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CONCISE REPORT

Variation of Class I HLA Antigen Expression Among Platelet Density Cohorts: A Possible Index of Platelet Age?

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Platelet alloantigens and other surface markers were studied in platelet cohorts of different mean density, using monoclonal and polyclonal probes. High density (HD) platelets expressed 12% more P1^{A1} molecules (46,942) than low density (LD) platelets (41,892). However, LD platelets carried 42% more HLA-A2 molecules (6,267 ± 184) than HD platelets (4,406 ± 232) ($P < .01$) and 55% more class I HLA antigens (17,034 ± 2,062 v 11,007 ± 2,190) ($P = .05$). The platelet subpopulations did not differ in their content of glycoprotein (GP)IIb/IIIa complex or Bak^a antigen. The difference in expression of class I HLA antigens on HD and LD platelets is consistent with two possibilities: either class I HLA molecules are acquired from plasma or they are released into plasma as platelets age in circulation. Accordingly, class I HLA molecules may provide a useful marker of platelet age.

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HUMAN PLATELETS are heterogeneous in size, buoyant density, functional properties, and organelle content. Much of this variation can be explained by events that occur during thrombocytopoiesis, but it is likely that other changes are superimposed as platelets age in circulation. The nature of these is controversial. There is presently no consensus as to the relationship, if any, between platelet size and platelet age.[1-3] It has been proposed that platelet density increases[2,4-7] and decreases[8-10] with age. Methodologic[11] and species[12,13] differences may account for some of these apparently discrepant observations.

Measurements of surface markers that may vary with age offers another approach to the study of blood cell aging in vivo. The possible relevance of such studies to platelets is suggested by recent reports that alloantigens of the P1^A and HLA systems may be acquired by or released from platelets in the circulation.[14-16] We therefore studied the expression of class I HLA, P1^{A1}, and other markers in platelet density subpopulations obtained by centrifugation through a continuous linear gradient of arabinogalactan. Evidence was obtained that the class I HLA determinants, in contrast to other surface markers, are expressed differently in high density (HD) and low density (LD) platelet cohorts.

MATERIALS AND METHODS

Venous blood was drawn from volunteers and anticoagulated with EDTA (4 mmol/L) and PGE_1 (0.4 μg/mL). Platelet-rich plasma (PRP), shown in previous studies to be representative of the total platelet population,[17] was obtained by differential centrifugation at 240 g for ten minutes at room temperature and the platelets were fractionated according to density within two hours.

A stock solution of 20% isosmotic arabinogalactan (Stractan; St Regis Paper Company, Tacoma, WA) was prepared as described by Corash et al.[18] A continuous gradient of 10 mL was formed in 9.16 × 3½ inch Ultraclear tubes (Beckman, CA) with a linear gradient maker. A 2.5 mL volume of PRP was layered on the Stractan gradient and centrifuged at 10,000 g for 45 minutes at 22°C in a Beckman SW 41Ti rotor. The Stractan concentrations used to form the gradients were 19% and 11% (density 1.084 and 1.045 g/mL, respectively) and contained PGE_1 (0.2 μg/mL). Platelet density subpopulations were recovered by piercing the bottom of the tube in a gradient fractionator and HD, middle density (MD), and LD platelets were collected. The three fractions contained approximately 13%, 73%, and 15% of the total platelets, respectively. Each platelet fraction was washed twice in PBS/EDTA, pH 6.8, containing 1% bovine serum albumin (BSA), resuspended in the same buffer, and counted. Mean platelet volume was determined in the platelet subpopulations,[17] and platelet content of protein (Markwell) and β-thromboglobulin (Amersham RIA Kit) was measured.

Preliminary studies showed that the platelet subpopulations did not contain detectable numbers of lymphocytes. For quantitation of platelet alloantigens, platelets from donors homozygous for P1^{A1} and Bak^a and heterozygous for HLA-A2 were saturated with alloantibodies specific for the P1^{A1}, Bak^a, and HLA-A2 antigens.[19] The number of IgG molecules bound was determined with saturating quantities of ^{125}I-labeled monoclonal antibody specific for the Fc fragment of IgG (HB-43; ATCC, Rockville, MD) and centrifugation through 20% Percoll as previously described.[19] HB-43 binds to IgG of all subclasses at a molar ratio of 1:1.[20] HLA-A,B,C antigens were similarly quantified by direct binding of a radiolabeled monoclonal antibody, W6/32 (ATCC) specific for a monomorphic determinant on class I HLA molecules. Membrane glycoprotein (GP) IIb-IIIa complexes were assayed using the monoclonal antibody AP-2 (courtesy of Dr T.J. Kunicki).[21] Activation of platelets during isolation was evaluated by the binding of radiolabeled monoclonal antibody S-12 (a gift from Dr Rodger McEver, San Antonio, TX) that recognizes a glycoprotein, GMP-140, carried on the alpha granule membrane and expressed only by activated platelets.[22]

RESULTS

Properties of HD and LD platelets are summarized in Table 1. HD and LD platelet fractions contained 11.2% ± 2.9% and 13.6% ± 5.6% of the total platelet populations, respectively. The mean volume of HD platelets (7.7 cubic

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Table 1. Characteristics of HD, LD, and Whole Platelet (WP) Populations (Mean ± 1 SD)

Platelet Cohort	Volume (n = 4) (μ^3)	Protein (n = 4) (mg/10^9 Platelets)	β-Thromboglobulin (n = 5) (μg/10^9 Platelets)
HD	7.7 ± 0.5	2.25 ± 0.39	47.25 ± 8.4
LD	6.6 ± 0.9	1.72 ± 0.32	30.07 ± 10
WP	7.4 ± 0.8	1.98 ± 0.22	39.3 ± 7.2

Table 3. Binding of ^{125}I-S12 to Resting and Activated HD, LD, and Whole Platelet (WP) Populations (Mean ± SEM)

Platelet Cohort	^{125}I-S12 Bound (Molecules/Platelet)	
	Resting (n = 6)	Activated (n = 6)*
HD	773 ± 64	12,758 ± 913
LD	1,394 ± 203 ($P < .05$)	6,674 ± 362 ($P < .01$)
WP	685 ± 82	8,544 ± 535

*Platelets were activated with human thrombin 0.5 U/mL at 37°C for 30 minutes.

microns) was about 16% greater than LD platelets (6.6 μm^3). However, HD platelets contained 24% more protein and approximately 40% more β-thromboglobulin than LD platelets.

As shown in Table 2, HD platelets carry approximately 12% more $P1^{A1}$ molecules (46,942 ± 1,258) than LD platelets (41,892 ± 1,666) ($P = .028$). However, LD platelets carry 42% more HLA-A2 molecules (6,267 ± 184) than HD platelets (4,406 ± 232) ($P < .01$). Using the probe W6/32, LD platelets were found to carry 55% more class I HLA molecules than HD platelets (17,034 ± 2,062 *v* 11,007 ± 2,190) ($P = .05$). HD platelets did not differ from LD platelets in binding AP-2, recognizing the GPIIb/IIIa complex, or anti-Bak^a, recognizing an alloantigen carried on GPIIa.

Resting LD platelets bound slightly more monoclonal S-12 (1,394 ± 203 molecules per platelet) than HD or total platelets (Table 3). After activation by human thrombin, S-12 binding increased markedly and HD platelets expressed significantly more binding sites (12,758) than LD platelets (6,674).

DISCUSSION

The continuous, isosmotic gradient of arabinogalactan provides a rapid and reproducible method for isolating platelet cohorts of selected mean density. As reported previously by others,[7,9] we found that the mean volume of HD platelets is approximately 16% greater than LD platelets, but that HD platelets contain 24% more protein and approximately 40% more β-thromboglobulin than LD platelets (Table 1). Only approximately 1.7% of total β-thromboglobulin was released from platelets during density gradient centrifugation. However, LD platelets showed evidence of minimal activation by the criterion of S-12 binding. It is not yet clear whether this is a consequence of in vitro manipulation or reflects low grade activation of LD platelets in vivo. After maximal activation of platelets with thrombin, binding of S-12 to HD and LD platelets increased 20- and 5-fold, a difference consistent with the larger number of alpha granules in HD platelets.[23-25] Activation of HD and LD platelets with thrombin did not significantly change the number of binding sites for anti-$P1^{A1}$, anti-HLA-A2, or anti-HLA-A,B,C (W6/32) (data not shown). Thus, differences in surface expression of the antigens recognized by these probes cannot be accounted for by activation of platelets in vitro or in vivo.

As summarized in Table 2, HD platelets carry 12% more $P1^{A1}$ antigen than LD platelets, but LD platelets carry 42% more HLA-A2 and 55% more HLA-A,B,C than HD platelets. HD and LD platelets express approximately the same number of Bak^a determinants and GPIIb/IIIa complexes. The HD platelets in the cohorts we studied were approximately 16% larger than LD platelets. The difference in surface density of class I HLA antigens on HD and LD platelets is even greater than indicated by the absolute number of molecules measured in that LD platelets have less surface area because of their slightly smaller size. The studies of thrombin-activated platelets show that minimal activation of LD platelets reflected by S-12 binding cannot account for the greater numers of class I HLA antigens they carry.

Our value for the average number of HLA-A,B,C, determinants on normal human platelets (approximately 15,000 per platelet) is much lower than the average value of 81,000 reported recently by Kao et al using radiolabeled Fab fragments from W6/32.[26] Each platelet can be expected to carry class I HLA molecules determined by six genes, two each at the HLA-A,B, and C loci. We previously showed that platelets from donors heterozygous for HLA-A2 carry 4,000 to 8,000 HLA-A2 molecules and that the number of class I HLA antigens other than HLA-A2 expressed on the platelet surface ranges from 1,000 to 6,000 for each gene.[19] HLA-C antigens are thought to be weakly expressed on platelets.[27] Thus, our finding of 15,000 binding W6/32 sites per platelet is consistent with estimates of the total number of HLA-A,B,C antigens on platelets obtained in other studies.[19] The

Table 2. Quantitative Analysis of Antigen Expression on HD, LD, and Whole Platelet (WP) Population

Platelet Cohort	Binding of Monoclonal Antibodies (Molecules/Platelet + SEM)				
	Pl^{A1} (n = 9)	Bak^a (n = 5)	HLA-A2 (n = 7)	HLA,A,B,C (n = 6)	GPIIb/IIIa (n = 5)
HD	46,942 ± 1,258*	23,728 ± 1,457	4,406 ± 232*	11,007 ± 2,190*	42,087 ± 2,495
LD	41,892 ± 1,666	22,297 ± 1,163	6,267 ± 184	17,034 ± 2,062	42,635 ± 2,330
WP	45,210 ± 1,949	21,936 ± 979	5,186 ± 197	15,372 ± 2,037	44,452 ± 1,970

Donors studied were heterozygous for HLA-A2, but homozygous for Pl^{A1} and Bak^a.

*$P \le .05$ comparison with LD values (paired *t* test).

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validity of our studies with W6/32 is supported by our finding that this probe recognizes an average of 111,564 class I HLA molecules on peripheral blood T lymphocytes (range, 128,147 to 94,982) a figure consistent with the value obtained by others.[28] Further studies will be required to explain the difference between our values for total platelet class I HLA antigens and those of Kao et al.

The significant difference in the number of class I HLA antigens expressed on HD and LD platelets, in contrast to the other markers studied (Pl^{A1}, Bak^a, GPIIb/IIIa complex), raises the question of whether HLA antigen expression changes as platelets age in the circulation. Class I HLA antigens are known to exist in plasma in a soluble form.[29] Platelets have been reported to bind these soluble HLA antigens when incubated in plasma[14,15] and it has been proposed that they acquire their class I HLA antigens in the circulation after being released by megakaryocytes. Class I HLA antigens of platelets appear to differ from those of other cells in being released readily by high concentrations of chloroquine at pH 5.5[30] and at neutral pH by hypertonic salt solutions.[31] In plasma, class I HLA antigens exist in two forms, one consisting of the entire integral membrane protein and one lacking the transmembrane region.[32] Together, these observations suggest two possibilities: either class I HLA antigens of platelets are acquired from plasma or they are released into plasma as platelets age in the circulation, perhaps as a consequence of proteolysis. Observations made by us[2] and several other groups[4,6,7] suggest that LD platelets are relatively younger than HD platelets in humans and thus support the latter possibility, ie, that HLA antigens are lost during the aging process. Regardless of whether HLA antigens are lost or acquired, measurement of surface class I HLA determinants may provide a useful index of platelet age. Studies to answer this question are now in progress.

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