10. B. Clarke, Origins and Development of Adaptation, London (1984).
11. J. Clench, S. Barton, V. Lenaxi. et al., Chronobiologia, 7, No. 2, 265 (1980).
12. W. Kubicek, J. N. Karnegis, and R. Patterson, Aerospace Med., 37, No. 12, 1208 (1966).

RADIO-ISOTOPIC PLATELET LABELING WITH MONOCLONAL ANTIBODY AGAINST MEMBRANE GLYCOPROTEIN IIb-IIIa. MEASUREMENT OF PLATELET ADHESION/AGGREGATION TO THE SUBSTRATE

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Adhesion and aggregation of platelets on the surface of an injured vascular wall are key reactions initiating thrombus formation. Methods of investigation of adhesion and aggregation are widely used to assess the functional state of platelets in patients with cardiovascular and hematologic diseases and to investigate the action of various drugs on platelets. To study aggregation in suspension, Born [4] suggested a method of recording aggregates based on the change in scattering of light in mixed platelet suspension. However, the method does not allow adhesion processes and aggregate formation to be studied on the substrate surface. To study these processes, microscopic and radio-isotopic methods are used. To study interaction of platelets with the surface active substrates stimulating adhesion, spreading, and aggregation of platelets are used. They include various types of collagens, fibronectin, and adhesive proteins. Mazurov and co-workers [1, 9] suggested that the platelets should first be activated by plasma inducers, and only after that should they be incubated with the substrate. In that case adhesion and aggregation were stimulated even on an inactive substrate. By the use of microscopic methods, the number of adherent platelets can be directly counted and the morphology of processes such as pseudopodium formation, changes in shape, and spreading of platelets can be studied in detail [1, 3, 9]. However, microscopic methods are difficult to carry out when quantitative measurements are needed. For quantitative assessment of the total number of platelets adherent to the substrate, radio-isotopic methods are the most convenient. Isotopes ^{51}Cr [5] and ^{111}In [6] are most frequently used to label platelets [6] ^{51}Cr is incorporated in the adenylate pool of the platelets [14], but the mechanism of binding of ^{111}In with platelets has not yet been elucidated.

In this paper we suggest a new method of labeling platelets, using ^{125}I-labeled monoclonal antibody (McAb) against the membrane glycoprotein IIb-IIIa complex. Labeled platelets were used to measure platelet adhesion/aggregation on the surface of the substrate. The method was tested in a study of antiplatelet activity of the antianginal drug trapidil and the antiatherogenic agent probucol.

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EXPERIMENTAL METHOD

Platelet-enriched plasma (PEP) and washed platelets (WP) were obtained from healthy human blood [2]. After washing the platelets were suspended in Tyrode-HEFES solution (137 mM NaCl, 2.7 mM KCl, 0.36 mM Na_2HPO_4, 0.1% glucose, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.4), containing 0.35% bovine serum albumin ("Sigma," USA). The platelets were labeled with ^{51}Cr after the first sedimentation, and were then washed twice as described previously [2]. In most experiments the platelet concentration in PEP was adjusted with autologous plasma to $2 \cdot 10^8$/ml, and in WP with Tyrode-HEPES solution to $2.5 \cdot 10^8$/ml. The monoclonal antibody VM16a was characterized in detail previously [2]. This antibody: 1) belongs to the IgCl(k) subclass; 2) is directed against the complex-specific glycoprotein IIb-IIIa epitope; 3) binds specifically with the platelet surface with $K_d = 7.9 \cdot 10^{-9}$ (1.2 μg/ml) and B_{max} = 56,000 molecules per platelet; 4) does not affect platelet aggregation induced by ADP, by thromboxane A_2 analog, and thrombin, but partially inhibits aggregation stimulated by collagen. The VM16a was labeled with ^{125}I with the aid of iodogen ("Pierce," USA) by the method suggested by Fraker and Speck [6], as described previously [2]. The radioactivity of the labeled antibody was usually 500-1000 cpm/ng protein. Determination of binding of VM16a with platelets in suspension ^{125}I-VM16a was incubated with 100 μl PEP ($2 \cdot 10^8$/ml) or WP ($2.5 \cdot 10^8$/ml) for 30 min at 37°C, after which the bound label was separated from free label by centrifugation through 20% sucrose as described previously [2]. To determine nonspecific binding, simultaneously with ^{125}I-VM16a, a 50-fold excess of unlabeled VM16a also was added to the platelets. To study the adhesion/aggregation reaction of platelets on the substrate surface, platelets in PEP or the suspension of WP were labeled with ^{125}I-VM16a. WP were labeled beforehand with ^{51}Cr. Labeled platelets were introduced in a volume of 200-250 μl into wells of Multiwell cultural plates ("Falcon," USA) with a diameter of 16.4 mm. Adhesion/aggregation was stimulated by the addition of inducers to the wells with platelets immediately before the beginning of incubation. Platelets in PEP were stimulated by ADP ("Sigma," USA) and the stable thromboxane A_2 analog U46619 ("Upjohn," USA), and those in the suspension of WP by ADP, U46619, and thrombin ("Sigma," USA) Phosphate-salt buffer was added to wells in which unstimulated adhesion was measured The plates were incubated for 30 min at 37°C with mixing on a horizontal shaker at a speed of 36 rpm. As was shown previously [2, 11], these are optimal conditions for the formation of large surface aggregates. Nonadherent platelets were washed off with Tyrode-HEPES solution and adherent platelets were extracted with 1 M NaOH for 2-3 h and radioactivity was counted (either ^{125}I only or both ^{125}I and ^{51}Cr) on Compugamma ("LKB," Sweden) counter. Each determination was conducted in two parallel wells.

EXPERIMENTAL RESULTS

The previously characterized antibody VM16a, directed against the membrane glycoprotein IIb-IIIa complex and not affecting platelet aggregation stimulated by ADP, the thromboxane A_2 analog U46619, and thrombin [2], was chosen to label the platelets. The glycoprotein IIb-IIIa complex is represented on the platelet surface in a larger number than other glycoproteins. On average, on the surface of one platelet there are 40,000-50,000 molecules of the complex [2, 7]. Labeling platelets with the use of McAb against glycoproteins IIb-IIIa therefore seemed to us to be more effective than by using antibodies directed against other membrane proteins. The absence of effects on platelet aggregation induced by the most important physiological inducers made it possible to use McAb VM16a as the label to activate platelets with these agonists.

The choice of optimal labeling conditions was based on the binding characteristics of ^{125}I-VM16a. Concentration and kinetic curves of binding of ^{125}I-VR116a with platelets in PEP are illustrated in Fig. 1a, b respectively. The fraction of bound McAb relative to the free McAb was maximal on the linear region of the concentration curve, i.e., in the region of concentrations up to 2.5 μg/ml, and was about 60-70% (Table 1). The binding curve then flattens out on a plateau and the percentage of bound antigen falls. Accordingly, we chose for labeling concentrations of ^{125}I-VM16a corresponding to the linear region, namely 0.5 or 1.0 μg/ml, depending on specific radioactivity of the antibody. It will clear from Fig. 1b that binding reached a maximum during the first 10 min, and remained unchanged thereafter until 40 min. On the basis of these observations, platelets were subsequently labeled with ^{125}I-VM16a in a concentration of 0.5 or 1.0 μg/ml for 30 min at 37°C. Under these conditions, with $2 \cdot 10^8$ platelets in PEP, binding

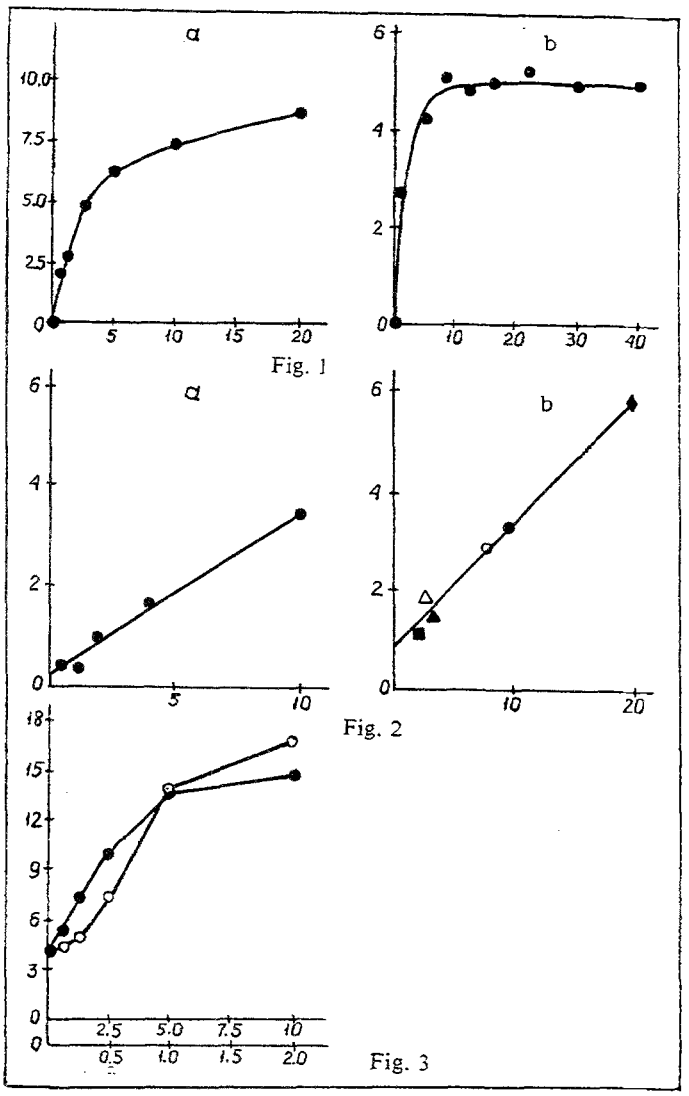

Fig. 1. Characteristics of binding of ^{125}I-VM16a with platelets: a) binding of ^{125}I-VM16a with platelets as a function of concentration 100 μl PEP ($2 \cdot 10^7$ platelets) were incubated with different concentrations of ^{125}I-VM16a for 30 min at 37°C and total binding of ^{125}I-VM16a was determined; b) kinetics of binding as function of concentration: 100 μl PEP ($2 \cdot 10^7$ platelets) was incubated with 1 μg/ml ^{125}I-VM16a at 37°C for different times and total binding of ^{125}I-VM16a was determined.

Fig. 2. Correlation of measurements of platelet adhesion/aggregation with the aid of ^{51}Cr and ^{125}I-VM16a. a) Adhesion/aggregation reaction carried out in the presence of 0.1 U/ml thrombin and with different concentrations of platelets from 0.3125 to $5 \cdot 10^8$/ml. Coefficient of correlation r = 0.995; b) adhesion/aggregation reaction carried out in the absence of inducers (filled square) or in the presence of 5 or 10 μM ADP (filled and empty triangles respectively) or 0.5 or 1.0 μM U46619 (filled and empty circles respectively), or 0.1 U/ml thrombin (filled lozenge). Concentration of washed platelets $2.5 \cdot 10^8$/ml. Coefficient of correlation r = 0.995. Intercepts of straight lines on ordinates characterize level of nonspecific sorption of ^{125}I-VM16a.

Fig. 3. Concentration curves of platelet adhesion/aggregation, stimulated by different doses of ADP and U46619. Platelets labeled in PEP with ^{125}I-VM16a and adhesion/aggregation reaction carried out in presence of different doses of PEP (0-10 μM) (filled circles) and U46619 (0-2 μM) (empty circles).

TABLE 1. Characteristics of Binding of ^{125}I-VM16a with Platelets

Binding of ^{125}I-VM16a	PEP		Blood, 1 μg/ml
	0.5 μg/ml	1 μg/ml	
Per cent of added dose	69	72	72
ng/10^6	2,0	4,0	4,9
Per cent of specific	88	89	—

TABLE 2. Action of Probucol, Trapidil, and PGE1 on Platelet Adhesion/Aggregation, Stimulated by ADP and U46619

Preparation	Adhesion/aggregation (in % of added)	
	ADP	U46619
Control	47±9	46±5
Probucol	40±9	46±6
Trapidil	18	16
PGE_1	12±1	4±1

with ^{125}I-VM16a amounted to about 70% of the added label, and the percentage of specific binding under these circumstances was about 90% The level of binding of ^{125}I-VM16a in a concentration of 0.5 μg/ml was 2 ng/10^6 platelets, whereas with a concentration of 1.0 μg/ml, the level of binding was doubled (linear region of the curve) and reached 4 ng/10^6 platelets (Table 1). It is important to note that similar binding characteristics were maintained when the platelets were labeled, not in PEP, but in whole blood, evidence of the high selectivity of binding and the absence of effects of other blood components on labeling efficacy (Table 1).

For quantitative determination of the level of adhesion and aggregation of platelets to a substrate, platelets labeled with ^{51}Cr are often used. We verified the level of correlation of this method with our proposed method of labeling with ^{125}I-VM16a. Experiments were carried out in a suspension of WP, for incorporation of ^{51}Cr during platelet labeling amounts to 10-20%, and for this reason, to carry out experiments with ^{51}Cr-labeled platelets, preliminary washing to remove the free label is necessary. ^{51}Cr-labeled platelets, washed to remove plasma, were incubated with ^{125}I-VM16a and adhesion was measured relative to ^{51}Cr and ^{125}I. In one experiment adhesion/aggregation was stimulated by thrombin with different concentrations of platelets (Fig. 2a), whereas in another the adhesion/aggregation reaction was carried out without inducers or in the presence of various agonists, namely ADP, U46619, and thrombin (Fig. 2b). In both types of experiments, high linear correlation was found between the ^{51}Cr and ^{125}I number. It can accordingly be concluded from these results that the use of ^{125}I-VM16a-labeled platelets yields adequate and readily interpreted results with quantitative measurements of adhesion/aggregation on the substrate surface.

Platelet adhesion/aggregation on the substrate surface in PEP was stimulated by ADP and U46619. In the region of ADP concentrations up to 5 μM and U46119 up to 1 μM the level of adhesion/aggregation rose and then flattened out on a plateau at concentration of ADP of 5-10 μm and U46619 of 1-2 μm (Fig. 3). According to the averaged results of several experiments, in the absence of inducers the number of platelets adherent to the substrate was 6 ± 1%, whereas on activation with 5 μM ADP and 1 μM U46619 it was 42 ± 9% and 46 ± 5% of the number of platelets added respectively (n = 4-5) The absolute number of adherent platelets was calculated from the specific radioactivity of the labeled platelets, determined on the basis of binding of ^{125}I-VM16a with platelets in suspension. In the absence of inducers the number of adherent platelets was about $3 \cdot 10^6$, whereas in the presence of ADP or U46619 it was about $20 \cdot 10^6$ per well. In some experiments, in order to estimate the level of nonspecific sorption of the antibody, we added ^{125}I-VM16a to plasma without platelets. The level of sorption under these conditions, i.e., in the presence of plasma, was less than 1% of the introduced label.

Our method was used to assess antithromocytic activity of the antianginal preparation trapidil [10] and of the antiatherogenic agent probucol [13]. As the control preparation, with a marked inhibitory effect, we used prostaglandin E_1 (PGE_1). Adhesion/aggregation was stimulated in PEP by ADP and U46619. The results showed that probucol does not affect the level of platelet adhesion/aggregation stimulated by either of these inducers, whereas trapidil depressed it, although by a lesser degree than PGE (Table 2).

The proposed method can therefore be used to measure platelet adhesion/aggregation not only in PEP but also in blood. It was shown by the use of the proposed method that the antianginal drug trapidil can effectively inhibit platelet–substrate interaction, stimulated by ADP and U46619, in agreement with previous observations showing that trapidil has an inhibitory action on platelets due to inhibition by cAMP phosphodiesterase [11].

REFERENCES

1. L. É. Atakhanova, A. V. Mazurov, É. R. Katsenovich, et al., Byull. Éksp. Biol. Med., No. 2, 135 (1990).
2. D. V. Vinogradov, T. N. Vlasik, O. G. Agafonova, et al., Biokhimiya, No. 5, 78 (1991).
3. R. D. Allen, L. R. Zacharski, S. T. Widirstky, et al., J. Cell Biol., **83**, 126 (n. d.).
4. G. V. R. Born and M. J. Cross, J. Physiol. (London), **168**, 178 (1963).
5. A. M. Dosne, L. Drouet, and E. Dassine, Microvasc. Res., **11**, 111 (1976).
6. P. J. Fraker and J. C. Speck, Biochem. Biophys. Res. Commun., **80**, 849 (sic).
7. M. H. Ginsberg, J. J. Loftus, and E. F. Plow, Thromb. Haemostas., **59**, 1 (1988).
8. A. P. Heyns, P. N. Badenhorst, H. Pieters, et al., Thromb. Haemostas., **42**, 1473 (1980).
9. A. V. Mazurov, V. L. Leytin, V. S. Repin, et al., Thromb. Res., **32**, 189 (1983).
10. A. V. Mazurov, M. Yu. Menshikov, V. L. Leytin, et al., FEBS Lett., **172**, 167 (1984).
11. A. V. Mazurov, V. E. Sinitsyn, and V. S. Repin, Thromb. Res., **52**, 573 (1988).
12. A. V. Mazurov, G. L. Idelson, M. V. Hachikyan, et al., Matrix, **9**, 214 (1989).
13. S. Parthasarathy, S. G. Young, J. L. Witztum, et al., J. Clin. Invest., **77**, 641 (1986).
14. M. Steiner and M. Baldini, Blood, 35, 727 (1970).