Silent" gene for serum cholinesterase. *Am. J. Human Genet.*, **16**: 180-188, 1964.
25) Goedde, H.W. and Altland, K.: Evidence for different "Silent" genes in the human serum

pseudocholinesterase polymorphism. *Ann. New York Acad. Sci.*, **151** Art 1: 540-544, 1967.
26) Omoto, K. and Goedde, H.W.: Pseudocholinesterase in Japan. *Nature*, **205**: 726-726, 1965.
27) Altland, K., Epple, F. and Goedde, H.W.: Pseudocholinesterase in Thailand and Japan. *Humangenetik*, **4**: 127-129, 1967.
28) Motulsky, A.G. and Morrow, A.: Atypical cholinesterase gene E_1^a: Rarity in Negroes and most Orientals. *Science*, **159**: 202-203, 1968.