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Co-selection of the *H63D* mutation and the *HLA-A29* allele: a new paradigm of linkage disequilibrium?

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Abstract The major histocompatibility complex (MHC) shows a remarkable conservation of particular HLA antigens and haplotypes in linkage disequilibrium in most human populations, suggesting the existence of a convergent evolution. A recent example of such conservation is the association of particular *HLA* haplotypes with the *HFE* mutations. With the objective of exploring the significance of that association, the present paper offers an analysis of the linkage disequilibrium between *HLA* alleles or haplotypes and the *HFE* mutations in a Portuguese population. Allele and haplotype associations between *HLA* and *HFE* mutations were first reviewed in a population of 43 hemochromatosis families. The results confirmed the linkage disequilibrium of the *HLA* haplotype *HLA-A3-B7* and the *HLA-A29* allele, respectively, with the *HFE* mutations *C282Y* and *H63D*. In order to extend the study of the linkage disequilibrium between *H63D* and the *HLA-A29*-containing haplotypes in a normal, random population, an additional sample of 398 haplotypes was analyzed. The results reveal significant linkage disequilibrium between the *H63D* mutation and all *HLA-A29*-containing haplotypes, favoring the hypothesis of a co-selection of *H63D* and the *HLA-A29* allele itself. An insight into the biological significance of this association is given by the finding of significantly higher $CD8^+$ T-lymphocyte counts in subjects simultaneously carrying the *H63D* mutation and the *HLA-A29* allele.

Keywords *HFE* · *HLA* · Linkage disequilibrium · MHC · $CD8^+$ T lymphocytes

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Introduction

The *HFE* gene is located on the short arm of Chromosome 6 (6p21.3) approximately 4 Mb telomeric to *HLA-A* (Feder et al. 1996). *HFE* encodes a 343 amino acid protein that exhibits significant amino acid identity to the HLA class-I molecules (Feder et al. 1996). A particular mutation in this gene has been associated with hereditary hemochromatosis (HH), a single nucleotide change at codon 282 resulting in an amino acid substitution of a cysteine for a tyrosine (*C282Y*) (Feder et al. 1996). Homozygosity for the *C282Y* mutation is present in the majority of HH Caucasian patients screened (for review see Merryweather-Clarke et al. 2000; Porto and De Sousa, 2000). A second mutation, resulting in a substitution of aspartic acid for histidine at position 63 (*H63D*), is found at polymorphic frequencies in control Caucasian populations and is present at lower frequencies in non-Caucasian populations (Cullen et al. 1998; Merryweather-Clarke et al. 1997). The highest allelic frequencies (>20%) of this mutation were found in the Iberian peninsula (Merryweather-Clarke et al. 1997; Porto et al. 1998; Sanchez et al. 1998).

In spite of the large physical distance between *HFE* and *HLA*, linkage disequilibrium has been demonstrated between particular *HLA-A* and *-B* and the *HFE* mutations. HH has been known for more than 20 years to be associated with the *HLA-A3* allele and with the *HLA-A3-B7* or *HLA-A3-B14* haplotypes in particular (Simon et al.

Table 1 Antigens and haplotypes found in linkage disequilibrium with the *HFE* mutations in chromosomes carrying *C282Y* or *H63D*. Statistical comparisons were done between the chromosomes carrying *HFE* mutations (*C282Y* or *H63D*) and the chromosomes without *HFE* mutations, using the chi-squared test. Significance levels (*P*) are indicated by *asterisks*

Associated HLA antigen / *HLA* haplotype	Chromosomes carrying the *HFE* mutations		Chromosomes not carrying *HFE* mutations (*n*=80)
	C282Y (*n*=44)	*H63D* (*n*=26)	
A3	19/44 (43%)*	1/26 (4%)	6/80 (7.5%)
B7	9/44 (20.5%)**	3/26 (11.5%)	4/80 (5%)
A3B7	7/44 (16%)*	0	1/80 (1.3%)
A29	0	7/26 (27%)*	2/80 (2.5%)
B44	8/44 (18%)	6/26 (23%)	9/80 (11%)
A29B44	0	3/26 (11.5%)[a]	1/80 (1.3%)

*$P<0.005$; **$P<0.02$

[a] Not tested, expected values <5 in two cells

1976). The strong linkage disequilibrium between the *C282Y* mutation and the extended haplotype containing the *HLA-A3* allele was a posteriori confirmed by others (Ajioka et al. 1997; reviewed in Jaswinska 2000; Thomas et al. 1998). More recently, linkage disequilibrium was found between the *H63D* and the *HLA-A29* allele in patients with non-classical forms of iron overload (Porto et al. 1998) and in control groups (Mullighan et al. 1998; Murphy et al. 1998).

In humans, linkage disequilibrium among MHC class I genes is known to be maintained over a large physical distance, but the nature of the underlying biological basis to explain this disequilibrium is not clear. The analysis of the linkage disequilibrium between *HFE* mutations and *HLA* offers a sound approach to clarify that question considering the physical distance of 4 Mb between the genes involved. In the present study, we explored the hypothesis that the presence of the *HFE* mutations may influence the maintenance of particular *HLA* haplotypes in linkage disequilibrium. To test this hypothesis, haplotype analysis involving *HLA-A* and *-B* alleles and *HFE* mutations was done first in a population of 107 members of 43 families from the north of Portugal, with various forms of iron overload, and also in a selected sample of 398 chromosomes from a normal, random population selected from the same geographical region. Strong linkage disequilibrium between *H63D* and the *HLA-A29* allele itself was confirmed. To address the question of the biological significance with regard to both our earlier work demonstrating a significant association between lymphocyte numbers and the phenotypic expression of iron overload in humans (reviewed in De Sousa et al. 2000; Porto et al. 1998, 2001), and in experimental models of iron overload (De Sousa et al. 1994; Levy et al. 2000; Fleming et al. 2001; Santos et al. 2000; Sproule et al. 2001; Ten-Elshof et al. 1999), we examined lymphocyte subpopulations in normal subjects based on the presence or absence of the *H63D* mutation and the *HLA-A29* allele. Significantly higher $CD8^+$ T lymphocyte counts were observed in subjects simultaneously carrying the *H63D* and the *HLA-A29* alleles.

Materials and methods

Data source

Haplotype analysis in families of patients with iron overload

HLA types and *HFE* genotypes from hemochromatosis patients and family members were accessed through the files from the Hemochromatosis Outpatient Clinic database at Santo António General Hospital, Porto, as described in previous studies (Porto et al. 1997, 1998). For the present analysis we selected all families in which both *HLA* typing and *HFE* genotyping were available. A total of 43 families was selected: 22 were families of patients with hereditary hemochromatosis linked to the *C282Y* mutation and 21 families from patients with non-classical forms of hemochromatosis. Haplotype assignment was done by segregation analysis. Within a family, each defined haplotype was taken only once. From the total of 43 families reviewed, only 150 haplotypes could be assigned with certainty (Table 1).

Haplotype analysis in control families according to the presence or absence of the H63D *mutation*

In order to test the linkage disequilibrium between the *H63D* mutation with the *HLA-A29* and *-B44* alleles and with the *HLA-A29-B44* haplotype in a normal population, an anonymous sample was selected from a random population of 587 unrelated *HLA* haplotyped family members of bone marrow recipients from whom DNA was available. *HLA* class I typing was done by standard serological methods (see Methods). Haplotypes were defined by family segregation. The information from each sample was kept in a database and the only accessed information regarding the samples was the record number and the corresponding *HLA* haplotypes, constituting the sampling base. From this base, all the samples carrying the *HLA-B44* allele and/or the *HLA-A29* allele were selected, corresponding to a total of 187 samples from unrelated subjects. These were genotyped for the *H63D* mutation (see below). In the case of homozygosity for the presence or absence of the *H63D* mutation, each chromosome was defined for the *HLA-A-B-HFE* haplotype (with no further studies). In the case of *H63D* heterozygosity, additional samples from other family members were genotyped in order to define the chromosomes carrying the *H63D* allele by family segregation. To attain this goal 210 additional samples from family members were tested for the *H63D HFE* mutation permitting the assignment of the 374 haplotypes from index cases and 24 additional haplotypes from family members, giving a total of 398 chromosomes (Tables 2, 3). The frequencies of the *HLA-A29* and *HLA-B44* alleles in the starting population (0.060 and 0.152, respectively) did not differ from those previously described in another population of 312 unrelated

Table 2 Frequencies of the *H63D* mutation according to the *HLA* haplotypes in the normal population, and respective linkage disequilibria (*D* and *D'*). *NS* Not significant

	Number	*H63D* frequency	Linkage disequilibrium		Level of significance
			D	*D'*	
HLA haplotypes					
All containing *B44*	162	0.216	0.004	0.012	NS
All containing *A29*	62	0.435	0.036	0.290	0.00003
Non*A29*-non*B44*	203	0.148	–0.043	–0.106	NS
HLA A29-B44	29	0.345	0.010	0.172	0.054
HLA A29-B[a]	33	0.515	0.026	0.394	0.00037
HLA A[b]*-B44*	133	0.188	–0.006	–0.023	NS

[a] Other *A29* containing haplotypes
[b] Other *B44* containing haplotypes

Table 3 Frequencies and 95% confidence intervals (*CI*) for *HLA A* and *-B* alleles and haplotypes in a large sample of normal chromosomes based on the absence (*H63D*$^-$) or presence (*H63D*$^+$) of the *HFE* mutation *H63D. N* Number of chromosomes; (frequency), allele frequency

	Chromosomes with or without *H63D*				Level of significance
	H63D$^-$ (*n*=316)		*H63D*$^+$ (*n*=82)		
	N (frequency)	95% CI	*N* (frequency)	95% CI	
Alleles					
HLA B44	127 (0.402)	[0.348, 0.456]	35 (0.427)	[0.320, 0.534]	NS
HLA A29	35 (0.111)	[0.076, 0.145]	27 (0.329)	[0.228, 0.431]	0.000004
Haplotypes					
HLA A29-B44	19 (0.060)	[0.034, 0.086]	10 (0.121)	[0.051, 0.193]	0.055
HLA A29-B[a]	16 (0.050)	[0.026, 0.075]	17 (0.207)	[0.119, 0.295]	0.000005
HLA A[b]*-B44*	108 (0.342)	[0.289, 0.394]	25 (0.305)	[0.205, 0.405]	NS

[a] Other *A29* containing haplotypes were as follows. In *H63D*$^-$ chromosomes: *A29-B45* (*n*=3), *A29-B14* (*n*=3), *A29-B49* (*n*=2), *A29-B8* (*n*=2), *A29*-B51 (*n*=2), *A29-B63* (*n*=1), *A29-B62* (*n*=1), *A29-B13* (*n*=1), *A29-B40* (*n*=1). In *H63D*$^+$ chromosomes: *A29-B51* (*n*=4), *A29-B45* (*n*=2), *A29-B8* (*n*=2), *A29-B7* (*n*=2), *A29-B37* (*n*=2), *A29-B35* (*n*=2), *A29-B60* (*n*=2), *A29-B13* (*n*=1)

[b] Other *B44* containing haplotypes were: in *H63D*$^-$ chromosomes: *A2-B44* (*n*=54), *A3-B44* (*n*=11), *A24-B44* (*n*=10), *A23-B44* (*n*=8), *A28-B44* (*n*=7), *A1-B44* (*n*=6), *A11-B44* (*n*=5); *A31-B44* (*n*=24), *A25-B44* (*n*=1), *A26-B44* (*n*=1), *A32-B44* (*n*=1), *A33-B44* (*n*=1), *A34-B44* (*n*=1); in *H63D*$^+$ chromosomes: *A2-B44* (*n*=10), *A11-B44* (*n*=5), *A24-B44* (*n*=3), *A23-B44* (*n*=2), *A28-B44* (*n*=3), *A1-B44* (*n*=1), *A31-B44* (*n*=1)

Table 4 Total lymphocyte, CD4$^+$ and CD8$^+$ T-cell counts in subjects according to the presence or absence of the *HLA-A29* and *HFE H63D* alleles. *NS* Not significant

HLA background	*HLA-A29*$^+$			*HLA-A29*$^-$		
HFE genotype	*H63D*$^+$ (*n*=14)	*H63D*$^-$ (*n*=7)	Level of significance	*H63D*$^+$ (*n*=33)	*H63D*$^-$ (*n*=43)	Level of significance
Total lymphocytes (x10^6 cells/ml)	2.37±0.55[a]	2.15±0.45	NS	2.19±0.48	2.04±0.55	NS
CD8$^+$ T lymphocytes (x10^6 cells/ml)	0.61±0.22	0.29±0.07	0.001	0.47±0.18	0.44±0.20	NS
CD4$^+$ T lymphocytes (x10^6 cells/ml)	1.00±0.26	0.89±0.31	NS	0.94±0.33	0.88±0.27	NS

[a] Arithmetic mean ± standard deviation

individuals from the north of Portugal (0.061 and 0.140, respectively) (Alves et al. 2001). As expected, the *HLA-A29* and *-B44* alleles were in linkage disequilibrium in the population studied (*D*=0.020; *D'*=0.392) (Imanishi et al. 1992). The selected sample was shown a posteriori to be also similar to the normal random Portuguese population in terms of the overall frequency of the *H63D* mutation (see results).

Analysis of total lymphocyte, CD4$^+$ and CD8$^+$ T-cell counts based on the presence or absence of the H63D *mutation and the* HLA-A29 *allele*

Total lymphocyte counts and those of CD4$^+$ and CD8$^+$ T cells from normal *HLA* and *H63D*-typed subjects included in previous studies (Arosa et al. 2000; Porto et al. 1997, 1998), were reviewed from the files of the Hemochromatosis Outpatient Clinic database at Santo António General Hospital, Porto. Exclusion criteria were the presence of positive viral markers for the following viruses: HCV, HBV, HIV I and II, and HTLV. Carriers of the *C282Y* mutation were also excluded. Ninety-seven subjects comprising normal random subjects (*n*=49) and family members of hemochromatosis patients without iron overload (*n*=48) were selected: 47 were carriers of the *H63D* mutation (of these 47, 14 were also carriers of the *HLA-A29* allele); 50 did not carry either of the two *HFE* mutations (of these 50, 7 were also carriers of the *HLA-A29* allele) (Table 4).

Statistical analysis

Haplotype analysis in families of patients with iron overload

The frequencies of the relevant HLA antigens and haplotypes were tested in groups of identified chromosomes carrying the

C282Y, *H63D* mutations in comparison with chromosomes not carrying these two *HFE* mutations (Table 1). The significance of the differences was tested using the chi-squared test with Yate's correction when the expected value in one cell was <5.

Haplotype analysis in control families based on the presence of the H63D *mutation*

Frequencies of the *H63D* mutation were compared among groups of chromosomes according to *HLA* type (Table 2). Linkage disequilibrium between the alleles was estimated by the standard *D* and *D'* values, where *D* is the difference between the observed and expected frequencies of the two alleles in the same chromosome, and *D'* is the estimated *D* value divided by the *D* maximum. The significance of the association was tested using the chi-squared test. The relative frequencies of the *HLA-A* and *HLA-B* alleles and *HLA-A-B* haplotypes were estimated in the two groups of chromosomes divided according to the presence or absence of the *H63D* mutation (Table 3); 95% confidence intervals were calculated for each proportion as well as for differences among proportions. Differences between proportions were tested using the approximation to the normal distribution.

Analysis of total lymphocyte counts, and CD4⁺ and CD8⁺ T cells based on the presence or absence of the H63D *mutation and the* HLA-A29 *allele*

In general, all parameters tested (total lymphocytes, $CD4^+$ and $CD8^+$ T lymphocytes) showed a normal distribution. The mean values and standard deviations were then calculated in the different groups according to the presence or absence of the *H63D* mutation and the *HLA-A29* allele. Differences between the means were tested using the *t*-test (Table 4).

Methods

HFE *genotyping*

The *HFE* genotyping was done using two commercial kits (Haemochromatosis gene mutation assay I and II, ViennaLab, Vienna, Austria). Briefly, sequences of exon 4 (for *C282Y*) or exon 2 (for *H63D*) of the *HFE* gene were amplified in vitro and terminally labeled with fluorescein as a reporter molecule. The amplification products were alkali-denatured, and 25 µl aliquots were selectively hybridized to allele-specific (wild type or mutant) oligonucleotide probes immobilized in two separate cavities of a microwell plate. After hybridization and stringent washes at 37°C, bound sequences were detected using a horseradish peroxidase-labeled anti-fluorescein antibody and color reaction with tetramethylbenzidine. The methodology as well as its validation on samples of known genotype (RFLP-typed) and the application for typing have been presented elsewhere (Oberkanins et al. 1998).

HLA-A *and* -B *typing*

HLA typing was performed in freshly collected venous blood samples by the standard complement-dependent micro-lymphocytotoxicity assay using a battery of sera which enabled the definition of the following HLA antigens: *A* locus: 1, 2, 3, 9 (23, 24, 25), 10 (26, 34), 11, 28, 29, 30, 31, 32 and 33; *B* locus: 5 (51), 7, 8, 12 (44, 45), 13, 14, 15 (62, 63), 16 (38, 39), 17 (57, 58), 18, 21 (49, 50), 22 (55), 27, 35, 37, 40 (60).

Peripheral blood T-cell phenotyping

Staining was done in whole peripheral blood cells, after erythrocyte lysis. Three milliliters of blood was fixed with an equal volume of formaldehyde (0.4%) for 4 min at 37 C. The red blood cells were then lysed (with 50 ml of lysis solution: 10 mM Tris, 0.15 mM NH_4Cl, pH 7.4, for 10 min at 37 C). Cells were washed twice in PBS supplemented with 0.1% NaAz and 2% of BSA (PBS-BSA). After the final washing 5×10^5 cells were stained in round-bottom 96-well plates in a total volume of 50 µl as follows: 25 µl of each appropriately diluted antibody was added to each well (anti-CD3-FITC, anti-CD4-PE, anti-CD8-PE; DakoPats-Denmark), and left for 30 min on ice in the dark, with gentle shaking. Cells were then washed twice with PBS-BSA and fixed in a final volume of 500 µl of PBS containing 0.1% paraformaldehyde and 0.1% NaAz. At least 2×10^4 lymphocytes were analyzed in Facscan (Becton and Dickinson) for determination of the percentage of $CD4^+CD3^+$, and $CD8^+CD3^+$ populations within the total lymphocytes. The total numbers of $CD4^+$ and $CD8^+$ T cells were then estimated from the total lymphocyte counts.

Results

Linkage disequilibrium between *HLA* haplotypes and *HFE* mutations in families of patients with iron overload

This analysis focused on the study of haplotypes involving *HFE*, *HLA-A* and *HLA-B*. For this purpose, segregation analysis was performed in 43 families allowing the identification of 44 chromosomes carrying the *C282Y* mutation, 26 chromosomes carrying the *H63D* mutation and 80 chromosomes carrying the wild-type *HFE* allele. The results are summarized in Table 1. In this population, the linkage disequilibrium between the *HLA-A3-B7* haplotype and the *C282Y* mutation was confirmed ($P<0.005$), as well as the linkage disequilibrium between the *HLA-A29* allele and the *H63D* mutation ($P<0.02$). The frequency of the haplotype *HLA-A29-B44* was higher in chromosomes carrying the *H63D* mutation than in wild-type alleles. The low number of representatives in two cells of chromosomes carrying the *HLA-A29-B44* haplotype does not allow us to measure a statistical significance. Therefore, we performed analysis of these alleles and haplotypes in a larger sample of non-hemochromatosis-associated chromosomes.

Haplotype analysis in control families based on the presence or absence of the *H63D HFE* mutation

The association of the *H63D* mutation with particular *HLA* alleles and haplotypes was analyzed in 187 samples from normal, unrelated subjects selected for the presence of the alleles *HLA-A29* and *-B44*, and in 210 of their relatives (see Materials and methods). In total, 398 chromosomes were defined. Of these, 82 were carriers of the *H63D* mutation and 316 were negative for the mutation. The overall frequency of the *H63D* mutation found in this selected population (0.206) was similar to that observed in the normal, random Portuguese population (Cardoso et al. 2001). However, the *H63D* frequency varied according to the presence of the *HLA-A29* allele (Table 2). Significantly higher *H63D* allele frequencies were found in all chromosomes with *HLA-A29* carrying

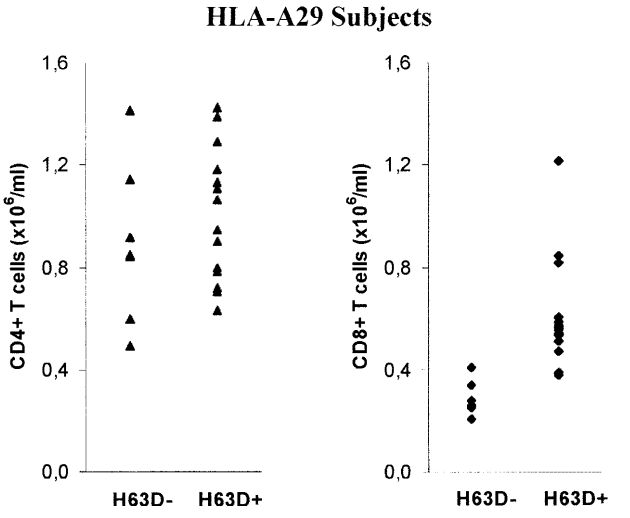

Fig. 1 CD4⁺ and CD8⁺ T cell subpopulations in *HLA-A29* subjects according to the presence (*H63D*⁺) or absence (*H63D*⁻⁻) of the *H63D HFE* mutation. Significantly higher CD8⁺ T cell numbers but not CD4⁺ T cell numbers were observed in *HLA-A29* subjects which were carriers of the *H63D* mutation. No significant differences in CD4⁺ and CD8⁺ T cell numbers were observed among subjects based on the presence or absence of any of the alleles alone (data not shown)

haplotypes (0.435, P=0.00003), particularly in those haplotypes carrying *HLA-A29* without *B44* (0.515, P=0.00037). No differences were seen in *H63D* frequencies in relation to the *HLA-B44* allele. A more detailed description of *HLA* alleles and haplotypes in chromosomes grouped according to the presence or absence of the *H63D* mutation is given in Table 3. No significant differences were observed for the *HLA-B44* frequencies in the two groups of chromosomes. A strong and significant enrichment of the *HLA-A29* allele and, consequently, all *HLA-A29*-carrying haplotypes, was observed in chromosomes carrying the *H63D* mutation (P<0.00001). The most common single haplotype is *HLA-A29-B44*, as expected by the linkage disequilibrium that exists between *HLA-A29* and *HLA-B44* in the general population, and also in this particular population (see Materials and methods). Therefore, the difference between the two groups of chromosomes in terms of the frequency of *HLA-A29-B44* is not so marked as for other *HLA-A29* containing haplotypes, which are uncommon in chromosomes without *H63D* (frequency=0.050), and significantly enriched in *H63D*-carrying chromosomes (frequency=0.207). In those haplotypes, no specific *HLA-B* allele was seen to be more significantly associated with *HLA-A29* (see Table 3). No differences were observed between the two groups of chromosomes for other *HLA-B44* containing haplotypes. Altogether, the results demonstrate the strong linkage disequilibrium between the *H63D* mutation and the *HLA-A29* allele itself (D=0.036; D'=0.290; P=0.00037) in a control Portuguese population, and do not confirm the hypothesis that the *H63D* mutation influences the linkage disequilibrium between the *HLA-A29* and *B44* alleles.

Analysis of total lymphocyte, and CD4⁺ and CD8⁺ T-cell counts based on the presence or absence of the *H63D* mutation and the *HLA-A29* allele

As a result of the strong linkage disequilibrium between the two distant alleles *HLA-A29* and *H63D*, we addressed the question of a possible interaction between *HFE* and *HLA* by examining lymphocyte numbers based on the presence or absence of the relevant alleles. The results are summarized in Table 4. Statistically significant, higher average numbers of CD8⁺ T lymphocytes were observed in subjects carrying both *HLA-A29* and the *H63D* mutation ($0.61\pm0.22\times10^6$ cells/ml) when compared with subjects carrying *HLA-A29* without an *HFE* mutation ($0.29\pm0.17\times10^6$ cells/ml) (Table 4). No effect was seen on CD4⁺ T cell numbers (Table 4). This result is illustrated in Fig. 1.

Discussion

The conjecture that some alleles would be associated more frequently than expected based on chance was put forward by Dausset some years ago (referred to in Dausset 1998). Many examples of linkage disequilibria have since been described among the alleles within the *HLA* system, apparently as normal polymorphisms. We confirm the linkage disequilibrium between the *HLA-A3-B7* and the *C282Y* mutation of the *HFE* gene, and demonstrate in a large sample of normal chromosomes the linkage disequilibrium between the *HLA-A29* allele and the *H63D* mutation. In the case of the *C282Y-A3-B7* linkage disequilibrium, a founder effect and a genetic drift are presently the most widely accepted explanations, assuming a recent age for the *C282Y* mutation (Ajioka et al. 1997; Thomas et al. 1998). In contrast, the *H63D* mutation is thought to be older in origin based on the high allele frequencies reported and a wider geographical distribution (reviewed in Merryweather-Clarke et al. 2000; Porto and De Sousa 2000). Thus, the maintenance of its linkage disequilibrium with *HLA-A29* needs a more elaborate explanation. In general, linkage disequilibrium is expected to diminish with recombination map distance (Abecasis et al. 2001; Cargill et al. 1999; Kruglyak 1999; Reich et al. 2001). Strong linkage disequilibrium at a large physical distances could be explained by: (1) a low recombination rate in the chromosomal region; (2) a strong founder effect by a recent allele/mutation; or (3) selection. The MHC-class I region has approximately one fifth of the expected recombination rate under the usual rule that 1 Mb corresponds to approximately 1 cM (Martin et al. 1995). This has been confirmed for a 6 Mb region containing *HFE* (Malfroy et al. 1997). However, in our view, it is improbable that the low recombination rate of the region could suffice to explain the strong linkage disequilibrium observed with this particular allele, knowing that both the *H63D* and *HLA-A29* alleles are common and distributed worldwide. This also argues against a recent founder effect. Thus, an additional alter-

native explanation could be the co-selection of this particular combination of alleles imposed by some biological advantage. In this context, the present finding of significantly higher numbers of $CD8^+$ T lymphocytes in *HLA-A29* subjects carrying the *H63D* mutation is of considerable interest. The influence of MHC-class I antigens on the setting of CD4:CD8 ratios and T-lymphocyte numbers is documented in mice (van Meerwijk et al. 1998), rats (Damoiseaux et al. 1999) and in humans (for review see Price et al. 1999). To our knowledge, this is the first demonstration of the impact of an *HLA-A* allele (*HLA-A29*) and a mutation in a non-classical MHC-class I gene located 4 Mb away (*H63D*) on lymphocyte numbers. The consistent finding of a phenotypic association between low lymphocyte numbers and high hepatic iron storage in HH patients (Porto et al. 1997, 1998, 2001) and in lymphocyte-defective knockout mice (De Sousa et al. 1994; Santos et al. 1996, 2000), led us to the present finding of significantly higher numbers of $CD8^+$ T cells in *HLA-A29* normal subjects carrying the *H63D* mutation. This observation may give us some insight into the mechanism whereby the lymphocytes could contribute to the regulation of iron metabolism. The postulated influence of the *H63D* mutation on the regulation of the transferrin receptor-mediated iron uptake (Feder et al. 1998) occurring in a specific MHC class I background could contribute both to the setting of $CD8^+$ numbers and to the regulation of transferrin iron loading. Activated T lymphocytes express transferrin receptors (Pattanapanyasat and Hoy 1991). Both activated and non-activated T lymphocytes synthesize ferritin but do not secrete (Dorner et al. 1980; Pattanapanyasat and Hoy 1991; Pollack et al. 1983). Lymphocytes could therefore act as a "mobile" and easily "mobilizable" iron-storage compartment protecting from iron-mediated toxicity, as originally postulated by De Sousa (1978). Finally, we could speculate that in human evolution individuals with higher numbers of $CD8^+$ T lymphocytes might have been better equipped to survive life-threatening viral epidemics. Further studies are currently being done to clarify the expression of -related proteins and genes in lymphocytes.

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