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Genetic Studies of Low-Abundance Human Plasma Proteins. VI. Polymorphism of Hemopexin

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SUMMARY

An analytical isoelectric focusing method in 3 M urea followed by immunoblotting has been devised to detect genetic and biochemical variation in the glycoprotein hemopexin (HPX) in human plasma or serum. HPX reveals extensive microheterogeneity with multiple major and minor components that are susceptible to neuraminidase treatment, suggesting that the observed biochemical variation is due to differences in sialic acid content between HPX isoproteins. However, charge differences that persist in HPX isoproteins following neuraminidase treatment suggest the presence of genetically determined HPX variation, and this is confirmed by population and family studies. HPX was found to be monomorphic, with an invariant pattern, in U.S. whites; but it is polymorphic in U.S. blacks, with three alleles controlled by a single locus, a situation that demonstrates an autosomal codominant pattern of inheritance. The *HPX*1*, *HPX*2*, and *HPX*3* allele frequencies in U.S. blacks are .941, .018, and .041, respectively.

INTRODUCTION

Human serum hemopexin (HPX) was discovered by Neale et al. (1958), and its purification and detailed characterization have been reported (Schultze et al. 1961; Heimburger et al. 1964; Muller-Eberhard 1970; Hrkal and Muller-Eberhard 1971). HPX is a β_1-glycoprotein that binds heme in an equimolar ratio and transports it to hepatocytes for iron conservation (Muller-Eberhard 1978;

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Smith and Morgan 1985). Human HPX consists of a single polypeptide chain with 439 amino acid residues containing six intrachain disulfide bridges (Hrkal and Muller-Eberhard 1971; Altruda et al. 1985; Takahashi et al. 1985). The reported molecular weight of HPX has varied between 57,000 and 80,000 (Schultze et al. 1961; Hayem-Levy and Hayez 1973), but recent structural studies have established the molecular weight of apohemopexin to be 63,000 (Takahashi et al. 1985). The carbohydrate moiety of the HPX molecule accounts for ~20% of its mass and consists of mannose, galactose, fructose, hexosamine, and sialic acid. There are six glycosylation sites on the HPX molecule—one oligosaccharide unit O-linked to galactosamine and five oligosaccharides N-linked to glucosamine (Takahashi et al. 1985). HPX shows internal homology in amino acid sequence, suggesting that it has evolved through gene duplication (Altruda et al. 1985; Takahashi et al. 1985).

Hemopexin is produced in the liver (Muller-Eberhard 1978), and in plasma its concentration is ~70 mg/100 ml (Foidart et al. 1983). However, its concentration fluctuates in certain pathological conditions such as hemolytic anemia, porphyrias, chronic neuromuscular diseases, cancer, etc. (Muller-Eberhard and Liem 1974). The elevation of HPX in plasma has been found to be due mainly to its increased synthesis, whereas low levels are apparently caused by enhanced catabolism (Foidart et al. 1983). By means of conventional starch-gel electrophoresis, HPX was found to be polymorphic in rabbit (Grunder 1966), swine (Imlah 1965; Baker 1967), sheep, mouflon, and goat (Stratil et al. 1984) but monomorphic in man (Stewart and Lovrien 1971).

In the present paper we describe an analytical isoelectric-focusing (IEF) technique followed by immunoblotting that can be used to detect biochemical and genetic variation in the HPX molecule. Analysis of plasma samples from U.S. whites and U.S. blacks indicates that HPX is polymorphic only in black populations.

MATERIAL AND METHODS

Blood Samples

Plasma samples from the unrelated white and black blood donors used in the present investigation were made available by the Human Genetics Laboratory. Heparinized or ethylenediaminetetraacetate plasma was collected and stored at −70 C prior to analysis. For segregation analysis 41 black trios (mother-child-father) were available for testing the genetic basis and mode of inheritance of HPX variation.

In a few cases plasma samples were desialylated overnight at room temperature by incubating 20 μl of plasma in 10 μl *Clostridium perfringens* neuraminidase (1 U/28 μl phosphate buffer, pH 6.8; Sigma) in a microdialysis apparatus (Bethesda Research Laboratories).

IEF and Immunoblotting

IEF experiments were carried out on an LKB 2217 Ultrophor electrofocusing unit with an LKB 2197 power supply and a Lauda RM6 cooling unit running

at constant 11–15 C. Thin-layer (0.5-mm) polyacrylamide gels of 5% (monomer = 4.85%; bis = 0.15%) were prepared in deionized water with the addition of 3 M urea. The pH gradient was established using either 2% pharmalyte, pH 4–6.5 (Pharmacia) or a combination of Pharmalyte, pH 4–6.5, and LKB ampholine, pH 5–7, in a 2:1 ratio. Gel polymerization was initiated by adding 30 μl of 0.1% riboflavin solution and exposure to fluorescent light, and gels were used the following morning. IM NaOH and IM H_3PO_4 solutions were used for the cathode and anode electrodes, respectively. Plasma samples absorbed on 5 × 4-mm Whatman 3MM wicks were applied near the cathode. IEF was done at a constant 1,500 V, with 25-W and 50-mA limits. Sample wicks were removed after 15 min, and IEF was allowed to continue another 2.15 h. For desialyzed samples, a higher-pH-range gel was prepared using LKB ampholine, pH 7–9. Other conditions of gel preparation and running conditions were the same as described above, except that total IEF time was 3.15 h. After the completion of the IEF run, proteins were transferred to a nitrocellulose membrane (0.45 μm) as described elsewhere (Kamboh and Ferrell 1986). In brief, partial protein blotting was achieved by means of capillary transfer to a nitrocellulose membrane for 10 min, followed by incubation with 5% (w/v) nonfat powdered milk for 45 min, incubation with goat anti-human HPX (Biomeda) at a 1:1,000–2,000 dilution for 30 min, followed by three 10-min washings in TBS buffer. Then the nitrocellulose was exposed for 30 min to rabbit anti-goat IgG conjugated with alkaline phosphatase at a 1:5,000 dilution, followed by three 10-min washings; and HPX bands were visualized using histochemical stain for the conjugated enzyme (Kamboh and Ferrell 1986).

Isoelectric points (pI's) of HPX isoproteins were estimated using an IEF marker kit after protein staining with Coomassie blue.

RESULTS

Initial efforts to obtain clear immunoreactive HPX bands on simple IEF gels yielded poorly resolved patterns, probably owing to the tendency of HPX to aggregate under mild conditions. However, IEF in the presence of 3 M urea resulted in a clear and discrete multiple-banded pattern with pI's of 5–6. The IEF-immunoblot pattern of the native HPX molecule was found to be invariant in U.S. whites and was characterized by four major and several minor bands. In addition to this common pattern, three other phenotypes were observed in plasma samples from U.S. blacks (fig. 1). Compared with the four major bands of the common type (fig. 1, lane 1), the pattern of the other rare four-banded type is shifted toward the anode (fig. 1, lane 4), with approximately equal displacement of each of the major bands. The third phenotype (fig. 1, lane 3) consists of an eight-banded pattern that is an exact combination of the two four-banded patterns. The fourth phenotype observed in U.S. blacks consists of the four major bands of the common phenotype in combination with a more anodal set of four discrete bands (fig. 1, lane 2). The electrophoretic pattern observed in these four phenotypes suggests the presence of a single structural locus coding for three allelic protein products. We propose that this locus be designated HPX, with the following three alleles: *HPX*1, HPX*2,* and *HPX*3.* In

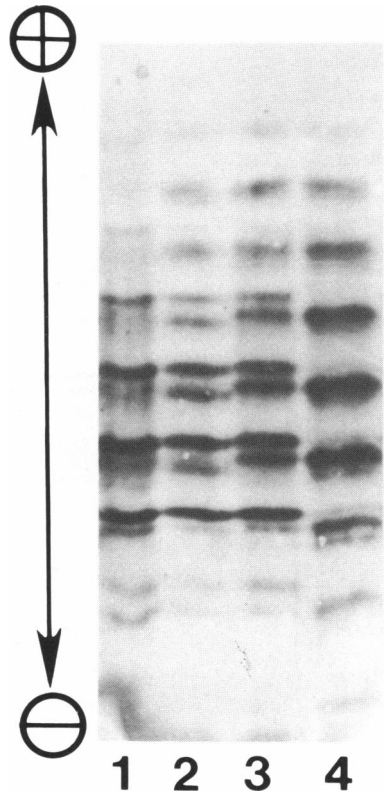

FIG. 1.—IEF in 5% polyacrylamide gel containing 3 M urea, pH 4–6.5. Lane 1, HPX 1-1; lane 2, HPX 2-1; lane 3, HPX 3-1; and lane 4, HPX 3-3.

order of decreasing pI's, the *HPX*1* allele controls the most basic products, *HPX*2* the intermediate ones, and *HPX*3* the most acidic products. A diagrammatic representation of the six possible phenotypes possible with these three alleles is shown in figure 2. Although we have not observed the 2-2 and 3-2 phenotypes, their products have been inferred on the basis of the existing four phenotypes.

To confirm the genetic mode of inheritance of the observed patterns, only black trios (mother-child-father) could be tested, because of the unavailability of large black pedigrees. Figure 3 shows the transmission of three HPX alleles in five informative trios, a transmission that is compatible with the assumption of autosomal codominant inheritance of three alleles at a single locus. The distribution of HPX phenotypes in U.S. blacks indicates no significant departure from the expected values as based on Hardy-Weinberg equilibrium (table 1) and provides further support that the observed variable IEF patterns are the products of a single structural locus.

The nature of biochemical and genetic heterogeneity observed in HPX is

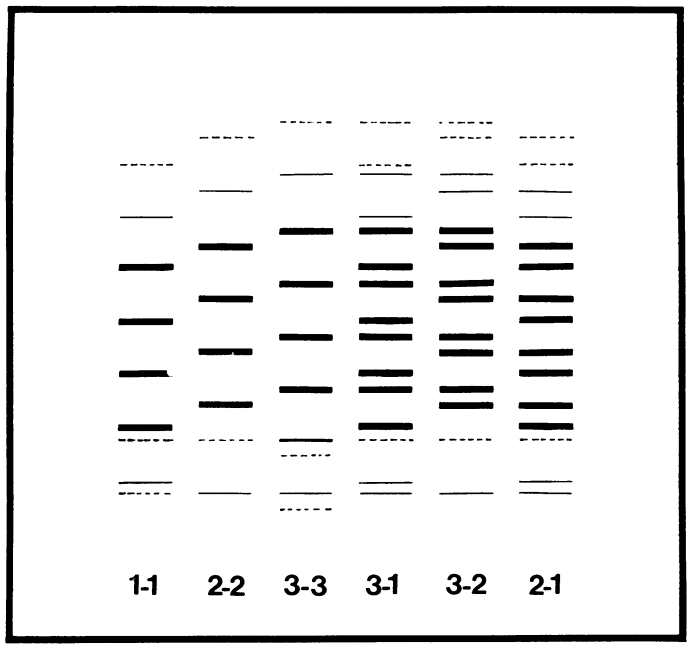

FIG. 2.—Diagrammatic representation of HPX phenotypes detectable on 5% IEF polyacrylamide gel containing 3 M urea, pH 4–6.5. Phenotypes 2-2 and 3-2 have not been observed in this investigation.

tested further by means of treating plasma samples of the presumed homozygote and heterozygote phenotypes with neuraminidase, followed by IEF immunoblotting. Figure 4 illustrates the asialo patterns of different HPX phenotypes that are focused at higher pH, with pI's 7–7.5. The asialo pattern is simplified into a single major band in homozygotes and into two major bands in heterozygotes, indicating that whereas the persisting difference in electrophoretic mobility is under genetic control, the molecular variation that yields the four-banded pattern of native HPX is due to different sialic acid content of the carbohydrate side chains of the HPX molecule. It is noteworthy that, compared with the HPX 1 pattern, the native patterns of the HPX 2 and HPX 3 phenotypes in some gels appear to be shifted toward the cathode, but the asialo patterns clearly indicate that both the HPX 2 and HPX 3 types are more acidic than the HPX 1 type. We have observed that, in contrast to the HPX 1 asialo pattern, the products of the HPX 2 and HPX 3 phenotypes are associated with minor anodal bands even after complete desialylation. This suggests that, in addition to sialic acid differences, there may be other posttranslational changes in the polypeptide chain of the HPX molecule.

DISCUSSION

We report a new genetic polymorphism, coded by a single HPX structural locus, discovered by means of a method that combines the analytical power of

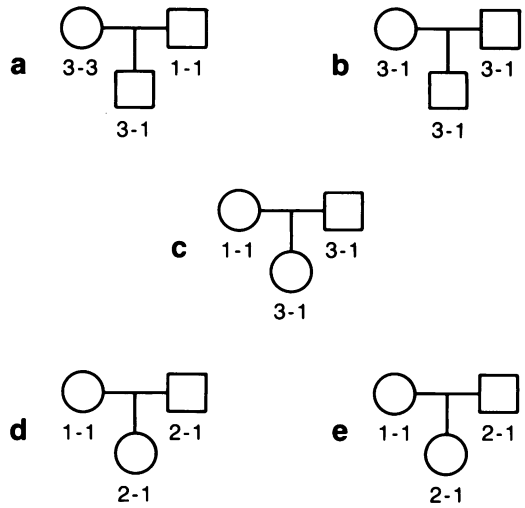

FIG. 3.—Five black trios (*a–e*) showing segregation of three HPX alleles. HPX phenotypes are indicated below sex symbols; females are represented by circles, males by squares.

IEF in urea and the sensitivity and specificity of immunoblotting. Separation of the glycoprotein under partial denaturing conditions that use 3 M urea, followed by its limited transfer to a nitrocellulose filter, appears to be critical to preserving the fine resolution of HPX. Both interindividual and intraindividual heterogeneity have been observed in HPX phenotypes. The native protein in plasma reveals an extensive microheterogeneity, with multiple major and minor bands having pI's of 5–6. As with other glycoproteins, this molecular heterogeneity is due to the presence of neuraminidase-susceptible sialic acid residues of HPX, and an asialo pattern that consists of a single major band provides support for this assumption. The second type of variation observed among individuals' plasma samples appears to be under genetic control, because even after deglycosylation this variation persists and can best be explained by the presence of three alleles—*HPX*1, HPX*2,* and *HPX*3*—at the HPX structural locus. The segregation of these three alleles follows a simple Mendelian pattern of inheritance. Plasma and serum samples gave identical phenotypic patterns that are unaffected by freezing and thawing. The genetic explanation of this variation is further strengthened by the observation that in vitro mixing of plasma samples from putative 1-1 and 3-3 homozygotes gave a pattern indistinguishable from that of the 3-1 heterozygote phenotype.

It is interesting to note in this context that all plasma samples from U.S. whites gave an invariant pattern, suggesting that the HPX locus is monomorphic (or nearly so) in whites. On the other hand, U.S. blacks show much more variation, with an estimated heterozygosity of 11.25%. Our present data suggest that HPX might be a uniquely black marker of potential importance in admixture and evolutionary studies. It would be of interest to screen additional black populations of known African origin to determine the exact distribution of the HPX marker. The observed frequencies in U.S. blacks may not repre-

TABLE 1

DISTRIBUTION OF HPX PHENOTYPES AND GENE FREQUENCIES IN UNRELATED U.S. BLACKS AND U.S. WHITES

POPULATION (*N*)	PHENOTYPES (Expected Values)				GENE FREQUENCIES		
	1-1	2-1	3-1	3-3	*HPX*1*	*HPX*2*	*HPX*3*
U.S. Blacks:							
Pittsburgh (109)	94 (94.48)	4 (3.65)	11 (10.35)	... (.28)	.931	.018	.051
Houston (85)	76 (75.27)	3 (2.88)	5 (6.56)	1 (.14)	.941	.018	.041
U.S. Whites:							
Pittsburgh (175)	175	...	...	...	1.00	...	...
Other (92)	92	...	...	...	1.00	...	...

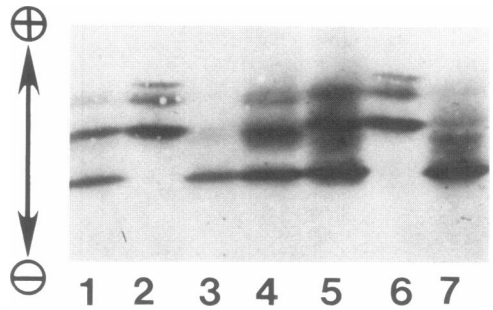

FIG. 4.—Desialyzed HPX patterns in 5% polyacrylamide IEF gel containing 3 M urea, pH 7–9. Lanes 1 and 5, HPX 3-1; lanes 2 and 6, HPX 3-3; lanes 3 and 7, HPX 1-1; and lane 4, HPX 2-1.

sent the true picture of HPX variation in blacks, because, as shown by other genetic markers, U.S. blacks have substantial white admixture. In the present study, we have not observed the suggested 2-2 homozygote and 3-2 heterozygote phenotypes. When the observed frequencies are taken into account, this is not surprising, since the expected frequencies of the 2-2 and 3-2 phenotypes are 1/3,000 and 1/1,000, respectively.

When conventional starch-gel electrophoresis has been used, the molecular heterogeneity of HPX has been observed in human plasma but HPX has been found to be genetically monomorphic (Stewart and Lovrien 1971). However, the possibility of detecting genetic polymorphism in human HPX was suggested by Morgan et al. (1974), who observed nearly equal mole ratios of the amino acids leucine and phenylalanine at position 3 in purified HPX from pooled serum. It is possible (1) that pooled serum might have contained samples from black individuals with the HPX variant and (2) that the charge differences observed here are due to an amino acid substitution, at position 3, for one of the HPX variants. The determination of the existence of an exact amino acid substitution between normal and variant HPX polypeptides may confirm this possibility. However, in view of the limited structural variation observed during the present investigation, the polymorphism suggested by Morgan et al. (1974) cannot be explained easily unless the purified pooled hemopexin came from a different population group, one in which HPX commonly is polymorphic. As well as in man, genetic polymorphism in HPX has been reported in some animal groups (Imlah 1965; Grunder 1966; Baker 1967; Stratil et al. 1984). The development of the present technique provides further opportunities to explore the extent of genetic polymorphisms in man and other animal species. Most placental mammals show immunological similarities in their antigenic determinants, and antiserum raised against one species could be used to visualize specific immunological reactions in related species.

To date, the HPX locus has not been assigned to any chromosome and the detection of new polymorphism will be helpful for linkage studies in families. In addition to its significance in population genetics, the HPX marker may also be useful in forensic serology and in disputed paternity cases among blacks, who are genetically heterogeneous at the HPX locus.

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