METHODS

Immunoenzyme Assay of Glycocalicin, Platelet Glycoprotein Ib Fragment. Evaluation of Platelet Turnover in Circulation and Differential Diagnosis of Thrombocytopenia

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We propose a method for measurement of plasma glycocalicin, a fragment of platelet membrane glycoprotein Ib. The concentration of glycocalicin is elevated in thrombocythemia and reduced in thrombocytopenia caused by insufficient platelet production, but not in immune thrombocytopenia due to enhanced platelet degradation. Thus, plasma content of glycocalicin is an indicator of platelet turnover. The proposed assay can be used for differential diagnostics of thrombocytopenia.

Key Words: *platelets; thrombocytopenia; thrombocythemia; glycocalicin; immunoenzyme assay*

Glycocalicin (GC) is a large (125 kD) extracellular proteolytic fragment of glycoprotein Ib, a major platelet membrane protein, receptor of Willebrand factor and thrombin on platelet membrane. GC is liberated from platelet membrane by different proteases, including some intracellular Ca^{2+} proteases released during platelet degradation. In the plasma of healthy donors CG was first detected in 1984 [3]. Plasma content of GC increases in some pathological states associated with enhanced platelet production and thrombocythemia and decreases in thrombocytopenia caused by reduced platelet formation. In thrombocytopenia caused by enhanced platelet degradation, the content of CG remains practically unchanged [2,8]. We assumed that the presence of GC in the plasma results from platelet destruction by the splenic reticuloendothelial system, while its content reflects the total platelet turnover in the circulation.

The aim of the present study was to develop an immunoenzyme assay for measuring plasma GC and to test this method for differential diagnostics of thrombocytopenia.

MATERIALS AND METHODS

GC was isolated from platelets by chromatography on WGA-Sepharose [9] with final purification by FPLC on TSK G-2000 column (LKB) instead of routine chromatography on thrombin-Sepharose column. GC was eluted with phosphate buffered saline (PBS), the purity of final preparation was controlled by 7.5% SDS-PAGE. Electrophoregram under reducing and non-reducing conditions showed single band about 125 kD, which corresponded to GC. The concentration of

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GC was measured using Pierce Kits and BSA as the standard.

Monoclonal antibody (MAB) MV16d to thrombin-binding site of glycoprotein Ib were generated and characterized previously [6]. Affinity-purified rabbit polyclonal antibodies to GC were kindly provided by Dr. M. Brendt (Baker Medical Research Institute, Melbourne).

For creating new MAB panel to GC (series SM), male BALB/c mice were consecutively (with 2-week intervals) immunized with the following antigens: 1) purified GC in complete Freund's adjuvant (50 μl, intraperitoneally); 2) GC in complete Freund's adjuvant (50 μl, intraperitoneally); 3) GC without adjuvant (50 μl, intraperitoneally); 4) plasma-free human platelets (5×10^8, intraperitoneally) [5,6]; 5) 50 μg GC (challenge dose was injected 3 days before hybridization). Hybridization and cloning were performed using standard methods [5,6]. Anti-GC antibodies were selected in 2 immunoenzyme tests using the following target antigens: 1) immobilized activated platelets; 2) purified GC. The first method was many times used for selecting antibodies to platelet membrane antigens [5], including MAB VM16d to glycoprotein Ib [6]. Selection of AMB with purified GC was carried out as follows. Polyclonal anti-GC antibodies (100 μl, 1μg/ml in PBS) was incubated in a 96-well Maxisorb plate (Costar) for 1 h at 37°C, washed 5 times with PBS containing 0.03% Tween-20 (PBS-Tween was used in all washout procedures). Nonspecific binding was blocked with 1% BSA (100 μl in PBS, 1 h, 37°C). GC (100 μl, 0.5 μg/ml in PBS) was added to each well, incubated for 40min at room temperature, and washed. The antigen was immobilized on polyclonal antibodies because preliminary experiments showed that immediate sorption of GC to plastic disturbed its interaction with some MAB. Hybridoma culture medium (100 μl) was added to each well, incubated for 30 min at 37°C, washed, and the plates were incubated with peroxidase-conjugated goat antimouse immunoglobulin antibodies (BioRad, 100 μl in PBS, 1:3000) for 30 min at 37°C and washed again. Bound peroxidase was determined with 1,2-phenylenediamine by absorbance at 492 nm (A_{492}). This selection procedure yielded 3 new MAB to GC: SM1B3, SM1B4, and SM3H2. To produce preparative amounts of these antibodies, producent clones were grown in the peritoneal cavity of BALB/c mice, followed by purification of antibodies from ascitic fluid [5,6].

MAB SM1B3 were labeled with ^{125}I-Na (Reakhim) using iodogen (Pierce) [5,6]. Competitive analysis was performed as follows: GC was immobilized in 96-well plates and nonspecific binding was blocked with BS as described above. The plates were incubated with ^{125}I-MAB SM1B3 (100 μl, 2.5 μg/ml in PBS) in the presence or absence of 15-fold excess of unlabeled MAB for 40 min at room temperature. Unbound antibodies were removed by 5-fold washout with PBS, bound label was eluted with 1 M NaOH, and radioactivity in each well was counted. Unlike MAB SM1B4 and MAB SM3H2, MAB VM16d did not compete with MAB SM1B3 for binding with GC. Binding of ^{125}I-MAB SM1B3 in the presence of unlabeled MAB SM1B4, SM3H1, and VM16d was 96, 18, and 41% of ^{125}I-MAB SM1B3 specific binding with GC (the difference between the total and nonspecific binding in the presence of an excess of unlabeled MAB SM1B3). Thus, MAB VM16d and SM1B3 were selected for GC immunoenzyme assay.

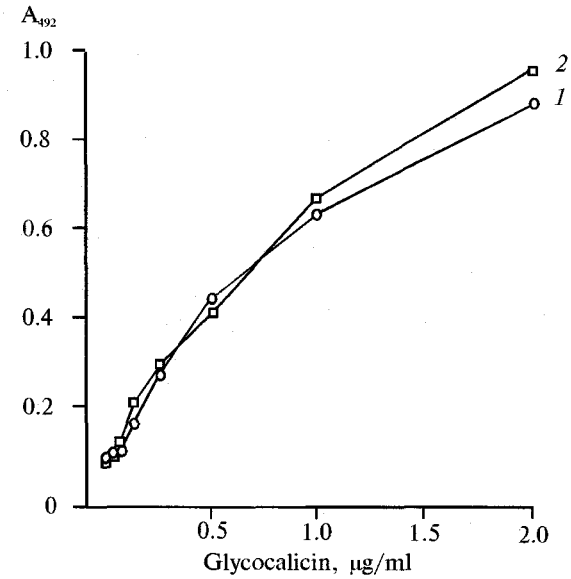

Fig. 1. Glycocalicin titration curves for pure preparation (*1*) and donor plasma (*2*) with glycocalicin concentration of 2 μg/ml. Enzyme-linked immunosorbent assay. Glycocalicin and plasma were diluted with phosphate buffered saline.

The GC determination system was based on sandwich enzyme-linked immunosorbent assay. MAB

TABLE 1. Plasma Concentration of Glycocalicin in Donors and Patients with Different Platelet Counts

Group	Platelets, $\times10^3$/μl	GC, μg/ml
Control (n=14)	233±11	2.29±0.18
Thrombocythemia (n=7)	1420±234	38.55±12.77*
Thrombocytopenia		
reduced platelet production (n=7)	26±8	0.43±0.09*
immune thrombocytopenia (n=14)	30±6	3.65±0.86+

Note. *p<0.001 compared with the control; +significant difference between two groups of patients with different thrombocytopenias (p=0.0178).

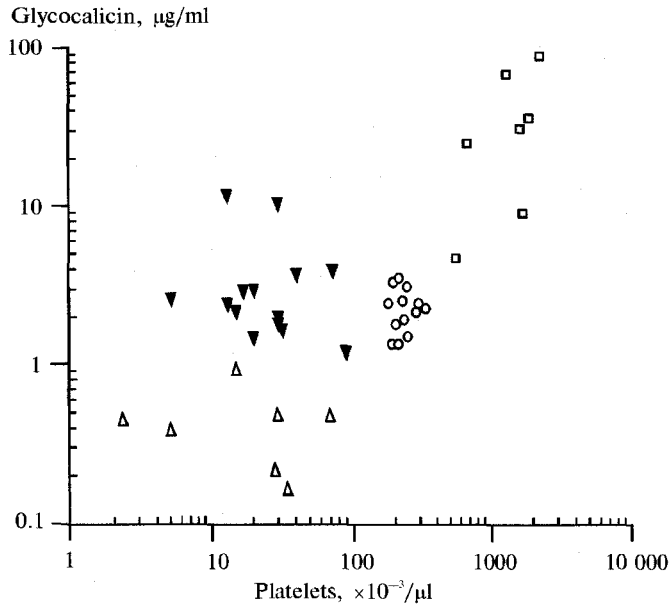

Fig. 2. Concentration of glycocalicin in plasma of donors and patients with different platelet counts. Donors (circles); patients with thrombocythemia (squares); patients with thrombocytopenia caused by reduced platelet production (open triangles) and with immune thrombocytopenia (dark triangles).

SM1B3 and VM16d (biotinylated with N-hydroxysuccinimido biotin (Sigma) according to the manufacturer protocol) were used as immobilizing and detecting antibodies, respectively. MAB SM1B3 (100 μl, 10 μg/ml in PBS) was added to a 96-well Maxisorb plate (Costar), incubated for 1 h at 37°C, washed 5 times with PBS-Tween, and nonspecific binding was blocked with 2% BSA in PBS (150 μl, 1 h, 37°C). The plates were incubated with plasma or purified GC in various dilutions (100 μl in PBS) for 40 min at room temperature, washed, incubated with biotinylated MAB VM16d (100 μl, 5 μg/ml in PBS-Tween) for 30 min at 37°C, washed again, and then incubated with streptavidin/peroxidase (IMTEK, Russia, 100 μl in PBS, 1:3000) for 30 min at 37°C. After washout, the concentration of bound peroxidase in wells was measured by the reaction with chromogen substrate as described previously.

Plasma with various platelet content was prepared from the blood of healthy donors and patients stabilized with 5% EDTA (pH 7.4) at a 1:9 ratio. The blood was centrifuged for 20 min at 2500*g* and room temperature, the plasma was collected and stored at -70°C. Before assay, the plasma was unfrozen and centrifuged 5 min at 10,000*g*. Measurements were repeated 2-3 times for each plasma sample with different dilutions.

Plasma GC was determined in healthy donors (control, n=14), patients with thrombocythemia (n=7, platelet content >500×10³/μl), patients with thrombocytopenia (platelet content <100×10³/μl) caused by reduced platelet production (n=7) and enhanced platelet degradation (n=14). The group of patients with thrombocytopenia associated with reduced platelet production comprised patients with low megakaryocyte content in the bone marrow (aplastic anemia, myelodysplastic syndrome) and one patient with disturbed platelet separation from megakaryocyte. The group of patients with thrombocytopenia associated with enhanced platelet degradation comprised patients with immune thrombocytopenia (acute and chronic idiopathic thrombocytopenic purpura, ITP). The patients were treated or observed in different hematological clinics in Moscow (Hematological Research Center, Russian Academy of Medical Sciences, Department of Hematology and Intensive Therapy of the Russian Medical Academy of Postgraduate Education, Republican Children Hospital, *etc.*)

RESULTS

Figure 1 shows the GC titration curve in PBS. The lower sensitivity limit for this method corresponds to GC concentration of about 0.1 μg/ml. This sensitivity is quite enough for measuring plasma GC (plasma GC concentration in healthy donors is about 2 μg/ml). Figure 1 also shows GC titration curve for donor plasma with GC concentration corresponding to the first value on the calibration curve. These two curves practically coincide, which confirms the absence of appreciable effect of the anticoagulant and plasma proteins on antigen-antibody interaction.

We used this method for measuring of plasma concentrations of GC in healthy donors and patients with different blood platelet content. In healthy donors, plasma concentration of GC was 2.29±0.18 μg/ml, which agrees with published data [2,3]. In thrombocythemia associated with enhanced platelet production this parameter increased, while in thrombocytopenia caused by reduced platelet production it significantly decreased in comparison with the control (Fig. 2, Table 1). A significant correlation was revealed between platelet content and plasma GC in these two groups (r=0.825, p<0.001, n=28, Fig. 2). In thrombocytopenia caused by enhanced platelet degradation (ITP) the content of GC in the plasma remained within normal despite reduced platelet count (Fig. 2, Table 1). In some patients with ITP (2 patients in acute phase) plasma content of GC was considerably elevated (Fig. 2), however, the differences between the mean values for ITP group and donors were insignificant (Table 1). It is known that in patients with ITP platelet production is not disturbed and thrombocytopenia results from enhanced platelet degradation due to sensitization with autoantibodies [1]. Since the rate of platelet degradation does not decrease in ITP, but even in-

crease in acute phase of the disease, the concentration of GC does not decline, because its release into circulation results from proteolytic cleavage associated with platelet degradation [3,8]. These findings suggest that the content of GC is proportional to the number of degrading platelets and, consequently, reflects platelet turnover in the circulation. Comparative study of patients with thrombocytopenia of different origin (caused by reduced production or enhanced degradation of platelets) revealed considerable (approximately 9-fold) differences in the content of plasma GC (Table 1, Fig. 2). These data suggest that the method proposed by us can be used for differential diagnostic of thrombocytopenia, which require different therapeutic strategies.

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REFERENCES

1. L. I. Idel'son, *Hemorrhagic Diseases and Syndromes* [in Russian], Moscow (1988), pp. 68-95.
2. J. H. Beer, L. Bochi, and B. Steiner, *Blood*, **83**, 691-702 (1994).
3. B. C. Coller, E. Kalomiris, M. Steinberg, *et al., J. Clin. Invest.*, **73**, 794-799 (1984).
4. G. A. Gamieson, T. Okumura, and M. Hasitz, *Thromb. Haemost.*, **42**, 1673-1678 (1979).
5. A. V. Mazurov, D. V. Vinogradov, N. V. Kabaeva, *et al., Ibid.*, **64**, 494-499 (1991).
6. A. V. Mazurov, D. V. Vinogradov, T. N. Vlasik. *et al., Thromb. Res.*, **62**, 673-684 (1991).
7. D. R. Phillips and M. Jakobova, *J. Biol. Chem.*, **252**, 5602-5605 (1977).
8. M. H. Steinberg, J. G. Kelton, and B. S. Coller, *N. Engl. J. Med.*, **317**, 1037-1042 (1987).
9. G. R. Wilcox, M. C. Brendt, P. A. Mehrabani, *et al., Platelets*, **2**, 45-50 (1991).