Application of the proteolytic enzyme papain in routine platelet serology

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The use of proteolytic enzymes is well established in red cell serology. These enzymes modify some antigen structures and remove sialic acid from the red cell membrane. Enzyme-sensitive structures have also been identified on the platelet membrane. The effect of papain, a proteolytic enzyme used widely in red cell serology, on the detection of various platelet alloantibodies was examined to determine its usefulness in platelet serology. Antisera with the specificities anti-HPA-la, -2b, -3a, -4a, -5a, -5b, and -Nak[a] were examined. HLA antibodies were also included. All sera were tested by a solid-phase red cell adherence technique in parallel with untreated platelets (UP) and platelets treated with papain (PP) for 15 minutes at 37°C. The reactivity of anti-HPA-2b was eliminated and that of anti-HPA-3a was either eliminated or almost eliminated with PP. Antisera specific for the other alloantigens tested reacted similarly or more strongly with PP compared with UP. These findings were confirmed by flow cytometry. The reactivity of HLA antibodies with PP was generally enhanced. Inactivation by papain of platelet alloantigens in the HPA-2 and HPA-3 systems, but not in other systems, may assist in resolving mixtures of platelet alloantibodies. Also, detection of weak antibodies of other specificities may be enhanced. The use of PP may be a simple and useful serologic tool for investigating platelet alloantibodies. ***Immunohematology*** 1995;11:140–142.

The use of proteolytic enzymes is well established in red cell serology for antibody detection.[1] These enzymes modify some antigen structures and remove sialic acid from the red cell membrane. This procedure may eliminate or enhance detection of corresponding antibodies. Enzyme treatment of red cells may therefore aid in the resolution of antibody mixtures and facilitate detection of weak antibodies.

Platelet alloantigens are located on membrane glycoproteins. It is known that glycoprotein Ib, on which the HPA-2 system antigens are located, is protease sensitive.[2] Sialic acid also contributes to the expression of HPA-3 system antigens.[3] These observations have not been applied to routine platelet antibody investigations.

The application of papain-treated platelets to the detection of platelet antibodies involving the HPA systems 1–5 and HLA antibodies was examined to determine its usefulness in platelet serology.

Materials and Methods

Platelet preparation

Freshly collected citrated whole blood was centrifuged at 150 g for 10 minutes, and the platelet-rich plasma was removed. The platelets, adjusted to a count of 50×10^9/L, were stored at 4°C in their own plasma for up to two days before testing.

Test sera

Sera that contained platelet-specific alloantibodies were obtained from patients with neonatal alloimmune thrombocytopenia, or from blood donors. In most of these sera there was no other detectable antibody. Six sera also contained HLA antibodies, which in each case were significantly weaker than the platelet-specific antibody. Sera from six patients with multispecific HLA antibodies were tested, along with sera from random patients not known to contain platelet antibodies. All sera were tested with papain-treated platelets (PP) and untreated platelets (UP).

Solid-phase red cell adherence (SPRCA) assay

This assay was performed as previously described.[4] Briefly, platelet monolayers were established on microwell strips (NUNC, Roskilde, Denmark) previously coated with a platelet membrane antibody (Dako, Denmark), by adding 50 μL of platelet suspension to the wells, centrifuging, incubating the strips for 15 minutes at 37°C, and then washing off excess platelets. Serum and a glycine solution were added to the platelet-coated wells and incubated for 15 minutes at 37°C. The wells were then washed, and IgG-sensitized red cells and anti-IgG (Commonwealth Serum Laboratories, Melbourne, Australia) were added. The strip wells were then centrifuged and the results interpreted. Effacement of indicator cells over the curved surface of the wells indicated a positive result, and a

button of agglutinated red cells indicated a negative result.

Flow cytometric immunofluorescence test

Fifty μL of platelet suspension were dispensed into microwells and washed × 2 with a solution of phosphate-buffered saline (PBS), pH 7.4, containing 380 mg/L EDTA and 0.2 percent bovine serum albumin (BSA). After adding 100 μL of test serum and incubating for 30 minutes at 37°C, the wells were washed and incubated with 100 μL of a 1:100 dilution of fluorescein isothiocyanate (FITC)-labeled anti-human Ig (Silenus Laboratories, Melbourne) for 30 minutes at 20–22°C. The platelets were then washed once and analyzed in a Coulter Epics Profile II flow cytometer. Analysis was performed using log forward and side scatter, and log fluorescence. The result was expressed as a percentage of fluorescing cells. The count analyzed 5,000 cells over a period of 30 seconds to 1 minute.

Papain treatment of platelets

For the SPRCA assay, 50 μL of papain solution (CSL Ltd., Melbourne) diluted with an equal volume of a glucose-citrate solution, Celpresol (CSL Ltd.), were added to platelet monolayers in microtiter plate wells. These were incubated for 15 minutes at 37°C, the papain discarded, and the wells washed once with PBS, pH 7.4, containing 0.2 percent BSA. For flow cytometry, the papain treatment was performed similarly using 50 μL volumes of a washed platelet suspension, and centrifuging to remove the papain and wash the cells.

Results

Table 1 shows the titers of various alloantibodies tested against platelets of appropriate phenotype by SPRCA assay. Both HPA-2b and HPA-3a antibody reactivity was eliminated or markedly reduced with PP. Other specificities tested displayed similar or enhanced reactivity. Of six sera containing multispecific HLA antibodies, the reactivity of five was enhanced by no more than two doubling dilutions, and one remained unchanged (data not shown).

Figure 1 shows the flow cytometry results obtained with examples of HPA-2b, HPA-3a, and Nak^a antibodies. The elimination of anti-HPA-2b reactivity using PP represented a reduction in percentage of fluorescing cells from 93.4 percent to 9.6 percent, and a reduction in reactivity with anti-HPA-3a using PP from 98.2 percent to 48.3 percent. The latter antibody, sample number 2

Table 1. Reactivity of platelet alloantibodies with papain-treated platelets

Antibody	Papain-treated* platelets	Untreated* platelets
HPA-1a		
1	32	16
2	256	64
3	256	32
4	512	64
5	512	128
6	64	8
7	1,024	256
8	16	16
9	4	4
HPA-2b		
1	0	8
2	0	8
3	0	32
HPA-3a		
1	0	64
2	2	64
3	0	32
HPA-4a	2	2
HPA-5a		
1	128	32
2	2	2
HPA-5b		
1	64	64
2	128	64
3	32	8
Nak^a	16	16

*Titer results expressed as reciprocals of doubling dilutions

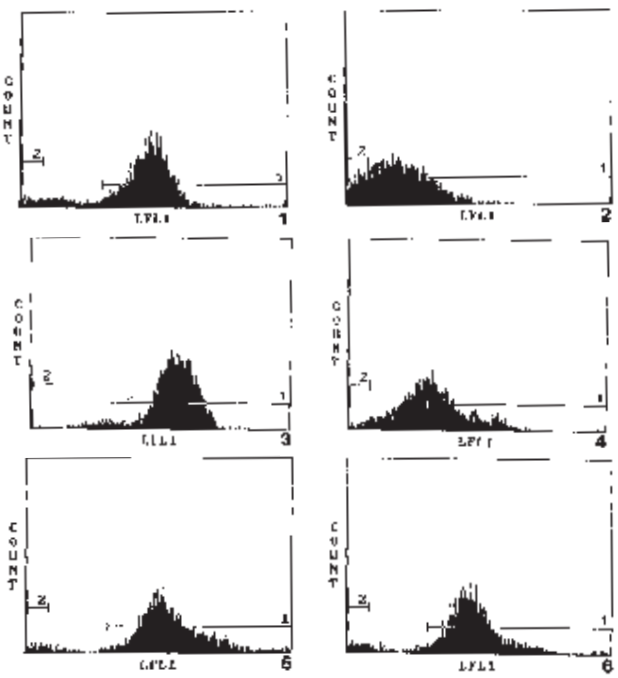

Fig. 1. Demonstration of the effect of papain treatment of platelets on alloantibody detection by flow cytometry.
LFL1 = log fluorescence.
Graphs 1 & 2: Anti-HPA-2b with untreated (1) and papain-treated (2) HPA-2b-positive platelets.
Graphs 3 & 4: Anti-HPA-3a with untreated (3) and papain-treated (4) HPA-3a-positive platelets.
Graphs 5 & 6: Anti-Nak^a with untreated (5) and papain-treated (6) Nak^a-positive platelets.

of that specificity shown in Table 1, retained a titer of 1:2 with PP. The percentage of fluorescence for anti-Nak[a] was similar for UP and PP, at 94.5 percent and 92.8 percent, respectively.

Of 57 serum samples from random patients not known to contain platelet antibodies (i.e., not previously tested), five reacted. For four of these, reactivity occurred with both PP and UP. The other reacted only with UP.

Discussion

The effect of papain on the detection of known platelet alloantibodies has not been established. It is known that protease-sensitive sites exist on the platelet membrane. The HPA-2 system antigens are located on that part of the GP1b chain cleaved by proteases,[5] and would explain the observed nonreactivity with PP of anti-HPA-2b.

Take et al.[3] demonstrated that desialylation of HPA-3a-positive platelets by neuraminidase resulted in variable reduced binding of anti-HPA-3a. The marked reduction of reactivity of anti-HPA-3a with PP in this study is consistent with those findings. However, it is possible that papain also removes some of the HPA-3a-specific peptide. Of the three examples of this specificity tested, reactivity was totally removed in two cases, and the titer was reduced from 64 to 2 in the other.

Conversely, reactivity of the HPA-1a antibodies and the examples of anti-HPA-5a and 5b, as demonstrated by titration studies, suggests that PP may enable detection of weak antibodies of these specificities that may otherwise be undetected using UP. In these cases, papain digestion of the platelet membrane may expose more antigen sites, or provide improved accessibility by altering the steric arrangement of relevant sites. Similar effects occur on the red cell membrane when exposed to proteases, enabling enhanced detectability of Rh antigens.[6]

The reactivity of the multispecific HLA sera was also generally enhanced. Although not available for this study, examination of single specificities of HLA antibodies is required to confirm these findings. It therefore remains to be demonstrated whether any HLA antibody specificities manifest reduced reactivity with PP.

The cause of the reactivity with the five sera not previously known to contain platelet antibodies was not determined. However, the observation that four sera showed reactivity with both PP and UP suggests that papain digestion of the platelet membrane does not result in increased nonspecific reactivity that has been associated with its use in red cell serology. The fifth serum reacted with only UP.

While this study used SPRCA and flow cytometry methods, PP should be able to be used with any of the conventional techniques applied to platelet antibody investigations. PP may therefore be useful in resolving mixtures of platelet alloantibodies when HPA-2 and HPA-3 system antigens are involved, and can provide a more sensitive test for detecting weak examples of some antibodies, e.g., in the HPA-1 and -5 systems.

This work suggests that papain treatment of platelets may be a useful and simple serological tool for investigating platelet alloantibodies.

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