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Biological and clinical significance of haptoglobin polymorphism in humans

MICHEL R. LANGLOIS and JORIS R. DELANGHE*

Haptoglobin is a hemoglobin-binding protein expressed by a genetic polymorphism as three major phenotypes: 1–1, 2–1, and 2–2. Most attention has been paid to determining haptoglobin phenotype as a genetic fingerprint used in forensic medicine. More recently, several functional differences between haptoglobin phenotypes have been demonstrated that appear to have important biological and clinical consequences. Haptoglobin polymorphism is associated with the prevalence and clinical evolution of many inflammatory diseases, including infections, atherosclerosis, and autoimmune disorders. These effects are explained by a phenotype-dependent modulation of oxidative stress and prostaglandin synthesis. Recent evidence is growing that haptoglobin is involved in the immune response as well. The strong genetic pressure favoring the 2–2 phenotype suggests an important role of haptoglobin in human pathology.

INDEXING TERMS: genetic variants • acute-phase proteins • anhaptoglobinemia • reference ranges • forensic medicine • interleukin-6 • hemoglobin • iron • free radicals • prostaglandin • ceruloplasmin • hemopexin • immune response

Haptoglobin (Hp) is an α_2-sialoglycoprotein with hemoglobin (Hb)-binding capacity *[1, 2]*.[1] The best-known biological function of Hp is capture of Hb to prevent both iron loss and kidney damage during hemolysis *[3]*. Hp is also a positive acute-phase protein and is characterized by a molecular heterogeneity with three major phenotypes: Hp 1–1, Hp 2–2, and the heterozygous Hp 2–1 *[1-4]*. Although Hp is found in serum of all mammals, this polymorphism exists only in humans *[1, 2]*. The geographic distribution of Hp phenotypes has been under a strong genetic pressure *[2]*. Functional differences have been described between the three phenotypes *[4, 5]*. Hp polymorphism appears to be related to immune response and to autoimmune and inflammatory disorders *[4]*.

Molecular Structure of Haptoglobin

GENERAL CHARACTERISTICS

The molecular variation in Hp was first suspected by Jayle and Judas in 1946 *[6]*. Using starch gel electrophoresis, Smithies identified the three major Hp types in 1955 *[7]*. These (pheno-)types are genetically determined by two alleles: Hp^1 and Hp^2 *[1, 2]*. The homozygote Hp^1/Hp^1 shows a single fast-migrating Hp 1–1 protein band on starch gel electrophoresis (Fig. 1). The homozygote Hp^2/Hp^2 has a series of slower-migrating bands. The heterozygote Hp^2/Hp^1 displays another series of slow bands and a weak Hp 1–1 band. This heterogeneity can be ascribed to differences in molecular mass. The slow-migrating bands are polymerized Hp forms and exist only in humans. In most animals, including the higher primates, Hp shows only a single band, corresponding to the human Hp 1–1 form.

Hp consists of two different polypeptide chains, the α-chain and the β-chain *[1, 2]*. The β-chain (40 kDa) is heavier than the

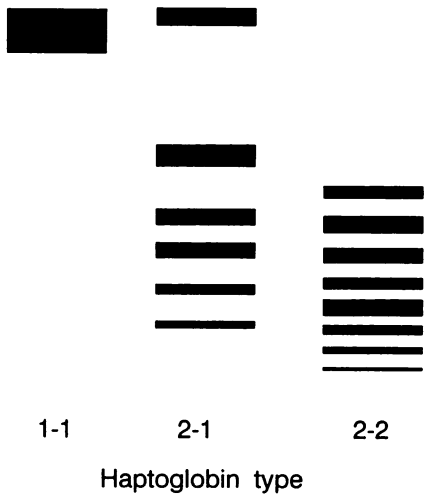

Fig. 1. Typical electrophoretic patterns of Hp phenotypes 1–1, 2–1, and 2–2 on starch gel electrophoresis of Hb-supplemented serum.

Central Laboratory, University Hospital Gent, De Pintelaan 185, B 9000 Gent, Belgium.
*Author for correspondence. Fax 32/9.240.49.85; e-mail jdelangh @ rug.ac.be.

[1] Nonstandard abbreviations: Hp, haptoglobin; Hb, hemoglobin; IL, interleukin; TNF, tumor necrosis factor; Hpr, haptoglobin-related protein; EDRF, endothelium-derived relaxation factor; RID, radial immunodiffusion; RA, rheumatoid arthritis; FHp, abnormally fucosylated haptoglobin; and CSF, cerebrospinal fluid.

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α-chain and is identical in all Hp types. After chemical reduction of Hp, starch gel electrophoresis shows three α-chains: α^{1S}- (S = slower), α^{1F}- (F = faster), and the slow-migrating α^2-chains. The Hp polymorphism arises from variant α-chains *[1, 2]*. The Hp 1–1 phenotype expresses only α^1-chains (8.9 kDa). α^2-Chains (16 kDa) are present in Hp 2–2 and Hp 2–1. Table 1 summarizes some physical characteristics of Hp phenotypes.

CHEMICAL STRUCTURE AND GENETICS

Two genetic loci are involved in Hp synthesis: Hp_α and Hp_β. The Hp_α gene, located on chromosome 16q22, consists of three structural alleles: Hp^{1F}, Hp^{1S}, and Hp^2 *[1, 2]*. As shown in Fig. 2, homozygote Hp 1–1 is a small protein (86 kDa) with formula $(\alpha^1\beta)_2$ ("monomeric form") *[4, 8]*. Both α-chains belong to the α^1-variety (α^{1F} or α^{1S}). Heterozygote Hp 2–1, $(\alpha^1\beta)_2 + (\alpha^2\beta)_n$ (n = 0, 1, 2, . . .), is characterized by polymerization *[4, 8]*. Hp 2–2 comprises higher molecular mass forms (>200 kDa) with formula $(\alpha^2\beta)_n$ (n = 3, 4, 5, . . .) *[4, 8]*.

The gene products of the Hp^{1F} and Hp^{1S} alleles differ by only one amino acid: The lysine in position 54 of the α^{1F}-chain is replaced by glutaminic acid in the α^{1S}-chain, the result of a point mutation in the original Hp^1 allele *[9]*. The amino acid sequence of the α-chains was published in 1968 *[10]*. The $Hp_\alpha{}^2$ allele originates from a fusion of a Hp^{1F} allele and a Hp^{1S} allele *[9]*, presumably by a nonhomologous crossing-over between the structural alleles during meiosis (intragenic duplication) *[1, 2, 11]*. The $Hp_\alpha{}^2$ gene exists only in humans. After the crossing-over between two $Hp_\alpha{}^2$ alleles, larger genes have formed, including the rare Hp "Johnson" type *[9]*. Other structural variants have been described, such as Hp "Carlberg" or Hp 2–1 "modified" *[1-3, 12]*. Duplication of the Hp^1 gene on chromosome 16 results in the Hp-related gene *Hpr [1, 13]*.

As a glycoprotein, Hp contains N-linked oligosaccharides attached to the β-chains *[14-17]*. These carbohydrate side-chains are characterized by terminal α2–6-linked sialic acid residues. A microheterogeneity of the carbohydrate moiety of Hp has been described *[15, 16]*. The Hb-binding capacity of Hp is attributed to the Hp β-chain *[11]*. However, concanavalin A-nonbinding fractions from Hp 2–1 and the tryptic glycopeptides III 1–1 and III 2–2 do not form an active complex with Hb *[16]*.

REGULATION OF HP SYNTHESIS

The *Hp* gene is expressed in hepatocytes *[1]*. Synthesis of Hp is considerably lower in fetal than in adult liver, the result of a difference in transcriptional rate *[1]*.

Expression of the *Hp* gene is absent in "anhaptoglobinemia" (Hp 0–0 phenotype), a condition present in ~1 in 1000 Caucasians *[2]*. In blacks, especially of West African origin (Nigeria, Cameroon), anhaptoglobinemia is more frequent (>30%) *[18]*. In the US, the frequency of Hp 0–0 in blacks is considerably less: 4% *[19]*. Hypohaptoglobinemia has also been reported in a few nonblack families carrying a "silent allele" with no gene product, Hp^0 *[3]*.

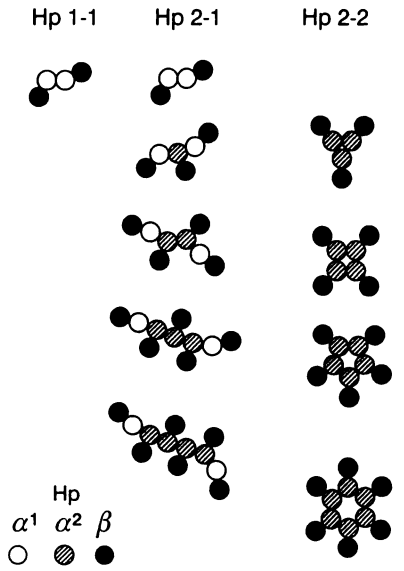

Fig. 2. Structural differences between Hp phenotypes.
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The hepatic synthesis of Hp is induced by cytokines, such as interleukin-6 (IL-6), IL-1, and tumor necrosis factor (TNF) *[1, 20]*. Three IL-6-responsive regulatory regions were identified on the human *Hp* gene promoter: A (−157), B (−111), and C (−61) *[1, 21]*. During the acute-phase reaction, a nuclear transcription factor, IL-6DBP, is induced by IL-6 *[21]*. IL-6DBP replaces proteins bound to regions A and C in the noninduced state *[1]*. Region B binds several nuclear proteins, all different from IL-6DBP, and forms complexes that are identical in induced and noninduced cells *[1]*. The physiological half-life of plasma Hp is estimated as 5.4 days *[22]*.

GEOGRAPHICAL DISTRIBUTION

The gene frequencies of Hp show marked geographical differences, with the lowest Hp^1 allele frequency in Southeast Asia and the greatest frequency in Africa and South America *[2]*. The $Hp_\alpha{}^2$ allele is estimated to have originated in India ~2 million years ago *[2]* and has since spread over the world under a strong genetic pressure, gradually displacing the monopoly of the Hp^1 allele. This suggests a selective advantage provided by the Hp^2 allele *[2]*. At present, the human species is in a state of transient

Table 1. Physical properties and reference values of Hp phenotypes.

	Hp 1-1	Hp 2-1	Hp 2-2
Structural formula	$(\alpha^1\beta)_2$	$[(\alpha^1\beta)_2 + (\alpha^2\beta)_n]$ (n = 0, 1, 2, . . .)	$(\alpha^2\beta)_n$ (n = 3, 4, 5, . . .)
Apparent molecular mass, kDa	86	86–300	170–900
Reference range in serum, g/L	0.57–2.27	0.44–1.83	0.38–1.50

Hp polymorphism *[2]*. In balanced polymorphism, the Hp^2/Hp^1 allele ratio will remain constant *[2]*.

The phenotypic distribution in the northwestern European population shows that ~16% of individuals are Hp 1–1, 48% Hp 2–1, and 36% Hp 2–2, which corresponds to allele frequencies of ~0.4 (Hp^1) and ~0.6 (Hp^2) *[2]*. Table 2 summarizes data on Hp phenotype distribution among various populations in the world *[12, 18, 19, 23-38]*. The Hp^1 allele frequency increases from Southeast Asia in the direction of Europe and Africa *[2]*. Secondly, an increasing Hp^1 frequency has been observed from Asia to America, passing through Alaska, the greatest value being in the Auracanian Indians of Chile *[2, 36, 37]*. The Hp^1 frequency also increases from Southeast Asia through Micronesia and Polynesia, reaching the highest values in Easter Island *[37]*.

HOMOLOGIES OF HP AND OTHER PROTEINS

Striking similarities are evident between the primary structure of the Hp β-chain and the serine proteases, a group of proteolytic enzymes that includes trypsin, chymotrypsin, thrombin,

Table 2. Geographical distribution of Hp phenotypes.

Population	% of population					Ref.
	Hp 1-1	Hp 2-1	Hp 2-2	Hp 0-0	Hp^1	
Caucasians						
North America						
Canada	21.1	50.5	28.4	0.0	0.46[a]	*18*
US (Seattle)	14.4	48.2	37.4	0.3	0.38	*19*
Europe						
Belgium (Gent)	13.0	53.0	34.0	0.0	0.40	*23*
France (Paris)	15.3	49.7	35.0	0.0	0.40	*24*
Germany (Baden Württemberg)	14.0	48.0	38.0	0.0	0.46	*25*
Hungary (Budapest)	12.6	47.2	40.2	0.0	0.36	*26*
Sweden (Umeå)	13.5	47.5	39.0	0.0	0.37	*27*
UK (Oxford)	10.1	55.5	31.7	0.0	0.43	*18*
Asia						
Iran (Moslem)	8.2	40.8	51.3	0.0	0.28	*28*
India (Hyderabad)	2.5	13.3	84.2	0.0	0.09	*29*
Australia						
Australia (Melbourne)	15.5	48.5	34.3	1.7	0.40	*30*
Blacks						
Africa						
Burundi (Hutu)	28.2	47.9	19.8	4.1	0.52	*24*
Burundi (Tutsi)	27.9	47.7	22.1	2.3	0.52	*24*
Nigeria (Yoruba)	53.5	11.1	3.0	32.3	0.59	*18*
Liberia/Ivory Coast	48.7	42.3	9.1	0.0	0.70	*18*
North America						
US (Seattle)	26.4	31.2	38.2	4.2	0.54	*19*
Mongoloids						
Asia						
Thailand (north central/northeast)	5.7	37.1	54.8	2.3	0.24	*31*
China (Han)	9.4	35.2	55.4	0.0	0.27	*32*
Taiwan (Taipei)	9.3	37.7	52.9	0.0	0.28	*33*
Japan (Akita/Fukuoka)	7.4	37.2	35.4	0.0	0.26	*34*
America						
Eskimos (Greenland)	6.6	45.9	47.2	0.0	0.26	*12*
US (Apache)	34.4	47.9	17.7	0.0	0.59	*35*
Southern Mexico/Guatemala	33.4	49.6	15.6	0.7	0.58	*35*
Chile (Mapucho Indians)	56.0	33.6	10.3	0.0	0.72	*36*
Chile (Pehuenche Indians)	62.8	34.5	2.7	0.0	0.78	*36*
Polynesians						
Easter Island	72.2	27.8	0.0	0.0	0.86	*37*
Aboriginals						
Australia (North Queensland)	2.0	31.7	66.3	0.0	0.18	*38*
Bushmen						
Botswana (Kalahari bushmen)	10.6	35.4	52.2	1.8	0.29	*33*

[a] Frequency of Hp^1 allele.

plasmin, elastase, and some complement factors *[14, 39]*. The amino acids in position 57 (His) and 195 (Ser), which are necessary for the activity of serine proteases, are replaced in the Hp β-chain by Lys and Ala, respectively *[14, 39]*. Despite the loss of proteolytic activities by Hp during the evolution of the Hp_β gene, the Hp β-chain has acquired Hb-binding capacity. Remarkably, the Hp β-chain has 53.6% homology with the plant lectin concanavalin A *[40]*.

A homology has been demonstrated in the Hp α domain with both the activation peptides of the serine proteases and the kringle domain found in thrombin, tissue plasminogen activator, and plasmin *[41]*. The greatest similarity is with the fifth kringle structure of plasminogen. Furthermore, homologous sequences in amino acids are seen near the α-β junction of Hp and tissue plasminogen activator *[41]*. There is also a homology between the primary structure of the Hp α-chain and the light chains of the gamma globulins *[10]*.

Functional Properties of Hp

BINDING HB

Hp forms a soluble complex with Hb, an oxygen-binding tetrameric ($\alpha_2\beta_2$) protein containing a protoporphyrin ring complexed with Fe^{2+} (heme). The binding of Hp with Hb is characterized by a very high affinity ($>10^{10}$ mol^{-1}) and stability *[42]*. The β globin chain of human Hb contains two specific binding sites for Hp, at amino acid residues β11–25 and β131–146, whereas the Hb α globin chain has one Hp-binding region, comprising residues α121–127 *[42]*. Hb αβ dimers bind stoichiometrically to Hp αβ subunits *[11]*. The Hp–Hb complex enhances the peroxidase activity of Hb *[7]*. The binding of myoglobin to Hp is relatively weak and quantitatively is much less important *[43]*.

After destruction of erythrocytes, free Hb in the circulation passes through the glomerular filter, and renal damage may occur. Hp reduces the loss of Hb and iron, because the Hp–Hb complex is not filtered through the glomeruli but is transported to the liver *[3]*. In physiological conditions, serum Hp is saturated when ~500–1500 mg/L free Hb is present *[25]*. The Hp–Hb complex is broken down in the parenchymal cells of the liver *[44, 45]*.

Hb binding depends not only on serum Hp concentration but also on Hp type *[46]*. The "clearance" of Hb, released into the circulation after intravascular hemolysis, is less effective in Hp 2–2 individuals *[46]*. Existing literature about the phenotype dependency of Hp–Hb binding is often confusing, if not conflicting. Because the α^1-chains of Hp are smaller than the α^2-chains, 1 g of Hp 1–1 contains more αβ subunits than 1 g of Hp 2–1 or Hp 2–2; therefore, 1 g of Hp 1–1 can bind more Hb than 1 g of one of the other phenotypes.

PROTECTION AGAINST FREE RADICALS

Free radicals such as superoxide ($O_2^{\cdot -}$) and hydroxyl ($^{\cdot}OH$) are extremely reactive molecules that can cause cell damage by peroxidation of membrane lipids *[47, 48]*. Free Hb promotes the accumulation of hydroxyl radicals, because iron can generate $^{\cdot}OH$ by means of the Fenton reaction: $H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^- + {}^{\cdot}OH$ *[49-51]*. Heme iron catalyzes the oxidation of low-density lipoproteins, which can damage vascular endothelial cells *[48]*. Oxidants generated by activated macrophages are involved in respiratory distress syndrome, acute tubular necrosis, and atherosclerosis *[52, 53]*. These dangers are reduced by the Hb-binding capacity of Hp *[54]*. However, Hp–Hb binding is phenotype-dependent (Table 3) *[46]*.

Breakdown of erythrocytes in the interstitial (e.g., intracerebral) fluid results in Hb-mediated $^{\cdot}OH$ formation. The distribution of highly polymeric Hp 2–2 proteins in extravascular fluids is restricted by their molecular mass *[4]*. Consequently, the antioxidative capacity of body fluids is less efficient in Hp 2–2 individuals *[4]*.

Hp-related protein (Hpr) appears to be involved in the free radical-mediated killing of trypanosomes *[55, 56]*. Endocytosis of the Hpr–Hb complex by the trypanosome causes iron toxicity through the formation of reactive free radicals *[55]*. Lipid peroxidation disrupts the lysosomal membranes, and the trypanosome is autodigested *[55]*.

INHIBITION OF NITRIC OXIDE

The highly reactive substance nitric oxide (NO) is produced by several types of human cells, including cytokine-activated macrophages *[57, 58]*. Large amounts of NO are cytotoxic and are associated with nonspecific defense against microorganisms *[58, 59]*. Pulses of low amounts of NO are involved in some regulatory events, such as maintaining vascular tonus. NO has been identified as the endothelium-derived relaxing factor (EDRF) *[60]*. Free Hb inhibits endothelium-dependent vasodilatation by means of a direct chemical interaction with EDRF *[61, 62]*. Endothelium-dependent relaxation of rabbit aorta strip preparations is rapidly inhibited by human plasma fractions containing Hp *[63]*. Purified Hp itself has no inhibitory effect on EDRF, but the Hp–Hb complex does *[63]*. This implies that, in the presence of Hp, EDRF has no intravascular downstream effect and that its physiological role is that of a local vasodilator *[63]*.

INHIBITION OF PROSTAGLANDIN SYNTHESIS

Hp is a member of the endogenous inhibitors of prostaglandin synthesis *[64, 65]*. As a consequence of Hb–Hp binding, the heme compounds that catalyze the oxidation of arachidonic acid by prostaglandin synthetase are removed *[4]*. The inhibitory effect of Hp on prostaglandin synthesis has important biological consequences, including an antiinflammatory action *[4]*. The inhibitory effects of Hp 2–2 and Hp 2–1 on prostaglandin synthesis are less pronounced than that of Hp 1–1 (Table 3) *[4]*.

Table 3. Functional properties of Hp phenotypes.

	Hp 1-1	Hp 2-1	Hp 2-2
Hemoglobin binding	Strong	Intermediate	Weak
Antioxidative capacity	Strong	Intermediate	Weak
Inhibition of prostaglandin synthesis	Strong	Intermediate	Weak
Angiogenic effect	Weak	Intermediate	Strong
Agglutination of *Streptococcus pyogenes* T4	—	Intermediate	Strong
Affinity towards CD22	Strong	Strong	Strong

In preterm infants with patent ductus arteriosus, use of prostaglandin synthetase inhibitors, such as indomethacin, has been proposed in an attempt to achieve medical ligation of the ductus [66]. Hp is a potent prostaglandin synthetase inhibitor but is absent from neonatal blood [12, 64, 65]. Accordingly, the use of Hp for the treatment of patent ductus arteriosus has been suggested [66].

BACTERIOSTATIC EFFECT

As a consequence of the capture of free Hb by Hp, heme iron is unavailable for bacterial growth [67]. An iron-restrictive environment established by Hp is part of the nonspecific defense against bacterial invasion. Rats inoculated intraperitoneally with pathogenic *Escherichia coli* and Hb are fully protected against lethality by simultaneous administration of Hp [68]. Some bacteria, e.g., *Neisseria meningitidis*, *Campylobacter jejuni*, *Bacteroides fragilis*, and *Vibrio vulnificans*, possess specialized iron-acquisition systems for survival in the host [69-72]. These microorganisms are capable of heme uptake from either Hb or the Hp–Hb complex.

ANGIOGENESIS

Angiogenesis plays an important role in a variety of physiological and pathological conditions, including tumor growth, wound healing, and chronic inflammatory diseases. Hp has been identified as one of the serum angiogenic factors required for proliferation and differentiation of endothelial cells in the formation of new blood vessels [73, 74]. Hp 2–2 is more angiogenic than the other phenotypes [73]. Increased serum Hp concentrations in chronic inflammatory and (or) ischemic conditions are important for tissue repair and promoting the growth of collateral vessels [73]. Furthermore, the neovascular growth-stimulating properties of Hp play a role in the development of maculopathy [75].

ANTIBODY-LIKE PROPERTIES

Köhler and Prokop have demonstrated that *S. pyogenes* group A, carrying the T4 antigen, can be agglutinated by human serum from Hp 2–2 and Hp 2–1 individuals, the Hp 2–2 serum having higher agglutination titers than the Hp 2–1 serum [76, 77]. This agglutination is performed by both Hp proteins, which behave like antibodies. In contrast, Hp 1–1 has no agglutination effect (Table 3). The agglutinating activity of Hp 2–2 and Hp 2–1 sera can be inhibited by adding Hp 1–1 serum before the test, leading Köhler and Prokop to postulate that Hp 1–1 represents a "blocking antibody" [76]. However, Hp is not a true antibody; it does not possess the highly variable antigen-binding sites characteristic for the Fab moiety of immunoglobulins. Also, Hp does not activate complement [77]. The agglutination of T4 antigen by Hp 2–2 and Hp 2–1 is probably mediated via binding with lectin-like structures.

INTERACTIONS WITH LEUKOCYTES

Hp has a negative effect on phytohemagglutinin-induced lymphoblast transformation [78], the inhibition being positively correlated with serum Hp concentration [78]. Hp also inhibits different forms of lectin-induced lymphocyte transformations [79, 80]. A lectin-like binding of Hp to lymphocytes has been postulated [4]. More recently, the β-chain of Hp has been demonstrated to bind to CD22, a B cell adhesion glycoprotein [81-83]. CD22 mediates B cell interactions with erythrocytes, T lymphocytes, monocytes, neutrophils, and endothelial cells by specific binding to glycoproteins with terminal α2–6-linked sialic acid residues [84-86]. Additionally, CD22 has a function in T cell activation via binding to CD45RO [87].

In human blood plasma, many glycoproteins with α2–6-linked sialic acids are present, but only IgM and Hp can selectively bind CD22 [81]. Hp inhibits the CD22 binding to TNF-α-activated endothelial cells of human umbilical veins [81]. Flow-cytometric analysis has shown that Hp types 1–1, 2–1, and 2–2 bind the cell surface of human B lymphocytes with equal affinity (Table 3) [88]. However, the saturation of CD22 molecules depends on Hp type because of differences in molar Hp concentrations required (Langlois M, et al., ms, submitted for publication).

A specific binding of Hp towards neutrophils has also been reported, in a demonstration that neutrophil respiratory burst activity can be inhibited by Hp [88]. Recent observations show that Hp is concentrated within granulocytes and monocytes and is exocytosed after neutrophil activation, suggesting that Hp concentrations may be enhanced locally at sites of inflammation to modulate granulocyte activity [89]. Apparently, Hp 1–1 is a ligand for the CD11b/CD18 integrin dimers on granulocytes and monocytes [90]. These integrins are involved in cell–cell and cell–matrix interactions, including binding to fibrinogen and to the cell surface molecule ICAM-1 (CD54) [82].

OTHER PROPERTIES

Hp exhibits an inhibitory effect on the activity of cathepsin B, a lysosomal protease [91]. In inflammatory processes and tissue injury where cathepsin B is liberated, increased Hp concentrations in plasma protect against active proteolysis.

Human gallbladder bile contains a group of concanavalin A-binding glycoproteins that have been reported to promote nucleation of cholesterol crystals [92]. At physiological concentrations in human bile (15 mg/L), Hp is a highly potent promoter of cholesterol crystallization and is potentially important in the formation of gallstones [92, 93].

Clinical Laboratory Aspects

MEASUREMENT OF HP CONCENTRATION

Initially, methods for determining Hp were based on enhancement of the peroxidase activity of Hb by Hp–Hb binding [94, 95]. Other methods are based on the altered spectrophotometric properties of Hp-bound Hb [96] or on the separation of the Hp–Hb complex from unbound Hb [5]. The results of these methods are expressed in Hb-binding capacity (grams or moles of Hb per liter of serum or plasma). Alternatively, serum Hp concentrations have been measured immunochemically by radial immunodiffusion (RID) [97, 98]. Because of such factors as the degree of polymerization, molecular mass, and diffusion rate, the RID method is dependent on Hp phenotype, and correction factors for each Hp phenotype have been applied to obtain the "true Hp concentration" [97, 98].

Immunonephelometric and immunoturbidimetric assays require much shorter time of analysis and allow the use of automated analyzers *[99-102]*, but Hp phenotype dependency has been observed in turbidimetric assays *[100]*. A microtiter ELISA system with *S. pyogenes* T4 antigen as solid phase has also been developed *[103]*.

DETERMINATION OF HP PHENOTYPE

Hp phenotype can be determined by starch gel electrophoresis of Hb-supplemented serum, followed by peroxidase staining *[7]*. Several studies of Hp phenotyping by means of isoelectric focusing in polyacrylamide gels containing urea or 2-mercaptoethanol, followed by immunoblotting, have also been reported *[34, 104]*. Hp phenotyping can also be performed with sodium dodecyl sulfate–polyacrylamide gel electrophoresis *[105]*. Immunoblotting with antibodies to Hp α-chain allows Hp subtypes and variants to be visualized more precisely *[34]*: Hp1F/Hp1F, Hp1S/Hp1S, and Hp1F/Hp1S (Hp 1–1); Hp2/Hp2 (Hp 2–2); and Hp1F/Hp2 and Hp1S/Hp2 (Hp 2–1).

Determination of Hp phenotypes and subtypes is commonly used in forensic medicine for paternity testing and individualization. The theoretical exclusion rate of the Hp system is ~0.184 *[106]*. This is rather high compared with the exclusion rate of blood group systems (ABO, Rh, MNSs, . . .). The Hp system therefore is a supplement to the blood groups and systems of human leukocyte antigens used in cases of disputed paternity *[106]*.

Further, the Hp system enhances the safety of the diagnosis of zygosity in twin studies. When only gender and blood group determinations are carried out, the probability of identity for dizygotic twins is 0.028 *[12]*. If Hp type is included, the probability decreases to 0.017 *[12]*.

Hp phenotyping may be necessary when uncorrected Hp concentrations obtained from RID or turbidimetric methods are below the reference range for healthy values *[100]*. In the case of a strongly hemolytic process, however, determination of Hp type will not influence the conclusion. Clinicians should be aware that some persons have no detectable Hp (anhaptoglobinemia), even when intravascular hemolysis is absent *[1-3]*. Hp 0–0 is rare in Caucasians and Mongoloids, but more frequent in some African populations (Table 2) *[18, 19]*.

REFERENCE VALUES

Since 1994, the concentration of Hp in serum can be expressed according to the new IFCC standardization *[107]*. The overall reference range of Hp in serum from adults is 0.38–2.08 g/L and remains constant throughout life *[25, 108]*. Reference values, however, differ between the three Hp types (Table 1) *[25]*. Small differences in Hp concentrations are observed between the genders (0.05–0.1 g/L, with values in females greater than in males; personal communication from a reviewer of this report).

No Hp can be detected in neonatal serum *[12, 109]* or in >50% of infants between ages 1 and 2 months *[12]*. By age 6 months, undetectable Hp is rare in Caucasians *[109, 110]*.

Serum Hp concentrations show no significant seasonal variation *[111]*. The between-subject variability in serum Hp is related to the status of various immunity markers (e.g., IL-6) *[111]*. Within-subject variability, on the other hand, is not related to IL-6 concentrations *[111]*.

EFFECTS OF HP POLYMORPHISM ON PERIPHERAL BLOOD ANALYTES

Associations between Hp 2–2 phenotype and high serum cholesterol concentrations have been reported but not confirmed *[112]*. Contrary relationships with high-density lipoprotein cholesterol have also been presented *[113, 114]*. Serum concentrations of LDL-cholesterol, triglycerides, and apolipoproteins are comparable for the three phenotypes *[114]*.

The gene encoding for lecithin:cholesterol acyltransferase is located near the *Hp* gene on chromosome 16 *[115]*. Subjects deficient in this enzyme show an increased Hp 1–1 frequency *[115]*. However, Hp type had no statistically significant effect on the degree of cholesterol esterification *[114]*.

Hp 2–2 is associated with higher serum albumin concentrations than are Hp 1–1 and Hp 2–1 *[4]*. Similar to Hp, albumin has an inhibitory effect on prostaglandin synthesis *[4]*. Concentrations of the copper-binding protein ceruloplasmin are also higher in serum from Hp 2–2 subjects *[4]*. Ceruloplasmin is an antioxidant, inhibiting the formation of superoxide radicals *[116]*. However, high concentrations of ceruloplasmin are unlikely to compensate for some effects of Hp 2–2 (i.e., less-efficient Hb-binding, protection from free radicals, or inhibition of prostaglandin synthesis) *[4]*.

Reference values for peripheral blood lymphocytes depend on Hp type, a result of the higher counts of circulating B lymphocytes and $CD4^+$ T cells in Hp 2–2 subjects (Langlois M, et al., ms. submitted for publication). However, we have observed no significant Hp-type-dependent variation in peripheral blood $CD4^+/CD8^+$ T cell ratio.

GLYCOSYLATION OF HP IN DISEASE

Abnormally fucosylated forms of the Hp β-chain (FHp) can be found in serum from patients with cancer, rheumatoid arthritis (RA), or alcoholic liver disease, including alcoholic cirrhosis *[117-120]*. FHp is useful for monitoring tumor burden and for discriminating between active and inactive RA: 94% of patients with active RA have high FHp vs only 5% in inactive RA *[119]*. A multiwell lectin-binding assay, using the fucose-specific lectin *Lotus tetragonolobus*, has been developed for measuring FHp in serum *[118]*. FHp gives fewer false-positive results than C-reactive protein does in cases of inactive RA *[119]*. However, the assay is time-consuming and not disease-specific, high concentrations of serum FHp being also found in some patients with osteoarthritis (10%) and seronegative polyarthritis (e.g., psoriatic arthritis, Reiter syndrome, and ankylosing spondylitis; 40%) *[119]*.

Clinical Applications

The concentration of Hp in serum decreases after intravascular hemolysis, whether immune (e.g., transfusion reactions), infec-

tious (e.g., malaria), hereditary (e.g., hemoglobinopathy), or mechanical (artificial heart valves, endocarditis, contact) *[25, 121]*. The amplitude of this decrease largely depends on the initial serum Hp concentration. After saturation of the Hb binding capacity, concentrations of serum hemopexin start to decrease, whereas the Hp concentration remains low (<0.3 g/L) *[25]*. Hemopexin binds free heme *[25]*. During intense hemolytic processes or in chronic hemolytic diseases, monitoring of serum hemopexin should be preferred to monitoring Hp *[25]*. In contrast to other markers for hemolysis (e.g., lactate dehydrogenase, potassium), Hp and hemopexin concentrations are not influenced by in vitro hemolysis.

Serum Hp concentrations decrease in malnutrition and chronic liver disease *[25, 121]*. The nephrotic syndrome may be associated with high or low concentrations of serum Hp, depending on the patient's Hp type and the supervening inflammation *[121]*. Hp 1–1 is small and therefore is excreted in urine of nephrotic syndrome patients, whereas Hp 2–1 and 2–2 are retained *[121]*.

Hp behaves like an acute-phase protein, its plasma concentration increasing in response to a variety of stimuli, e.g., infection, neoplasia, pregnancy, trauma, acute myocardial infarction, and other inflammatory reactions *[25, 121]*. Hyperhaptoglobinemia is also observed in inflammatory psychiatric disorders, such as major depression *[122]*. A positive relationship between serum Hp concentrations and immune activation (e.g., number of neutrophils, monocytes, and activated T cells) observed in major depression was explained by hypersecretion of IL-6 *[123]*. Unlike Hp, hemopexin is not an acute-phase protein. When hemolysis is associated with acute-phase reaction, mon-

Table 4. Clinical consequences of Hp polymorphism.

	Consequences	RR[a]	Ref.
Infections and vaccinations			
Tuberculosis	Hp 2-2 overrepresented among patients with advanced destruction and dissemination	1.23	*26, 124, 125*
Vaccination	Hp 2-2: stronger antibody response to typhus and tetanus vaccination		*126, 127*
	Hp 2-2: lowest antibody titers after vaccination against influenza and hepatitis B		*23, 128*
Viral hepatitis	Increased Hp^1 allele frequency in patients with chronic hepatitis B		*29*
	Hp 1-1: increased risk for chronic hepatitis C	1.55	*129*
HIV	Hp 2-2 associated with higher 5-year mortality (40% vs 20% in Hp 1-1 and 2-1)		*130*
Allergies	Hp 1-1 overrepresented in allergic contact dermatitis and allergic rhinitis	1.48	*4, 131*
	Decreased Hp 2-1 frequency in family history of bronchial asthma	0.60	*132*
Autoimmune diseases	Hp 2-2 overrepresented in family history of rheumatoid arthritis	1.37	*133*
	Hp 2-2 overrepresented in systemic lupus erythematosus	1.43	*134*
	Decrease of Hp 1-1 frequency in immune complex nephritis		*4*
Malignancies	Hp 1-1 overrepresented in breast cancer and cervix carcinoma	1.72	*135, 136*
	Low Hp 2-2 frequency in adenocarcinoma of the lung	0.66	*137*
	Low Hp 2-2 frequency in bladder carcinoma	0.76	*138*
	Excess of Hp 2-1 in patients with family history of ovarian carcinoma	1.25	*139*
Hematological diseases	Increased Hp^1 allele frequency in children from ABO-incompatible parents		*140, 141*
	High Hp 2-2 frequency in retinal detachment (hemorrhages)		*142*
	Hp 1-1 associated with sickle cell disease in US blacks	1.85	*143*
	Hp 1-1 frequency increased in acute myeloid leukemia,	1.58	
	acute lymphoid leukemia,	1.67	
	and chronic myeloid leukemia	1.46	*30*
	Hp 2-2 frequency decreased in IgA-myeloma	0.67	*144*
Cardiovascular diseases			
Sodium sensitivity	Hp 1-1 subjects are more sodium-sensitive than Hp 2-1 or 2-2	1.69	*145, 146*
Essential hypertension	Hp 1-1 is overrepresented	1.96	*105*
	Hp 2-2 patients need more intensive and more complex drug treatment, and show higher prevalence of coronary artery disease	1.34	
	and peripheral arterial occlusive disease	1.55	*114*
	Hp 2-2 patients show higher risk of developing refractory hypertension	1.51	*147*
Pregnancy-induced hypertension	Women with a Hp 2-2 type show higher risk	1.83	*148*
Coronary artery diseases	Hp 2-2 patients have more severe myocardial infarctions and complications (30%)		*149*
	Coronary lesions more pronounced in Hp 2-2 among coronary artery grafting patients		*150*
Psychiatric disorders	Major depression associated with increased Hp^1 allele frequency		*151*
	Hp 2-2 overrepresented in familial epilepsy, affective psychoses, and alcohol and drug abuse		*4*

[a] RR: relative risk = observed number for Hp type at risk/expected number for Hp type at risk.

itoring the changes in hemopexin concentrations is again preferable to monitoring Hp *[25]*.

Clinical Consequences of Hp Polymorphism

Hp polymorphism has an effect on the prevalence of many life-shortening conditions (Table 4) *[4, 23, 26, 29, 30, 105, 114, 124-151]*. The Hp 2–2 phenotype is overrepresented in autoimmune diseases, and Hp 2–2 subjects are characterized by a higher immune reactivity. Investigators have explained these observations by an insufficient inhibition of the prostaglandin-mediated inflammatory reaction, which should be taken into consideration when evaluating the immunogenicity of vaccines in different ethnic groups *[4]*. However, the immune reactivity depends primarily on the nature of the antigen, given that Hp 1–1 individuals show the highest antibody titers after hepatitis B or influenza vaccination *[23, 128]*. Remarkably, the antibody response to *Salmonella typhi* O-antigen is considerably reduced in hemolytic disease (e.g., acute malaria) *[152]*.

In families where the father is ABO-incompatible with the mother, children show a higher frequency of the Hp^1 allele than in families in which the parents are ABO-compatible *[140, 141]*. This has been explained as reflecting the frequency of deaths from hemolytic disease of the newborn when there is ABO incompatibility, and the fact that the product of the Hp^1 allele is more efficient than that of Hp^2 in removing dissolved Hb from the plasma *[141]*. Similarly, the Hb liberated intravascularly during retinal hemorrhages in Hp 2–2 patients is not eliminated completely because of the low Hb-binding capacity of Hp 2–2, resulting in further retinal complications *[142]*.

Associations between Hp phenotypes and atherosclerotic disorders have been demonstrated (Table 4). In aortic fatty streaks and fibro-fatty lesions, plasma proteins (including albumin and Hp β-chain) have been detected that are not present in healthy aortic intima *[153]*. However, it remains unclear whether the role of Hp in development of atheromatous lesions can be attributed to inhibition of local oxidative effects or to modulation of immune and inflammatory reactions *[154-156]*. The presence of a genetic influence (e.g., Hp type) is not surprising in families with high rates of acute myocardial infarction—apart from classical risk factors such as smoking, hypertension, or high serum cholesterol concentrations.

Hp type has an effect on acute inflammatory reactions associated with depression (Table 4). Increased prostaglandin E_2 concentrations are found in cerebrospinal fluid (CSF) from patients with unipolar depression *[157, 158]*. The "triggering" of postsynaptic receptors has been postulated to be influenced by prostaglandins *[4]*. As previously mentioned, the diffusion of Hp polymers into the CSF compartment depends on the molecular mass of the polymer, and Hp 2–2 concentrations in CSF are low *[4]*. The increased frequency of Hp 2–2 in familial epilepsy has been attributed to less-efficient inhibition of brain-lipid peroxidation *[159]*.

Hp polymorphism was a potent selective factor during the evolution of human beings *[2]*. The higher Hb-binding capacity of Hp 1–1 and the association of the Hp^2 allele with higher immune reactivity contributed to the selection and worldwide distribution of Hp alleles *[126]*. The balance between Hp^1 and Hp^2 alleles is achieved by the superior ability of Hp^1 to remove free Hb and the superior ability of Hp^2 to form antibodies. Turowska et al. *[160]* found that, in the elderly, the prevalence of Hp 1–1 and the appearance of anhaptoglobinemia are considerably increased. This observation supports the view that life expectancy may differ according to Hp phenotype.

Since the discovery of the molecular heterogeneity of Hp, many clinical studies have suggested effects of Hp polymorphism on a broad range of pathological conditions. Recent insights in the immunological function of Hp provide a theoretical background for the observed differences. In the future, further exploration of the role of Hp in the immune system will lead to a better understanding of the effect of Hp polymorphism in disease and will eventually contribute to a better-tailored treatment.

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