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The Polymorphic Forms of α_1-Acid Glycoprotein of Normal Caucasian Individuals*

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α_1-Acid glycoprotein (2, 3), one of the most highly purified human plasma proteins, appears polymorphic on starch gel electrophoresis near its isoelectric point. When isolated from pooled normal blood it reveals seven bands (4). Some of the chemical and physical chemical properties of the polymorphic forms of this protein have been found to be very similar, whereas significant differences were observed with respect to others (5).

In the present paper, studies on the genetic aspects of the polymorphism of α_1-acid glycoprotein are described. Specifically, this investigation has been carried out to determine whether normal Caucasian individuals possess different α_1-acid glycoprotein traits characterized by different numbers of bands of the starch gel electrophoretic patterns.

Methods

The following apparently healthy Caucasian individuals each donated fifty ml of blood drawn by venipuncture: 53 adults who were between 19 and 54 years old and selected at random in the Boston area; 15 members of a Caucasian family (P) (ages, 8 to 66 years); 23 members of another Caucasian family (C); and 18 pairs of Caucasian twins (ages, 13 to 18 years).

After the blood clotted, the sera were separated, shell-frozen, and stored at $-20°$ C until subsequent fractionation was started. The identity or nonidentity of the nine pairs of twins was established by analyses of thirteen different blood groups.[1]

For the fractionation of the sera, Cohn's method 10 (6, 7) was modified so that, in the first step, the so-called Fractions I to V were rendered insoluble simultaneously. The second step involved precipitation of Fraction VI of which the major component is α_1-acid glycoprotein. The reagents used for this modified method were described earlier (6, 7). The fractionation was carried out as follows: Twenty-five ml of reagent A (6, 7) was placed in a 500-ml Erlenmeyer flask and cooled until partially frozen. For convenience dry ice and ethanol were used as cooling media. By stoppering the flask care was taken to exclude carbon dioxide. Thereafter the flask was placed in a $-5°$ C bath. Under gentle swirling of the partly frozen reagent, 25 ml of serum precooled to 0° was slowly added. It was found advantageous to stir the resulting suspension with a thermometer in order to follow any change in temperature. In a separate flask 75 ml of reagent A, 125 ml of reagent A′ (6, 7), and 20 ml of zinc reagent (6, 7) were mixed, cooled until partially frozen, and, again under gentle swirling, added to the above mentioned diluted and partially precipitated serum. The resulting suspension was kept at $-5°$ C for at least 60 minutes, then centrifuged at $-5°$ C and 4,500 rpm for 30 minutes. The clear supernatant solution was collected in a 1,000-ml Erlenmeyer flask placed in the $-5°$ C bath. Its pH, after fourfold dilution with 0.01 M NaCl, should be 5.80 ± 0.02. For the precipitation of Fraction VI, 10 ml of 1 M barium acetate, precooled to $-5°$ C, was added with gentle stirring of the supernatant solution of Fractions I to V followed by 130 ml of 95% ethanol precooled to $-60°$ C. The obtained suspension was placed in a $-10°$ C bath. On the following day most of the supernatant solution was decanted and the remaining suspension centrifuged at $-5°$ C and 4,500 rpm for 40 minutes. The obtained residue, Fraction VI, was carefully suspended at $-5°$ C in the remaining small volume of supernatant solution and then dissolved at 0° by ad-

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[1] Carried out at the blood bank of Massachusetts General Hospital in Boston under the direction of Dr. Morten Grove-Rasmussen.

dition of a neutralized 0.1 M solution of ethylenediaminetetraäcetic acid precooled to 0°. The resulting clear solution of Fraction VI was quantitatively transferred to a prewashed dialysis membrane (Visking tubings, 8/32), dialyzed against several changes of cold distilled water for approximately 48 hours, and then lyophilized. The weight of Fraction VI averaged 25 mg, an amount agreeing with that obtained by large-scale fractionation (2).

The isolation of α_1-acid glycoprotein in the homogeneous state from Fraction VI was achieved by chromatography on Amberlite IRC-50 as described earlier (8). An appropriate glass tubing, 0.8 cm in diameter and 30 cm long, was filled in the conventional way with Amberlite IRC-50 to give a column of 12 cm. The resin had to be equilibrated exhaustively against pH 5.20, 0.05 M sodium citrate buffer before use. After the flow rate was adjusted to one drop of effluent (0.05 ml) per 1 minute, Fraction VI was dissolved in 1 ml of the same buffer and applied to the column; this was followed by two washings of 0.5 ml each. The first two ml of effluent was discarded. The subsequent 15 ml containing approximately 80% of the α_1-acid glycoprotein was dialyzed against cold distilled water for 48 hours and then lyophilized. The resulting α_1-acid glycoprotein preparations, averaging 11 mg, were stored in well-closed tubings at −20° C until starch gel electrophoresis was carried out. Many preparations were analyzed by electrophoresis at pH 8.6 in ionic strength 0.1 citrate-diethyl-barbiturate buffer and appeared homogeneous. Further, when the amount of glycoprotein permitted it, starch gel electrophoresis at the same pH in borate was also performed. Again homogeneity was observed.

Starch gel electrophoresis (175 v, 70 ma, 6 hours) was performed at pH 2.90 in ionic strength 0.02 phosphate buffer (5). The pH of the buffer was adjusted so that none of the protein zones moved toward the anode. For each analysis 2.0 mg of protein was used.[2] The size of the starch gel block (21 × 125 × 0.6 cm) permitted simultaneous analysis of four specimens. For the evaluation of the starch gel patterns it proved to be of particular help to include in every set of analyses as control the same amount of the same preparation of pooled α_1-acid glycoprotein. After the electrophoresis the starch gel was sliced with a razor blade into three or four equally thick parts. Amido black 10B was used as stain. The center parts were used for the evaluation of the patterns. Under the chosen conditions, the fastest band of the control sample should migrate 4.5 cm from the trough of application. The number of bands of each pattern and the band with the highest color intensity were recorded. The evaluation of the patterns of the two families and the twins was carried out in a double-blind study.

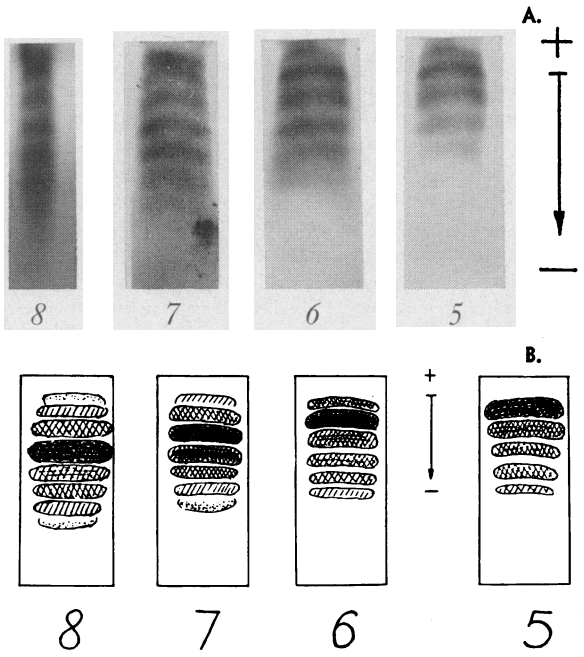

FIG. 1. A. REPRESENTATIVE PHOTOGRAPHS OF α_1-ACID GLYCOPROTEIN PATTERNS OF NORMAL CAUCASIAN ADULTS WITH 5, 6, 7, AND 8 BANDS. Starch gel electrophoresis at pH 2.9. Direction of electrophoretic movement as indicated by the arrow. B. DRAWINGS OF A.

Results

A. α_1-Acid glycoprotein patterns of normal white adults

α_1-Acid glycoprotein derived from 53 normal white adults and analyzed by starch gel electrophoresis at pH 2.9 resulted in patterns with 5, 6, 7, and 8 bands (Figure 1 A, B). The relative incidence of these patterns was found to be 4, 36, 49, and 11%, respectively (Table I). A further interesting observation was made with regard to the position of the band that contained the maximal amount of protein: the higher the total number of bands of a pattern, the higher was the apparent

[2] Because of the low concentration of α_1-acid glycoprotein in blood and because no specific stain is available for this protein, α_1-acid glycoprotein must be isolated before starch gel electrophoresis can be carried out. In an earlier paper (5) a series of control experiments was described demonstrating that the polymorphism is not an artifact due to the isolation procedure utilized. In addition α_1-acid glycoprotein subjected to ultracentrifugation at pH 3 sedimented with a coefficient of 2.9 S, a value identical with that observed at neutrality under otherwise the same conditions. This finding excluded the possibility that this glycoprotein dissociates at acid pH values into subunits leading to polymorphism, thus being in agreement with the observation that at pH 2 and below a single band is observed on starch gel electrophoresis.

electrophoretic mobility of the band with the largest amount of protein. Patterns with 5 bands revealed the maximal amount of protein essentially at the slowest moving zone. The mentioned maximum of patterns with 6 bands was almost equally distributed between the second and third zones. Half of the patterns with 7 bands showed the maximum at the third zone, whereas the maximum of the other patterns was found at the second or fourth zone. Patterns with 8 bands revealed the maximum at the third or fourth zone. The significance of this positive but incomplete correlation is difficult to establish on the basis of available data. Difficulties were occasionally encountered in locating the maximum exactly, accounting perhaps for part of the scatter of this distribution. Therefore, we did not attempt to establish the location of the maxima of the patterns reported in sections B and C. The slowest moving zone of all patterns always exhibited the same apparent electrophoretic mobility. Further, the corresponding bands of the patterns obtained during the same run also exhibited the same apparent electrophoretic mobilities.

B. The α_1-acid glycoprotein patterns of the members of two Caucasian families

Fifteen members of a family whose genealogical tree is given in Figure 2 (family P) were analyzed for their α_1-acid glycoprotein patterns. The pattern of the father showed 5 and that of the mother 6 bands. The patterns of their six daughters also revealed 6 bands, except one that showed only 5. The pattern of the husband of the daughter, age 39, revealed 7 bands. Unfortunately it was not possible to obtain blood from the husbands of the other two married daughters. The corresponding patterns of the grandchildren showed 5, 6, or 7 zones.

TABLE I

Relative incidence of the α_1-acid glycoprotein patterns of normal white adults

Number of bands of the α_1-acid glycoprotein patterns	5	6	7	8
Number of cases	2	19	26	6
Relative incidence of the patterns, %	4	36	49	11

Twenty-three members of family C (Figure 3) were analyzed for their α_1-acid glycoprotein patterns. Unfortunately, the father of this family had passed away before the study was initiated. The mother's α_1-acid glycoprotein patterns revealed 6 bands. The patterns of four daughters and one son showed the same number of bands. Two sons and the remaining two daughters had 7-band patterns and one son an 8-band pattern. The husbands of the married daughters showed α_1-acid glycoprotein patterns with 7 and 8 zones. The patterns of the nine investigated grandchildren revealed patterns with 6 and 8 bands.

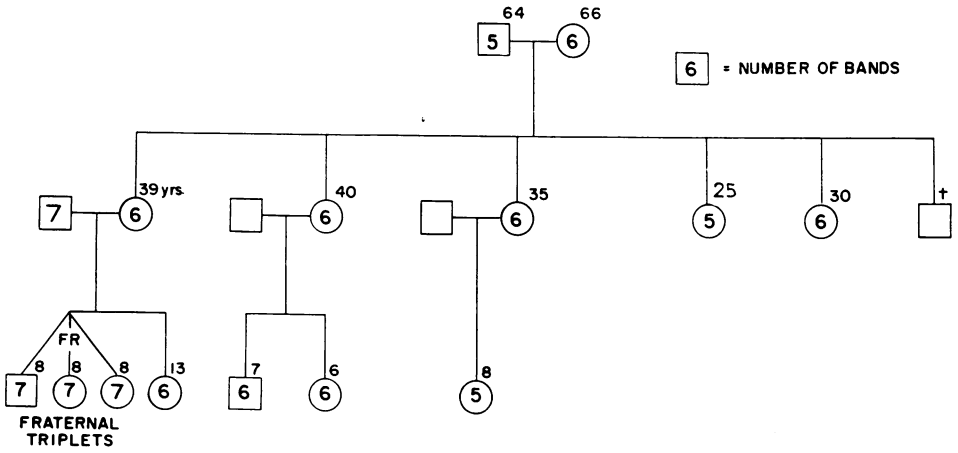

FIG. 2. GENEALOGICAL TREE OF THE CAUCASIAN FAMILY P WITH α_1-ACID GLYCOPROTEIN PATTERNS REVEALING MAINLY SIX BANDS. The number in the squares and circles indicates the number of bands of the α_1-acid glycoprotein patterns. The number above the squares and circles indicates the age of the individual.

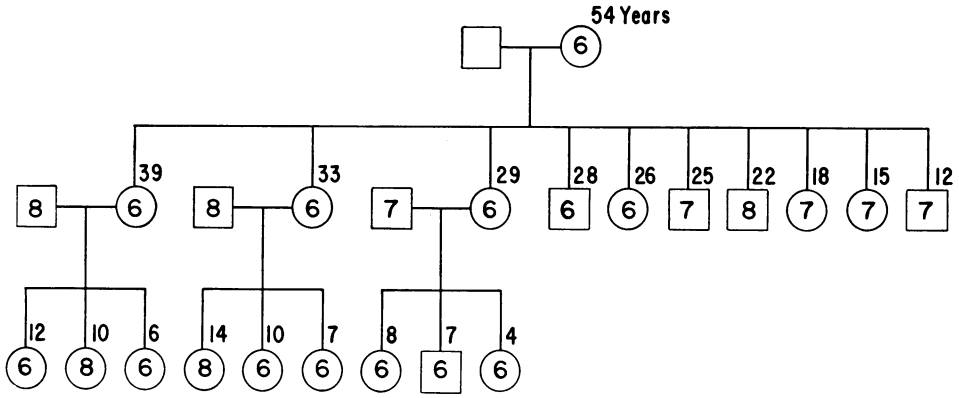

FIG. 3. GENEALOGICAL TREE OF THE CAUCASIAN FAMILY C. The number in the squares and circles indicates the number of bands of the α_1-acid glycoprotein patterns. The number above the squares and circles indicates the age of the individual.

TABLE II

α_1-Glycoprotein patterns of eighteen pairs of normal white twins observed at pH 2.9

Twin no.	Subject	Age	Sex	Number of bands of patterns	Zygosity
1A	GA	18	M	6	I
1B	GD			6	
2A	BF	13	F	6	I
2B	BW			6	
3A	HC	17	M	7	I
3B	HW			7	
4A	TJ	17	F	7	I
4B	TJ			7	
5A	GJ	18	F	6	F
5B	GJ			6	
6A	GD	13	M	7	F
6B	GD			7	
7A	CP	15	F	6	F
7B	CM			7	
8A	VA	18	F	6	F
8B	VP			7	
9A	CC	16	F	6	I
9B	CC			6	
10A	EM	17	M	7	F
10B	EJ			7	
11A	HB	18	F	7	F
11B	HC		M	8	
12A	MJ	13	F	8	I
12B	MJ			8	
13A	MJ	13	F	7	I
13B	MM			7	
14A	BS	15	F	7	I
14B	BS			7	
15A	FJ	10	M	6	I
15B	FC			6	
15C	FR			6	F
16A	KD	16	M	7	F
16B	KD			6	
17A	AG	14	F	8	F
17B	AG		M	8	
18A	DG	17	F	6	F
18B	DJ				

C. α_1-Acid glycoprotein patterns of identical and fraternal twins

Nine of the 17 pairs of twins and one set of triplets used in this study were identical as judged from 13 different blood groups (Table II). The fraternal twins, pair numbers 5, 6, 7, 8, 10, 11, 16, 17, and 18, and the fraternal triplets differed with respect to 1, 2, or 6 blood groups. The α_1-acid glycoprotein patterns of the identical twins were the same for each pair. Four pairs of twins revealed 6 bands each, four showed 7 bands each, and one 8 bands. Of the eleven pairs of fraternal twins, pair numbers 7, 8, and 20 and the fraternal triplets each exhibited a pattern with 6, two with 7 bands each, and another with 8 bands. Of the remaining four pairs, three showed the same patterns, namely a pattern with 6 and a pattern with 7 bands, and the fourth pair revealed a 7- and an 8-band pattern.

D. Control experiments

In control experiments varying amounts of pooled α_1-acid glycoprotein (2.00, 1.75, 1.50, and 1.25 mg) were applied to troughs of the same size that were also used for all analyses described above. The number of zones observed on the obtained patterns was 7, 7, 7, and 6, respectively. Thus, these results exclude the possibility that the different number of bands could be due to the presence in the α_1-acid glycoprotein preparations of varying amounts of salts. Since for every analysis 2.0 mg of α_1-acid glycoprotein was weighed out, the observed 6-band patterns could

not be due to a low concentration of glycoprotein. Further, the reproducibility of the pattern of the same preparation was discussed earlier (5).

α_1-Acid glycoprotein was isolated from donations of blood of two individuals that were obtained three different times at intervals of 3 months. The three patterns of each individual revealed the same number of bands, namely, 6 and 7 bands, respectively.

Discussion

Genetically determined variants of a large number of normal plasma proteins including haptoglobin, transferrin, and γ-globulins have been well established (9–12). α_1-Acid glycoprotein, which is also polymorphic (4, 5), appears to be another member of this class of blood constituents. Evidence is presented in this paper that the different starch gel electrophoretic patterns of the latter protein are most probably due to genetically determined variants.

The α_1-acid glycoprotein patterns of normal white individuals of the Boston area showed either 5, 6, 7, or 8 zones and occurred with a relative incidence of 4, 36, 49, and 11%, respectively, as judged by the present relatively limited series of analyses. It may be concluded from these data that the four α_1-acid glycoprotein patterns are encountered at a constant frequency, which, however, may be different in different races, suggesting transmission of genetically determined polymorphic forms of this plasma constituent. Further studies are required to establish whether or not different frequencies of the mentioned patterns exist with regard to sex.

Strong evidence for the presence of genetically determined polymorphic forms was obtained from the study of the α_1-acid glycoprotein patterns of 15 members of a Caucasian family. The parents revealed a pattern with six and another with five zones. Four of their five daughters showed patterns with 6 and one a pattern with 5 bands. If these patterns were not genetically transmitted and occurred by chance according to the above mentioned frequency, three patterns with 7 bands would be expected among these offspring, but no pattern with 7 bands was observed. Further, since the probability of obtaining a set of five patterns with 6 bands each, plus one pattern with 5 bands is extremely small, it was concluded that the patterns of this family strongly suggest genetic transmission of the polymorphic forms of α_1-acid glycoprotein. Further support for this concept was derived from the patterns of the third generation. Some of the offspring of the daughter whose husband had a pattern with 7 bands also showed patterns with 7 bands.

The α_1-acid glycoprotein patterns of nine children of family C showing essentially 6 or 7 bands suggest that their father most probably would have had a 7-band pattern. The patterns of the grandchildren were identical with one of their parent's patterns. The concept of the genetically transmitted polymorphic forms of α_1-acid glycoprotein is thus further supported by these data.

Additional evidence for the transmission of the genetically determined polymorphism of α_1-acid glycoprotein was obtained from the study of 17 pairs of twins and a set of triplets. The α_1-acid glycoprotein patterns were the same for each of the nine pairs of identical twins. Although the parents of these twins were not investigated, it is unlikely that the parents of each pair of identical twins would also show the same patterns. The patterns observed with pairs of fraternal twins are in agreement with the transmission of genetically determined polymorphism of α_1-acid glycoprotein. Therefore, the genetic transmission of the polymorphic forms of α_1-acid glycoprotein appears to be firmly established.

Summary

α_1-Acid glycoprotein was isolated, by fractionation by a modified procedure of Cohn's method 10 and subsequent chromatography on Amberlite IRC-50 of the obtained Fraction VI, from serum of 53 normal Caucasian adults of the Boston area, 15 and 23 members of two additional Caucasian families, each representing three generations, and from 18 pairs of Caucasian twins, five of whom were demonstrated to be identical as judged by 13 different blood groups. The isolated glycoprotein preparations were analyzed by starch gel electrophoresis at pH 2.9.

Four types of α_1-acid glycoprotein patterns were observed. They revealed 5, 6, 7, and 8 bands and occurred at a relative incidence of 4, 36, 49, and 11%, respectively.

The polymorphism of α_1-acid glycoprotein is most probably genetically determined. The α_1-acid glycoprotein patterns of 15 and 23 members, respectively, of two families and of the nine pairs of identical twins are compatible with the concept of transmission of genetically determined polymorphic forms of this blood protein.

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References

1. Schmid, K., and J. P. Binette. Polymorphism of α_1-acid glycoprotein. Fed. Proc. 1961, **20**, 383.
2. Schmid, K. Preparation and properties of serum and plasma proteins. XXIX. Separation from human plasma of polysaccharides, peptides and proteins of low molecular weight. Crystallization of an acid glycoprotein. J. Amer. chem. Soc. 1953, **75**, 60.
3. Weimer, H. E., J. W. Mehl, and R. J. Winzler. Studies on the mucoproteins of human plasma. V. Isolation and characterization of a homogeneous mucoprotein. J. biol. Chem. 1950, **185**, 561.
4. Schmid, K., and J. P. Binette. Polymorphism of α_1-acid glycoprotein. Nature (Lond.) 1961, **190**, 630.
5. Schmid, K., J. P. Binette, S. Kamiyama, V. Pfister, and S. Takahashi. Studies on the structure of α_1-acid glycoprotein. III. Polymorphism of α_1-acid glycoprotein and the partial resolution and characterization of its variants. Biochemistry 1962, **1**, 959.
6. Lever, W. F., F. R. N. Gurd, E. Uroma, R. K. Brown, B. A. Barnes, K. Schmid, and E. L. Schultz. Chemical, clinical, and immunological studies on the products of human plasma fractionation. XL. Quantitative separation and determination of the protein components in small amounts of normal human plasma. J. clin. Invest. 1951, **30**, 99.
7. Schmid, K., E. C. Rosa, and M. B. MacNair. Fractionation of the proteins of human synovial fluid and plasma. J. biol. Chem. 1956, **219**, 769.
8. Schmid, K., M. B. MacNair, and A. F. Bürgi. The chromatographic separation and purification of acidic proteins on carboxylated ion exchange resins. J. biol. Chem. 1958, **230**, 853.
9. Gitlin, D., and C. A. Janeway. Genetic alterations in plasma proteins of man *in* The Plasma Proteins, F. W. Putnam, Ed. New York, Academic Press, 1960, vol. 2, p. 407.
10. Biochemistry of Human Genetics, Ciba Foundation Symposium, G. E. W. Wolstenholme and C. M. O'Connor, Eds. Boston, Little, Brown, 1959.
11. Cleve, H., and A. G. Bearn. The group specific component of serum: genetic and chemical considerations. Progr. med Genetics 1962, **2**, 64.
12. Schmid, K. Preparation, properties, and structure of plasma glycoproteins *in* The Hexosamines, R. W. Jeanloz and E. A. Balazs, Eds. New York, Academic Press, in press.