Haptoglobins and Rheumatic Fever*

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The haptoglobins are polymorphic α_2 -globulins which combine with free haemoglobin (Polonovski and Jayle, 1940). The usual method of haptoglobin typing with starch-gel electrophoresis reveals three phenotypes designated Hp 1-1, 2-1, and 2-2 (Smithies, 1955). A fourth phenotype in which no haptoglobin bands are detected has been classified Hp 0. Special methods reveal 15 possible haptoglobin phenotypes which are produced by the action of five autosomal, allelic, co-dominant genes (Hp^{1F} , Hp^{1S} , Hp^{2FF} , Hp^{2FS} , Hp^{2SS}) at a single locus (Connell *et al.*, 1962; Nance and Smithies, 1963). This polymorphism has been studied in many populations. Both racial and regional variations in gene frequencies have been reported (Parker and Bearn, 1961). There is no known reason for such variations, but it is suspected that the Hp^1 and Hp^2 genes are now or have in the past been influenced by one or more selective factors. Evidence for this hypothesis has been presented in a study (Workman *et al.*, 1963) in which the Hp^1 gene frequency differences between the West African and American negro populations could not be accounted for on the basis of gene migration alone. Their evidence suggests that the haptoglobin genes have different adaptive values in the two populations and that the polymorphism may possibly be unstable in the populations studied.

The level of haptoglobin in the blood is influenced by a variety of factors. These include inflammation, haemolysis, hormone administration, administration of pyrogens, and probably genetic constitution (Nyman, 1959). Variations related to sex (Nyman, 1959), age (Jayle and Moretti, 1962), and haptoglobin phenotype (Smith and Owen, 1961) have also been found. Mean levels of haptoglobin in relation to phenotype in decreasing order are: Hp 1-1 > Hp 2-1 > Hp 2-2.

Rheumatic fever is a disease which produces inflammatory changes in connective tissue. During the acute attack serum haptoglobin levels are markedly elevated (Owen *et al.*, 1964). This is probably due to the release of a material from the inflamed tissue which stimulates the hepatic synthesis of haptoglobin (Borel *et al.*, 1964). No information concerning the haptoglobin levels of individuals who have recovered from the acute attack of rheumatic fever is available. The present study was done to compare the distributions of haptoglobin phenotypes and haptoglobin levels of a suitable control population with those of a group of adults who had had one or more documented episodes of rheumatic fever or clinically proved rheumatic heart disease some time in the past.

Materials

The specimens used in this investigation were collected for a study of blood groups and secretor types in a rheumatic and non-rheumatic population (Dublin *et al.*, 1964). The character and source of the population of adult subjects and method of collection and handling of the specimens are described in that report. Since the spouse of each rheumatic-fever patient served

as a control, and since there is a preponderance of female patients on the registers of two of the sources of index cases, there is a much higher proportion of females in the rheumatic population and the similar preponderance of males in the control population. This difference is taken into account in the analysis of the data on quantitative haptoglobin.

After the earlier study the samples available were stored at -20°C . until they were examined in the present investigation.

Methods

Haptoglobin phenotyping was performed with horizontal starch-gel electrophoresis (Smithies, 1955) with the buffer system described by Poulik (1957). 0.1 ml. of plasma was mixed with 0.05 ml. of a haemoglobin solution (20 mg./ml.). Material from this mixture was taken up on 10-by-5-mm. pieces of Whatman No. 3 filter paper and inserted into a slot cut in the gel. After electrophoresis the gel was sliced and the cut surfaces were exposed to a benzidine-peroxide solution. This was prepared by dissolving 0.8 g. of benzidine base in 30 ml. of 95% ethanol, adding 2.5 ml. of glacial acetic acid, and, immediately before use, 5 ml. of 3% H_2O_2 . The haptoglobin phenotypes were recorded after maximum development of the haptoglobin bands.

Specimens with bands too faint to type were then subjected to electrophoresis with the use of Whatman No. 17 filter paper, which will absorb more plasma than the routinely used No. 3 paper. If the pattern of haptoglobin bands was still too weak to be typed, 0.1 ml. of the specimen mixed with 0.05 ml. of haemoglobin solution was placed in a collodion sac (Schleicher and Schuell), at the bottom of which was a 10-by-5-mm. piece of Whatman No. 17 filter paper. The solution was concentrated by ultrafiltration until all the concentrate was taken up on the filter paper. In this manner the specimens were subjected to approximately fivefold concentration. Specimens without detectable haptoglobin bands after this procedure were phenotyped as Hp 0.

Haptoglobin levels were measured by the method of Smith and Owen (1961) on all paired rheumatic and non-rheumatic subjects, provided that haemolysis was not visible in either serum specimen. To ensure accuracy of measurement a further control was added. A single serum specimen was divided into 100 (0.7-0.8 ml.) portions. The haptoglobin level of this specimen was determined in duplicate with each set of eight unknown specimens analysed. If measurements on the control specimen fell outside the accepted limits of error ($\pm 2.5\%$) the unknown specimens in that group were reanalysed. Specimens with haptoglobin levels less than 40 mg./100 ml. of serum were remeasured at a 2:5 rather than a 1:5 dilution. Specimens with levels above 250 mg./100 ml. of serum were remeasured at a 1:10 dilution.

Results

Table I summarizes the results of the haptoglobin phenotyping. Four hundred and eight specimens from rheumatic and an equal number from non-rheumatic subjects were

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examined. Three individuals of the rheumatic group and one of the non-rheumatic group had haptoglobin patterns that did not correspond to known haptoglobin types. One specimen had a faint band in the Hp 1-1 region and multiple faint bands in the Hp 2-2 region. Another had a heavy band in the Hp

TABLE I.—Distribution of Haptoglobin Phenotypes in 408 Rheumatic and 408 Non-rheumatic Subjects

Population	Haptoglobin Phenotype										Gene Frequency	
	1-1		2-1		2-2		0		Unclassified		Hp ¹	Hp ²
	N	%	N	%	N	%	N	%	N	%		
Rheumatic	45	11.0	183	44.9	167	40.9	10	2.5	3	0.7	0.346	0.654
Non-rheumatic	51	12.5	193	47.3	162	39.7	1	0.2	1	0.2	0.363	0.637

1-1 region and faint bands in the Hp 2-2 region (see Chart). Two other specimens (not shown on the Chart) had only one or two faint bands in the Hp 2-2 region even after fivefold concentration of the serum.

Diagram of a starch-gel electrophoresis comparing the patterns of two atypical haptoglobin phenotypes with those of the three common haptoglobin phenotypes.

The only significant difference between the rheumatic and non-rheumatic groups is in the increased frequency of individuals of type Hp 0 in the rheumatic population (Table I). The Hp 0 frequency in the control population (0.20%) is similar to the frequency (0.19%) previously reported in a Caucasian population by Galatius-Jensen (1958). The finding of 10 Hp 0 subjects in the rheumatic population of 408 individuals (2.5%) would not be expected on the basis of chance variation ($0.02 > P > 0.01$). No clinical evidence for haemolysis or liver disease was present in any of these Hp 0 individuals at the time of the study. Persons of type Hp 0 and those with unclassified haptoglobin phenotypes were omitted in calculating the gene frequencies of the populations. The gene frequencies conform to those expected in populations of European origin when calculated in this manner.

The rheumatic population was divided into groups, according to the predominant signs that the subjects had during their acute illness. This was based on information obtained from clinical records compiled during an earlier study (Dublin *et al.*, 1964). The frequency of the Hp 1-1 phenotype in a subpopulation of 54 rheumatic individuals who had Sydenham's chorea as the only acute manifestation of rheumatic fever was more than twice as great as the remainder of the rheumatic population (see Table II). The difference between these groups is statistically significant ($0.05 > P > 0.02$). A further subdivision of the rheumatic population according to the presence or absence of rheumatic heart disease revealed that among 82 rheumatic individuals without a history or physical finding of rheumatic heart disease there were no Hp 0 individuals. It is of interest that the 10 rheumatic individuals with the Hp 0 phenotype were found among the 326 individuals who had

either physical findings or a history of rheumatic heart disease, or both. None of the 82 individuals with a history of rheumatic fever but without cardiac sequelae was phenotyped Hp 0. The difference between these groups is not statistically significant ($0.3 > P > 0.2$).

TABLE II.—Distribution of Haptoglobin Phenotypes in Rheumatic Subjects who Presented only with Sydenham's Chorea Compared with the Remainder of the Rheumatic Group

	Haptoglobin Phenotype								Total	Gene Freq.
	1-1		2-1		2-2		0			Hp ¹
	N	%	N	%	N	%	N	%		
Chorea without A.R.F.	11	20.4	17	31.5	25	46.3	1	1.8	54	0.38
Other rheumatics ..	34	9.7	166	47.6	140	40.1	9	2.6	349	0.34

Quantitative Haptoglobin

Table III summarizes the results of the measurements of the haptoglobin levels. A total of 212 specimens were suitable for examination. The range of haptoglobin levels in 106 normal individuals was 24-260, with a mean of 87.8 (S.D. = ± 42.3), and in 106 rheumatics was 11-220, with a mean of 77.5 (S.D. = ± 40.5). Because the mean haptoglobin levels of men are reported to be somewhat higher than that of women, the data were analysed separately for each sex. Rheumatic and non-rheumatic females showed only a slight difference in mean haptoglobin levels.

TABLE III.—Mean Haptoglobin Levels of Rheumatics and Non-rheumatics, Male and Female

Group	N	Mean Hp Level*	S.D.	Range*
Rheumatic	106	77.5	40.5	11-220
Non-rheumatic	106	87.8	42.3	24-260
Rheumatic, female	75	76.5	40.6	11-220
Non-rheumatic, female	31	69.3	26.4	24-125
Rheumatic, male	31	80.1	40.6	13-182
Non-rheumatic, male	75	95.4	45.3	26-260

* Haptoglobin level is expressed in mg. of haemoglobin bound/100 ml. of plasma.

In agreement with reports in the literature we found that below age 50 the haptoglobin level was independent of age. We also verified that there is an increase in the mean haptoglobin level above age 50 (114 ± 58). The mean haptoglobin level in the male rheumatic group, all of whom were below age 50, was significantly lower than the mean level of the males in the control group (95.4 ± 45.3). This significance remains even when those males over 50 years of age are excluded (90.9 ± 41).

Four of the 11 Hp 0 specimens were unsuitable for measurement of haptoglobin levels because of haemolysis or insufficient plasma. Hp levels on the remaining seven specimens ranged from 9 to 12 mg. haemoglobin bound/100 ml. plasma. This substantiates the finding of others that upon the addition of haemoglobin there may be enhanced peroxidase activity in plasma even in the absence of benzidine-staining activity of starch gel.

As a consequence of the increased frequency of Hp 0 specimens in the rheumatic population it seemed indicated to examine both populations for the frequency of hypohaptoglobinaemia as defined by the haptoglobin level.

In choosing a suitable level indicative of abnormality we took into consideration the following facts. First, those individuals whose phenotypes were determined with difficulty had levels below 29. Secondly, in the normal control population only four individuals had haptoglobin levels below 30: one was Hp 2-2 and three were Hp 2-1. The lowest level in an Hp 1-1 individual was 53. Shinton *et al.* (1965), who used the same method that we have, chose a haptoglobin level of 40 as the index of abnormality because they found only one individual out of 110 normals with haptoglobin level below this point. The mean haptoglobin level for their population, however, was

110, which is slightly higher than ours. Finally, we noted that Nyman (1959), using a different method for measuring haptoglobin levels, found three individuals in her control group with levels below 30. We have therefore chosen a haptoglobin level of 30 or lower as abnormal for persons of type Hp 2-2 or Hp 2-1 and 40 or lower as abnormal for persons of type Hp 1-1.

These criteria were used to determine the number of hypohaptoglobinaemic individuals in the rheumatic and non-rheumatic populations (Table IV). Four times as many hypohaptoglobinaemic individuals were found in the rheumatic

TABLE IV.—Distribution of Hypohaptoglobinaemia According to the Presence or Absence of Rheumatic Fever

Haptoglobin Level	Rheumatics		Non-rheumatics	
	N	%	N	%
Hypohaptoglobinaemic ..	16	15.1	4	3.8
Not hypohaptoglobinaemic ..	90	84.9	102	96.2
	N = 106		N = 106	

Haptoglobin level < 30.

as in the non-rheumatic population. This difference is statistically significant ($0.01 > P > 0.001$). There is a slightly greater frequency of hypohaptoglobinaemia in the female rheumatic group (18%) than in the male (13%), but the difference is not statistically significant. There is, furthermore, a twofold to threefold greater frequency of hypohaptoglobinaemia in the rheumatics of each sex than in the non-rheumatics, but these differences are also not statistically significant. All of the hypohaptoglobinaemic rheumatic individuals were found in the portion of the rheumatic population with rheumatic heart disease.

No significant differences in mean haptoglobin levels were noted when the rheumatics were grouped according to major clinical signs. Though there were slight differences in mean haptoglobin levels ranging from 72 to 90, the numbers of subjects in the groups were small and the haptoglobin levels in any given group varied widely.

A comparison of the frequency of non-secretors was made in the normohaptoglobinaemic and hypohaptoglobinaemic members of both groups. Four hypohaptoglobinaemic (23.5%) and 63 normohaptoglobinaemic (39.6) individuals were non-secretors. There is a higher frequency of secretors in the hypohaptoglobinaemic group but the difference between the groups is not significant.

Discussion

The data presented here indicate that hypohaptoglobinaemia (including Hp 0) is found more often in a population of individuals who have had rheumatic fever and rheumatic heart disease than in a group of normal controls. The basis for this difference is not clear. An abnormally reduced level of plasma haptoglobin is a well-known sequel of acute or chronic haemolysis, or severe liver disease (Allison and ap Rees, 1957; Nyman, 1959). There was no evidence of these disorders in the hypohaptoglobinaemic individuals in our populations. On the other hand, the data might be interpreted to suggest that in some persons a lowered haptoglobin level is a consequence of having rheumatic fever or rheumatic heart disease. Since haptoglobin levels were not available on these subjects prior to their illness, we cannot definitely implicate this hypothesis. Another view, which is attractive and also consistent with the data presented here, is that hypohaptoglobinaemia may be a factor in producing susceptibility in some subjects to rheumatic fever and more particularly to rheumatic heart disease.

Despite the wide range of normality of the haptoglobin level, studies by Nyman (1959) showed that the haptoglobin level of a given individual is relatively constant. Furthermore, twin studies by Bayani-Sioson *et al.* (1962) support the hypothesis

that the haptoglobin level is inherited. One might reason that the inheritance of an abnormally low level of haptoglobin in itself produces, or is associated with something else which produces, increased susceptibility to rheumatic fever and rheumatic heart disease. Since such a small proportion (15.1%) of the rheumatic population is hypohaptoglobinaemic, this characteristic has somewhat limited applicability as an indicator of susceptibility to rheumatic fever. Until more is known of the actual metabolic interrelation between haptoglobin and connective tissue and the actual genetic control of the haptoglobin level, it would be premature to suggest ways in which rheumatic fever might influence haptoglobin levels or in which haptoglobin level might influence susceptibility to rheumatic fever.

In addition to the part that haptoglobin has been shown to play in the catabolism of haemoglobin (Yamaguchi *et al.*, 1961), it probably has other, as yet unknown, functions. Studies of patients who have persistent *anhaptoglobinaemia* due to chronic haemolysis, or presumed due to genetic factors, have revealed that they may become hyperhaptoglobinaemic during acute illness (Whitten, 1961; Laurell and Grönvall, 1962). This evidence and the fact that hypohaptoglobinaemic individuals appear to have no particular difficulty in catabolizing haemoglobin lend support to the hypothesis that binding of haemoglobin, resulting in the promotion of its catabolism, is probably only one physiological function of haptoglobin.

It is also not possible to draw conclusions from the increase in the frequency of the Hp 1-1 phenotype in choreic individuals. This is especially true since so little is known of the pathogenesis of chorea itself. The increase in Hp 1-1 frequency is not sufficient to produce a significant difference in *Hp*¹ gene frequency in choreics and other rheumatic groups. The explanation of the association, if it is supported by further investigations, will have to await more knowledge of the other biological functions of the haptoglobins.

The "atypical" or unclassified haptoglobin phenotypes noted here do not correspond to any already described (Beckman, 1959; Robson *et al.*, 1964). Those with one or two faint bands would be classified Hp 0 by Sutton and Karp (1964). They also comment that the usual Hp 1-1 phenotype can develop slower fainter bands which do not correspond to known type 2 Hp bands. Two of our atypical types may be examples of the latter phenomenon. This finding has not been checked on fresh specimens, and so no attempt has been made to fit our typical types in with the classification proposed by Robson *et al.* (1964).

Summary

Haptoglobin phenotypes of 408 rheumatic and 408 non-rheumatic individuals were determined. An increased frequency of the Hp 0 type was noted in the rheumatic population, and an increased frequency of the Hp 1-1 phenotype was found in the group of rheumatic subjects who presented with chorea as the sole manifestation of acute rheumatic fever. All the Hp 0 individuals in the rheumatic group had rheumatic heart disease.

Haptoglobin levels were determined on specimens from 106 rheumatic and 106 non-rheumatic subjects. A definite increase in the frequency of hypohaptoglobinaemia was noted in the rheumatic group.

The Hp 0 and hypohaptoglobinaemic subjects were confined to the group of rheumatics who had had cardiac sequelae.

It is not clear whether hypohaptoglobinaemia is a consequence of rheumatic fever and rheumatic heart disease in some individuals or whether it may be an indicator of susceptibility of some persons to rheumatic fever.

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Folic-acid Deficiency in Rheumatoid Arthritis: Relation of Levels of Serum Folic-acid Activity to Treatment with Phenylbutazone

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Anaemia is a well-recognized complication of rheumatoid arthritis. The anaemia is usually hypochromic and normocytic, and, though the mechanism responsible for its production is obscure, factors such as ineffective utilization of iron for erythropoiesis, haemolysis, and alimentary bleeding are probably important (Roy *et al.*, 1955; Alexander *et al.*, 1956). Macrocytic anaemia was considered rare until Partridge and Duthie (1963) reviewed the haematological data of more than 2,000 patients with the disease. They found megaloblastic anaemia in 1.38% of patients with rheumatoid arthritis compared with 0.27% in patients attending their clinic for other diseases with musculo-skeletal symptoms. Though Partridge and Duthie (1963) considered that deficiency of vitamin B₁₂ was the cause of the megaloblastic anaemia, Gough *et al.* (1964) found evidence of folic-acid deficiency in other patients with this disease.

Gough *et al.* (1964) assessed the folic-acid status of 46 patients with rheumatoid arthritis by microbiological assay of serum folic-acid activity and the estimation of the urinary excretion of formimino-glutamic acid (Figlu). Low serum levels of folic-acid activity were found in one-third of patients, and biochemical evidence of folic-acid deficiency in the form of increased excretion of Figlu was found in nearly three-quarters of them. These authors excluded from their survey patients taking anticonvulsant drugs, barbiturates, and phenylbutazone, because these drugs had been reported to produce megaloblastic anaemia (Mannheimer *et al.*, 1952; Badenoch, 1954; Hawkins and Meynell, 1958; Robson and Lawrence, 1959). The case of megaloblastic anaemia following the administration of phenylbutazone reported in the literature was from this unit. However, as this patient has since developed alcoholic cirrhosis, and because no further reports of the development of megaloblastic anaemia following the administration of this drug have appeared in the literature, a study of the effect of phenylbutazone on folic-acid metabolism has been made. The serum levels of folic-acid activity were estimated in two groups of patients with rheumatoid arthritis, one receiving treatment with

phenylbutazone and the other not receiving this drug. The results were also compared with those from patients with other forms of arthritis and rheumatism, both treated and untreated, and with results from normal people.

Material and Methods

Eighty patients (58 women, 22 men) were studied. Fifty had received phenylbutazone or oxyphenbutazone for at least three months and 30 had not been treated with these drugs for more than one year. There were 21 patients with rheumatoid arthritis in the treated group and 20 in the non-treated group. The remaining patients had a variety of diagnoses: osteoarthritis, intervertebral disk degeneration, gout, ankylosing spondylitis, and Pager's disease.

Each patient was examined and details of the duration and activity of the disease, drugs used in treatment, and dietary habits recorded. Blood was taken for haematological studies—haemoglobin estimation, red-cell indices, and blood film (Dacie, 1956), serum level of folic-acid activity (Herbert, 1961), and serum level of vitamin B₁₂ (Hutner *et al.*, 1956).

Results

Serum Folic-acid Activity.—The distribution of the levels of serum folic-acid activity is shown in Fig. 1. The level of serum folic-acid activity in 60 normal subjects ranged from 2.3 to 18.6 mμg./ml., with a mean of 7.5 mμg./ml. Levels of 2 mμg./ml. or lower were found in 13 of 41 patients with rheumatoid arthritis (31.7%). Of the patients with reduced levels eight were receiving phenylbutazone or oxyphenbutazone (eight of 21, or 38.1%), and five were not (five of 20, or 25%). This difference was not statistically significant ($P > 0.05$). The means of the levels of serum folic acid in patients with rheumatoid arthritis treated with phenylbutazone (3 mμg./ml.) and not treated (3.2 mμg./ml.) were also not significantly different. However, as a group the levels of patients with rheumatoid arthritis were significantly lower than those of the normal subjects.

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