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The assay system is sensitive to 0.2  $\mu$ g or less of serum Hp. Furthermore, it can detect determinants that are not recognized by double diffusion studies.

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# Immunogenetics of Human Haptoglobins

## I. THE ANTIGENIC STRUCTURE OF NORMAL Hp PHENOTYPES

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**ABSTRACT** The various antigenic determinants of serum haptoglobins (Hp) may be studied semiquantitatively in a radioimmunoassay system. Using three antisera of overlapping specificities, we constructed an antigenic model of the various Hp phenotypes comprising four distinct determinants. This adds a new determinant, situated near the hemoglobin binding site, to the three determinants reported in the literature.

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### INTRODUCTION

Three common variants of human haptoglobin (Hp) can be distinguished by starch gel electrophoresis. These types differ in containing alternate structural forms of a polypeptide chain,  $hp\alpha$ , controlled by allelic genes at the  $H\beta$  locus (1). The three phenotypes, designated Hp 1-1, Hp 2-1, and Hp 2-2, all have another polypeptide chain,  $hp\beta$  (1, 2), under the control of a gene locus for which the designation  $B\beta$  has been proposed (3). All common Hp types have a strong affinity for hemoglobin (Hb); the Hb binding site in Hp appears to be located on the  $hp\beta$ -chain (4). Early studies on the immunologic characteristics of Hp did not reveal differences among the three phenotypes, or between free and Hb-bound Hp (5, 6). Recently, however, with the use of more discriminating anti-

sera both these categories of antigenic differences have been demonstrated by Korngold (7, 8) and by Eichmann, Deicher, and Cleve (9). The latter group has proposed the following terminology: (A) represents the antigenic determinant present in all common Hp types and in their Hb complexes. (B) is a determinant which is present only in free Hp of all types but which is no longer reactive in Hb-Hp complex. Finally, (C) is a type-specific determinant present in Hp 2-1 and Hp 2-2, but not in Hp 1-1.

Rare Hp variants with uncommon electrophoretic patterns (10-14), with anomalous Hb binding properties (12, 13), or with immunologic abnormalities (15) have now been reported. It has become desirable to redefine the criteria for identifying a new protein variant as Hp. This need is especially apparent in the case of Hp variants with altered or insignificant Hb binding capacity, since this property is most commonly used in the detection of Hp. In such cases, the use of immunologic techniques offers certain advantages. The method of Ouchterlony double diffusion has been employed in the works cited to define the three antigenic determinants of Hp. This method requires the use of relatively large amounts of Hp and of antiserum, and is not ideally suited to the detection of quantitative antigenic differences among immunologically related proteins. The present paper describes the application of a radioimmunoassay technique to the semiquantitative analysis of the Hp antigenic determinants, and postulates the presence of a hitherto undetected determinant.

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## METHODS

### Reagents for immunoassay

Normal sera of the phenotypes Hp 1-1, Hp 2-1, and Hp 2-2 were used. Hemoglobin binding capacity was determined by the peroxidase activation method (16) using at least six different dilutions for each sample. Purified Hp was prepared by DEAE-cellulose chromatography (17) followed by gel filtration on Sephadex G-100. Hb-Hp 2-1 complex was obtained by the method of Cloarec and Moretti (18) and was further purified by gel filtration on Sephadex G-100. A highly purified sample of Hp 2-1 was iodinated by the chloramine-T method (19) using 4.6 mg Hp, 800  $\mu$ c carrier-free Na<sup>125</sup>I, and 300  $\mu$ g chloramine-T. Exposure to the oxidant was kept down to 90 sec to minimize denaturation of the protein. Unreacted <sup>125</sup>I was removed by exhaustive dialysis. Under these circumstances, the efficiency of iodination was about 10% and a specific activity of roughly 15  $\mu$ c/mg protein was achieved. Radioactivity was measured in a well-type scintillation counter.

Dilutions of human sera and of Hp-<sup>125</sup>I were made in Tris-buffered saline consisting of 0.14 M NaCl in 0.05 M tris (hydroxymethyl) aminomethane hydrochloride, pH 8.2. All antisera were diluted in rabbit globulin from normal rabbit serum. To prepare this we precipitated the serum globulins in 2.05 M neutralized ammonium sulfate and reconstituted them in Tris-buffered saline in a concentration of 15 mg/ml.

Hp-rich rabbit serum (Hb binding capacity 4 mg/ml) was obtained from animals previously treated with a subcutaneous injection of turpentine (20).

Three rabbit antisera (I, II, and III) containing antibodies of different specificities were used. The ability of these sera to recognize the antigenic determinants (A), (B), and (C) by Ouchterlony double diffusion technique is summarized in Table I. The antisera types I and III were the generous gift of Dr. Leonhard Korngold and have been described in detail by him (7, 8). All antisera were absorbed completely with Hp-free human serum obtained from a patient shortly after a he-

TABLE I  
Specificities of the Antisera Used

Antiserum	Determinants		
	(A)	(B)	(C)
I	+	+	-
II	+	-	-
III	+	+	+

molytic episode. An aliquot of antiserum I was further partially absorbed with Hb-Hp complex. The complex-absorbed antiserum, designated Ia, showed a 33% fall in titer, as determined by the titration method described below. Ouchterlony double diffusion was performed in 1% agar, 0.05 M barbital buffer, pH 8.6. Acrylamide gel electrophoresis was done by the method of Davis (21).

### Radioimmunoassay

The method employed is based on the procedure of Farr (22). Pilot experiments showed that free and antibody-bound Hp could be quantitatively separated by differential precipitation in 1.45 M ammonium sulfate at 0°C. When equivalent amounts (see below) of antiserum and of Hp-<sup>125</sup>I were used, 90% of the radioactivity was recovered in the precipitate. When comparable amounts of nonimmune rabbit globulin were substituted for the antiserum, only 5% of the radioactivity was precipitated. About 10% of the radioactivity did not precipitate even in the presence of excess antiserum.

*Calibration of antisera.* Each antiserum was titrated by adding increasing amounts of Hp-<sup>125</sup>I to aliquots of antiserum and determining the radioactivity remaining in the supernatant fluid (unbound antigen) when the mixture was made 1.45 M in ammonium sulfate. Fig. 1 shows a typical curve. A clear-cut break in the curve occurs when the amount of Hp exceeds the binding capacity of the antiserum. This point (E) in the curve was taken to indicate equivalent amounts of antiserum and of

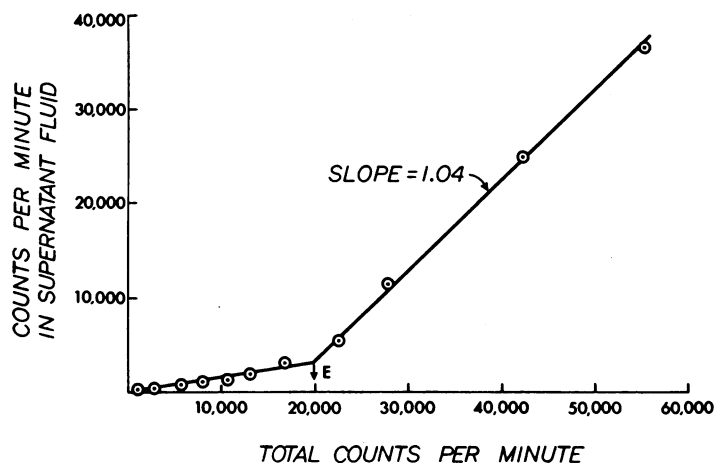


FIGURE 1 Calibration of antibody (antiserum I) with radioactive Hp. Increasing amounts of Hp-<sup>125</sup>I were added to constant volumes of antiserum. The free Hp-<sup>125</sup>I was recovered in the supernatant fluid after precipitation with 1.45 M ammonium sulfate. The first portion of the curve shows a constant fraction of radioactivity remaining free even in the presence of excess antibody. The second portion shows the quantitative recovery of the excess Hp in the supernatant fluid once all of the antibody is bound. The junction of the two curves (point E) marks the amount of Hp which is equivalent to the antiserum.

Hp-<sup>125</sup>I which were then used in all subsequent experiments.

**Test system.** The immunoassay was based on the ability of the test material to block the subsequent binding of Hp-<sup>125</sup>I by antiserum. Increasing amounts of the unknown serum in volumes of up to 150  $\mu$ l were incubated at 20°C with 200- $\mu$ l aliquots of antiserum. After 1 hr, 50  $\mu$ l Hp-<sup>125</sup>I, equivalent to the initial capacity of the antiserum, was added and the mixture incubated an additional hour at 20°C. 1.5 ml Tris-buffered saline was added to achieve a convenient volume. Saturated, neutralized ammonium sulfate was then mixed into a final concentration of 1.45 M. After 0.5 hr at 0°C, the mixture was centrifuged at 4000 g for 10 min and the radioactivity in the supernatant fluid was determined by counting a 1 ml aliquot. The quantities of reactants used were chosen such that about 70,000 cpm were introduced into each tube. The equivalent amount of antiserum was diluted to 200  $\mu$ l with nonimmune rabbit globulin. Sera to be assayed were first diluted in Tris-buffered saline to an Hb binding capacity of 0.03–0.04  $\mu$ g/ $\mu$ l. Although all sera were not diluted to the same concentration, the Hb binding capacity of each diluted specimen was accurately known. Increasing volumes of serum up to 150  $\mu$ l (0–6  $\mu$ g Hp) were used to construct the immunoassay curves. The amount of serum added, expressed in terms of its Hb binding capacity, was plotted against the per cent of total radioactivity remaining in the supernatant fluid. In each tube the amount of protein supplied by the antiserum was on the order of 50  $\mu$ g. Additional protein was provided by the nonimmune rabbit globulin as a carrier for the coprecipitation of antibody by ammonium sulfate. The results were independent of the total protein concentration over a wide range, but increasing amounts of antibody-bound Hp-<sup>125</sup>I remained in the supernatant fluid when the protein concentration fell below 0.5 mg/ml of the final mixture. The amount of non-immune rabbit globulin used provided a final protein concentration of 1.45 mg/ml.

In a second set of experiments, the effect of binding of Hb on the antigenic determinants of Hp was studied using a modification of the immunoassay. Single concentrations of Hp and of antiserum, representing equivalent amounts, were used; full immunoassay curves were not constructed. The blocking effect of free Hp (1.2  $\mu$ g) was compared with that of a corresponding amount of

Hb-Hp complex. The latter was formed by adding 1.9  $\mu$ g Hb to 1.2  $\mu$ g Hp, thus insuring that only the complex would participate in the subsequent antigen-antibody reaction. After incubation with antiserum, 150  $\mu$ g rabbit Hp was added to bind all traces of free Hb. (This precaution was found to be necessary since any excess Hb left after this stage of the experiment would finally bind to the Hp-<sup>125</sup>I and obscure the results.) The degree of blocking achieved by Hp and by Hb-Hp was then ascertained in the usual way by the reaction with Hp-<sup>125</sup>I. As control, 1.9  $\mu$ g Hb, previously complexed by 150  $\mu$ g rabbit Hp, was added to the human Hp, thus preventing the formation of Hb complex with the Hp being tested. Both antisera Ia and II were used in parallel experiments.

## RESULTS

### *Immunoassay of the common Hp phenotypes.*

The reliability of the system is achieved by insuring the purity of the Hp-<sup>125</sup>I and the specificity of the antiserum. Discontinuous acrylamide electrophoresis of the labeled Hp showed only the bands of Hp 2-1. Immunoelectrophoresis and Ouchterlony double diffusion against unabsorbed anti-human serum showed only one precipitin band corresponding to Hp. The antisera used, when tested by immunoelectrophoresis and by the Ouchterlony technique against whole serum, also gave one precipitin band corresponding to pure Hp. These findings indicate that only Hp or antigenically related material will compete with the Hp-<sup>125</sup>I for antibody binding.

Reproducible results for each phenotype were obtained with sera from different individuals. On repeated examinations, values were in agreement to within 5%. Larger variations were observed with the use of different preparations of labeled Hp and with variations in the concentrations of the reactants. These parameters were kept constant within each set of experiments presented in this and in the following paper.

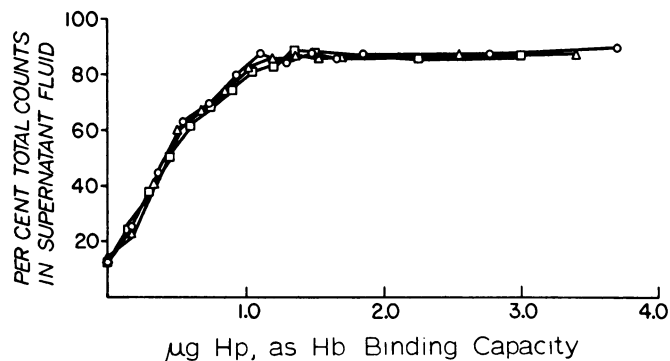


FIGURE 2 Radioimmunoassay curves of the three common Hp phenotypes with antiserum II. The amount of Hp used is expressed in terms of its Hb binding capacity. The ordinate represents the blocking effect achieved. ○—○, Hp 1-1; □—□, Hp 2-1; △—△, Hp 2-2.

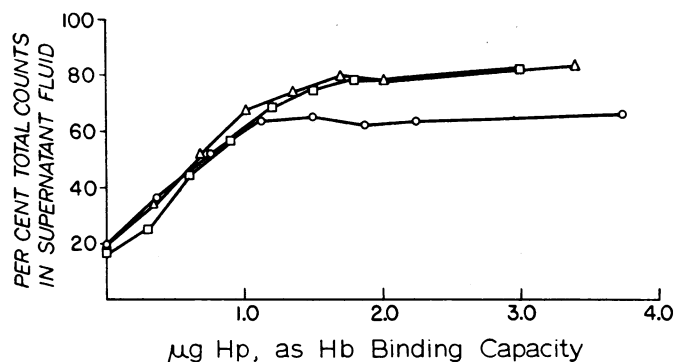


FIGURE 3 Radioimmunoassay curves of the three common Hp phenotypes with antiserum III. ○—○, Hp 1-1; □—□, Hp 2-1; △—△, Hp 2-2. The antigenic deficiency of Hp 1-1 is demonstrated as the inability of this protein to achieve maximum inhibition of binding of Hp-<sup>125</sup>I 2-1.

Fig. 2 shows the results of immunoassay obtained using sera of phenotypes Hp 1-1, Hp 2-1, and Hp 2-2 with antiserum II. This antiserum, it will be recalled, reacts identically with all three phenotypes on double diffusion plates. The three immunoassay curves are also identical in their configuration.

When antiserum III is used, distinct differences are noted between Hp 1-1 on the one hand, and Hp 2-1 and Hp 2-2 on the other (Fig. 3). The latter two are closely similar in their reaction. Hp 1-1, however, although not distinguishable from the other two types at low Hp concentrations, reaches a lower plateau (by 20%). Thus, a number of antibody molecules remain which are not reactive with the determinants present on Hp 1-1; these antibodies are directed against the type-specific determinant (C) present in Hp 2-1 and Hp 2-2.

*Effect of Hb binding.* Using antisera Ia and II, we compared the antigenic determinants of

Hb-Hp 2-1 complex with those of free Hp 2-1 (Table II). The complex showed reduced ability to compete for the antibody binding sites. This amounted to 49 and 28% reduction, with antisera Ia and II, respectively. When Hb was first complexed with rabbit Hp and then added to serum so that no free Hb was present to bind the serum Hp, no reduction in blocking ability was noted. This control rules out a nonspecific effect of Hb and indicates that the reduction in the antigenic determinants of Hp depends on its actual binding to Hb.

Experiments were performed to rule out the possible instability of the antigen-antibody complexes as a factor in the results obtained. Aliquots of antiserum I and of antiserum II were incubated with equivalent amounts of Hp-<sup>125</sup>I or of Hb-Hp-<sup>125</sup>I complex. One set of duplicates from each of the four combinations was then incubated with unlabeled Hp and precipitated with 1.45 M ammonium sulfate (note the reverse order of addition of the radioactive and of the cold Hp). The second set of duplicates was precipitated directly without addition of cold Hp. There was no difference in the amount of radioactivity remaining in solution when the two sets were compared. This was taken to mean that once Hp or Hb-Hp is bound to antibody, it does not (under the conditions of the immunoassay) exchange with Hp added subsequently.

## DISCUSSION

*General considerations.* The calibration curve (Fig. 1) shows that a constant fraction of the radioactivity (11% in this case) remains in the supernatant fluid even in the presence of excess antibody. Once the antibodies are fully bound,

TABLE II  
Effect of Hb on the Antigenic Determinants of Hp

Sample	Antiserum	Blocking effect	Deficiency
			with respect to Hp
		%	%
Hp 2-1	Ia	79	—
Hp 2-1 and Hb	Ia	40.5	49
Hp 2-1 and Hb-rabbit Hp*	Ia	80	0
Hp 2-1	II	86	—
Hp 2-1 and Hb	II	61.5	28
Hp 2-1 and Hb-rabbit Hp*	II	86	0

\* Hb previously incubated with excess rabbit Hp and fully bound to it.

however, all of the additional radioactivity remains in the supernatant fluid. The quantitative recovery of the free radioactive Hp in the fluid is indicated by the final slope of the curve, which is close to unity. The observation that when only nonimmune rabbit globulin was used instead of antiserum about 95% of the radioactivity remained in solution is further support for this suggestion. The break in the curve (*E*) determines unambiguously the amount of radioactive Hp which is equivalent to the antiserum used.

The fact that Hp-anti-Hp complexes precipitate indicates that the Hp molecule contains a multiplicity of antigenic determinants. The rather large size of the Hp molecules [mol wt = 100,000 for the smallest, Hp 1-1 (23)] suggests that several different kinds of determinants will be present in these proteins. Antisera from different rabbits, such as the three used in this study, will contain antibodies to some of these determinants and not to others. Comparison of immunoassay curves using different antisera will thus permit the study of specific determinants in each Hp phenotype.

The amount of radioactivity in the supernatant fluid (unbound Hp-<sup>125</sup>I) is a function of the number of antibodies blocked by the test Hp. When a test Hp lacks one or more determinants compared to another Hp, it will have a lower *maximum* blocking effect. This difference in the maximum blocking effect has the same connotation as a spur on double diffusion plates. Difference in the number of antigenic determinants per unit of Hb binding capacity will be expressed as differences in the initial slope, in the general configuration of the curve, and in the value of the smallest amount of Hp which will achieve the maximum blocking effect. In addition, slight alterations in the sites which would modify the association constant for the binding of specific antibody would also affect the initial slope predominantly.

*Normal determinants of Hp.* The data presented can be interpreted in terms of antigenic models of Hp 1-1, Hp 2-1, and Hp 2-2. Both on Ouchterlony plates and in the immunoassay (Fig. 2) the three common phenotypes behave identically in their reactions with antiserum II. This antiserum, then, detects a number of determinants, collectively designated as (A), which are common to all normal Hp phenotypes.

The reactions of the three phenotypes with antiserum III are consistent with Korngold's demonstration (7) that this antiserum can distinguish Hp 1-1 from the other two common phenotypes on double diffusion plates. The low maximum blocking effect of Hp 1-1 is the manifestation of its antigenic deficiency. The additional determinant (C) in Hp 2-1 and Hp 2-2 may be related either to a structural configuration on the hp2 $\alpha$ -peptide of these two Hp phenotypes, or to new configurations formed as the result of polymerization (9).

The data presented in Table II point to the antigenic deficiency of Hb-Hp complex as compared to Hp. The results obtained with antiserum Ia are consistent with a similar deficiency which can be detected by double diffusion studies. With antiserum II, however, the observed effect of Hb in the immunoassay is unexpected since a corresponding deficiency of the Hb-Hp complex is not apparent on double diffusion plates when antiserum II is used. Two explanations are possible for this discrepancy:

(a) The effect may be explained by assuming that an exchange occurs between Hp-<sup>125</sup>I and the Hp or Hb-Hp already bound to antibody. If this exchange is more rapid when the initially bound antigen is Hb-Hp rather than Hp, then less Hp-<sup>125</sup>I remains unbound in the former case and an *apparent* deficiency of the Hb-Hp complex may be inferred. The experiment in which the order of the addition of the antigens is reversed shows that there is no displacement of either Hp or of Hb-Hp once it is bound to antibody. The findings make the exchange hypothesis untenable.

(b) The alternate hypothesis, which is the one adopted here, is that antiserum II does in fact detect a Hb-sensitive determinant in Hp. Two questions immediately arise, namely, is this determinant the same as (B), and, why is this effect not apparent on double diffusion plates? The data presented so far do not bear on the first question; additional findings discussed in the second paper of this series (24), however, indicate that the determinant in question is separate and distinct from (B). This second Hb-sensitive determinant will be designated (D). The evidence referred to consists of the demonstration that in two rare Hp variants (B) is lost whereas (D) is retained.

The failure of the double diffusion method to

recognize (D) suggests that the antibody in antiserum II directed against (D) does not form an insoluble complex with Hp. A spur, representing the (D)-anti-(D) reaction, will therefore not be visible on double diffusion plates. The determinant (D) can nevertheless be detected by the immunoassay since here even soluble antigen-antibody complexes are precipitated by ammonium sulfate. The present antigenic model comprising the four determinants is a tentative and incomplete one. Further extension of the model may be expected when other antisera with specificities other than those encountered here reveal the presence of new determinants on Hp. Furthermore, abnormal Hp phenotypes with immunological deficiencies will help to define new determinants (24).

If the immunoassay system is to be used for the quantitation of normal Hp, care should be taken that the antiserum employed reacts identically with all three phenotypes, such as an antiserum of the type I or II. The sensitivity of the method greatly exceeds that of others currently in use (16, 25-28). Under the conditions described, 0.2  $\mu\text{g}$  Hp (the content of about 0.2  $\mu\text{l}$  normal plasma) may be reliably assayed. By employing labeled Hp of higher specific radioactivity, even smaller amounts of Hp may be measured. The method may be of use in quantitating Hp in body fluids (e.g. urine) where this protein occurs in very small concentrations (29).

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