Re-Evaluation of the Haptoglobin Reference Values with the Radial Immunodiffusion Technique

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The reference values of the three main types of serum haptoglobin Hp 1-1, Hp 2-1, and Hp 2-2, as determined by radial immunodiffusion and with phenotype determination on polyacrylamide gel electrophoresis have been re-evaluated for both sexes. For that purpose about 500 serum samples were collected from normal, healthy Dutch volunteers. The relative occurrence of the three main types of serum haptoglobin in Dutch men and women was found to be comparable to that reported for other whites, with the Hp 2-1 phenotype predominating in the Dutch population and phenotype Hp 0-0 being absent. The observed overall reference range of haptoglobin for the Dutch population, irrespective of the phenotype, was 0.50–3.30 g/L serum.

As reported by other investigators we found that the reference values for the three main types of haptoglobin significantly differed (Hp 1-1: 1.40 ± 0.51; Hp 2-1: 2.10 ± 0.76; and Hp 2-2: 1.65 ± 0.73 g/L serum, mean ± SD).

However, in contrast to the data available in the literature, the reference values of haptoglobin for men and for women, as determined by the radial immunodiffusion technique, were not significantly different.

KEY WORDS: haptoglobin, phenotypes, serum, radial immunodiffusion, reference values, sex differences

Introduction

Haptoglobins, first described in 1938 (1), are mucoproteins of the α_2 globulin class, capable of interacting with hemoglobin or globin to form stable complexes. Heterogeneity exists within the haptoglobins, which is genetically controlled by the presence of two allelic autosomal genes, Hp^1 and Hp^2 (2). Three main types of haptoglobins are found in human populations (3), which have been designated as haptoglobins 1-1, 2-1 and 2-2. They can be distinguished from one another by the patterns they give in gel electrophoresis. There are multiple subtypes of haptoglobin. Furthermore, a third gene of type 0 (Hp^0) has been postulated to account for the complete absence of haptoglobin in some population groups (4–6).

Haptoglobins prevent the build-up of free hemoglobin in plasma by binding the quantity released from the red cells, thereby preventing loss of iron via the kidney (7).

Haptoglobin levels are frequently elevated in inflammatory diseases, carcinoma and tissue necrosis. Elevated levels are sometimes found in diabetes mellitus, renal disease and endocrine imbalance. Depressed levels are found following episodes of intravascular hemolysis, in anemia, malaria, liver disease, and in mononucleosis and as a result of transfusion of incompatible blood. Nyman (8) has published an extensive review of the clinical aspects of haptoglobins.

The available methods to estimate haptoglobin in serum can be divided into several groups, viz. (a) measurement of the peroxidase activity of the haptoglobin-hemoglobin complex, (b) electrophoresis of haptoglobin and the complex, (c) gel filtration, (d) immunodiffusion, (e) differential acid denaturation of hemoglobin and the complex, followed by spectrophotometry, and (f) immunonephelometry.

In recent years, more and more laboratories have come to rely upon immunological techniques for the estimation of haptoglobin. Of these techniques single radial immunodiffusion is a technically simple method measuring directly the haptoglobin concentration (9–12). In this technique the square of the diameter of the ring of the immunoprecipitate, which is formed during the diffusion of the protein, is proportional to the concentration of the antigen haptoglobin. As the haptoglobin phenotypes have distinctly different molecular weights and therefore different diffusion rates, the immunochemical determination of haptoglobin is phenotype-dependent (13). It has also been reported that on the average males have significantly higher levels of serum haptoglobin than females (8). This difference should be consistent with the fact that males tend to have a greater mass of circulating hemoglobin (14). The influence of the phenotype in the quantitative determination of haptoglobin with the radial immunodiffusion technique and the different average value for both sexes prompted us to determine reference values. To our knowledge these values, determined with the Mancini technique (9) have not yet been presented for the three main types Hp 1-1, Hp 2-1 and Hp 2-2 with respect to the two sexes.

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Material and methods

Reagents and equipment

Disc electrophoresis was performed by Desaga System Havana (C. Desaga GmbH, FRG) on vertical polyacrylamide gels (PAGE). Acrylamide was obtained from Serva (Heidelberg, FRG), and recrystallized from chloroform. *N,N'*-methylenebisacrylamide and *N,N,N',N'*-tetra-methylenediamine were purchased from Eastman Kodak Co. (Rochester, NY, USA).

All other chemicals were of analytical grade and were obtained from E. Merck AG (Darmstadt, FRG) or JT Baker Chemicals (Deventer, The Netherlands).

Methods

For the radial immunodiffusion technique, commercially prepared immunodiffusion plates (M-Partigen®) and human protein standard serum B (cat. no. RDT 02) were obtained from Behringwerke AG (Marburg/Lahn, FRG). The plates contain monospecific polyvalent antiserum, produced by immunizing rabbits with purified human haptoglobins.

The human protein standard serum B consists of three standard solutions with different haptoglobin concentrations. These standards were run on every immunodiffusion plate together with the unknown samples. A control serum sample was regularly used for quality control. A diffusion time of 48 h was allowed at room temperature, after which time a sharply confined precipitin ring of the antigen-antibody complex was obtained.

The concentration of haptoglobin in the unknown samples was read from a calibration curve in which the square of the diameter of the precipitin ring was plotted against the haptoglobin concentration using the standard solutions as references. The haptoglobin concentrations obtained in this way are relative amounts. In the radial immunodiffusion technique the variation in size of the antigen protein molecule (different phenotypes) causes the diffusion coefficient and hence the size of the precipitin ring to vary (9). This should be compensated for by the factors given by the manufacturer of the plates.

These factors are determined empirically. For the plates used in our study these factors were 0.6 (type 1-1), 1.3 (type 2-1) and 1.5 (type 2-2) respectively, according to Behringwerke AG. The present technique is limited by the need to rely on these factors and to determine the phenotype before an accurate value can be given (13).

The haptoglobin phenotypes were determined by disc-PAGE, with the method of Smith *et al.* (15), except that 7.5% instead of 5% gel was used.

After electrophoresis the gels were placed overnight in a 1 g/L solution of human hemoglobin in 9 g/L NaCl. The hemoglobin bound to haptoglobin in the gel was stained by incubation at room temperature in a freshly prepared solution of *o*-dianisidine (1.05 g/L) in 0.15 *M* acetate buffer (pH 4.7), containing 0.2 mL 30% (v/v) H_2O_2 per 100 mL. The gels were allowed to stay in the staining solution until brown bands were clearly visible (20–30 min) and were then removed and washed with distilled water. A large number of very sharp bands were obtained which, however, in spite of their adequate resolution, were sometimes difficult to identify, due to the low concentration of haptoglobin. The number of bands with their travelling distances is specific to the haptoglobin phenotype (16). An example of the banding patterns of the three main types of haptoglobin seen in our study is shown in Figure 1.

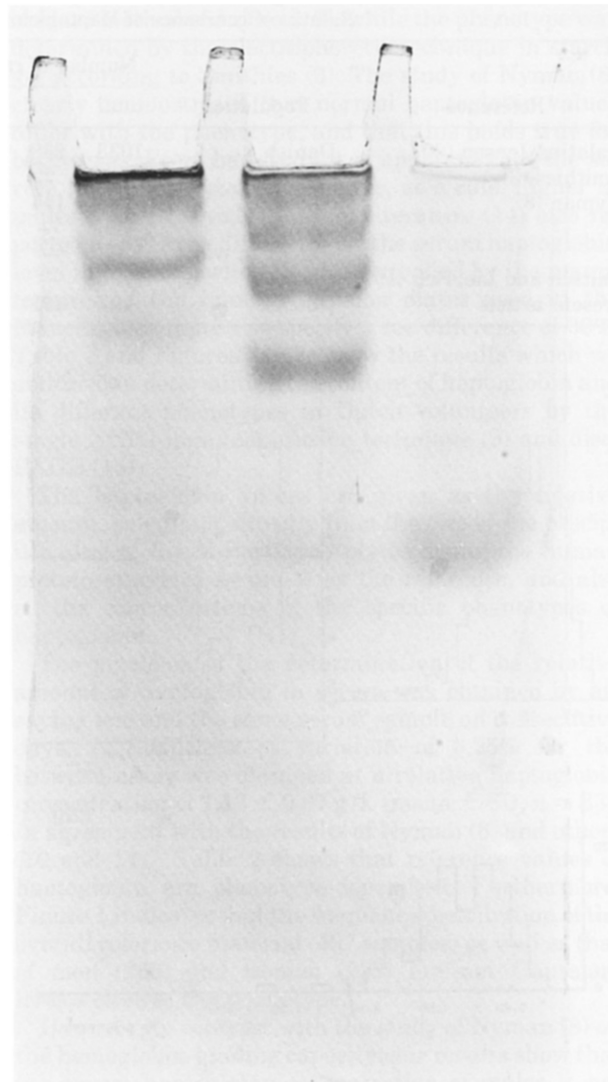


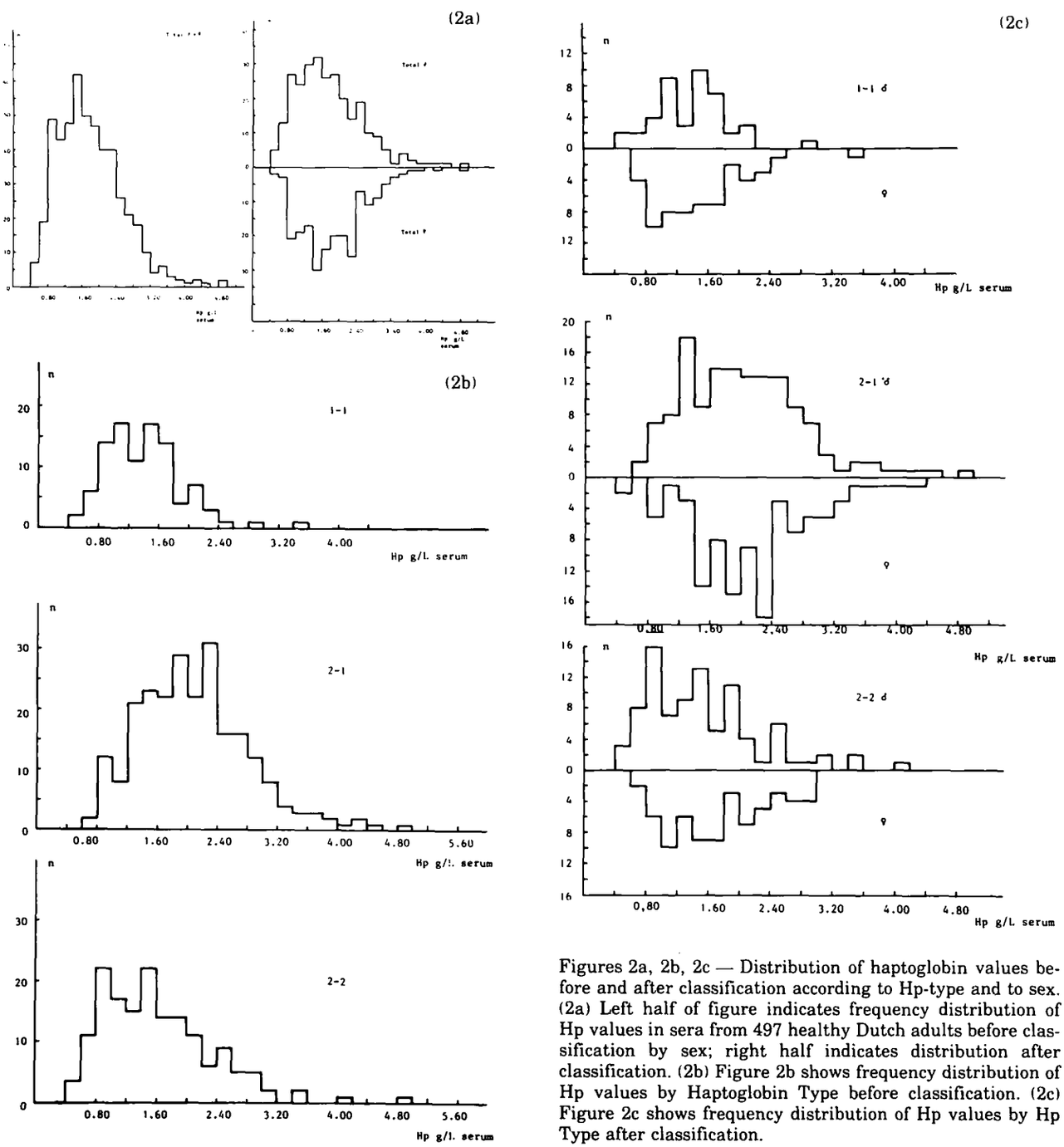
Figure 1 — The banding patterns of the three main types of haptoglobin as observed on PAGE. From left to right: type 2-2, type 2-1 and type 1-1.

Samples

Sera were prepared from blood samples collected from veins of normal, healthy Dutch volunteers of both sexes (age: 18 to 65 years) directly into "Venoject evacuated tubes" (Terumo Co., Tokyo, Japan). Volunteers

TABLE 1
Relative Occurrence of Haptoglobin-Types in Different White Populations

Reference	Population	Number of cases			Type 1-1%			Type 2-1%			Type 2-2%			Type 0-0%
		total	♂	♀	total	♂	♀	total	♂	♀	total	♂	♀	
Galatius-Jensen (20)	Danish	1033	238	795	17	17	17	47	47	47	36	36	36	0
Smithies (3)	Canadian	49	22	27	21	31	11	50	45	55	29	22	33	0
Nyman (8)	Swedish	228	144	84	21	15	28	47	47	35	32	38	37	0
	French	406	—	—	15	—	—	50	—	—	35	—	—	0
	American white	68	—	—	13	—	—	59	—	—	28	—	—	0
Baitsch and Liebrich (19)	German	700	—	—	19	—	—	51	—	—	30	—	—	0
present article	Dutch	497	268	229	20	16	24	48	51	46	32	33	30	0



Figures 2a, 2b, 2c — Distribution of haptoglobin values before and after classification according to Hp-type and to sex. (2a) Left half of figure indicates frequency distribution of Hp values in sera from 497 healthy Dutch adults before classification by sex; right half indicates distribution after classification. (2b) Figure 2b shows frequency distribution of Hp values by Haptoglobin Type before classification. (2c) Figure 2c shows frequency distribution of Hp values by Hp Type after classification.

TABLE 2
Reference Values for and Relative Occurrence of Haptoglobin Types in the Dutch Population

	Hp type	*n*	%	Mean	SD	Reference Range[1]
Total	—	497	100	1.59	0.76	0.50–3.30[2]
	1-1	98	20	1.40	0.51	0.70–2.30
	2-1	239	48	2.10	0.76	0.90–3.60
	2-2	160	32	1.65	0.73	0.60–2.90
Men	—	268	100	1.51	0.75	0.50–3.00[2]
	1-1	43	16	1.39	0.46	0.80–2.10
	2-1	137	51	2.06	0.79	0.90–3.60
	2-2	88	33	1.56	0.74	0.60–2.60
Women	—	229	100	1.68	077	0.60–3.30[2]
	1-1	55	24	1.40	0.56	0.70–2.30
	2-1	105	46	2.13	0.92	0.90–3.40
	2-2	69	30	1.76	0.73	0.70–2.90

[1]Values are given in g/L serum.
[2]Values as calculated directly from the immunoprecipitin ring (untyped). The conversion factors were 0.6 for type 1-1, 1.3 for type 2-1 and 1.5 for type 2-2 respectively, in accordance with the information of the manufacturer.

were selected who had normal renal function and absence of metabolic and hematological disease.

For the assessment of the reference values of haptoglobin the distribution-free method described by Rümke and Bezemer (17) was used. The limits of percentiles were set at 2.5 and 97.5% with a reliability of 95%.

Results and discussion

PHENOTYPE DISTRIBUTION

A number of papers have been published on the relative occurrence of haptoglobin groups within different populations (for reviews see refs. 8 and 18). For example, Hp[1] predominates in the populations of Africa and tropical America, but is seldom encountered in Asia (19).

The haptoglobin phenotype 1-1 is more common among coloured people; in Europe phenotype 2-1 is found in at least 40% of the population, while in Asia phenotype 2-2 predominates.

In Table 1 the percentage of haptoglobin types observed in our group are given and compared to those reported for other whites. The distribution of the three phenotypes in the Dutch population seems not to diverge from that in other white populations. Also, in our group no type 0-0 has been observed. It is concluded that the results presented in this paper are representative of other whites. Perhaps with the exception of type 1-1, there seems to be no difference between Dutch males and females (cf. the Danish group, Table 1) regarding the relative occurrence of the haptoglobin phenotypes.

REFERENCE VALUES

Nyman (8) was the first (1959) to report extensively on normal values of haptoglobin. She determined the hemoglobin-binding capacity of serum by the peroxidase method of Jayle (1, 2), while the phenotype was determined by the electrophoretic technique in starch gel according to Smithies (3). The study of Nyman (8) clearly demonstrated that normal haptoglobin values differ with the phenotype, and that this holds true for both sexes. From her study it is apparent that the serum haptoglobin concentration is, as a rule, higher in males than in females. Recent literature (14) also reported a (30%) sex difference for the serum haptoglobin level in humans, while the data supplied by the manufacturer of the immunodiffusion plates used in the Mancini technique also specify a sex difference of 30%. Table 2 and Figures 2a–2c show the results which we obtained in determining the content of haptoglobin and its different phenotypes in Dutch volunteers by the single radial immunodiffusion technique (9) and disc-PAGE (15).

The haptoglobin values are given as the relative amount, calculated directly from the size of the precipitin ring of the M-Partigen® plates, using the human protein standard serum B as the reference, and also as the concentrations of the specific phenotypes of haptoglobin.

The precision of the determination of the relative amount of haptoglobin in serum was obtained by assaying one and the same serum sample on consecutive days. A coefficient of variation of 6.25% for the between-assay was obtained at a relative haptoglobin concentration of 1.12 ± 0.07 g/L (mean ± SD, n = 33). In agreement with the results of Nyman (8) and others (10 and 11), Table 2 shows that reference values of haptoglobin are phenotype-dependent. Furthermore, Figure 1 indicates that the frequency distribution of the overall reference material (497 samples) as well as that of men (268) and women (229) are not Gaussian, irrespective of the phenotype.

However, in contrast with the study of Nyman (8) on the hemoglobin-binding capacity, our results show that the serum haptoglobin concentration of males is not higher than that of females, in fact it is lower if anything. As a general rule it can be stated that haptoglobin levels of the two sexes as determined by radial immunodiffusion do not differ. To our knowledge this observation has not been presented earlier in such clear-cut form by giving frequency distributions, means, SD's and reference ranges in normal sera of both sexes. As the method and plates applied in this study are quite common in clinical laboratories, one can imagine that haptoglobin reference values incorporating a difference of about 30% between male and female values, are widely used.

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