GENETIC STUDY OF HEMOGLOBIN VARIANTS IN MICE (*MUS MUSCULUS*)

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Three alleles, *Hbb^s*, *Hbb^d* and *Hbb^p*, have been reported at the *Hbb* locus in mice, which is considered to determine the structure of hemoglobin β -chain polypeptide. These alleles can be distinguished by the different electrophoretic behavior of their corresponding hemoglobin molecules, but it is not fully understood how such different patterns are derived from the three alleles. *Hbb^s* and *Hbb^d* are found in most inbred strains and also in many feral populations. Whereas, *Hbb^p*, discovered by Morton (1962, 1966), is present only in an inbred strain AU/SsJ and in the Asian feral mice *Mus musculus castaneus* (Gilman 1974). This paper describes two additional mouse strains carrying the rare allele *Hbb^p*, and some evidence for the genetic mechanism controlling three electrophoretic patterns of mouse hemoglobins.

MATERIALS AND METHODS

Mice from five strains, C57BL/6J, A/He, AU/SsJ, PON and Mol-A were used in this study. C57BL/6J, A/He and AU/SsJ were imported from the United States. PON is a inbred strain derived from F₁ mice of C57BL/6J×Pink which is a Japanese fancy mouse stock maintained by Laboratory of Animal Genetics, Nagoya University. Mol-A (*Mus musculus molossinus*) is a strain originating from the Japanese feral mice and has been followed over 18 generations of full-sib matings.

Blood from individual mice was collected in heparinized saline. The blood cells were sedimented (3,000 g, 10 min) and washed 3 times with isotonic saline. The cells were lysed with 3 volumes of water, and the stroma was removed by centrifugation (10,000 g, 15 min). The clear supernatant was used as fresh hemolysate. Alkylation of hemoglobin with maleic anhydride was carried out in a similar manner described by Petras and Martin (1969).

Electrophoresis was carried out in a horizontal gel system using hydrolyzed starch (Connaught, Canada) at a concentration of 14%. In preparing the buffer, a stock solution at pH 8.5 was made, consisting of 120 g of Tris-aminomethane and 60 g of boric acid in a liter of water. The stock solution was diluted in 5 vol for the bridge buffer and in 20 vol for the gel buffer. In each case, electrophoresis was performed with a constant voltage of 10 V/cm across the gel for 16 hr at 4°C.

Electrophoresis for the hemoglobin polypeptide chains was carried out at pH 1.9 by the method of Muller (1960).

RESULTS

When the fresh hemolysates from five mouse strains were electrophoresed on a starch gel in Tris-borate buffer at pH 8.5, three hemoglobin patterns were observed as shown in Fig. 1. The hemolysate from C57BL/6J showed a single anodal band, called single hemoglobin (Hbb^s). The hemolysate from A/He showed two bands, one major band at the same position of s hemolysate's and one minor band migrating more slowly than the major. That is known as diffuse hemoglobin (Hbb^d). The hemolysates from AU/SsJ, PON and Mol-A also revealed two bands (Hbb^p); the position of the major band corresponded to that from the d hemolysate, but the minor band migrated more slowly than that of d .

Electrophoretic separation of fresh hemolysates could not be applied completely for the genetic analysis of Hbb locus, since the hemolysate of heterozygous Hbb^d/Hbb^s or Hbb^p/Hbb^s mouse is indistinguished from that of homozygous Hbb^d/Hbb^d or Hbb^p/Hbb^p mouse respectively. Petras and Martin (1969) and Hutton (1969) resolved this problem by alkylation of hemoglobin. After maleic anhydride treatment, the both major and minor hemoglobins of d migrated more rapidly. The migration rate of major and minor hemoglobins of p was also increased by the treatment; the major hemoglobin migrated at the same position as that of d , but the minor appeared far separated from the major. Although the single hemoglobin of s was slightly broadened by the treatment, the migration rate was not affected. By employing both the fresh and

Fig. 1. Photograph of mouse hemoglobins electrophoresed on a starch gel in Tris-borate buffer at pH 8.5. Samples and their Hbb genotypes are fresh hemolysates from (1) C57BL/6J, Hbb^s/Hbb^s , (2) PON, Hbb^p/Hbb^p , and (3) A/He, Hbb^d/Hbb^d , and maleic anhydride treated hemolysates from (4) B57BL/6J, (5) PON, and (6) A/He.

Table 1. Breeding Data for *Hbb* Locus

Parental genotypes	Total	Progeny genotypes						χ^2	<i>P</i>
		<i>ss</i>	<i>ps</i>	<i>pp</i>	<i>dp</i>	<i>dd</i>	<i>ds</i>		
<i>ss</i> × <i>pp</i>	10	—	10	—	—	—	—		
<i>ps</i> × <i>pp</i>	42	—	20	22	—	—	—	0.10	>0.50
<i>ps</i> × <i>ss</i>	30	12	18	—	—	—	—	1.20	>0.20
<i>ps</i> × <i>ps</i>	53	19	25	9	—	—	—	3.94	>0.10
<i>dd</i> × <i>pp</i>	9	—	—	—	9	—	—		
<i>dp</i> × <i>pp</i>	13	—	—	6	7	—	—	1.59	>0.20
<i>dp</i> × <i>dd</i>	51	—	—	—	30	21	—	0.08	>0.50
<i>dp</i> × <i>dp</i>	49	—	—	13	24	12	—	0.08	>0.95
<i>dp</i> × <i>ss</i>	20	—	12	—	—	—	8	0.80	>0.20

Table 2. Linkage Test between *Hbb* and *c* Loci

Parental genotypes	Total	Progeny genotypes				χ^2	<i>P</i>
		$\frac{Hbb^d c}{Hbb^d c}$	$\frac{Hbb^d c}{Hbb^p C}$	$\frac{Hbb^d c}{Hbb^d C}$	$\frac{Hbb^d c}{Hbb^p c}$		
$\frac{Hbb^d c}{Hbb^d c} \times \frac{Hbb^d c}{Hbb^p C}$	51	20	28	1	2	42.25	<0.01

Table 3. Linkage Test between *Hbb* and *p* Loci

Parental genotypes	Total	Progeny genotypes				χ^2	<i>P</i>
		$\frac{Hbb^p p}{Hbb^p p}$	$\frac{Hbb^p p}{Hbb^s P}$	$\frac{Hbb^p p}{Hbb^p p}$	$\frac{Hbb^p p}{Hbb^s P}$		
$\frac{Hbb^p p}{Hbb^p p} \times \frac{Hbb^p p}{Hbb^s P}$	42	19	16	3	4	19.14	<0.01

alkylated hemolysates as electrophoretic samples, crosses to determine the genetics of the three hemoglobin patterns were conducted (Table 1). The breeding data clearly proved that *Hbb^s*, *Hbb^d* and *Hbb^p* are multiple and codominant allelic genes.

Two coat color genes, *c* (albino) and *p* (pink-eye dilution) are known to be on chromosome 7 (Linkage group I). These color genes were checked for the linkage relationships with *Hbb* locus. As shown in Tables 2 and 3, the breeding data indicated positive linkage among them. The recombination frequencies are 5.9% between *Hbb* and *c*, and 16.7% between *Hbb* and *p*.

After incubation for 48 hr at 37°C, the hemolysates were electrophoresed (Fig. 2). Although the single hemoglobin of *s* was slightly broadened, no change was observed in the mobility. Whereas, the mobilities of major and minor hemoglobins of *d* were markedly lowered. The mobility of major hemoglobin of *p* was also lowered, but it was not apparent whether or not the mobility of the minor hemoglobin was changed. The mobilities in the incubated hemolysates were protected by addition of 2-mer-

Fig. 2. Photograph of mouse hemoglobins electrophoresed on a starch gel in Tris-borate buffer at pH 8.5. Samples are fresh hemolysates from (1) C57BL/6J, (2) PON, and (3) A/He, incubated hemolysates for 48 hr at 37°C from (4) C57BL/6J, (5) PON, and (6) A/He, and incubated hemolysates together 2-mercaptoethanol from (7) C57BL/6J, (8) PON, and (9) A/He.

Fig. 3. Photograph of α - and β - chains of mouse hemoglobins electrophoresed on a starch gel in formate buffer at pH 1.9. Samples are (1) single hemoglobin of C57BL/6J, (2) major hemoglobin of PON, (4) minor hemoglobin of A/He, and (5) minor hemoglobin in of PON.

captoethanol to a concentration of 0.1%.

Vertebrate hemoglobins are known to consist of two α - and two β -chain polypeptides. After fresh *s*, *d*, and *p* hemolysates were electrophoresed on a starch gel in Tris-borate buffer at pH 8.5, the five kinds of hemoglobins (single hemoglobin of *s*, major and minor hemoglobins of *d*, and major and minor hemoglobins of *p*) with 1 mm wide pieces were clipped from the gel. These clipped gels were filled up in a starch gel for the chain separation (pH 1.9) and the hemoglobins were re-electrophoresed to resolve their α - and β -chains. As shown in Fig. 3, the α -chains in all hemoglobins showed the same electrophoretic mobility. The mobility of β -chains, however, was different in some points. It was observed that, respectively, the chains of single hemoglobin

of *s*, major hemoglobins of *d* and *p*, and minor hemoglobins of *d* and *p* migrated faster toward the cathode.

DISCUSSION

Hbb^s and *Hbb^d* alleles are distributed widely in inbred mouse strains, and many workers (Popp and St. Amand 1960; Heinecke and Wagner 1964; Petras 1967; Selander and Yang 1969; Selander *et al.* 1969) have reported that the feral mice in the United States, Jamaica, Venezuela, Germany, and Denmark carry both or either of the two alleles. In contrast, *Hbb^p* was reported to be found only in an inbred strain AU/SsJ, and recently, Gilman (1974) described four feral mice in the Thai species *Mus castaneus* which gave the hemoglobin patterns corresponding to that of a 50:50 mixture of *d* and *p* hemolysates. Furthermore, he suggested that the hemolysates of the Japanese feral mice *Mus musculus molossinus* were similar to the *d* hemolysates. Our study has demonstrated that the rare allele *Hbb^p* is also present in two mouse strains, PON and Mol-A. PON is a strain derived from the Japanese feral mice. Minezawa and Kondo (personal communication) have examined the gene frequencies of *Hbb* locus in several feral populations in Japan. According to them, Japanese feral mice are polymorphic for *Hbb^d* and *Hbb^p*, and feral mice in Aichi Prefecture are almost fixed for *Hbb^p*. Since the ancestors of Mol-A were captured in Aichi Prefecture, the strain must have carried *Hbb^p* through the inbreeding course.

To date, two mechanisms have been discussed to explain the difference of electrophoretic patterns of hemoglobins between the *s* and *d* hemolysates. The first explanation is that the hemoglobin molecule of *d* is broadly electrophoresed as a result of polymerization of the hemoglobin. The second is that the *d* hemolysate contains two different hemoglobin molecules which are not well separated by electrophoresis. Our data exclude the first way and support the second.

No change was observed in the single hemoglobin of *s* after incubation for 48 hr at 37°C. Since the migration rate of incubated hemoglobin of *d* was slower than those of fresh major and minor hemoglobins, both the major and minor hemoglobins must polymerize under these conditions. The behavior of the polymerized major hemoglobin of *p* was similar to that of *d*, but it was not apparent whether or not polymerization of the minor component occurred. Bonaventura and Riggs (1967) reported that the β T-2 peptide of single hemoglobin has a glycyl residue at position β 13, but that a cysteinyl residue is present at this position in diffuse hemoglobin. The cysteinyl residue at this position has been interpreted to be responsible for diffuse electrophoretic pattern through the formation of intermolecular disulfide bonds (Riggs, 1965, 1967). In fact, Bonaventura (1969) observed that the diffuse hemoglobin polymerized from 3.9s to 9.2s after 72 hr at 37°C, and that the polymerization was reversed by addition of 2-mercaptoethanol. We have also recognized this phenomenon in starch gel electrophoresis. The advantage with this method is the ability to compare each band of the fresh hemoglobins with each band of the incubated. Certainly, the major and minor hemoglobins of *d* polymerized, but the migration rate of the incubated hemoglobin differed from those of

fresh major and minor. The polymerization behavior of the major hemoglobin of *p* can be also considered to be similar to that of *d*.

It is undoubted that the single hemoglobin of *s* differed from each of the major and minor hemoglobins of *d* and *p*, and that the difference of hemoglobins between *d* and *p* is present in the minor hemoglobin. Hutton *et al.* (1962, 1962) previously found two different β -chains in some inbred strains with *d* hemolysate. Also, Gilman (1972, 1974) isolated two different hemoglobins from *d* and *p* hemolysates, and pointed out the existence based on the structurally different β -chains in both hemolysates. Furthermore, Popp (1972), and Popp and Bailiff (1972) determined the complete amino acid sequence of β -chain from single hemoglobin of *s*, and major and minor hemoglobins of *d*. However, it was not fully understood how three electrophoretic patterns of hemoglobins are caused by the difference of β -chain alleles. We confirmed by chain separation and electrophoresis that the different electrophoretic mobilities of the five kinds of hemoglobins (single hemoglobin of *s*, major and minor hemoglobins of *d*, and major and minor hemoglobins of *p*) are due to difference in their β -chains. Moreover, it has been suggested, by genetic analysis and by linkage tests, that all differences among three electrophoretic patterns are derived from multiple allelic genes at one locus, which determines the structure of hemoglobin β -chains. Therefore, the genes specifying the major and minor hemoglobins of *d* and *p* must be closely linked on chromosome 7. The duplication and subsequent differentiation of β -chain allele could have resulted in the existence of two different hemoglobins in *d* and *p* hemolysates.

SUMMARY

Electrophoretic patterns of adult hemoglobins from two mouse strains, PON and Mol-A, are controlled by *Hbb^d*, an allele found only in a strain, AU/SsJ. Fresh hemolysate from *Hbb^s/Hbb^s* mouse possesses a single hemoglobin, and the hemolysates from *Hbb^d/Hbb^d* and *Hbb^p/Hbb^p* mice contain two different hemoglobins in unequal amounts. The hemoglobin of *s* homolysate is different from each of the major and minor hemoglobins of *d* and *p*, and the difference of hemoglobins between *d* and *p* is observed in the minor hemoglobin. All differences among these are derived from multiple allelic genes at one locus, which determines the structure of the hemoglobin β -chain polypeptide.

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