Am J Hum Genet 35:1107–1116, 1983

Determination of Genotypes of Human Aldehyde Dehydrogenase *ALDH*$_2$ Locus

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SUMMARY

Virtually all Caucasians have two major aldehyde dehydrogenase isozymes, $ALDH_1$ and $ALDH_2$, in their livers, while approximately 50% of Japanese and other Orientals are "atypical" in that they have only $ALDH_1$ and are missing $ALDH_2$. We previously demonstrated the existence of an enzymatically inactive but immunologically cross-reactive material (CRM) in atypical Japanese livers. Among 10 Japanese livers examined, five had $ALDH_1$ but not $ALDH_2$ isozyme. These are considered to be homozygous atypical at the *ALDH*$_2$ locus. Four had both $ALDH_1$ and $ALDH_2$ components detected by starch gel electrophoresis, that is, they are apparently usual. However, biochemical and immunological studies revealed that three of these four livers contained CRM. These three livers should be heterozygous atypical in the *ALDH*$_2$ locus, that is, genotype $ALDH_2^1/ALDH_2^2$. A Japanese liver, as well as control Caucasian livers, had no CRM, and they must be homozygous usual $ALDH_2^1/ALDH_2^1$. Although the number of liver specimens examined is limited, the frequencies of three genotypes determined in this study are compatible with the values calculated based on the genetic model that two common alleles $ALDH_2^1$ and $ALDH_2^2$ for the same locus are codominantly expressed in Orientals.

The remaining liver had only $ALDH_2$ isozyme and was missing $ALDH_1$. This type was not previously found in Caucasians and Orientals. The two-dimensional crossed immunoelectrophoresis revealed the existence of a CRM corresponding to $ALDH_1$ in this liver. The abnormality can be considered to be due to structural mutation at the *ALDH*$_1$ locus producing a defective $ALDH_1$ molecule, although other possibilities such as post-translational modifications are not ruled out.

Received January 4, 1983; revised March 4, 1983.

This work was supported by grant HL-29515 from the National Institutes of Health.

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INTRODUCTION

Liver aldehyde dehydrogenase (aldehyde: NAD oxidoreductase, E.C.1.2.1.3, ALDH) plays a major role in alcohol metabolism. Two major and several minor isozymes exist in the livers of mammals, including man. One of the major isozymes, designated as $ALDH_1$, is associated with slower anodal electrophoretic mobility, a low K_m for NAD, and a high K_m for acetaldehyde. Another major isozyme, designated as $ALDH_2$, is associated with faster anodal electrophoretic mobility, a high K_m for NAD, and a low K_m for acetaldehyde [1–4]. $ALDH_1$ isozyme is of cytosolic origin and $ALDH_2$ of mitochondrial origin in several animal species, and also presumably in man [1, 4, 5].

Human $ALDH_1$ and $ALDH_2$ isozymes are both tetrameric forms, but no common subunit exists in the two isozymes [6, 7]. The two isozymes immunologically cross-react, but are not identical to each other [8, 9]. All Caucasians thus far examined had both $ALDH_1$ and $ALDH_2$ isozymes [10]. By contrast, approximately 50% of Orientals are "atypical" in that they have only $ALDH_1$ component and are missing $ALDH_2$ isozyme [10, 11]. It was proposed that the absence of $ALDH_2$ isozyme in many Orientals could be due to gene deletion [11]. However, our study demonstrated the existence of an enzymatically inactive but immunologically cross-reactive material (CRM) in atypical Oriental livers [8]. Caucasian livers do not contain CRM. CRM was purified to homogeneity and characterized [9]. Molecular weight, amino acid composition, and immunological characteristics of CRM were indistinguishable from that of active $ALDH_2$ isozyme, but differed from $ALDH_1$ isozyme. These findings support the genetic model that CRM is an abnormal defective protein resulting from a mutation of the *$ALDH_2$* locus. In this model, some Orientals who have both $ALDH_1$ and $ALDH_2$ components are expected to be heterozygous in the *$ALDH_2$* locus and others are homozygous usual. We report here determination of genotypes of the *$ALDH_2$* locus.

MATERIALS AND METHODS

Human Livers

Autopsy livers from Caucasians and Japanese were stored at −65°C before use.

Enzyme and Protein Assay

Liver specimens were homogenized with equal amounts of 25 mM phosphate buffer, pH 6.0, containing 1 mM ethylenediaminetetraacetate and 10 mM 2′-mercaptoethanol. The homogenates were centrifuged at 20,000 *g* for 15 min. Aldehyde dehydrogenase activity was assayed in 0.1 M glycine-NaOH buffer, pH 8.6, containing 1 mM pyrazole, 0.1 mM NAD, and 0.5 mM acetaldehyde at 25°C. One unit of enzyme activity was defined as the amount required to convert 1 μmol of the substrate per min. For the study of the enzyme inhibition by disulfiram, the enzyme solution was dialyzed against 20 mM phosphate buffer, pH 7.0, containing 1 mM EDTA overnight at 4°C. The dialyzed enzyme was incubated in the reaction buffer with or without 5 μM disulfiram for 2 min at 25°C prior to starting enzyme reaction with acetaldehyde and NAD. Protein concentration was assayed by the method of Lowry et al. using bovine serum albumin as standard [12].

Complement Fixation

Antibody against $ALDH_2$ was produced in rabbits by immunizing the animals with purified $ALDH_2$, as described [8]. The antibody was salted out with 2 M $(NH_4)_2SO_4$ and

further purified through DEAE-cellulose. The micro-complement fixation was performed by a modification of the method of Wasserman and Levine [13]. The reaction mixture contained 0.2 ml of sample solution of various dilutions, 0.2 ml of standardized complement, 0.2 ml of standardized antibody solution, 0.6 ml of isotonic veronal buffer containing 5 mM $MgCl_2$, 0.15 mM $CaCl_2$, and 0.1% gelatin. The mixture was incubated at 4°C for 18 hrs, and 0.2 ml of standardized sheep red cells (5×10^7 cells/ml) was added to the reaction mixture. After incubation at 37°C for 60 min, the degree of hemolysis was spectrophotometrically determined. The complement fixation titer was defined as a dilution of sample solution that caused 50% inhibition of hemolysis.

Electrophoresis

Starch gel electrophoresis was carried out in 12% starch gel (Electrostarch, Madison, Wis.) using 10 mM sodium phosphate buffer, pH 7.2. Rocket immunoelectrophoresis was carried out in 1% agarose using veronal buffer system at pH 8.6 [14].

Separation of CRM from Active Enzyme Components

An aliquot of liver extract (2 ml) was applied to a 5′-AMP-Sepharose 4B column (0.5 × 2 cm) equilibrated with 25 mM phosphate buffer, pH 6.0, containing 1 mM ethylenediaminetraacetate and 2 mM 2′-mercaptoethanol at 4°C. The column was washed with 2 ml of the equilibration buffer. The active enzyme remained absorbed to the column during the sample application and the washing. The pass-through and the washing fractions (total 4 ml) were pooled and subjected to complement fixation test and rocket immunoelectrophoresis.

5′-AMP-Sepharose 4B was obtained from Sigma, St. Louis, Mo. Complement and sheep red cells were obtained from Colorado Serum, Denver, Co. Disulfiram (tetraacetylthiuram disulfide) was obtained from Sigma and recrystallized from methanol.

RESULTS AND DISCUSSION

Phenotypes Determined by Starch Gel Electrophoresis

Among 10 Japanese liver specimens examined, five had only an $ALDH_1$ component and were missing an $ALDH_2$ component. These are "atypical" livers that were found in about 50% of Japanese and other Orientals by previous investigators [10, 11]. The total absence of $ALDH_2$ component in the atypical livers should be the consequence of atypical homozygous status, $ALDH_2^2/ALDH_2^2$, in the genetic model proposed.

Four Japanese livers had both $ALDH_1$ and $ALDH_2$ isozymes, that is, these were apparently usual in starch gel electrophoresis (fig. 1). However, some of them are likely to be heterozygous in the *ALDH*$_2$ locus. These four livers, together with a control Caucasian liver (C) and an atypical Japanese liver (J_2), were subjected to further examination.

One Japanese liver had only $ALDH_2$ component and was missing ALDH isozyme. Such a phenotype was not previously found in Caucasians and Orientals. Study of this unusual type is described in the APPENDIX.

Genotypes Determined by Immunological Method

It was demonstrated that atypical Japanese livers contained CRM, but usual Caucasian livers had no such material [8, 9]. Therefore, it is expected that heterozygous atypical livers, $ALDH_2^1/ALDH_2^2$, contain CRM in addition to active

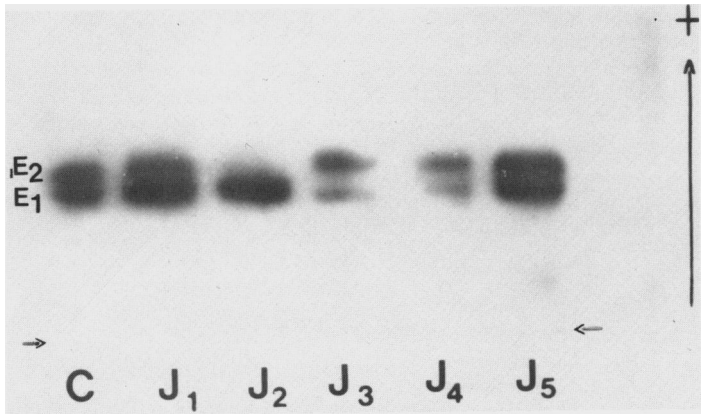

FIG. 1.—Starch gel electrophoresis patterns of liver extracts. Electrophoresis was carried out in 12% starch gel using phosphate buffer system, pH 7.2, at 9 V/cm for 4 hrs. The isozymes were stained for enzyme activity. *C*: Control Caucasian liver; J_1–J_5: Japanese livers; E_1: $ALDH_1$; E_2: $ALDH_2$; ↑: sample origin.

$ALDH_1$ and $ALDH_2$ components, but homozygous usual livers, $ALDH_2^1/ALDH_2^1$, have no CRM.

Rocket immunoelectrophoresis patterns of the liver extracts are shown in figure 2. In a control Caucasian liver extract (C), a precipitin peak related to $ALDH_2$ component was almost as high as that of $ALDH_1$, and the two peaks appeared to be superimposed in the particular electrophoresis condition used, while the corresponding precipitin peak was much lower in an atypical Japanese liver (J_2). This result is compatible to the previous finding that the amount of CRM in

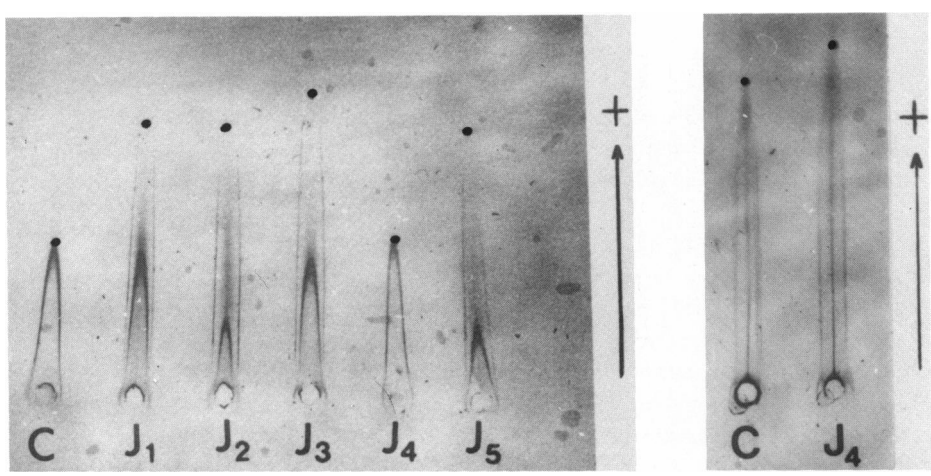

FIG. 2.—Rocket immunoelectrophoresis patterns of liver extracts. Electrophoresis was carried out in 1% agarose gel containing anti-$ALDH_2$ antibody using a veronal buffer system, pH 8.6, at 10 V/cm for 3 hrs. Immunoprecipitin peaks were stained with Coomassie Brilliant Blue R. *Sample designations* are the same as those given in figure 1. *Black dots* indicate top of the peaks. *A (left):* Approximately 5 μl of the diluted extracts (1:3) were placed in wells. *B (right):* Approximately 5 μl of the diluted extracts (1:1) were placed in wells.

TABLE 1

ALDEHYDE DEHYDROGENASE ACTIVITY AND IMMUNOLOGICAL ACTIVITY OF LIVER EXTRACTS

	Extracts before AMP-Sepharose				Extracts treated with AMP-Sepharose		
Liver specimen	Protein (mg)	Enzyme activity (U)	Disulfiram inhibition (%)	Immunological activity (total titer)	Enzyme activity (U)	Immunological activity (total titer)	Genotype
J_1	72	0.64	68	44×10^3	< 0.01	6.4×10^3	$ALDH_2^1/ALDH_2^2$
J_2	103	0.82	91	40×10^3	< 0.01	7.2×10^3	$ALDH_2^2/ALDH_2^2$
J_3	115	0.76	51	48×10^3	< 0.01	8.8×10^3	$ALDH_2^1/ALDH_2^2$
J_4	88	0.50	...	34×10^3	< 0.01	$< 1 \times 10^3$	$ALDH_2^1/ALDH_2^1$
J_5	120	0.90	66	50×10^3	< 0.01	4.4×10^3	$ALDH_2^1/ALDH_2^2$
C	59	0.47	49	32×10^3	< 0.01	$< 1 \times 10^3$	$ALDH_2^1/ALDH_2^1$
Four controls	...	...	54 (63–43)	...	...	...	$ALDH_2^1/ALDH_2^1$

NOTE: Protein content, enzyme activity, and immunological activity given in the table are the values in 2 ml of the extracts before AMP-Sepharose treatment or in 4 ml of the eluates recovered from the AMP-Sepharose columns. Immunological activity was expressed as total complement fixation titer, that is, (titer) × (vol, ml). Samples J_1, J_2, J_3, J_4, and J_5 are Japanese livers that correspond to the livers shown in figure 1. Sample C is a Caucasian liver shown in figure 1. Four control livers are all from Caucasians. Values of disulfiram inhibition for the four control livers are the average and range (in parentheses). Genotypes are presumed based on the starch gel electrophoresis patterns (fig. 1), and the complement fixation activities and rocket immunoelectrophoresis patterns (fig. 4) of the extracts treated with 5′-AMP-Sepharose 4B.

atypical livers quantified by crossed immunoelectrophoresis was much lower than that of $ALDH_2$ isozyme in usual livers [8]. One Japanese liver (J_4) exhibited a precipitin pattern that was similar to that of Caucasian liver (C), while three other Japanese livers exhibited precipitin patterns that were intermediate between the usual Caucasian's (C) and the atypical Japanese's (J_2). From these results, it is likely that J_4 is homozygous usual, $ALDH_2^1/ALDH_2^1$, and that J_1, J_3, and J_5 are heterozygous atypical, $ALDH_2^1/ALDH_2^2$.

To achieve more conclusive genotype determination, CRM and active enzyme components were separated from each other by the treatment with 5′-AMP-Sepharose 4B. After the treatment, liver extracts had no, or very low, aldehyde dehydrogenase activity (table 1). The treated extracts of J_1, J_2, J_3, and J_5 had high complement fixation titers, while the titers of C and J_4 were substantially lower (table 1). In rocket immunoelectrophoresis, the treated extracts of J_1, J_2, J_3, and J_5 exhibited double precipitin peaks, that is, a higher peak that is unique in these four samples, and a lower peak that is common to all six samples (fig. 3). The common low peak is likely to be related to residual active enzyme and/or denatured enzyme that leaked out from the 5′-AMP-Sepharose 4B, while the higher peak must be related to CRM. From these results, one can conclude that J_1, J_3, and J_5 are really heterozygous $ALDH_2^1/ALDH_2^2$, and that J_4 is homozygous usual $ALDH_2^1/ALDH_2^1$.

Attempt for Genotype Determination Based on Disulfiram Inhibition

$ALDH_1$ isozyme is strongly inhibited by a low concentration of disulfiram, while $ALDH_2$ isozyme is resistant against the disulfiram inhibition [2, 3]. Under an appropriate inhibition condition, enzyme activity of atypical liver extracts that contained only $ALDH_1$ isozyme would be strongly inhibited and the activity of homozygous usual liver extracts would be partially inactivated. The degree of inhibition of heterozygous atypical $ALDH_2^1/ALDH_2^2$ livers is expected to be in between these two types. In fact, Harada et al. reported that the activity of

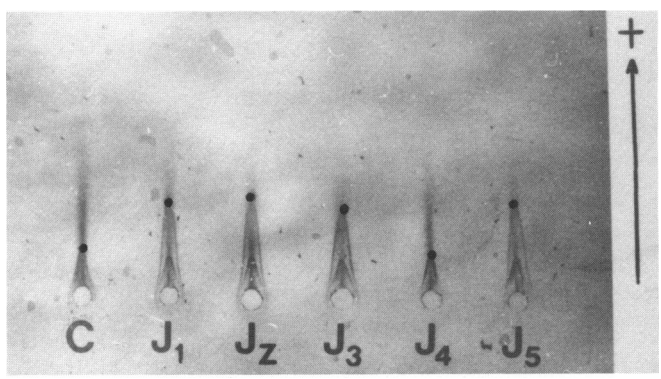

FIG. 3.—Rocket immunoelectrophoresis patterns of liver extracts treated with 5′-AMP-Sepharose 4B. Approximately 5 μl of eluates from the affinity column were placed in wells. Condition of electrophoresis and *sample designations* are the same as those given in figure 2. *Black dots* indicate top of the peaks.

the atypical liver extracts were more strongly inhibited than that of the usual livers by the reagent [15].

As expected, enzyme activity of an atypical Japanese liver (J_2) was inhibited more than 90% by 5 μM disulfiram, and the activity of the usual Caucasian livers was less severely inhibited. The enzyme activity of Japanese livers with presumed genotype $ALDH_2^1/ALDH_2^2$ was, on the average, more severely inhibited than that of the usual Caucasian livers. However, a substantial overlap in the degree of disulfiram inhibition was found between these two groups (table 1). We conclude that reliable genotype determination cannot be made based on the degree of enzyme inhibition by disulfiram.

Genotypes of $ALDH_2$ *Locus*

Based on the genetic model, that is, the usual $ALDH_2^1$ gene for active $ALDH_2$ isozyme and the atypical $ALDH_2^2$ gene for CRM, in the same autosomal locus, and codominant expression of these two genes in heterozygous state, genotypes of the $ALDH_2$ locus can be calculated from the Hardy-Weinberg equation. Although the number of Japanese livers examined is rather limited, figures of genotypes experimentally determined matched with the calculated values (table 2).

Since it is extremely difficult to obtain liver specimens from related individuals, the mode of inheritance of the atypical character was not previously determined. Goedde et al. reported that the ALDH types can be determined by isoelectric focusing of hair-root extracts [16]. However, thus far, pedigree analysis using their method is too limited to warrant the mode of inheritance. Our present study provides sufficient evidence for the Mendelian inheritance and codominant expression of the two allelic genes, $ALDH_2^1$ and $ALDH_2^2$, in man.

A high frequency (50%) of absence of $ALDH_2$ isozyme in Orientals was correlated to the high incidence (60%–80%) of acute alcohol intoxication in these populations by Goedde et al. [10]. On the other hand, Stamatoyannopoulos et al. postulated that the racial differences in alcohol sensitivity could be due to the rapid acetaldehyde formation by superactive atypical liver alcohol dehydrogenase controlled by the ADH_2 locus, found in about 90% of Orientals [17]. In our view, it is more

TABLE 2

GENOTYPES OF $ALDH_2$ LOCUS OF JAPANESE POPULATION

GENOTYPES	FREQUENCY	
	Calculated	Found
$ALDH_2^1/ALDH_2^1$	.08	.11 (1/9)
$ALDH_2^1/ALDH_2^2$	.40	.33 (3/9)
$ALDH_2^2/ALDH_2^2$	.52	.56 (5/9)

NOTE: The frequencies are calculated based on the observed population frequency (52%) of the absence of $ALDH_2$ isozyme in Japanese [9], assuming the Mendelian mode of inheritance of two allelic genes, $ALDH_2^1$ and $ALDH_2^2$, which are codominantly expressed in the heterozygous state.

likely that these two genetic factors are superimposed in alcohol-sensitive Orientals [6, 7]. Mizoi et al. found that the blood acetaldehyde level was nearly 10 times higher in alcohol-sensitive individuals, but found no difference in the maximum blood alcohol level and the rate of alcohol elimination between alcohol-sensitive and -nonsensitive individuals [18]. By contrast, the rate of alcohol elimination was suppressed in artificially induced alcohol sensitivity by administration of disulfiram or calcium carbamide [19]. Therefore, absence of $ALDH_2$ isozyme alone cannot fully account for the natural alcohol sensitivity with the normal rate of alcohol elimination. An additional genetic factor, that is, superactive alcohol dehydrogenase, could elevate the acetaldehyde level, thus compensating for the decrease in ALDH activity, and maintain the rate of ethanol elimination unchanged in alcohol-sensitive individuals. The degree of acute alcohol intoxication is known to be heterogeneous [17]. Subjects with $ALDH_2^2/ALDH_2^2$ in association with the atypical superactive ADH_2^2 enzyme (homozygous ADH_2^2/ADH_2^2 or heterozygous ADH_2^1/ADH_2^2) would be most sensitive, and subjects with $ALDH_2^1/ALDH_2^2$ in association with the atypical ADH_2^2 enzyme would be moderately sensitive to alcohol. Based on the gene frequencies of atypical $ALDH_2^2$ and atypical ADH_2^2, approximately 75% of Japanese belong to such types. Although other genetic and environmental factors may be involved in the acute alcohol intoxication, the figures are comparable to the observed frequency of alcohol-sensitive subjects in Japanese and other Orientals.

ACKNOWLEDGMENT

We are indebted to Prof. S. Miwa, University of Tokyo, for providing the autopsy Japanese livers.

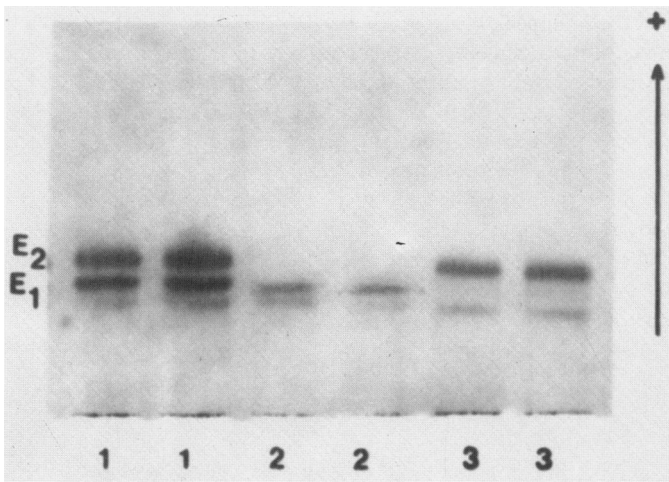

FIG. 4.—Starch gel electrophoresis patterns of liver extracts. Electrophoresis condition was the same as that given in figure 1. *1*: Control Caucasian liver; *2*: atypical Japanese liver that lacks $ALDH_2$ isozyme; *3*: a new unusual liver that lacks $ALDH_1$ isozyme.

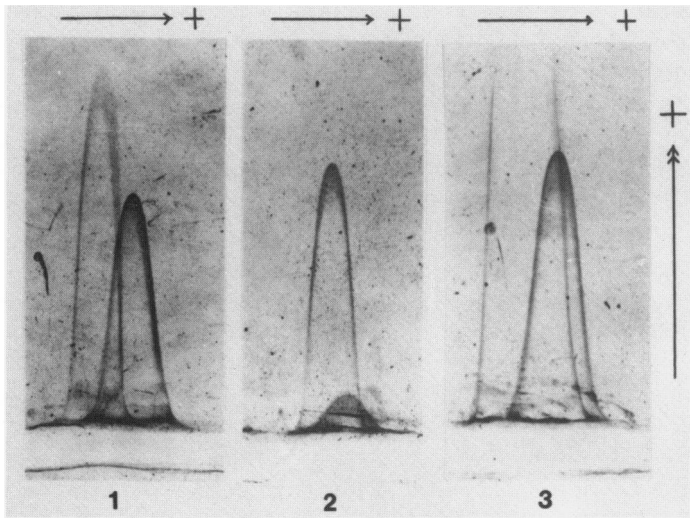

FIG. 5.—Patterns of crossed immunoelectrophoresis of liver extracts. *First dimension*: Starch gel electrophoresis under the same condition as that of figure 4. *Second dimension*: Immunoelectrophoresis in agarose gel containing anti-ALDH$_2$ antibody using a veronal buffer system at pH 8.6; 4 V/cm, 16 hrs. *Sample designations* are the same as those given in figure 4.

APPENDIX
A POSSIBLE STRUCTURAL VARIANT OF HUMAN CYTOSOLIC ALDEHYDE DEHYDROGENASE WITH DIMINISHED ENZYME ACTIVITY

In the course of study of liver aldehyde dehydrogenase abnormalities in Japanese individuals, a new phenotype that lacked ALDH$_1$ isozyme component was detected in starch gel electrophoresis (fig. 4). An absence of ALDH$_1$ isozyme was not previously observed either in Orientals or in Caucasians [10, 11].

In two-dimensional crossed immunoelectrophoresis, a large precipitin peak was observed in addition to a precipitin peak that is correlated to ALDH$_2$ isozyme, indicating an existence of CRM with no (or diminished) enzyme activity in this liver (fig. 5). From its peak position, CRM seemed to be slightly more acidic than the active ALDH$_1$ isozyme. The liver was obtained from a 70-year-old Japanese male who died by renal failure with the complication of pneumonia. He had had diabetes mellitus since middle age and also suffered from pulmonary tuberculosis and Parkinsonism.

The observed phenotype could have originated by homozygous abnormality at the *ALDH*$_1$ locus. However, liver samples of his relatives were not available, and it is impossible to establish the mode of inheritance of the abnormality. Starch gel electrophoresis patterns of other liver enzymes examined, that is, alcohol dehydrogenase isozymes and glucose-6-phosphate dehydrogenase, were the same as that commonly observed in Japanese individuals. The cause of abnormality, whether it was due to a genetic mutation or to post-translational modification, may be settled by examining the structure of the active ALDH$_1$ obtained from usual livers and that of the inactive CRM obtained from this unusual liver.

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