Immunogenetics of Human Haptoglobins

II. Hp BELLEVUE, A CROSS-REACTING MUTANT

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ABSTRACT Using a radioimmunoassay method in conjunction with double diffusion studies, we characterized the antigenic determinants of the three Hp Bellevue phenotypes. An antigenic model based on these data indicates that each phenotype comprises a heterogeneous group of proteins with properties depending on their content of normal ($hp\beta$) and of mutant ($hp\beta$ -Bellevue) chains of haptoglobin. The loss of the hemoglobin binding capacity and of a specific Hb-sensitive antigenic determinant is a consequence of the structural alteration in $hp\beta$ -Bellevue and is expressed to various extents by the populations of proteins containing this chain. It is suggested that those molecules with a preponderance of mutant β -chains are without significant hemoglobin binding capacity and are degraded more slowly in vivo than the ones capable of hemoglobin binding.

INTRODUCTION

A new series of human serum haptoglobins (Hp), designated Hp Bellevue, was recently described (1). This included three phenotypes showing the same variations in the $hp\alpha$ -polypeptide chain as do the three common Hp variants, but each one being, in addition, heterozygous for a new $hp\beta$ -chain called $hp\beta$ -Bellevue. The three new variants are called Hp 1-1 Bellevue, Hp 2-2 Bellevue, and Hp 2-1 Bellevue. Anomalous hemoglobin (Hb) binding by these three was suggested on immunologic grounds and was attributed to the structural mutation in the $hp\beta$ -chain, which appears to be the site of Hb binding (2).

This study describes an examination of the antigenic determinants and the Hb binding properties of the three Bellevue phenotypes, using the radioimmunoassay technique presented in the preceding paper (3). Antigenic models of these mutant haptoglobins are developed, and the presence in these phenotypes of Hp molecules lacking in Hb binding capacity and in some of the specific antigenic determinants is demonstrated.

METHODS

Sera of the phenotypes Hp 1-1 Bellevue, Hp 2-2 Bellevue, and Hp 2-1 Bellevue were those reported previously (1). The Hb binding capacities of these sera were 4, 25, and 740 $\mu\text{g/ml}$, respectively. The other human sera and haptoglobin preparations, antisera, general methods and radioimmunoassay technique have been described in the first paper of this series (3). For ease of reference, a brief description of the antisera employed is given here.

Antiserum I distinguishes Hp of all normal phenotypes from their hemoglobin (Hb) complexes. Antiserum Ia is prepared by partial absorption of antiserum I with Hb-Hp complex to enhance its discriminating ability. Antiserum II reacts in a qualitatively identical manner with Hb and Hb-Hp on Ouchterlony plates, but can distinguish quantitatively between them by immunoassay. Antiserum III differentiates Hp 1-1 from Hp 2-1 and Hp 2-2.

The antigenic determinants already described (3) are: (A) a number of determinants common to all normal Hp and Hb-Hp complexes; (B) an Hb-sensitive determinant which is not reactive once Hp is bound to Hb; (C) a type-specific determinant which is present only on the polymer fractions of Hp 2-1 and of Hp 2-2; and (D) a second Hb-sensitive determinant present on all normal Hp molecules. (The overlapping specificities of the antisera for these and one other antigenic determinant to be described below are given as part of Table IV.)

The presence or absence of the type-specific determinant (C) and of the Hb-sensitive determinants (B) and (D) in Hp 1-1 Bellevue and Hp 2-2 Bellevue was es-

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established by a modification of the immunoassay technique. The protein being studied for the presence of the determinant was mixed with an Hp known to lack the same determinant [Hp 1-1 for (C) and Hb-Hp complex for (B) and (D)]. If the deficiency of the known sample was corrected by the addition of the unknown, it was concluded that the latter contained the particular determinant under consideration. The amount of each Hp used was that which by itself gave the maximum blocking effect characteristic of it. Appropriate controls were included in each experiment and will be presented in the data.

Immunoelectrophoresis and Ouchterlony double diffusion were performed by standard methods (4). Dried plates were stained for protein with amido black, and for peroxidase activity with benzidine.

RESULTS

Immunoelectrophoresis

Samples of Hp 2-1 and of the three Bellevue phenotypes were mixed with Hb in excess of their Hb binding capacity. The samples were then subjected to electrophoresis on duplicate agar plates and precipitin lines were developed with antiserum II. One plate was stained with amido black and the other with benzidine. The results are shown in Fig. 1. All sera give clearly visible precipitin arcs with the protein stain, but there is little or no peroxidase activity associated with the arcs of Hp 1-1 Bellevue and Hp 2-2 Bellevue. This finding suggests that the amount of Hb bound by these two Hp phenotypes is negligible.

Radioimmunoassay

Reactions with antiserum II. Fig. 2 shows the reaction of the Bellevue sera with antiserum II. In contrast to the identical reactions obtained with the three common Hp types (Fig. 2 in reference 3), striking differences are observed among the three Bellevue phenotypes. *Hp 2-1 Bellevue:* This variant is essentially similar to normal phenotypes in the general configuration of its immunoassay curve and in the maximum blocking effect it can achieve. *Hp 1-1 Bellevue:* The initial slope of the curve for this variant is strikingly steeper than that for Hp 2-1 Bellevue or the normal phenotypes. That is to say, Hp 1-1 Bellevue has only a fraction of the Hb binding capacity of antigenically equivalent Hp 2-1 Bellevue or of normal Hp. The maximum blocking effect of Hp 1-1 Bellevue is, however, comparable to that of normal Hp. *Hp 2-2 Bellevue:* This variant also exhibits diminished Hb binding per unit antigenic activity. Moreover, the maximum blocking effect of this variant, even at great antigen excess, is significantly below normal (60%, as compared to 82% for normal). This is interpreted to mean that there are antigenic determinants present in normal Hp which are lacking in Hp 2-2 Bellevue.

Type-specific determinants of the Bellevue variants. Using antiserum III, which can distinguish determinants not present in Hp 1-1, we made the

FIGURE 1 Immunoelectrophoresis of normal and Bellevue haptoglobin phenotypes in the presence of excess Hb. Antiserum II was used. Duplicate plates are stained with amido black (left) and with benzidine (right). Note that the precipitin bands in Hp 1-1 Bellevue and Hp 2-2 Bellevue stain weakly or not at all with benzidine.

FIGURE 2 Radioimmunoassay curves of the three Bellevue phenotypes with antiserum II. The amount of Hp used is expressed in terms of its Hb binding capacity. The ordinate shows the extent to which the antibody is blocked. ●—● Hp 1-1 Bellevue; ■—■ Hp 2-1 Bellevue; ▲—▲ Hp 2-2 Bellevue. The curve for Hp 2-1 Bellevue closely resembles that for normal phenotypes (Fig. 2 in reference 3). The other Bellevue variants have a steep initial portion suggesting low Hb binding capacity per unit antigenic content. Hp 2-2 Bellevue, moreover, is antigenically deficient, as shown by the low plateau it can achieve.

following observations: Hp 2-1 Bellevue is similar to Hp 2-1 and Hp 2-2 (Fig. 3 in reference 3) in general configuration and maximum blocking effect. Hp 2-2 Bellevue and Hp 1-1 Bellevue both have a considerably steeper slope, as they do with antiserum II, but here *both* variants show antigenic deficiency, reflected in a low maximum blocking effect of 56–58%, as compared to 68% for Hp 1-1 and 85% for Hp 2-1 and Hp 2-2.

Table I shows the complementary effect of some serum mixtures. The maximum blocking due to individual sera is given in the first four values. Three points are evident from the effects of mixing: The antigenic deficiency of Hp 1-1 is not corrected by the addition of Hp 1-1 Bellevue. Hp 2-2 Bellevue, on the other hand, although itself antigenically deficient, can almost completely correct the deficiency of Hp 1-1. Hp 2-2, which is not antigenically deficient, is not affected by the addition of either Bellevue serum.

Effect of Hb binding on the antigenic determinants of the Bellevue variants. Table II shows the effect of Hb on the maximum blocking effect of the three Bellevue phenotypes compared to the effect on normal Hp.

The binding of Hb by Hp 2-1 Bellevue produces an antigenic deficiency amounting to 12% with antiserum II and 23% with antiserum Ia. These are about one-half the deficiencies produced in the

common types. More striking is the fact that the presence of Hb has *no effect* on the antigenic determinants of Hp 1-1 Bellevue and Hp 2-2 Bellevue.

The status of the (B) and (D) determinants, which are Hb-sensitive in normal phenotypes, was further investigated by mixture experiments. Table III shows the effect of the addition of the Bellevue phenotypes on the antigenic deficiency of Hb–Hp 2-1 complex. Parallel experiments were performed with antisera I and II. All reactions

TABLE I
Type-Specific Determinants of Normal and
Bellevue Haptoglobins

Sample	Maximum blocking of antiserum III
Hp 1-1	68%
Hp 2-2	85
Hp 1-1 Bellevue	58
Hp 2-2 Bellevue	56
Hp 1-1 + Hp 1-1 Bellevue	68
Hp 1-1 + Hp 2-2 Bellevue	81
Hp 2-2 + Hp 1-1 Bellevue	82
Hp 2-2 + Hp 2-2 Bellevue	88

The antigenic deficiency of Hp 1-1, compared to Hp 2-2, is in the determinant (C). This determinant can be provided by the addition of Hp 2-2 Bellevue but not by Hp 1-1 Bellevue.

TABLE II
Effect of Hemoglobin Binding on the Antigenic Determinants of Normal and Bellevue Haptoglobins

Sample	Antiserum II		Antiserum Ia	
	Block- ing	Defi- ciency	Block- ing	Defi- ciency
	%	%	%	%
Hp 2-1	86		79	
Hp 2-1 + Hb	61	28	40	49
Hp 2-2	81		79	
Hp 2-2 + Hb	67	18	42	47
Hp 1-1	80		79	
Hp 1-1 + Hb	62	23	29	61
Hp 2-1 Bellevue	75		63	
Hp 2-1 Bellevue + Hb	65	12	48	23
Hp 2-2 Bellevue	34		32	
Hp 2-2 Bellevue + Hb	35	0	33	0
Hp 1-1 Bellevue	62		41	
Hp 1-1 Bellevue + Hb	61	0	41	0

The quantitative immunologic deficiency produced in the presence of Hb is expressed as per cent of the antibody blocking effect of the unbound haptoglobin.

before the addition of Hp-¹²⁵I were carried out in the presence of excess Hb which was later completely bound by the addition of a large excess of rabbit Hp. The data indicate that both Hp 1-1 Bellevue and Hp 2-2 Bellevue can completely correct the antigenic deficiency of Hb-Hp complex as measured with antiserum II, but can only par-

TABLE III
Dissociation of the (B) and (D) Determinants in the Bellevue Phenotypes

Sample	Antiserum II		Antiserum I	
	Block- ing	Defi- ciency	Block- ing	Defi- ciency
	%	%	%	%
Hp 2-1	75		79	
Hp 2-1 + Hb	64	15	36	54
Hp 2-1 + Hp 1-1 Bellevue + Hb	76	0	54	32
Hp 2-1 + Hp 2-2 Bellevue + Hb	77	0	56	28

The amount of each Hp used here is that which would give maximal blocking of the antibodies ("equivalent" amounts). Using larger amounts did not alter the results. The per cent deficiency of mixtures is calculated with respect to the figure for Hp 2-1.

tially correct it when antiserum I is used. The partial correction with antiserum I was constant and could not be increased by using larger amounts of Hp 1-1 Bellevue or of Hp 2-2 Bellevue.

Double diffusion studies

Fig. 3 shows that insofar as the determinants detected by antiserum II are concerned, Hp 2-1 and Hp 1-1 Bellevue are *qualitatively* identical. Hp 2-2 Bellevue is antigenically deficient, as shown by the spur pointing to the wells at 6 and 10 o'clock.

Fig. 4 shows the comparison of Hp 2-1 and Hp 2-1 Bellevue and of their Hb complexes, using antiserum I. As the spurs indicate, both of these types have determinants that are covered in normal Hb-Hp complex (wells at 12, 2, and 10 o'clock); this is the (B) determinant. In Hb-Hp 2-1 Bellevue complex, however, this determinant is *not* covered, as shown by the reaction of identity among the wells at 6, 8, and 10 o'clock. In the free state, the two haptoglobins are immunologically identical. These data indicate that in Hp 2-1 Bellevue the (B) determinant is present, but is not covered in the presence of Hb.

The reactions of Hp 1-1 Bellevue and Hp 2-2 Bellevue with antiserum I are shown in Fig. 5. Both these types are deficient with respect to Hp

FIGURE 3 The reaction of Hp 1-1 Bellevue and Hp 2-2 Bellevue with antiserum II. Hp 1-1 Bellevue is not distinguishable from normal Hp by antiserum II, but Hp 2-2 Bellevue is antigenically deficient.

FIGURE 4 The reactions of Hp 2-1 Bellevue and of its Hb complex with antiserum I. Hb-Hp 2-1 complex is antigenically deficient with respect to both Hp 2-1 and Hp 2-1 Bellevue, whereas Hb-Hp 2-1 Bellevue complex is not.

2-1, but are identical with Hb-Hp 2-1 complex, thereby suggesting the lack of the (B) determinant in both these mutant types.

Because of the short supply of antiserum III, Ouchterlony patterns with this antiserum were

FIGURE 5 Reactions of Hp 1-1 Bellevue and Hp 2-2 Bellevue with antiserum I. Both these types are antigenically deficient with respect to Hp 2-1, but not with respect to Hb-Hp 2-1 complex. This identifies their deficiency as the lack of the determinant (B).

not studied. Judgment about qualitative differences was therefore based on the special modification of the immunoassay already described.

Two children in the family had the phenotype Hp 2-2 Bellevue. The behavior of their sera in the Ouchterlony diffusion plates and the immunoassays described above were identical.

DISCUSSION

With the exception of Hp 1-1 and possibly also of Hp 1-1 Bellevue, the proteins considered in this study show molecular heterogeneity in that they comprise multiple polymer components. The discussion that follows is concerned with the total antigenic endowment and the Hb binding property of all these components within each phenotype, not of individual components. Indeed, Korngold (5) and Eichmann, Deicher, and Cleve (6) have shown that the type-specific determinant (C) is present on the polymers of Hp 2-1 but is missing in the component of this phenotype which corresponds to Hp 1-1. As will be suggested below, a different order of heterogeneity may exist among the components of the Hp Bellevue phenotypes.

Reactions with antiserum III. The type-specific determinant (C) is present in Hp 2-1 Bellevue as is evident from the similarity between the immunoassay curves of this variant and of Hp 2-1, using antiserum III. Although the immunoassay curve for Hp 2-2 Bellevue reaches a low maximum value (56%, compared to 85% for Hp 2-2) the mixture experiments make it clear that this Hp also contains the (C) determinant. Table I shows that the qualitative immunological deficiency of Hp 1-1 (low maximum blocking effect) which is due to the lack of (C) is completely corrected by the addition of Hp 2-2 Bellevue. On the other hand, Hp 1-1 Bellevue must lack this same determinant since comparable amounts of this variant cannot correct the deficiency of Hp 1-1. These conclusions are consistent with the analogy between each Bellevue variant and its normal counterpart.

Reactions with antiserum II. Both the immunoassay (Fig. 2) and the Ouchterlony plate (Fig. 3) indicate that with respect to the determinants detected by antiserum II, Hp 2-2 Bellevue is antigenically deficient, whereas Hp 1-1 Bellevue is not. Since antiserum II can detect only the determinants (A) and (D), the deficiency of

Hp 2-2 Bellevue must be in one of these. The mixture experiments (Table III) show that Hp 2-2 Bellevue can provide the determinant (D), which is missing in Hb-Hp complex. The deficiency of Hp 2-2 must therefore be in (A). It will be recalled that (A) was defined as including several determinants, all of which are present on free and Hb-bound Hp of the three normal phenotypes. The particular member of this group which Hp 2-2 Bellevue lacks will be designated (A₁) (see Table IV).

The immunoassay shown in Fig. 2 further points out a strikingly reduced ability of Hp 1-1 Bellevue and Hp 2-2 Bellevue to bind Hb, reflected in the very steep initial slope of the immunoassay curves for these two phenotypes. The same conclusion may be drawn from the immunoelectrophoretic pattern (Fig. 1) where the lack of peroxidative activity reflects the absence of significant Hb in the precipitin arcs of these two Hp types. The Hp levels in the Hp 1-1 Bellevue and Hp 2-2 Bellevue sera, measured as Hb binding capacity, are 4 and 25 $\mu\text{g}/\text{ml}$, respectively. But since these haptoglobins have markedly diminished affinity for Hb, the figures underestimate the actual amount of Hp protein in these sera. The levels are, nevertheless, very low even after this correction.

Effect of Hb binding. All three Bellevue phenotypes show immunologic evidence of a qualitatively abnormal Hb binding. These abnormalities probably arise from the structural mutation of the $\text{hp}\beta$ -chain affecting the Hb binding site and the determinants situated in its vicinity.¹ Hp 2-1 Bellevue possesses the determinant (B), as is shown by its reaction of identity with Hp 2-1 and of partial identity with Hb-Hp complex (Fig. 4). The effect of Hb binding, however, is very different in the two phenotypes. Whereas Hb-Hp 2-1 complex is deficient in the determinant (B), this determinant is still present in at least some molecules of Hp 2-1 Bellevue even after the addition of excess Hb.

The findings with Hp 1-1 Bellevue and Hp 2-2 Bellevue are more striking. Table II shows that the addition of Hb has *no effect* on the antigenic

endowment of these phenotypes as detected by either antiserum I or II. This finding could be explained by postulating an inability on the part of these variants to bind Hb. This view is supported by the data from immunoelectrophoresis (Fig. 1) and immunoassay (Fig. 2). There is reason to believe, however, that aside from lack of Hb binding, there is also a lack of the antigenic determinant (B) normally associated with the Hb binding site. The double diffusion patterns with antiserum I (Fig. 5) show that both these Bellevue variants are deficient with respect to Hp 2-1, but not with respect to Hb-Hp 2-1 complex. Their deficiency is therefore in the determinant normally covered by Hb binding, namely (B). This is also evident from the data presented in Table III: both these phenotypes can correct the deficiency of Hb-Hp as detected by antiserum II, which is in the determinant (D); they must therefore possess (D). The correction of the deficiency detected by antiserum I, which is in (B) and (D), is only partial and is attributable to their content of (D). These two Bellevue phenotypes may therefore be said to have (D)

TABLE IV
Antigenic Models of the Normal and the Bellevue Variants of Haptoglobin

	A	A ₁	B	C	D	Hemoglobin binding site
Phenotype	<i>Antigenic Determinants</i>					
Hp 1-1	+	+	+	-	+	+
Hp 2-1	+	+	+	(+)	+	+
Hp 2-2	+	+	+	+	+	+
Hp 1-1 Bellevue	+	+	-	-	+	-
Hp 2-1 Bellevue	+	+	(+)	(+)	+	(+)
Hp 2-2 Bellevue	+	-	-	+	+	-
Antiserum	<i>Antibodies</i>					
I	+	+	+	-	+	
II	+	+	-	-	+	
III	+	+	+	+	+	

The upper panel shows the presence or absence of the five antigenic determinants in the normal and the Bellevue phenotypes, as well as the ability of the protein to bind Hb. (+) signifies presence of the determinant or of the Hb binding site in some, but not all, protein molecules within the phenotype. The lower panel defines the three antisera used in this study in terms of their recognition of the five determinants.

¹ The nature of the structural alteration (point mutation, deletion, etc.) is not known. It is also unclear whether the mutation involves the Hb binding site and its vicinity directly, or by an allosteric effect through a distant structural change.

but not (B). The dissociation between these two Hb-sensitive determinants lends support to the individuality of (D) as distinct from (B), as was postulated in the preceding paper (3).

The status of the five antigenic determinants in the three normal and the three Bellevue phenotypes, and of the antibody content of the three antisera, is presented diagrammatically in Table IV.

Some of the unexpected properties of the three Bellevue haptoglobins must now be considered. The relation among these three variants is analogous to the relation among normal Hp phenotypes. It may, therefore, be reasonably expected that if Hp 2-1 Bellevue can bind Hb, then so should the other two phenotypes, Hp 1-1 Bellevue and Hp 2-2 Bellevue. These two, in fact, bind little or no Hb. Although the data presented here do not explain this finding, a hypothesis may be presented which is amenable to experimental verification. Two points should be brought out before considering this hypothesis: (a) Haptoglobins are degraded in the body through two different routes. One is the removal of free haptoglobin by mechanisms shared with other plasma proteins, and the other is the more rapid turnover of that fraction of Hp which has bound to Hb. The sum of these two gives an over-all Hp half-life of 4 days (7). Available data (8, 9) indicate that the daily turnover of Hb-Hp complex accounts for a sizable portion, perhaps the major part, of Hp degradation. This is consistent with the very low Hp levels observed with relatively small increase in Hb turnover (10).

(b) The Bellevue haptoglobins are heterozygous with respect to their $hp\beta$ -chains (1). With the choice of two kinds of $hp\beta$, three kinds of Hp polymers (or of the one molecular species in the case of Hp 1-1) would be made, namely those having only normal $hp\beta$ -chains, or only mutant ones, or having some of each. In this heterogeneous population, those molecules containing a sufficient proportion of normal $hp\beta$ -chains would be expected to have some Hb binding capacity, whereas the few with predominantly abnormal $hp\beta$ would be molecules with negligible Hb binding capacity. If the rate of Hp synthesis is low, so that it is close to the rate of Hb-Hp turnover, then almost all of the molecules in the heterogeneous Bellevue components *with* Hb binding capacity will be rapidly removed, leaving the molecules without Hb binding capacity to be de-

graded by the slower protein turnover mechanism. The circulating Hp molecules under these circumstances will consist predominantly, or even exclusively, of the nonfunctioning species, as is the case with the two phenotypes Hp 1-1 Bellevue and Hp 2-2 Bellevue. That the donors of these two types in fact do have low rates of Hp synthesis relative to Hp removal is evident from their very low levels of Hp (even if measured in terms of antigenic equivalence, rather than Hb binding capacity). This would imply that if the rates of Hp synthesis in the donors of these two Bellevue variants were increased, for instance as a result of infection (10), then the Hb-binding species of their Hp would be demonstrable in their sera. It is of interest that when blood was collected from the proband (Hp 2-1 Bellevue) whose serum had considerable Hb binding capacity, he had a fever of inflammatory origin and may have been synthesizing Hp at a higher rate. The idea of the differential turnover of Hp molecules with different chain contents is in a sense analogous to the concept of differential turnover of red blood cells with different proportions of Hb S and Hb F, as proposed by Bradley, Brawner and Conley (11).

In the particular cases of Hp 1-1 Bellevue and Hp 2-2 Bellevue reported here, the heterozygous carrier phenotypically resembles the homozygote for $hp\beta$ -Bellevue as a consequence of the differential Hp turnover. The abnormal Hp molecules lack significant Hb binding capacity and also lack the structural features responsible for the Hb-sensitive determinant (B). This is consistent with a structural mutation in the $hp\beta$ -chain involving the Hb binding sites and its immediate vicinity.

The observation that Hp 2-1 Bellevue does possess the determinant (B) and that this determinant is not completely covered by Hb binding (Table II and Fig. 4) is also consistent with the idea of heterogeneity of these Hp molecules. In some of the molecules with both normal and mutant $hp\beta$ -chains, the mutant chains may interfere with Hb binding by normal chains, and may yet leave the (B) determinant in the latter exposed. Consequently, in the presence of Hb, some molecules will be devoid of (B), either to begin with or as a result of Hb binding, while another set of molecules will retain the reactivity of their (B) determinant. An earlier observation (1) sup-

FIGURE 6 Heterogeneity of Hp 2-1 Bellevue. Tracing of original photograph from a previous publication (1). In the presence of Hb, Hp 2-1 Bellevue can be physically separated into two components in one of which the (B) determinant is still reactive.

ports this point. The spur formed by the interaction of Hp 2-1 and Hb-Hp 2-1 with antiserum I fuses with the main precipitin arc. By contrast, the "spur" formed between Hb-Hp 2-1 Bellevue [which contains (B)] and Hb-Hp 2-1 [which lacks (B)] is physically separate from the main precipitin arc throughout its length (Fig. 6). Two physically separable types of molecules are therefore present in Hb-Hp 2-1 Bellevue. Some lack (B) and fuse with the arc of Hb-Hp 2-1; others retain (B) even in the presence of Hb. The antigenic model depicted in Table IV stresses this high order of heterogeneity of Hp 2-1 Bellevue.

The presence of a heterogeneous population of molecules with different antigenic and Hb binding properties is probable in other rare Hp phenotypes. This may be particularly true of those variants in which Hb binding is qualitatively or quantitatively altered (12-14). These variants would be suitable for analysis by the approach presented. In the case of Hp 2-1 Marburg, Shim, Jin, and Cleve (15), using the double diffusion method, have already shown heterogeneity of the molecules with respect to the Hb-sensitive (B) determinant.

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