Hemochromatosis: Association of Severity of Iron Overload with Genetic Markers

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James C. Barton[1], Leigh Harmon[2], Charles Rivers[2], Ronald T. Acton[2]

ABSTRACT: We postulated that the severity of iron overload in homozygous hemochromatosis probands is related to the expression of HLA-A3 or D6S105 allele 8. Therefore, we used these markers to characterize Alabama hemochromatosis probands and normal control subjects. We then quantified the blood removed by phlebotomy to exhaust body iron stores and maintain normal serum ferritin concentrations in our hemochromatosis probands. Induction and maintenance phlebotomy requirements were significantly greater in presumed HLA-A3 homozygotes or in D6S105 allele 8 homozygotes than in homozygous probands lacking these markers. Intermediate values were observed in probands who were HLA-A3 or allele 8 heterozygotes, respectively. We also analyzed data from males and females separately. Among subjects of the same sex, the induction and maintenance phlebotomy requirements in subjects presumed to be HLA-A3 homozygotes or in allele 8 homozygotes were greater than those of other groups. Our results support the hypothesis that the severity of iron overload in hemochromatosis is determined predominantly by genetic factors, and provide evidence that two or more mutations for hemochromatosis exist. However, the design of our study does not permit a distinction to be made between allelic and locus heterogeneity for the hemochromatosis gene(s).

Keywords: human leukocyte antigen (HLA) typing, microsatellite DNA markers, hemochromatosis, iron, iron overload

INTRODUCTION

Hemochromatosis is an autosomal recessive disorder which affects approximately 0.5% of Caucasians of European descent (1). Iron absorption in homozygotes is inappropriately high for body iron content, and many subjects have progressive iron deposition that causes injury to the liver, joints, pancreas, heart, and other organs (2). The hemochromatosis gene is linked to the human leukocyte antigen (HLA) region on chromosome 6, particularly the HLA-A locus, and a majority of homozygotes express HLA-A3 (3, 4). Based on our informal observations, we postulated that the severity of iron overload in homozygous hemochromatosis probands is related to expression of HLA-A3 and/or allele 8 at the microsatellite locus D6S105 (5). To test this hypothesis, we first determined the frequencies of these genetic markers in homozygous hemochromatosis probands and normal control subjects from Alabama to permit comparisons with findings from other geographic areas, and to estimate the location of the hemochromatosis gene(s) on chromosome 6. We then compared the severity of iron overload and parameters of iron metabolism in subgroups of Alabama probands stratified according to their HLA-A3 and D6S105 allele 8 phenotypes. Our results provide additional evidence that the severity of iron overload in hemochromatosis is determined predominantly by genetic factors. Our results also support the hypothesis that two or

[1] Department of Medicine, Brookwood Medical Center, Birmingham, Alabama;
[2] Immunogenetics Program and Departments of Microbiology, Epidemiology, and Medicine, University of Alabama at Birmingham, Birmingham, Alabama.
Reprint requests to: Dr. James C. Barton, Suite G-105, 2022 Brookwood Medical Center Drive, Birmingham AL 35209,
phone (205)877-2888, fax(205)877-2039

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more mutations for hemochromatosis exist, but do not permit a distinction to be made between allelic and locus heterogeneity.

METHODS

Patient Population and Definition of Hemochromatosis

The performance of this study was approved by the Research and Human Use Committees of Brookwood Medical Center and the University of Alabama at Birmingham. We used the working diagnostic criterion for hemochromatosis of the American College of Pathologists: elevated transferrin saturation (≥60% males, ≥50% females) on at least two occasions in the absence of other known causes (2). Iron overload was assessed using serum ferritin concentrations, analysis of hepatic biopsy specimens, and quantity of iron removed by phlebotomy, as described below in detail. Elevated values of hepatic iron index (≥1.9) were not used as a diagnostic criterion (2), and the hepatic iron index was not usually quantified in our patients because of the lack of availability of sufficient additional liver tissue to permit atomic absorption spectrometric analysis. Requiring an elevated hepatic iron index as a diagnostic criterion causes a selection bias for subjects with heavier iron loading, and tends to exclude hemochromatosis homozygotes who are younger, females, and subjects who possibly have hemochromatosis gene mutations which cause lesser degrees of increased iron absorption than found in "classical" cases. However, because some heterozygotes also develop iron overload (2), we cannot exclude the possible entry of some heterozygotes into our study. Subjects not included in our therapeutic phlebotomy analyses included those who had undergone community blood donation >5 units, received excessive medicinal iron, or consumed excess ethanol, or who had hemolytic anemia, porphyria cutanea tarda, hepatitis C, or other disorders known to affect iron absorption and/or metabolism (6-9). Unrelated hemochromatosis homozygotes (probands) diagnosed during routine medical care delivery in the greater Birmingham area were included; no screening of any group to diagnose hemochromatosis probands was performed. Normal subjects were randomly recruited from the same geographic area. All probands and normal control subjects were Caucasians.

HLA and D6S105 Phenotyping

HLA phenotypes of the A and B loci were determined by the microdroplet lymphocytotoxicity assay (10) using National Institutes of Health antisera, antisera from our own screening programs, those obtained by exchange with other laboratories, and trays obtained from One Lambda (Canoga Park, CA). Each specificity was assessed using at least two antisera. We defined presumed HLA-A3 homozygotes to be those subjects whose only identifiable HLA-A phenotype was HLA-A3. D6S105 phenotyping was performed using DNA isolated from peripheral blood buffy coats by inorganic salt extraction (11). Microsatellite alleles at D6S105 were amplified using the primers and conditions previously described (12). Primers were end-labeled with ^{32}P. Alleles were separated by electrophoresis on a 6% denaturing polyacrylamide gel containing an M13 sequencing ladder (Amersham Life Sciences, Arlington Heights, IL), and visualized by autoradiography. Allele size was determined by reference to the M13 sequencing ladder. The autoradiographs were read independently by two individuals; if the results were not concordant, the sample was processed again and re-typed.

Characterization of Iron Overload in Hemochromatosis Probands

Therapeutic phlebotomy. Excess iron in hemochromatosis homozygotes can be quantified by phlebotomy (13), and the rate of iron accumulation is age-dependent (14). To measure the severity of iron overload and its relative rate of accrual, we *a*) quantified induction phlebotomy as the units of therapeutic phlebotomy initially required to

Table 1. Frequencies of HLA-A3, -B7, and -B14 Phenotypes in Alabama Caucasian Subjects

HLA Phenotype	Hemochromatosis Probands, % (*n*)	Normal Control Subjects, % (*n*)	*p**	Odds Ratio
A3	62.5 (80/128)	27.4 (361/1,318)	0.00001	3.0
B7	42.2 (54/128)	25.6 (338/1,321)	0.00075	2.0
B14	18.0 (22/128)	6.4 (84/1,314)	0.00002	3.1
A3, B7	37.5 (48/128)	3.6 (48/1,318)	0.00001	15.9
A3, B14	10.2 (13/128)	1.1 (14/1,318)	0.00001	10.5

* value of *p* in comparison with normal control subjects.

reduce the serum ferritin concentration to ≤20 ng/ml after diagnosis; *b*) defined the therapeutic phlebotomy index = units of induction therapeutic phlebotomy ÷ age (years); and *c*) quantified maintenance phlebotomy as units of phlebotomy required to maintain the serum ferritin concentration at ≤50 ng/ml during the first year after completion of induction phlebotomy. One unit of blood is equivalent to ~200 mg of elemental iron (2).

General clinical evaluation. Medical history and examination pertinent to iron metabolism, and quantification of serum iron concentration, transferrin saturation, and serum ferritin concentration were performed by routine clinical methods (15). Liver specimens obtained by biopsy were prepared and evaluated for hepatocyte iron grade, cirrhosis, and other hepatic disorders as previously described (15). Many subjects underwent evaluations for arthropathy, diabetes mellitus, other endocrinopathy, and cardiac dysfunction, as appropriate (16-19).

Statistical Methods

The data set for HLA phenotype frequency analyses consisted of observations from 128 homozygous hemochromatosis probands and 1,318 normal control subjects. The D6S105 phenotype was determined in forty-five probands and in 95 normal control subjects, each randomly chosen. Descriptive results are expressed as the mean ± 1 S.D.; age in years, units of phlebotomy, and the corresponding S.D. were rounded to the nearest integer for presentation in tables. Comparative results were obtained using t-tests, Chi-square analysis, Fisher exact test, and Woolf's odds ratio, as appropriate (20, 21). A value of $p < 0.05$ was defined as statistically significant.

RESULTS

HLA and D6S105 Phenotyping

HLA-A3, -B7, and -B14 phenotypes were significantly more frequent among our probands than among normal control subjects (Table 1). Likewise, co-expression of HLA-A3 and -B7 and of HLA-A3 and -B14 occurred with significantly greater frequencies in hemochromatosis probands than in normal control subjects (Table 1). D6S105 allele 8 was observed in 73.3% (33/45) of hemochromatosis probands and in 34.7% (33/95) of normal control subjects ($p = 0.00005$, odds ratio = 5.2). Twenty-three (51.1%) of the hemochromatosis probands expressed both HLA-A3 and D6S105 allele 8. However, 22.2% of our probands expressed neither HLA-A3 nor D6S105 allele 8.

Relationships of HLA and D6S105 Phenotypes and Severity of Iron Overload

Forty-three of our 45 hemochromatosis probands who underwent D6S105 typing also completed a therapeutic phlebotomy program. Among

Table 2. Clinical Features of Hemochromatosis Probands and HLA-A Phenotype*

	Presumed HLA-A3 Homozygotes (n = 9)	HLA-A3 Heterozygotes (n = 16)	HLA-A3 Negative (n = 18)
Age, years	49 ± 14	48 ± 11	47 ± 10
Induction phlebotomy, units	41 ± 34	23 ± 19†	16 ± 10†§
Therapeutic phlebotomy index	0.87 ± 0.80	0.49 ± 0.37†	0.39 ± 0.35†§
Maintenance phlebotomy, units	3 ± 2	2 ± 1	2 ± 1†
Serum iron, µg/dL	219 ± 51	199 ± 36	194 ± 59
Transferrin saturation, %	85 ± 18	73 ± 21	68 ± 24
Serum ferritin, ng/ml	1,160 ± 1,012	931 ± 1,182	773 ± 659
Hepatocyte iron grade 3 or 4, %	66.7	56.3	61.1
Hepatic cirrhosis, % (n)	56 (*5*)	19 (*3*)	33 (*6*)
Arthropathy, % (n)	44 (*4*)	31 (*5*)	22 (*4*)
Diabetes mellitus, % (n)	11 (*1*)	0	6 (*1*)
Hypogonadism, % (n)	33 (*3*)	0	0
Cardiomyopathy, % (n)	11 (*1*)	0	0

* Results are expressed as mean ± SD (range). All parameters except therapeutic phlebotomy data represent the findings at the time of diagnosis of hemochromatosis.
† value of $p < 0.05$ in comparison with presumed HLA-A3 homozygotes.
§ value of $p < 0.05$ in comparison with presumed HLA-A3 heterozygotes.

these, probands presumed to be homozygous for HLA-A3 required significantly more therapeutic phlebotomy to reduce serum ferritin to ≤20 ng/ml and had significantly higher values of therapeutic phlebotomy index than did probands who lacked HLA-A3 expression. Probands heterozygous for HLA-A3 had intermediate values (Table 2). Corresponding results were observed when maintenance phlebotomy requirements were tabulated (Table 2). These 43 subjects were then re-stratified according to D6S105 phenotype. Probands homozygous for D6S105 allele 8 required significantly more therapeutic phlebotomy and had significantly higher values of therapeutic phlebotomy index than did probands who lacked allele 8; probands heterozygous for allele 8 had intermediate values (Table 3). Corresponding results were also observed for maintenance phlebotomy among these subjects (Table 3). Other parameters of iron metabolism and overload in these respective groups revealed similar trends, but the differences were not statistically significant (Tables 2, 3). We also analyzed data from males and females separately. Among subjects of the same sex, the induction and maintenance phlebotomy requirements and therapeutic phlebotomy indices in subjects presumed to be HLA-A3 homozygotes or in allele 8 homozygotes were greater than those of other groups (Tables 4, 5). Consistent with previous observations, males had approximately twice as much mobilizable iron, on the average, as females (22).

DISCUSSION

Expression of HLA-A3 was observed in 62% of our Alabama probands, and is in agreement with corresponding values of 59%, 62%, 78%, and 79% reported for homozygotes in Utah, Australia,

Table 3. Clinical Features of Hemochromatosis Probands and D6S105 Phenotype*

	Allele 8 Homozygotes (n = 12)	Allele 8 Heterozygotes (n = 20)	Allele 8 Negative (n = 11)
Age, years	47 ± 11	47 ± 11	51 ± 10
Induction phlebotomy, units	37 ± 35	23 ± 13	12 ± 6†§
Therapeutic phlebotomy index	0.81 ± 0.77	0.52 ± 0.34†	0.25 ± 0.13†§
Maintenance phlebotomy, units	3 ± 2	2 ± 1	2 ± 1
Serum iron, μ/dL	191 ± 47	213 ± 36	194 ± 67
Transferrin saturation, %	75 ± 24	78 ± 19	65 ± 23
Serum ferritin, ng/ml	1,264 ± 1,495	746 ± 523	829 ± 734
Hepatocyte iron grade 3 or 4, %	58.3	60.0	72.7
Hepatic cirrhosis, % (*n*)	42 (*5*)	15 (*3*)	55 (*6*)
Arthropathy, % (*n*)	33 (*4*)	25 (*5*)	36 (*4*)
Diabetes mellitus, % (*n*)	8 (*1*)	0	9 (*1*)
Hypogonadism, % (*n*)	25 (*3*)	0	0
Cardiomyopathy, % (*n*)	8 (*1*)	0	0

* Results are expressed as mean ± SD (range). All parameters except therapeutic phlebotomy data represent the findings at the time of diagnosis of hemochromatosis.
† value of $p < 0.05$ in comparison with D6S105 allele 8 homozygotes.
§ value of $p < 0.05$ in comparison with D6S105 allele 8 heterozygotes.

Table 4. Phlebotomy Requirements, Sex, and HLA-A3 in Homozygous Hemochromatosis Probands*

	Presumed HLA-A3 Homozygotes	HLA-A3 Heterozygotes	HLA-A3 Negative
Males (*n*)	5	11	12
Induction phlebotomy, units	53 ± 39	29 ± 21	19 ± 10†
Therapeutic phlebotomy index	1.13 ± 1.01	0.59 ± 0.40	0.48 ± 0.38†
Maintenance phlebotomy, units	4 ± 3	3 ± 1	2 ± 1†
Females (*n*)	4	5	6
Induction phlebotomy, units	25 ± 23	11 ± 4§	11 ± 10§
Therapeutic phlebotomy index	0.54 ± 0.31	0.28 ± 0.17	0.23 ± 0.22§
Maintenance phlebotomy, units	2 ± 1	1 ± 1§	1 ± 1†§

* There were no significant differences in mean age of probands among these groups.
† value of $p < 0.05$ in comparison with presumed HLA-A3 homozygotes.
§ value of $p < 0.05$ in comparison with corresponding data from males.

Table 5. Phlebotomy Requirements, Sex, and D6S105 Allele 8 in Homozygous Hemochromatosis Probands*

	Allele 8 Homozygotes	Allele 8 Heterozygotes	Allele 8 Negative
Males (*n*)	7	13	8
Induction phlebotomy, units	48 ± 40	28 ± 12	14 ± 6†
Therapeutic phlebotomy index	1.04 ± 0.93	0.64 ± 0.34	0.30 ± 0.13†§
Maintenance phlebotomy, units	4 ± 2	3 ± 1†	2 ± 0†§
Females (*n*)	5	7	3
Induction phlebotomy, units	22 ± 22‡	15 ± 10‡	12 ± 6
Therapeutic phlebotomy index	0.47 ± 0.33‡	0.44 ± 0.42‡	0.25 ± 0.13‡
Maintenance phlebotomy, units	2 ± 1‡	1 ± 1‡	1 ± 0‡

* There were no significant differences in mean age of probands among these groups.
† value of $p < 0.05$ in comparison with D6S105 allele 8 homozygotes.
§ value of $p < 0.05$ in comparison with D6S105 allele 8 heterozygotes.
‡ value of $p < 0.05$ in comparison with corresponding data from males.

Wales, and France, respectively (1,3,5,23). HLA-A3 was detected in 22% to 29% of normal subjects in these geographic areas (1,3,5,23), in comparison with 27% in our Alabama normal control subjects. However, co-expression of HLA-A3, -B7 was detected in 38% of Alabama hemochromatosis probands, in comparison with values of 22%, 25%, and 45% observed in Australian, French, and Welsh subjects with hemochromatosis, respectively (23-25). Thus, our Alabama probands and normal control subjects are similar to those from other geographic areas with respect to HLA-A3 expression. However, co-expression of HLA-A3, -B7, often considered to be part of the ancestral hemochromatosis ancestral haplotype (23,26), is more prevalent among Alabama probands than among hemochromatosis homozygotes in some other population groups. HLA-A3 and D6S105 allele 8 have both been reported to be on the ancestral haplotype (23,26). Because these two alleles are in linkage disequilibrium, one would expect that both would be associated with hemochromatosis. However, this haplotype was not observed in all patients, and some patients possessed only one of these markers.

The results of some studies suggest that the gene lies between the HLA-A and HLA-B loci (27). Others suggest a location telomeric to the HLA-A locus (28-30). That 28% of our probands lacked expression of HLA-A3, -B7 and HLA-A3, -B14 implies that the gene does not lie within the ~1500 kb interval defined by the HLA-A and -B loci (31). Among our probands, the association of D6S105 allele 8 with hemochromatosis was stronger than with HLA-A3, but less so than in Australian and Welsh patients (5,23). Among these latter subjects, a hemochromatosis candidate gene region of ~3000 kb extends from centromeric of the HLA-A locus to the telomeric limit of the D6S105 allele 8 locus (5,23,26). That 22% of our patients expressed neither HLA-A3 nor D6S105 allele 8 suggests that the hemo-chromatosis gene is not within this interval and, perhaps, lies telomeric to the D6S105 locus. More extensive microsatellite marker typing in the Welsh population also indicates that the hemochromatosis gene locus is telomeric to D6S105 (32). Taken together, these genetic marker data support the hypothesis that allelic and/or locus heterogeneity for hemochromatosis occurs on Ch6p. This would explain the association of genes at extreme ends of a rather large region of 5 cM (32), and observa-

tions that patients with two copies of the ancestral haplotype defined by a ~3000 kb region (D6S248: D6S105) tend to store more iron than subjects heterozygous for the ancestral haplotype (26). If there were only one hemochromatosis gene, the severity of iron overload in probands homozygous for all markers that define the ancestral haplotype should not differ from that of probands who do not possess all of the markers. Based on comparisons with other common autosomal recessive disorders, it seems likely that there are multiple mutations which cause hemochromatosis (33). Recently, a gene with structural similarities to the MHC class I family has been identified which contains two missense alterations, one of which was homozygous in 83% of hemochromatosis patients (34). However, 12% of hemochromatosis subjects possessed neither of these two mutations (34). The region to which the mutations were mapped lies more than 500 kB distal to the D6S105 marker (35). The association of HLA-A3 and markers such as D6S105 allele 8 with the hemochromatosis gene(s), separated by a relatively large amount of DNA, is due to linkage disequilibrium that exists in the Ch6p region surrounding the major histocompatibility complex. Taken together, these data also support the hypothesis that allelic and locus heterogeneity occur among hemochromatosis genes.

Excessive iron absorption and consequent iron overload are direct phenotypic expressions of hemochromatosis, particularly in homozygotes. However, iron absorption is also influenced by dietary iron content and other nutritional variables, blood loss, and hormonal factors (36). We have demonstrated significant differences in the severity of iron overload among unrelated probands stratified according to their expression of phenotypes HLA-A3 or D6S105 allele 8. These results are best explained by the existence of two (or more) hemochromatosis mutations which augment iron absorption by the intestine to different degrees. In β2 knockout mice, increased iron absorption from ordinary diets and the development of iron overload is also attributed to the effects of a multigene family located in the major histocompatibility complex (MHC), which corresponds to the HLA region of the human genome (37). Among our male probands, the severity of iron overload was greater, on the average, than in females, in accordance with hormonal and other sex-associated differences in iron intake, absorption, and loss (36). However, significant differences in iron overload among subjects stratified by genetic markers could still be discerned. Further, the predominant factor in determining the concordance of iron overload severity in HLA-identical sibling hemo-chromatosis homozygotes is also genetic (38). Taken together, these observations suggest there is allelic and/or locus heterogeneity of the hemochromatosis gene(s), and that the iron absorption and phenotypic variability among hemochromatosis probands is more attributable to genetic influence than to other factors.

The serum ferritin concentration, hepatocyte iron grade, and hepatic iron index measure the relative severity of iron overload in hemochromatosis homozygotes. Among our groups of probands stratified by genetic markers, the mean ferritin concentrations and hepatocyte iron grades were not significantly different. However, these parameters indicated trends which corresponded with the significant differences in mean units of phlebotomy and mean phlebotomy iron indices. Among Australian hemochromatosis patients who expressed two copies of a common, ancestral haplotype, there was a significantly greater mean hepatic iron index in comparison with those heterozygous for or those without this haplotype. Further, there were differences between the hepatic iron indices of Australian males and females (26) which correspond to the differences in units of blood removed by phlebotomy in our male and female Alabama probands stratified by genetic markers. Therefore, the hepatic iron index may distinguish among groups of hemochromatosis patients stratified by genetic markers, but the other commonly measured parameters of iron stores appear to be less sensitive and less specific than quantification of excess body iron by phlebotomy.

We evaluated target organ injury characteristic of iron overload, and found that cirrhosis and

arthropathy occurred with similar frequencies in all probands stratified by HLA or D6S105 phenotypes. Thus, iron overload is sufficiently great in many probands at the time of routine clinical diagnosis to cause these complications (2). Diabetes mellitus occurred among a minority of probands in all phenotype groups, consistent with observations in recent case series (2). Although deposition of iron in the pancreatic islet cells may cause or exacerbate diabetes mellitus (39), this complication is more strongly associated with a family history of diabetes mellitus than with hemochromatosis (40,41). Hypogonadism and cardiomyopathy are often associated with severe iron overload, particularly among younger subjects (42-44). Our results suggest that probands positive for HLA-A3 or D6S105 allele 8 phenotypes could be at greater risk for these complications. Altogether, statistically significant differences in target organ manifestations of iron overload among probands stratified by HLA or D6S105 phenotypes possibly occur. However, greater numbers of subjects than were analyzed in the present study would be required to substantiate this.

Our data were derived from analysis of older, largely symptomatic, individuals diagnosed to have hemochromatosis during routine medical care. Subjects with hemochromatosis identified by screening programs are, on the average, younger and less likely to have symptoms and/or iron overload (2). Therefore, study of such homozygotes might reveal smaller differences in units of blood removed by phlebotomy and therapeutic phlebotomy index than we observed in our probands. However, our results suggest that analysis of HLA-A3 and D6S105 phenotypes of young, asymptomatic homozygotes could permit identification of those subjects at greatest risk to develop severe iron overload.

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