Hemoglobin Agenogi, a Slow-Moving Hemoglobin Resembling Hb E, Distributed in Izumo District*

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Hemoglobin surveys of recent several years are revealing that there are a great variety of abnormal hemoglobins distributed in Japan despite the rarity of hemoglobinopathy.[1] Four types of hemoglobin M[2-5] causing hereditary nigremia and Hb Ube-1[6], an unstable hemoglobin giving rise to "hereditary Heinz body anemia", are the noteworthy examples of the abnormal hemoglobins found in this country. However, the majority of them are associated with neither clinical symptoms nor hematological abnormalities. Among these hemoglobins causing no symptoms is listed Hb Agenogi, a slow-moving hemoglobin which was discovered in Agenogi-cho, Shimane prefecture, in 1965. It bore, electrophoretically and chromatographically, a considerable resemblance to Hb E[7][8] a representative slow-moving hemoglobin heavily distributed among the people living in the South-Eastern Asia. Detailed chemical analysis, carried out in our laboratory, however, disclosed that it was not identical with Hb E but a new abnormal hemoglobin which had never been recorded before.

About two years had elapsed until a similar hemoglobin was detected in a family inhabiting Izumo-city, Shimane prefecture. Study of the amino acid substitution demonstrated its identity with Hb Agenogi in spite of the absence of marital relationship between the two families in Agenogi-cho and Izumo-city.

This paper aims to report the results of our chemical analysis of Hb Agenogi together with those of some investigations on its functional and clinical significance.

CASE RECORD

Hb Agenogi was discovered in February 1965, from a patient (aged 66) with lung tuberculosis and diabetes mellitus. In January 1965, the patient had been admitted to Shimane National Sanatorium in Agenogi-cho, Shimane prefecture, with the diagnosis (by X-ray and bacteriological examination) of lung tuberculosis.

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He had had jaundice some twenty-five years ago, and "apical catarrh" twenty years ago. He had been diagnosed as diabetes mellitus three years before admission. Routine hematological and blood chemical examination (Table 1 : Agenogi family) revealed slight depletion with slight decrease in serum albumin to globulin ratio and cholinesterase activity. Fasting blood sugar was within normal limits. Slight leukocytosis with slight neutrophilia and monocytosis was noted, but anemia was absent. Abnormal hemoglobin which was the same as that seen in the patient was found also in one of his two daughters who were apparently healthy (Fig. 1).

Table 1: Abstracts of the laboratory data

	Agenogi family			Izumo family								
	II 4	III1	III3	I 2	II1	II2	II3	II4	II5	II6	III1	III2
Hematocrit (%)	44.5				29	43	39	39	31.5	45	34.5	34
Hemoglobin (mg/dl)	12.8			8.6	9.4	16.1	14.3	11.5	11.0	16.1	12.5	12.7
R B C (X 10^4)	497				390	465	427	347	349	473	422	421
Serum glucose (mg/dl)	94			98	106	200	114	93	76	94	92	98
Serum cholinesterase (Δ pH)*	0.66			0.19	0.58	0.71	0.73	0.54	0.65	0.62	0.55	0.58
Abnormal hemoglobin (%)	44.5	40.3	—	—	—	47.0	46.0	—	40.6	—	—	31.0
Hb A_2 (%)							0.74		1.8			1.7
Hb F (%)						1.9	2.4		3.4			
Alkali-resistant hemoglobin (%)	2.1					1.8	1.0		1.2			1.62
Ferrohemoglobin solubility (%)	96.1					88.0	94.6		98.1			92.7

* Takahashi-Shibata's method; normal range: 0.75-1.20

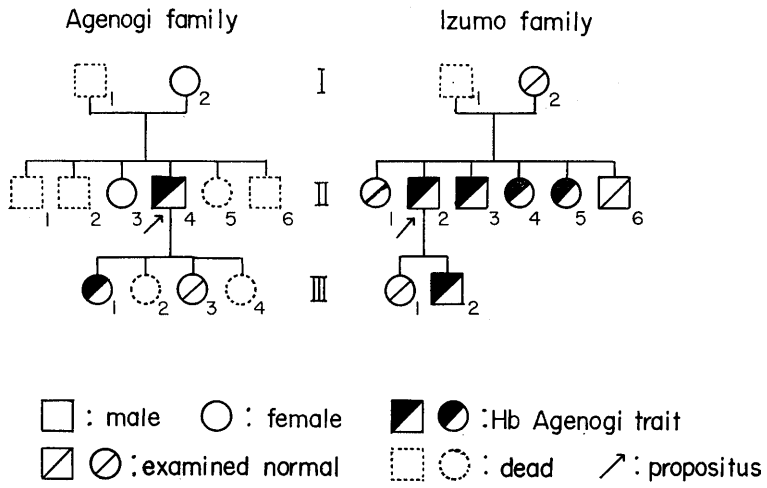

Fig. 1. The pedigrees of Agenogi and Izumo fmily.

Nearly two years later, Takeda who was performing a screening test for hemoglobinopathy found the second family (Izumo family) with similar abnormal hemoglobin in Izumo-city, Shimane prefecture. The propositus was a 44-year-old man with diabetes mellitus. Clinically he was unremarkable, though fasting blood sugar was distinctly elevated.

Systemic blood chemical and routine hematologic examination were made on nine members of the second family (Table 1 : Izumo family, Fig. 1). All of them showed slight decrease in serum cholinesterase activity. Questionable hyperglycemia was noted in siblings II-1 and II-3. I-2 had an advanced cancer of the gallbladder. III-2 had had lung tuberculosis when he was five years old. Moderate anemia was noted on I-2 and II-1 and II-4, II-5 and III-2 showed a slight anemia. Jaundice was absent in all members except I-2 who was jaundiced because of biliary obstruction. The abnormal hemoglobin was found in three of the five siblings and one of the two children of the propositus.

METHOD

Hemolysates were prepared by the conventional procedure, in which red cells were washed with saline solution, hemolysed with water, and shaken with toluene to remove the stroma. Detection of the abnormal hemoglobin was done by agar gel electrophoresis (pH 8.6 and 7.0)[9]. Huisman and Prins's technique[10] was followed in Amberlite IRC 50 chromatography. Carboxymethyl cellulose chromatography was conducted according to Huisman, Martis and Dozy[11]. The content of the abnormal hemoglobin, Hb A_2 and Hb F in the hemolysates was determined by scanning the agar gel plate after electrophoresis. The content of Hb F was also examined by alkali denaturation test of Singer, Chernoff and Singer[12]. The solubility of deoxygenated hemoglobin was studied by Goldberg's procedure[13].

The abnormal hemoglobin was separated and purified by starch block electrophoresis[14] (pH 8.6) of the hemolysate. Hb A_2 was obtained by the same method from normal adult hemolysates. The chain anomaly was examined by the hybridization test with canine hemoglobin[15], urea dissociation paper electrophoresis[16] and by the method invented by us recently[17], in which the hemolysate was treated with p-chloromerculibenzoic acid (PCMB) and the α and β subunits were subsequently separated by starch gel electrophoresis.

Globin was prepared by Anson-Mirsky's method[18]. Fingerprints were obtained by Ingram's[19] or Baglioni's[20] procedure after tryptic hydrolysis of the heat-denatured globin (trypsin/globin ratio 1/100 by weight, pH 8.0 for 4h at 37°C). The peptide spots were eluted and hydrolized with 6N HCl for 20h at 105°C.

The amino acid composition of the hydrolysates was studied qualitatively in the same way as done in fingerprinting, or quantitatively by Yanagimoto's Amino Acid Analyzer Model LC-5[21)22)]. The tryptic "core" which precipitates at pH 6.4 was further purified by repeated hydrolysis with trypsin for two hours (each) three times.

N-terminal amino acid residues were determined by DNP method using Sanger and Thompson's technique[23)] and two dimensional paper chromatography. DNP-peptides were hydrolized with 6N HCl for 8 hours or 20 hours, and α-DNP amino acids were extracted by ethylether. Toluene-pyridine-ethylenechlorhydrine-0.8 N ammonia (100 : 36 : 60 : 60 by volume) was used for the first ascending irrigation, and buthanol-acetic acid-water (3 : 1 : 1) for the second which enabled development in the direction vertical to the first.

RESULTS

Agar gel electrophoresis (pH 8.6 and 7.0) of the hemolysate disclosed the presence of four different hemoglobins, namely Hb A, Hb Agenogi, Hb A_2 and Hb F. Hb Agenogi was slower in anodal migration than Hb A and faster than Hb A_2 on agar gel electrophoresis at pH 8.6. However, it was faster than Hb A and Hb F at pH 7.0 (Fig. 2). Abnormal hemoglobin content was about 45% both in the Agenogi and Izumo family (Table 1). Concentration of Hb A_2 and that of Hb F were normal. Solubiliy of the deoxygenized hemoglobin was within the normal range. Hb Agenogi was slow-moving on starch block electrophoresis (pH 8.6 and 7.0). It was nearly equal to Hb A_2 or Hb E in migration at pH

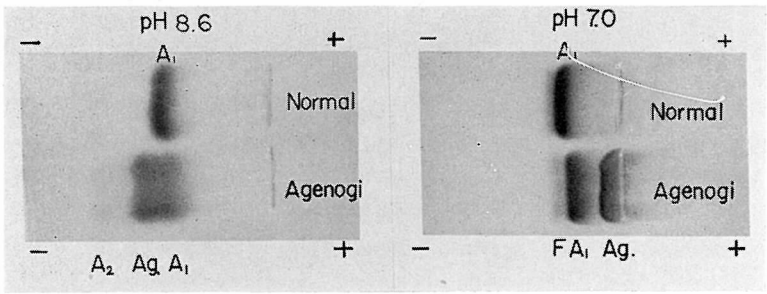

Fig. 2. Agar gel electrophoresis of the hemolysate (pH 8.6 and 7.0).
A_1: Hb-A_1; A_2: Hb-A_2; F: Hb-F; Ag: Hb Agenogi. HbAgenogi is slower than Hb-A_1 and faster than Hb-A_2 in anodal migration at pH 8.6. It is, however, faster than Hb-A_1 and Hb-F at pH 7.0; therefore differentiation from Hb-E (which does not separate from Hb-A_1 at pH 7.0) can be made by this technique.

8.6 (Fig. 3). So, contamination with Hb A_2 was inevitable on purification of the abnormal hemoglobin by this technique.

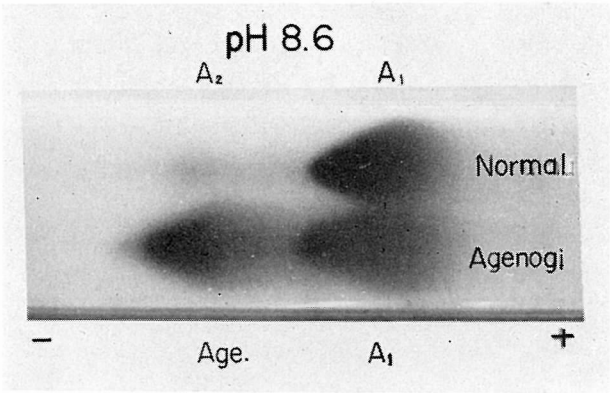

Fig. 3. Starch block electrophoresis of the hemolysate (pH 8.6). Hb Agenogi is equal to Hb-A_2 in anodal migration. It cannot be differentiated from Hb-E by this technique.

The abnormal hemoglobin descended the column of Amberlite IRC 50 resin behind Hb A. It emerged from CM-cellulose column later than Hb A_2.

In hybridization test with canine hemoglobin (α_2^{can} β_2^{can}), the hybrid appearing on the anode side representing α_2^{can} $\beta_2^{Agenogi}$ (produced by the β chain of the abnormal hemoglobin and the α chain of canine hemolgobin). was distinctly slower in electrophoretic migration than the corresponding hybrid α_2^{can} β_2^{A} (formed from the β chain of normal Hb A and canine α chain).

Starch gel electrophoresis of the PCMB-treated Hb Agenogi revealed the presence of an abnormal β chain which migrated much slower than normal β chain. Its α chain was electrophoretically identical with that of Hb A (Fig. 4).

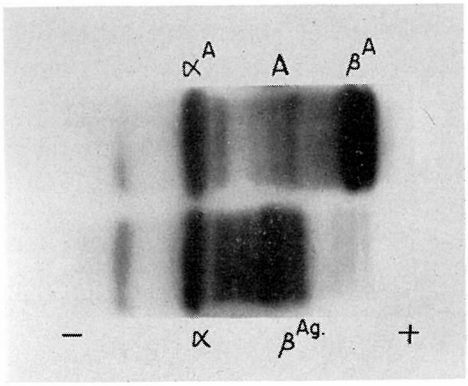

Fig. 4. Starch gel electrophoresis of PCMB-treated Hb A (upper) and Hb Agenogi (lower). The β chain of Hb Agenogi (β^{Ag}) is distinctly slower than that of normal Hb A (β^{A}) and is situated very close to the undissociated Hb A (A). Its α chain (α) is equal in electrophoretic migration to the normal α chain (α^{A}).

Urea dissociation paper electrophoresis of Hb Agenogi globin was also consistent with slow-moving β chain anomaly. The fingerprint (according to Ingram) of Hb Agenogi globin was characterized by the appearance of an abnormal spot which was clearly visible above the peptide spots Nos. 15-16 (Fig. 5). However, all the peptide spots pertaining to Hb A were seen on the Hb Agenogi fingerprint.

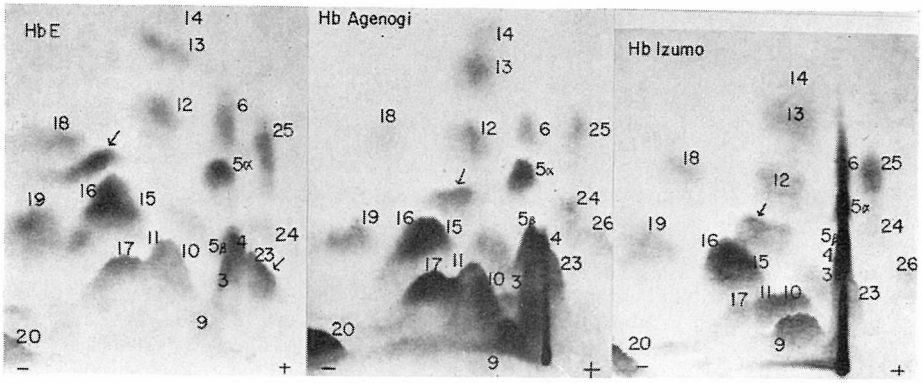

Fig. 5. Fingerprint by Ingram's method of the whole globin of Hb Agenogi contrasted with that of Hb E. Arrows indicate the abnormal spots. Note that the Hb Izumo fingerprint is essentially identical with the Hb Agenogi fingerprint.

These characteristics were recognized also by Baglioni's fingerprinting, in which the abnormal spot was seen indistinctly separated from the peptide spot No. 15. With nihydrin the abnormal spot showed a transient yellow coloration suggestive of N-terminal glycyl. The spot was negative for histidine, tyrosine and arginine by specific color tests[24]. N-terminal glycyl was confirmed by DNP method: only DNP-glycine was demonstrated in the ether-soluble fraction. This abnormal spot was free of contamination of tryptic peptides derived from Hb A_2 because the corresponding area was vacant in the Hb A_2 fingerprint. DNP-valine and DNP-leucine were demonstrable from the dinitrophenylated "core" of Hb Agenogi, and no DNP-glycine could be detected. N-terminal valine, leucine and glycine of the normal Hb A "core" were confirmed by the same method.*

Amino acid analysis of the abnormal spot by acid hydrolysis disclosed a peptide which was composed of alanine, glycine, leucine, lysine, phenylalanine, serine and threonine. This was confirmed by automatic quantitative amino acid analysis (Table II).

*These findings are consistent with the findings of Botha, Beale, Isaacs and Lehmann[25] that αTp X and αTp XI are distributed both in the soluble and "core" fractions, so that N-terminal leucine (91 and 100) and valine (93) for α chain core are expected to be detected.

DISCUSSION

It is apparent from the experimental results described above that Hb Agenogi is a hemoglobin of β chain anomaly resembling Hb E ($\alpha_2\beta_2^{26Lys}$)[7] closely in electrophoretic and chromatographic behavior except for one point that Hb Agenogi is separable from Hb A (Hb E can not be separated) by agar gel electrophoresis at pH 7.0. Accordingly, this hemoglobin is not identical with Hb E. However, it is expected that one of the acidic amino acid residues in the β chain of Hb Agenogi may be substituted for by a basic amino acid, for instance, lysine in substitution for glutamic acid as seen in Hb E.

However, on looking at the fingerprints one notices remarkable differences between these hemoglobins. Firstly, there is only one abnormal spot seen on the Hb Agenogi fingerprint, whereas two abnormal spots, the "cathodal" and the "anodal", are present on the Hb E fingerprint. Hb Agenogi's abnormal spot corresponds to the "cathodal" one of Hb E (lying below the spot No. 18 and above the spots Nos. 15, 16 and 19), although it appears to be slightly more biassed to the anode side. Secondly, the abnormal spot of Hb Agenogi is negative for arginine when examined by specific color test, making a sharp contrast to the "cathodal" abnormal spot of Hb E which is arginine-positive. Thirdly, the peptide spot No. 26 of Hb Agenogi (corresponding to the "anodal" abnormal spot of Hb E) is arginine-positive, just like the same peptide of Hb A, whereas the "anodal" abnormal spot of Hb E lacks arginine residue.

As has been stated above, the fingerpint of Hb Agenogi is featured by the presence of all the peptide spots pertaining to Hb A without any apparent omission in spite of the presence of an abnormal spot. This suggests that the peptide of the abnormal spot might originate from the "core" of the β chain rather than from the soluble tryptic peptides of the same chain; if substitution were in the part of soluble tryptic peptides of Hb Agenogi, its fingerprint should lack some spot representing the normal tryptic peptides as exemplified by Hb E in which peptide spot No. 26 is missing in its proper place. This presumption was substantiated, in our study, by the amino acid analysis of the abnormal peptide. Namely, N-terminal glycyl, two threonyl, phenylalanyl, alanyl, leucyl, seryl and lysyl residues were demonstrated. (Table II). This composition is in

Table II: Amino acid composition of the peptide of the abnormal spot of the fingerprint from Hb Agenogi as compared with the known amino acid sequence of the N-terminal portion of the "core" of β chain.

N-terminal portion of the	83	84	85	86	87	88	89	90	91
"core" of β chain	Gly -	Thr -	Phe -	Ala -	Thr -	Leu -	Ser -	Glu -	Leu...
Abnormal spot; estimated residues per molecule	Gly -	(Thr,	Phe,	Ala,	Leu,	Ser) -	Lys		
Agenogi	0.78	1.74	0.82	1.22	1.03	1.22	1.06		
Izumo	0.34	1.83	1.19	1.07	1.05	1.11	0.68		

complete accordance with that of the first portion of the "core" of the β chain[26] encompassing the amino acid sequence from glycine (83 β) to glutamic acid (90 β) apart from the absence of glutamic acid and presence of lysine instead. As a corollary, substitution of lysine for glutamic acid (90 β) which will result in liberation of a soluble peptide from the "core" of the β chain by tryptic digestion is conceivable for Hb Agenogi (in this respect, Hb Agenogi is similar to Hb D_{Ibadan}[27]). The fact that DNP-glycine was not demonstrable by the N-terminal analysis of the "core" furnishes an additional evidence for the substitution of lysine for glutamic acid (90 β). Leucine is the amino acid residue situating next to glutamic acid (90 β) in the core of the β chain, and this is destined to form the N-terminal of the core of the β chain of Hb Agenogi on account of the substitution of lysine for glutamic acid (90 β).

For the past several years the abnormal hemogobins all over the world have been increasing in number and, inferentially, at present variants of no less than 50 have been identified by elucidation of the primary structure[28][29]. However, so far as we know, no abnormal hemoglobin of substitution at 90 β has been described. It is therefore stated that Hb Agenogi ($\alpha_2\beta_2^{90\,Lys}$) is a new abnormal hemoglobin.

Amino acid substitution of lysine for glutamic acid will be the result of one step base substitution (thymine for cytosine) at only one point of the 440 base sequence of the DNA code[30].

Substitution of lysine for glutamic acid has been recorded in several abnormal hemoglobins, namely Hb C ($\alpha_2\beta_2^{6\,Lys}$)[31], Hb E ($\alpha_2\beta_2^{26\,Lys}$)[32], Hb O_{Arabia} ($\alpha_2\beta_2^{121\,Lys}$)[33][34], Hb $O_{Indonesia}$ ($\alpha_2^{116\,Lys}\beta_2$)[33], Hb Siriraj ($\alpha_2\beta_2^{7\,Lys}$)[35], Hb $F_{Galveston}$ ($\alpha_2\gamma_2^{5\text{ or }6\,Lys}$)[36], and the new one, Hb Agenogi ($\alpha_2\beta_2^{90\,Lys}$). They are thought to be easily discriminated by fingerprinting, and will also be differentiated by means of combined use of several systems of electrophoresis. Discrimination of Hb Agenogi from Hb E by agar gel electrophoresis is such an example.

Oxygen equilibrium of Hb Agenogi was studied by Imai of the Kotani Laboratory of Osaka University School of Basic Engineering. It has a slightly decreased oxygen affinity; pO_2 for a half saturation was about 40 % higher than normal Hb A at 30°C and pH 7.5* (Fig. 6). It showed slightly increased Bohr effect as well as temperature effect. Its heme-heme interaction expressed by "n" of Hill's equation was normal. Functional deviation of this magnitude is not likely to cause pathophysiological problem. Minor abnormality in oxygen equilibrium curves of Hb Agenogi is very similar to those of Hb E[37].

*0.1 % hemoglobin solution in phosphate buffer was used in his study, and oxygen equilibrium study under physiological conditions (in whole blood) has never been done.

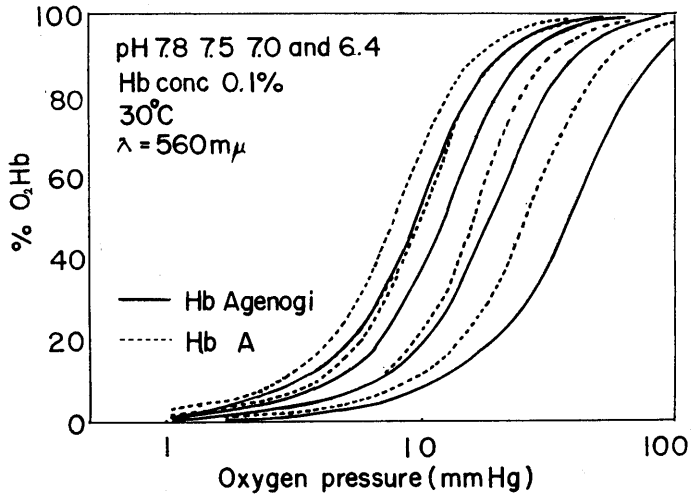

Fig. 6. Oxygen equilibrium curve of Hb Agenogi, redrawn from the unpublished data of Dr. K. Imai.

Occurrence of mild diabetes mellitus and subnormal serum cholinesterase activity as well as mild anemia are unrelated to the presence or absence of the abnormal hemoglobin. The heterozygosity for Hb Agenogi is presumed to cause no clinical symptoms. Nevertheless, this does not necessarily imply that the Hb Agenogi homozygosity is also without clinical manifestation, since homozygosities for Hb C and Hb E are associated with mild to moderate hemolytic anemia[8] though heterozygosities for the genes of Hb E is free of clinical or hematological abnomality.

So far as the results obtained by our family study, it is not certain that the Agenogi and Izumo families have originated from a common ancester. Although marital relation was not demonstrable, they are the natives of the same district: the situations are comparable with the two families of Hb Hikari ($\alpha_2\beta_2{}^{61\,ASPNH_2}$)[38] reported by Shibata and his associates, both of which were the natives of Tottori prefecture in ancestry without any demonstrable marital relationship inbetween.

SUMMARY

An abnormal hemoglobin resembling Hb E was discovered in two possively unrelated families living in Shimane prefecture, Japan. Study of the amino acid substitution has demonstrated that it is a new variant of Hb A. Its molecular formula can be expressed by $\alpha_2\beta_2.^{90Lys}$ This new abnormal hemoglobin, named Hb Agenogi, seems to cause no clinical symptom though it has slightly lowered oxygen affinity.

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