Beta-2 microglobulin-free HLA class I heavy chain (FHC) A3 and/or A30 soluble products contribute only minimally to serum FHC expression

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Summary

No monoclonal antibodies (mAbs) are presently available to measure the total amount of β2-microglobulin-free HLA class I heavy chain (FHC) in sera. The available ELISA-based double determinant immunoassay (DDIA), established to measure FHC, uses two mAbs (TP25.99 and HC-10) that recognize a monomorphic determinant expressed on all HLA-B/C FHC products and a determinant expressed only on some HLA-A FHC products. This restricted reactivity implies that, in addition to HLA-B/C, HLA-A FHC products are also detected in individuals bearing HLA A3 and/or A30 allotypes. The aim of this study was to establish whether such restriction results in the detection of low FHC levels in individuals lacking HLA A3 and/or A30 allospecificities. The FHC mean concentration (± SD) in 294 healthy blood/bone marrow donors (HBDs) was 0.24 (± 0.2) mg/l. The grouping of HBDs according to their HLA-A FHC product reactivity with one, both or no mAbs did not result in any statistically significant differences (Mann–Whitney test: $P > 0.05$) between their median FHC concentrations. Since the absence of differences in their FHC levels was not attributable to a difference in the percentage distribution of HLA allotypes associated with high or low HLA-B/C FHC expression, our results indicate that FHC HLA A3 and/or A30 products detected in DDIA by these two mAbs only minimally contribute to FHC serum expression and that the assay is not limited by the failure to detect HLA-A FHC products in $A3^-$ and/or $A30^-$ individuals.

Introduction

HLA class I antigens are heterodimeric molecules composed of a 44-kDa polymorphic heavy chain (HC) glycoprotein associated with a 12-kDa monomorphic chain referred to as β2-microglobulin (β2m). Three soluble forms derived from these surface antigens have been detected in serum: β2m, β2m-associated HLA class I HC (HLA-I) and β2m-free HLA class I HC (FHC).

Like the first two forms, the levels of FHC are related to tumour mass and proliferation rate in haematological malignancies (Perosa *et al*., 1999a) and immune system activation, as indicated by enhancement of FHC levels during viral infections (Migliaresi *et al*., 2000) or acute graft rejection after liver transplantation (Rebmann *et al*., 1999). Furthermore, the ability of HLA class I and FHC to inhibit CD8-mediated cytotoxicity (Puppo *et al*., 1997) suggests that they have a regulatory function, although the metabolic pathways controlling FHC and HLA secretion may be different (Demaria *et al*., 1994; Perosa *et al*., 1999b).

An enzyme-linked immunosorbent assay (ELISA)-based double determinant immunoassay (DDIA) has been established to measure FHC (Perosa *et al*., 1999b). It uses two monoclonal antibodies (mAbs) (TP25.99 and HC-10) that recognize a monomorphic determinant expressed on all HLA-B/C FHC products and a polymorphic determinant only expressed on some HLA-A FHC products. This restricted reactivity implies that, in addition to HLA-B/C, HLA-A FHC products are detected in individuals bearing HLA-A3 and/or A30 allotypes. To assess whether this differential reactivity has a major effect on FHC levels, we measured FHC in sera from 294 healthy blood/bone marrow donors (HBDs) grouped according to their HLA-A reactivity with one, both or no mAbs. The absence of significant differences in the median FHC concentrations of these groups suggests that FHC products derived from HLA-A3 and/or A30 only minimally contribute to FHC serum expression.

Subjects, materials and methods

Subjects

Serum samples were collected from 294 HLA class I-typed HBDs and stored at –80 °C until they were used.

Cells

Cultured human B lymphoid cells C1R-sB7, secreting large amounts of soluble (s) B7-β2m heterodimer, were

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Table 1. Statistics of the cohort

				Age		FHC	
Subjects	No.	%	M/F ratio	Median	Mean ± SD	Mean ± SD	Median (range)
Total	294	100	0.9	33	35.13 ± 12.17	0.24 ± 0.2	0.19 (0–1.5)
High secretors[a] (FHC ≥ 0.44 mg l^{-1})	48	18.6	1.1	31	33.38 ± 12.8	0.61 ± 0.21	0.54 (0.44–1.5)
Low secretors[b] (FHC ≤ 0.04 mg l^{-1})	20	7.7	0.66	28.5	31.8 ± 9.67	0.02 ± 0.01	0.023 (0–0.04)

[a] Mean + 1 SD.
[b] Mean – 1 SD.

kindly provided by Dr F. C. Grumet (Stanford University, Stanford, CA) (Hiraki *et al.*, 1994) and grown in RPMI 1640 medium supplemented with 10% foetal calf serum (complete medium) and 5 mM L-glutamine (Seromed, Biochrom KG, Berlin, Germany).

Conventional reagents, mAbs and immunoassay to measure FHC

Electrophoresis reagents were purchased from Bio-Rad Laboratories (Hercules, CA). Unless otherwise specified, all other chemicals were purchased from BDH Chemical (Poole, UK).

Mouse anti-αv/β3 mAb VF27-312 (IgG1) (Hamby *et al.*, 1996), mAb HC-10 (an IgG2a) to a determinant preferentially expressed on the FHC of HLA-B, -C and HLA-A3, A10, A28, A29 and A30-33 (Stam *et al.*, 1986; H. Grosse-Wilde, Institute of Immunology, University Hospital of Essen, Essen, Germany, personal communication) and mAb TP25.99 (an IgG1) to a determinant expressed on HLA class I (A, B and C locus molecules) and on all HLA FHC alleles except A2, A10, A29, A31-33, A68.1, A69 and B73 were developed and characterized as described (Tanabe *et al.*, 1992; Desai *et al.*, 2000).

Peroxidase-conjugated avidin and xeno-antisera to mouse IgG (H + L) were purchased from Jackson Immunoresearch Laboratories (Avondale, PA). Polyclonal mouse IgG (mIgG) was purchased from Sigma Chemicals Co. (St. Louis, MO).

Purification of mAbs from ascites by sequential precipitation with caprylic acid and ammonium sulphate or by affinity chromatography on protein G-Sepharose (Pharmacia-LKB, Biotech AB, Uppsala, Sweden) and assessment of purity by SDS-PAGE were performed as previously described (Laemmli, 1970; Perosa *et al.*, 1990). The mAb concentration was determined by a bicinchoninic acid assay (BCA) (Pierce, Rockford, IL). Purified mAbs were coupled to biotin using the biotin-N-hydroxysuccinimide ester (Sigma Chemicals Co.) (Streicher *et al.*, 1986).

Purification of standard soluble FHC from C1R spent medium and DDIA to measure FHC in sera, using purified mAb TP25.99 and biotinylated HC-10 as capture and detect reagents, respectively, was performed as previously described (Perosa *et al.*, 1999b).

Statistical analyses

Analyses were performed using version 2.0 of the GraphPad PRISM software (GraphPad, San Diego, CA). The significance of the difference between the median of the variables of two groups was determined by the non-parametric Mann–Whitney *U*-test. The significance of the association between high or low FHC concentration and HLA-B and -C allotypes was calculated by Fisher's exact test, using a cut-off of FHC > mean + 1 SD (high) and FHC ≤ mean − 1 SD (low), respectively. A P-value < 0.05 with a relative risk > 1 was accepted as significant.

Results and Discussion

To determine whether FHC levels in individuals who lack HLA-A allospecificities detectable with both mAbs are lower than in those who express them, we measured serum FHC in 294 HBDs and correlated their FHC levels to mAb HLA-A-restricted specificity. Eight-fold serial dilutions (starting dilution 1 : 4) of serum from each HBD were tested for FHC to give an absorbance binding corresponding to the linear portion of the standard curve. The characteristics of our cohort are indicated in Table 1.

The mean serum FHC concentration (± SD) was 0.24 ± 0.2 mg l^{-1}. The high- and low-secretor groups were subjects with a mean FHC ≥ 0.44 mg l^{-1} (mean + 1 SD) and a mean FHC < 0.04 mg l^{-1} (mean − 1 SD), respectively. As in our previous study (Perosa *et al.*, 1999b), Fisher's exact test (contingency test analysis) applied to each allotype group indicated that low secretion was associated with HLA-B17 (P = 0.011), and high secretion with B35 (P = 0.0017) and Cw4 (P = 0.008) (data not shown). Subjects were then grouped according to their HLA-A FHC allotype reactivity with the mAbs (Table 2): group A, subjects with at least one FHC HLA-A allele recognized by both mAbs (all HLA-A3 and/or A30); group B, HLA-A2 subjects, and it is noteworthy that FHC bearing this allotype did not react with either mAb; groups C and D, subjects with at least one FHC-bearing allotype reactive with TP25.99 and HC-10, respectively; group E, subjects with one HLA allospecificity reacting with TP25.99 and the other with HC-10.

The mean FHC concentration of group A was expected to be the highest, as both HLA-B/C and HLA-A FHC

Table 2. Absence of statistically significant differences between FHC median concentrations in subjects grouped according to all possible combinations of the reactivity of their HLA-A allotype products with TP25.99 and HC.10 mAbs, respectively

Group (denomination)	No. of subjects	mAb reactivity with FHC-bearing HLA-A (A′/A″) allotypes		FHC (median/mean ± SD)	*P*[a]
		mAb TP25.99	mAb HC-10		
A[b]	73	+/–	+/–	0.15/0.25 ± 0.26	
		–/+	–/+		
		+/+	+/+		
B[c]	24	–/–	–/–	0.21/0.25 ± 0.17	
C	100	+/–	–/–	0.17/0.22 ± 0.19	NS[d]
		–/+	–/–		
		+/+	–/–		
D	56	–/–	+/–	0.2/0.27 ± 0.21	
		–/–	–/+		
		–/–	+/+		
E	41	+/–	–/+	0.19/0.22 ± 0.14	
		–/+	+/–		

[a] Mann–Whitney test.
[b] HLA-A3 and/or A30 positive individuals.
[c] HLA-A2 individuals.
[d] FHC median concentration of each group vs. the others was not statistically different ($P > 0.05$).

Table 3. Distribution of alleles statistically associated with high (B35) or low (B17) FHC serum expression or in linkage disequilibrium with B35 in subjects grouped according to their HLA allotype reactivity with the mAb used in DDIA

HLA	Linkage disequilibrium (HLA; value Δ x 1000)	FHC serum expression[a]	No. (%)[b]				
			Groups (denomination)[c]				
			A	B	C	D	E
Bw35	—	↑	26 (35)	2 (8.3)	42 (42)	23 (41)	17 (41)
Cw4	B35; 116	↑	28 (38.3)	3 (12.5)	44 (44)	21 (37)	11 (26)
B17	—	↓	7 (9.5)	2 (8)	1 (1)	3 (5.3)	4 (9.7)

[a] Determined by Fisher's exact test and the Mann–Whitney test to be statistically associated with high (↑) or low (↓) FHC serum expression.
[b] Number and percentage of allele expression.
[c] Grouped according to their HLA-A (A′/A″) allotype reactivity with the mAb used in DDIA (legend in Table 2).

products are detected in this group. Conversely, the same concentration in group B or D donors was expected to be the lowest, because of the lack of detection of HLA-A FHC products. Lastly, the FHC median concentration in groups C and E was also expected to be lower than in A, unless dimerization between the two HLA-A FHC products occurred (Hiraki *et al*., 1994).

However, the median FHC concentration was, surprisingly, found to be lowest in group A, whereas it was highest in group B (Table 2). The Mann–Whitney test, in contrast, showed that the five FHC median concentrations did not differ, suggesting the absence of any relationship between HLA-A restriction of mAb binding and FHC levels, provided these had not been conditioned by a heterogeneous group distribution of HLA-B/C allotype associated with high or low FHC serum expression.

To rule out this possibility, the expression percentage of these alleles was examined. Table 3 shows that the 'low FHC secretor' B17 was equally distributed in groups A (9.5%), B (8%), D (5.3%) and E (9.7%), and the 'high FHC secretor' B35 or Cw4 in groups A (35%), C (42%), D (41%) and E (41%). The lowest percentage of $B17^+$ subjects was detected in group C (1%), and the lowest percentage of $B35^+$ (8.3%) and $Cw4^+$ (12.5%) in group B. This low B35 percentage in group B is presumably unrelated to its high mean FHC concentration (0.25 ± 0.17 mg l^{-1}), since this was higher than in groups C (0.22 ± 0.19 mg l^{-1}) and E (0.22 ± 0.14 mg l^{-1}), which included 42 and 41% of $B35^+$ individuals, respectively. A statistical bias generated by the limited number of individuals in group B and the fact that they were all HLA-A2 is unlikely, since after the grouping of B with D the median FHC was still statistically similar to that of group A (data not shown).

Therefore, HLA-A3 and/or A30 FHC products may only constitute a minor component of total serum FHC and their non-detection in $HLA\text{-}A3^-$ and/or $A30^-$ subjects does not result in an underestimation of FHC measured in DDIA.

These data also provide indirect evidence for a depressed serum expression of HLA-A3 and/or A30 FHC.

Whether this reflects a phenomenon general to HLA-A FHC products remains to be determined, as mAbs recognizing monomorphic determinants of these molecules have not yet been described. Alternatively, as for HLA class I (McDonald & Adamashvili, 1998), there might be an association of some FHC HLA-A allotypes with decreased expression of the corresponding product. If this were the case, then the mechanism(s) controlling the shedding of FHC would be different from that of HLA-I, as an association with reduced serum expression of this molecule has only been reported in individuals bearing HLA-A2 (Adamashvili *et al*., 1996) and not A3 and/or A30.

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