Positive Darwinian Selection Promotes Charge Profile Diversity in the Antigen-binding Cleft of Class I Major-Histocompatibility-Complex Molecules[1]

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Certain major-histocompatibility-complex (MHC) loci are highly polymorphic, and the mechanism of maintenance of this polymorphism remains controversial. Recent studies of the pattern of nucleotide substitution at MHC loci have produced strong evidence that this polymorphism is maintained mainly by positive Darwinian selection that operates on the antigen recognition site (ARS) of the MHC molecule. The ARS of the class I MHC consists of three subregions: (1) the binding cleft, (2) T-cell-receptor-directed residues, and (3) outward-directed residues. Here we report that the rate of nonsynonymous nucleotide substitution is much higher in the binding cleft than in the other ARS subregions. Furthermore, nonsynonymous nucleotide substitutions that result in a change of residue side-chain charge occur significantly more frequently than expected by chance. We conclude that the main target of positive selection on the class I MHC molecules is the binding cleft of the ARS and that this selection acts primarily to promote diversity among alleles with respect to the pattern of residue side-chain charges (charge profile) in the binding cleft. These results provide additional support for the hypothesis that MHC polymorphism is maintained by overdominant selection relating to antigen-binding capacity and thus to disease resistance.

Introduction

Classical class I major-histocompatibility-complex (MHC) glycoproteins are expressed on all nucleated cells in vertebrates and function to present intracellularly processed foreign peptides to cytotoxic T cells, thereby triggering the cytotoxic reaction against cells infected by intracellular pathogens (Klein 1986; Lawlor et al. 1990). These molecules are highly polymorphic in mice, humans, and many other species, and numerous hypotheses have been proposed to explain this polymorphism (Nei and Hughes, accepted). Recent evidence has favored the hypothesis that class I MHC polymorphism is maintained by overdominant selection (heterozygote advantage) relating to the antigen-binding function of the molecule. This hypothesis was originally proposed by Doherty and Zinkernagel (1975) on the basis of their observation that different MHC class I allelic products differ in their ability to present different foreign peptides to T cells. Thus they argued that, in a population exposed to numerous

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pathogens, individuals heterozygous at MHC loci would have an advantage. Foreign peptides are bound and presented to T cells in a region of the class I MHC molecule known as the antigen recognition site (ARS), consisting of two α helices bordering a β-pleated sheet (Bjorkman et al. 1987*a*, 1987*b*). When Hughes and Nei (1988) compared rates of nucleotide substitution in the ARS and in other regions of human and mouse class I MHC genes, they found that the rate of nonsynonymous substitution exceeds that of synonymous substitution in the ARS. In other gene regions, they found that the rate of synonymous substitution exceeds that of nonsynonymous substitution, as is true of most genes. This unusual pattern of nucleotide substitution is evidence of positive Darwinian selection acting on the ARS and supports the hypothesis that MHC polymorphism is due to overdominant selection (Hughes and Nei 1988).

In the present paper, we extend the analysis of nucleotide substitution in class I MHC by analyzing rates of synonymous and nonsynonymous substitution separately for three functionally distinct subregions of the ARS. In addition, we examine the extent to which patterns of amino acid replacement in the ARS are nonrandom with respect to amino acid side-chain properties. Monos et al. (1984) noted that amino acid differences between class I MHC allelic products often involve charge differences. Since charged residues are known to play a major role in molecular recognition, Monos et al. suggested that differences with respect to the pattern of residue charges in different MHC molecules might be functionally significant. Garrett et al. (1989), in comparing crystallographic structures for two allelic products at the *HLA-A* locus, also drew attention to the potential importance of differences in the pattern of amino acid residue charges in accounting for differences that allelic products show in their abilities to bind different foreign peptides. To determine whether natural selection acts on this aspect of the ARS, we developed a method to test whether amino acid replacements involving a charge change occur at a greater rate than expected by chance, and we applied this method to the analysis of class I MHC DNA sequences from the human and the mouse.

DNA Sequences Analyzed

The class I MHC molecule consists of three extracellular domains (α_1, α_2, and α_3), a transmembrane portion, and a cytoplasmic tail. The α_3 domain (encoded by exon 4 of the gene) is highly conserved; in vivo it associates noncovalently with β-2 microglobulin (Klein 1986). The α_1 and α_2 domains (encoded by exons 2 and 3, respectively) include the 57 residues which compose the ARS (Bjorkman et al. 1987*a*, 1987*b*). Parham et al. (1988) divided the ARS into three functionally distinct subregions: (1) the *binding cleft,* in which foreign peptides are believed to be bound (29 residues), (2) *TCR-directed residues,* which are directed toward the T cell receptor (TCR) and presumably interact with it (18 residues), and (3) *outward-directed residues,* which point away from both the binding cleft and the TCR (10 residues). In the present paper we analyzed rates of nucleotide substitution in these three ARS subregions and in the remainder of the α_1 and α_2 domains for 44 alleles from the human *HLA-A, -B,* and *-C* loci (Cianetti et al. 1989; Ellis et al. 1989; Mizuno et al. 1989; Ooba et al. 1989; Parham et al. 1989) and for 10 alleles from the mouse *H-2K* and *-L* loci (Kuhner and Goodenow 1989).

Results

Rates of Nucleotide Substitution in ARS Subregions

In pairwise comparisons among alleles at human and mouse class I MHC loci, we estimated the rate of synonymous substitution per synonymous site (d_S) and the

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rate of nonsynonymous substitution per nonsynonymous site (d_N) in the three ARS subregions and in the remainder (outside the ARS) of $\alpha_1-\alpha_2$. We used Nei and Gojobori's (1986) method (also see Nei 1987, pp. 73–76) to estimate d_S and d_N; and we estimated the standard errors (SEs) of mean d_S and mean d_N by Nei and Jin's (1989) method. In all cases, d_N is higher in the binding cleft than in all other regions; and in both human and mouse, mean d_N in the binding cleft is significantly higher than mean d_S (table 1). In the human data, mean d_N in the binding cleft is more than twice as high as mean d_N in the TCR-directed residues and is more than seven times as high as that in either the outward-directed residues or the remainder of $\alpha_1-\alpha_2$. Similarly, in the mouse, mean d_N in the binding cleft is ~1.8 times as high as that in the TCR-directed residues and is more than five times as high as that in either the outward-directed residues or in the remainder of $\alpha_1-\alpha_2$.

In the TCR-directed residues, d_N is somewhat elevated in comparison with that in either the outward-directed residues or the remainder of $\alpha_1-\alpha_2$. In this subregion, d_N is always higher than d_S, but this difference is not statistically significant in most cases (table 1). The number of codons in this subregion is small, and thus the SEs of d_S and d_N are large. However, d_N in the TCR-directed residues is generally significantly lower than d_N in the binding cleft (table 1). In the outward-directed residues, d_N is very similar to that in the remainder of $\alpha_1-\alpha_2$. These results clearly show that positive selection is focused mainly on the binding cleft of the ARS.

In the human data, d_S is very low in both the TCR-directed and outward-directed residues (table 1). The reason for the unusually low d_S values in these regions is not clear; a highly biased pattern of codon usage in these subregions in human alleles (unpublished data) seems to be partly responsible. There is no reduction of d_S in these subregions in the mouse.

In the comparison between human and mouse alleles, d_N remains highest in the binding cleft; but in this case d_N is no longer significantly different from d_S in either the binding cleft or any other ARS subregion. The main reason for this seems to be a leveling off of d_N in the binding cleft. In the interspecific comparison, the ratio of d_N in the binding cleft to d_N in the TCR-directed residues is only ~1.4:1, as opposed to an average ratio of about 2:1 in intralocus comparisons (table 1).

Conservative and Radical Amino Acid Replacements

If positive selection with respect to foreign peptide binding capacity operates in the binding cleft, it is expected that the types of amino acid replacement occurring in this cleft will not be random. Given the importance of charged residues in protein-protein binding (Monos et al. 1984), we predicted that selection relating to foreign peptide binding should favor allelic variation in the pattern of residue charges (charge profile) in the binding cleft. Under this type of selection, nonsynonymous nucleotide substitutions causing charge changes are expected to occur more often than if nonsynonymous substitution occurred at random. To test this hypothesis, we developed a method to compute rates of conservative and radical nonsynonymous substitution with respect to a given amino acid property, extending Nei and Gojobori's (1986) method of estimating rates of synonymous and nonsynonymous nucleotide substitution.

Nei and Gojobori's method divides nucleotide sites into synonymous and nonsynonymous sites. If both synonymous and nonsynonymous substitutions occur at a site with certain probabilities, the site is divided into fractional synonymous and nonsynonymous sites with the corresponding probabilities. In our new method for studying protein evolution, some qualitative side-chain property of amino acids [e.g., charge,

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Table 1
Mean ± SE d_S and d_N in Different Regions of Human and Mouse Class I MHC Genes

	Region[a]							
	Binding Cleft (N = 29)		TCR Directed (N = 18)		Outward Directed (N = 10)		α_1–α_2 (N = 125)	
Locus (no. of alleles)	d_S	d_N	d_S	d_N	d_S	d_N	d_S	d_N
Human:								
A (16)	5.9 ± 3.7	15.1 ± 2.8**	2.3 ± 1.9	8.7 ± 2.7	0.0 ± 0.0	1.1 ± 1.1†††	3.1 ± 1.1	1.3 ± 0.4†††
B (20)	9.0 ± 4.1	23.3 ± 3.0**	1.5 ± 1.6	7.8 ± 2.4*,†††	1.3 ± 1.4	2.9 ± 2.1†††	5.2 ± 1.3	2.2 ± 0.5†††
C (8)	5.9 ± 3.6	11.6 ± 2.5	0.0 ± 0.0	3.8 ± 2.0†	0.0 ± 0.0	0.0 ± 0.0†††	7.1 ± 1.5	2.9 ± 0.6†††
Mean	7.6 ± 3.8	19.4 ± 2.8***	1.7 ± 1.1	7.8 ± 2.4†††	0.7 ± 0.8	2.6 ± 1.4†††	4.6 ± 1.3	1.9 ± 0.5**,†††
Mouse:								
K (5)	12.8 ± 6.1	31.6 ± 5.2*	14.6 ± 7.4	17.4 ± 4.4†	19.1 ± 13.0	7.2 ± 4.2†††	7.1 ± 1.9	5.4 ± 0.9†††
L (5)	10.5 ± 5.1	25.5 ± 5.0*	3.2 ± 3.6	12.8 ± 4.2	22.3 ± 16.7	6.0 ± 4.0††	6.9 ± 1.9	5.2 ± 0.9†††
Mean	11.8 ± 4.8	31.5 ± 4.1**	8.9 ± 5.8	15.1 ± 4.3††	20.7 ± 15.0	6.6 ± 4.1†††	7.6 ± 1.9	5.3 ± 0.9†††
Human vs. mouse	31.0 ± 11.0	46.9 ± 6.0	20.0 ± 12.0	33.1 ± 7.6	53.7 ± 29.2	19.0 ± 8.9††	32.4 ± 5.8	13.6 ± 1.9**,†††

[a] N = number of codons compared. d_S is significantly different from d_N in the same region at the 5% level (*), 1% level (**), or 0.1% level (***). d_N is significantly different from d_N in the binding cleft at the 5% level (†), 1% level (††), or 0.1% level (†††). Mean numbers of synonymous and nonsynonymous sites, respectively, are as follows: binding cleft (20.0 and 67.0), TCR directed (13.0 and 41.0), outward directed (8.1 and 21.9), and α_1–α_2 (92.2 and 282.8).

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polarity, hydrophobicity, etc. (Taylor 1986)] is considered. Each nonsynonymous nucleotide site (or fractional nonsynonymous site) is assigned to one of two categories: conservative nonsynonymous sites or radical nonsynonymous sites. At a conservative nonsynonymous site, each possible nonsynonymous substitution will lead to an amino acid replacement that is conservative with respect to the property of interest, whereas at a radical nonsynonymous site each possible nonsynonymous substitution will lead to an amino acid replacement that is radical. If some fraction of the possible nonsynonymous substitutions is conservative and the other fraction is radical, fractional values are given. For example, the second nucleotide position of the codon CTG (encoding the neutral amino acid residue leucine) is a nonsynonymous site. Two of the three possible nucleotide substitutions at this site lead to conservative amino acid replacements with respect to charge (these substitutions are CCG and CAG, encoding proline and glutamine, respectively, which are both neutral). One possible substitution (CGG, which encodes arginine, a positively charged residue) is radical with respect to charge. Thus, with respect to charge, the first nucleotide position of AAC is counted as two-thirds conservative nonsynonymous site and one-third radical nonsynonymous site.

Once the number of conservative and radical nonsynonymous sites are obtained by this procedure for each of the two sequences compared, the proportion of conservative and radical nonsynonymous substitutions (denoted by p_{NC} and p_{NR}, respectively) is computed by dividing the number of conservative substitutions by the average number of conservative sites for the two sequences and by dividing the number of radical substitutions by the average number of radical sites, respectively. In the present case, p_{NC} and p_{NR} are not transformed into distance measures by Jukes and Cantor's (1969) formula because the assumption for this distance is not satisfied (Nei 1987, pp. 63–66). (A computer program for this method is available on request.) If amino acid replacements take place at random with respect to the property of interest, p_{NC} is expected to be equal to p_{NR}. If the replacements conserve the property, we have $p_{NC} > p_{NR}$; whereas if they promote radical changes we have $p_{NR} > p_{NC}$.

Table 2 shows mean p_{NC} and p_{NR} with respect to charge, for comparisons among alleles at human and mouse class I MHC loci. p_{NR} consistently exceeds p_{NC} in the binding cleft, and the difference is statistically significant for overall means in both human and mouse. By contrast, in the other ARS subregions and in the remainder of α_1–α_2, p_{NC} and p_{NR} are not significantly different. p_{NC} and p_{NR} were also computed with respect to polarity (table 2). In this case, however, mean p_{NC} and mean p_{NR} are nearly the same for all ARS subregions, including the binding cleft. Therefore, nonsynonymous substitutions seem to have occurred at random with respect to polarity. Similar analyses with respect to residue hydrophobicity and volume likewise revealed no departure from random substitution (data not shown).

Discussion

Our results show that selection promotes charge profile diversity in the binding cleft among different alleles. In the case of the class II MHC, experimental evidence suggests that the motif of a foreign peptide recognized by a particular MHC molecule is a particular pattern of side-chain charges (Sette et al. 1989). This is consistent with the hypothesis that charge profile diversity in the binding cleft is a major cause of allelic variation in peptide binding capacity (Garrett et al. 1989).

Many hypotheses have been advanced to explain MHC polymorphism (Nei and Hughes 1990). Most of these fall into three categories: (1) nonselective hypotheses,

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Table 2
Mean ± SE p_{NC} and p_{NR} with Respect to Charge and Polarity in Different Regions of Human and Mouse Class I MHC Genes

	Region[a]							
	Binding Cleft (N = 29)		TCR Directed (N = 18)		Outward Directed (N = 10)		α_1–α_2 (N = 125)	
Locus (No. of alleles)	p_{NC}	p_{NR}	p_{NC}	p_{NR}	p_{NC}	p_{NR}	p_{NC}	p_{NR}
Charge:								
Human:								
A (16)	10.5 ± 3.2	18.8 ± 4.8	8.1 ± 3.6	7.9 ± 3.3	2.6 ± 2.4	0.0 ± 0.0	1.3 ± 0.5	1.4 ± 0.6
B (20)	15.8 ± 2.9	28.0 ± 4.9*	8.9 ± 4.4	6.2 ± 2.8	5.7 ± 4.7	0.8 ± 0.7	2.1 ± 0.6	2.3 ± 0.8
C (8)	9.2 ± 2.7	12.6 ± 4.1	4.0 ± 2.9	3.3 ± 2.4	0.0 ± 0.0	0.0 ± 0.0	2.5 ± 0.8	3.3 ± 0.9
Mean	13.4 ± 2.7	23.3 ± 4.6**	8.2 ± 3.7	6.6 ± 2.9	4.1 ± 3.0	0.4 ± 0.4	1.8 ± 0.6	2.1 ± 0.8
Mouse:								
K (5)	21.8 ± 4.3	32.8 ± 7.3	21.0 ± 6.6	10.4 ± 3.8	6.0 ± 4.8	6.9 ± 4.6	4.4 ± 1.1	6.3 ± 1.4
L (5)	16.6 ± 4.0	30.8 ± 7.1	12.9 ± 6.2	10.8 ± 4.2	14.5 ± 9.4	1.3 ± 1.4	4.3 ± 1.1	5.9 ± 1.5
Mean	19.2 ± 4.2	31.8 ± 7.2*	17.0 ± 6.4	10.6 ± 4.0	10.3 ± 7.1	4.1 ± 3.0	4.4 ± 1.1	6.1 ± 1.5
Polarity:								
Human:								
A (16)	12.7 ± 3.0	14.7 ± 4.2	7.2 ± 3.0	9.0 ± 4.2	1.9 ± 1.9	0.0 ± 0.0	0.0 ± 0.4	2.2 ± 0.8*
B (20)	21.2 ± 3.2	16.6 ± 4.2	4.2 ± 2.3	11.8 ± 4.5	0.8 ± 0.7	5.5 ± 4.5	1.9 ± 0.6	2.7 ± 0.8
C (8)	9.2 ± 2.7	13.9 ± 4.6	2.5 ± 2.1	5.4 ± 3.5	0.0 ± 0.0	0.0 ± 0.0	2.8 ± 0.7	2.9 ± 0.9
Mean	17.2 ± 3.0	15.7 ± 4.3	5.1 ± 2.5	10.3 ± 4.1	1.1 ± 1.2	3.1 ± 2.6	1.3 ± 0.6	2.5 ± 0.8
Mouse:								
K (5)	23.4 ± 4.4	29.8 ± 7.2	14.0 ± 4.0	15.6 ± 6.4	5.8 ± 4.2	8.2 ± 6.3	5.1 ± 1.1	5.3 ± 1.5
L (5)	21.2 ± 4.6	21.1 ± 5.8	12.1 ± 4.4	10.8 ± 5.2	9.1 ± 5.7	0.0 ± 0.0	5.6 ± 1.2	3.9 ± 1.2
Mean	22.3 ± 4.5	25.5 ± 6.5	13.1 ± 4.2	13.2 ± 5.8	7.5 ± 5.0	4.1 ± 3.2	5.4 ± 1.2	4.6 ± 1.4

[a] N = number of codons compared. p_{NC} is significantly different from p_{NR} in the same region at the 5% level (*) or 1% level (**). With respect to charge, mean numbers of conservative and radical nonsynonymous sites, respectively, were as follows: binding cleft (44.2 and 22.8), TCR directed (16.0 and 25.0), outward directed (8.6 and 13.2), and α_1–α_2 (156.4 and 126.6). With respect to polarity, mean numbers of conservative and radical nonsynonymous sites, respectively, were as follows: binding cleft (46.1 and 20.9), TCR directed (24.9 and 16.1), outward directed (12.6 and 9.2), and α_1–α_2 (172.1 and 110.9). Amino acids were categorized with respect to qualitative properties according to the method of Taylor (1986). With respect to charge, there were three categories: positive (H, K, and R), negative (D and E), and neutral (all other amino acids). Any change of category was considered a radical change. With respect to polarity, there were two categories: polar (C, D, E, H, K, N, Q, R, S, T, W, and Y) and nonpolar (all others). With respect to hydrophobicity, there were two categories: hydrophobic (A, C, F, G, H, I, K, L, M, T, V, W, and Y) and nonhydrophobic (all others). With respect to volume, there were two categories: small (side-chain volume <60 Å^3) and large (all others).

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which usually invoke a high mutation rate (Bailey and Kohn 1965) or gene conversion (López de Castro et al. 1982); (2) hypotheses of balancing selection due to maternal/fetal interaction (Clarke and Kirby 1966) or mating preference (Yamazaki et al. 1976); and (3) hypotheses of balancing selection based on disease resistance (Doherty and Zinkernagel 1975). Comparison of DNA sequences from different mammalian species has shown that the mutation rate in the MHC is not particularly high (Hayashida and Miyata 1983; Hughes and Nei 1988, 1989*a*, 1989*b*). Gene conversion can be a source of variation in the MHC (Weiss et al. 1983), but this factor alone cannot account for MHC polymorphism. MHC polymorphism is caused mainly by nonsynonymous substitutions in the ARS (Hughes and Nei 1988, 1989*b*), particularly in the binding cleft (as noted in the present paper); and a random process such as gene conversion cannot generate such a highly specific pattern of allelic differences.

As regards selective models of MHC polymorphism, it is possible that the model of maternal/fetal incompatibility could explain an enhancement of the rate of nonsynonymous substitution in MHC genes (Flaherty 1988). However, it is unlikely that under this model nonsynonymous substitutions would be focused in the binding cleft. It has been proposed that the production of maternal antibodies to fetal class I MHC molecules has a beneficial effect on fetal growth and survival, though this has not been experimentally established (James 1965, 1967; Clarke 1971; Wegmann 1984). If such a mechanism were to operate, it would produce selection for amino acid substitution in regions of the class I molecule that serve as epitopes for maternal antibodies. However, serological evidence suggests that epitopes for antibodies against MHC molecules are scattered throughout the α_1 and α_2 domains rather than being concentrated in the ARS (Bjorkman et al. 1987*a;* Nathenson et al. 1986). Therefore, our results are not consistent with the maternal/fetal interaction hypothesis. For further discussion of this hypothesis, see the work of Nei and Hughes (accepted).

The mating-preference hypothesis is based on the observation that rodents use olfaction to discriminate between urines of conspecifics differing only at MHC loci (Yamazaki et al. 1979, 1983; Singh et al. 1987). Breakdown products of MHC molecules are found in the urine, and these or odorants bound by them presumably make such olfactory discrimination possible. It is conceivable that urine odors are due at least in part to peptides bound by MHC breakdown products in the urine; if so, selection relating to discrimination of MHC genotypes might affect the binding cleft of the class I molecule. However, the evidence that mice use the MHC as a basis for mate choice is actually very poor (reviewed in Nei and Hughes, accepted). Furthermore, mating preference on the basis of olfaction cannot be a general explanation of MHC polymorphism, since polymorphism is high in species such as the chicken and the human, which have poor olfactory capabilities. In humans, there is in fact no evidence that MHC types play a role in mate choice (Rosenberg et al. 1983).

The present results, because they show that positive selection on class I MHC molecules is focused mainly on the binding cleft and affects primarily the amino acid property most important in peptide binding, favor the hypothesis that MHC polymorphism is maintained by overdominant selection due to disease resistance (Doherty and Zinkernagel 1975; Hughes and Nei 1988, 1989*b;* Takahata and Nei 1990). These results also help to account for one aspect of class I gene evolution that was previously difficult to explain. Hughes and Nei (1988) observed that, in comparisons between class I genes of distantly related species, there is a tendency for the rate of nonsynonymous substitution in the ARS to level off. One possible explanation for this finding is the occurrence of forward and backward substitutions at nonsynonymous sites

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(Hughes and Nei 1988). If selection acts mainly to promote allelic diversity with respect to the charge profile of the binding cleft, certain nonsynonymous substitutions will occur with greater frequency than others. As a consequence the frequency of forward and backward substitution will be enhanced, thereby leading to a leveling off of d_N in comparisons of distantly related species.

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