Inhibition of Platelet Aggregation by Monoclonal Antibodies against Glycoprotein IIb-IIIa Complex

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Monoclonal antibodies CRC64 are obtained against Ca^{2+}-dependent glycoprotein IIb-IIIa complex of the platelet membrane which possess the ability to inhibit completely fibrinogen-dependent platelet aggregation. CRC64 is directed against the epitope formed by the glycoprotein IIb-IIIa complex and does not interact with proteins isolated after platelets are treated with ethylenediamine tetraacetate. Complete, reproducible blockade of platelet aggregation caused by 5 μM adenosine diphosphate is noted in an MCA concentration of 3 μg/ml, while in the case of a stronger inductor, namely 1 U/ml thrombin, platelet aggregation is inhibited in a concentration of 5 μg/ml. $F(ab')_2$ fragments are also able to inhibit platelet aggregation completely and are usually effective in concentrations lower than native monoclonal antibodies.

Key Words: *monoclonal antibodies; platelets, aggregation; glycoprotein IIb-IIIa complex*

The main platelet reactions, namely adhesion and aggregation, are mediated via membrane glycoproteins (GP). The Ca^{2+}-dependent GP IIb-IIIa complex plays a key role in the process of aggregation. After activation of platelets by thrombin, adenosine diphosphate (ADP), collagen, and other agonists, the complex changes its conformation and becomes capable of high-affinity binding with fibrinogen as well as with fibronectin and Willebrand factor. The interaction of these proteins, but primarily of fibrinogen, with the surface of activated platelets is an event mediating the adhesion of platelets and the formation of aggregates [6,10].

The GP IIb-IIIa complex is a major and high-immunogenic protein of the platelet surface. A large number of monoclonal antibodies (MAB) against this protein have been obtained during the last 10 years. Some of them have been able to inhibit fibrinogen binding and thereby to block platelet aggregation [2,4,5]. Results of experiments carried out on animals have shown that such antibodies may be used as antithrombic preparations [3,7].

A method of obtaining and characterizing new MAB against GP IIb-IIIa effectively inhibiting platelet aggregation is described in the present study.

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MATERIALS AND METHODS

Platelet-enriched serum and serum-washed platelets were obtained as described previously [1].

Female BALB/c mice were immunized with washed platelets according to a scheme not used previously. Platelets at 2.5×10^8 per mouse were administered i.p. with Freund complete adjuvant prior to the experiment and on the 7th day; 2.5×10^8 platelets per mouse were administered i.p. in Tyrode/Hepes solution on the 12th day, and on the 16th and 18th day 2.5×10^8 platelets per mouse were administered i.p. in Tyrode/Hepes solution and the same number of platelets were injected i.v. Hybridization was performed 3 days after the last immunization. The confluence and cloning of cells were performed according to [9] using mouse myeloma X63-Ag 8.653 as partner lines. In the

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first stage of screening clone-producing MAB were selected according to the capacity to bind with platelets. MAB-platelet binding was recorded using a method of immunoenzyme assay described previously in detail [8]. Hybridized cells synthesizing MAB against platelets were cloned using the method of limiting dilutions and then cultivated in BALB/c mouse peritoneal cavity. Purification and isolation of MAB from ascitic fluid as well as determination of isotypes were performed as described previously [1]. Purity of antibodies was controlled with polyacrylamide gel SDS-electrophoresis (PAGE). MAB CRC54 recognizing GP IIb-IIIa but not inhibiting platelet aggregation were used as a control [8], while MAB 8D8 against monkey B apoprotein, which do not interact with platelets, were the control in immunoprecipitation.

Binding of ^{125}I-labeled MAB with platelets was studied as described previously [1].

Determination of the MAB antigen was performed using immunoprecipitation with ^{125}I-surface-labeled platelet lysate, obtained after treatment with Triton X-100, as initial material. Immunoprecipitation was carried out according to a method described previously [1].

F(ab')$_2$ fragments of antibodies were obtained by pepsin hydrolysis [1].

RESULTS

One hundred twenty-five MAB-producing clones binding with platelets, including 15 antibodies able to inhibit to a lesser or greater degree the ADP-aggregation of platelets, were screened. CRC64 MAB which completely and repeatedly blocked platelet aggregation were chosen for further investigation. After cloning and culturing in mouse peritoneal cavity, MAB were purified and characterized. It was shown that CRC64 MAB pertain to the IgG1 subclass and F(ab')$_2$ fragments of CRC64 were obtained by pepsin hydrolysis. The effect of purified CRC64 MAB and F(ab')$_2$ fragments on platelet aggregation intensified by the physiologically most important inductors ADP and thrombin was checked in the next stage. It was found that CRC64 MAB as well as its F(ab')$_2$ fragments are able to block platelet aggregation completely (Figs. 1, 2). The complete inhibition of aggregation stimulated by 5 μM ADP was achieved when the MAB concentration was 3 μg/ml. A lower concentration of MAB yielded partial inhibition of

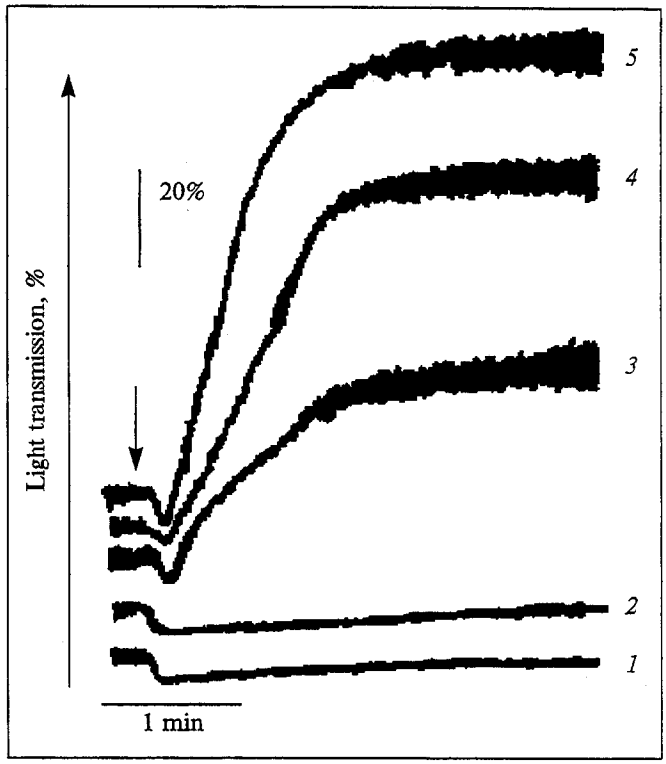

Fig. 1. Effect of CRC64 antibodies and F(ab')$_2$ fragments of CRC64 on ADP-induced platelet aggregation. Platelet-enriched serum (2.5×10^8 platelets/ml) was placed in an aggregometer cuvette, incubated for 2 min with CRC64 at 3 μg/ml (*1*) or 2 μg/ml (*3*), F(ab')$_2$ fragments of CRC64 at 2 μg/ml (*2*) or 1.0 μg/ml (*4*), or control antibodies CRC54 at 5 μg/ml (*5*), after which aggregation was induced by the addition of 5 μM ADP. Here and in Fig. 2: an arrow shows the moment of inductor addition.

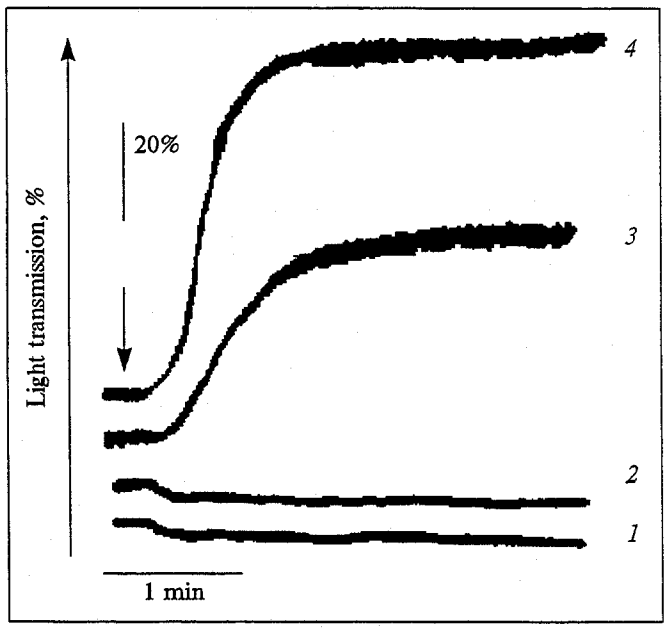

Fig. 2. Effect of CRC64 antibodies on aggregation of washed platelets induced by 1 U/ml of thrombin. Serum-washed platelets (3×108/ml) were placed in an aggregometer cuvette, incubated for 2 min with CRC64 at 5 μg/ml (*1*) or 2 μg/ml (*3*), F(ab')2 fragments of CRC64 at 4 μg/ml (*2*), or control antibodies CRC54 at 5 μg/ml (*4*). Aggregation was induced by the addition of 1 U/ml thrombin.

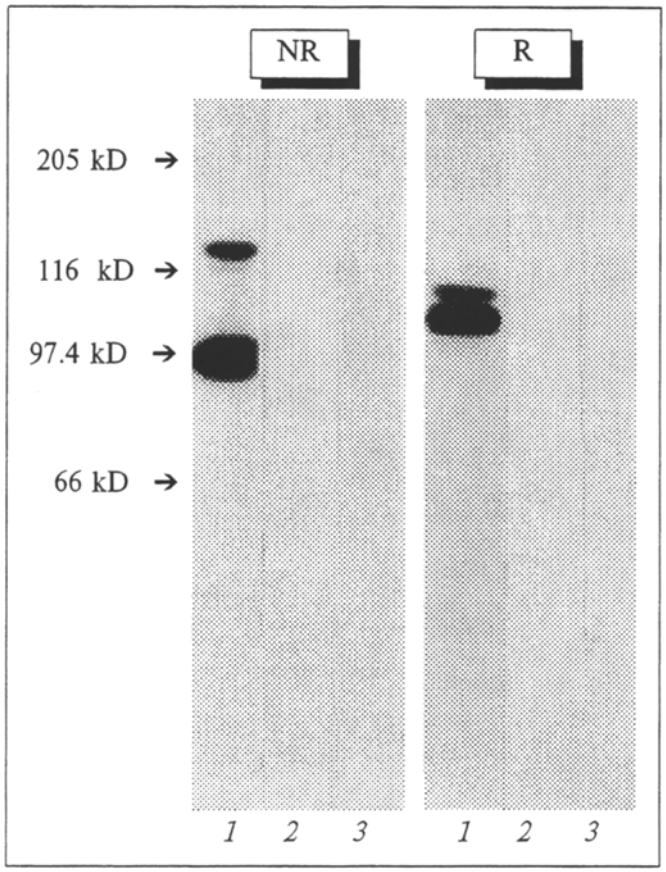

Fig. 3. Immunoprecipitation of CRC64 MAB from ^{125}I-labeled platelet lysate. Radioautograph. 7.5% PAGE after SDS-electrophoresis under nonreducing (*NR*) and reducing (*R*) conditions. Washed platelets were incubated for 20 min at 37°C at the presence of 2 mM $CaCl_2$ and 1 mM $MgCl_2$ (*1*) or 5 mM EDTA (*2*), after which the platelets were lysed with Triton X-100 and immunoprecipitation was performed as described previously [1]. *1*) GP IIb and IIIa precipitated by CRC64 MAB in the presence of 2 mM $CaCl_2$ and 1 mM $MgCl_2$; *2*) in the presence of 5 mM EDTA CRC64 does not precipitate any proteins; *3*) the track of control MAB 8D8, which did not bind with platelets. Arrows point to the position of markers of molecular weight: 205 kD for myosin, 116 kD for galactosidase, 97.4 kD for phosphorylase B, and 66 kD for serum bovine albumin.

aggregation. $F(ab')_2$ fragments of MAB blocked ADP aggregation in concentrations from 2 to 5 μg/ml (Fig. 1). Thrombin in high doses (more than 0.5 U/ml) is the most powerful inductor of platelets. As is evident from Fig. 2, CRC64 MAB are able to inhibit completely platelet aggregation induced by 1 U/ml thrombin. Complete inhibition is noted at 5 μg/ml CRC64. $F(ab')_2$ fragments of CRC64 at 4 μg/ml completely blocked thrombin-induced aggregation. Thus, $F(ab')_2$ fragments usually produce a blocking effect in lower concentrations than native MAB. It should be noted that complete inhibition of aggregation was noted at different MAB concentrations in different donors. In the case of ADP aggregation the variations were from 0.5 to 3.0 μg/ml CRC64.

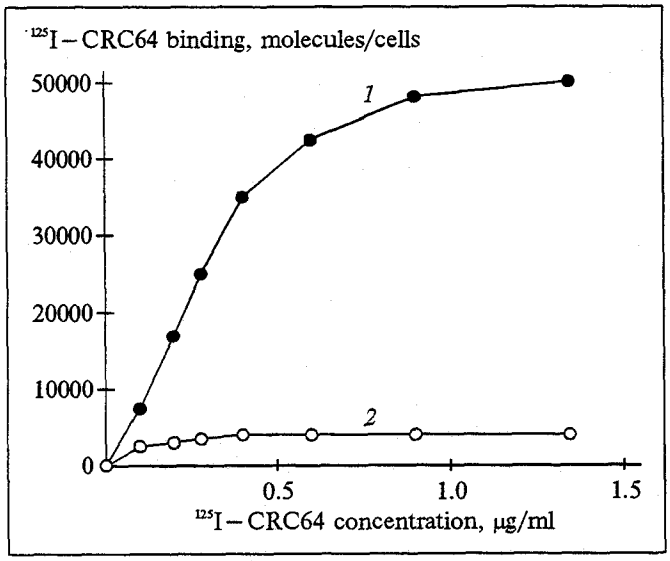

Fig. 4. ^{125}I-labeled CRC64 binding with washed platelets. Washed platelets at 3×10^8/ml were incubated for 20 min at 37°C with 2 mM $CaCl_2$ and 1 mM $MgCl_2$ (*1*) or 5 mM EDTA (*2*), after which ^{125}I-CRC64 was added in the indicated concentrations either without (total binding) or with 20-fold nonlabeled CRC64 (nonspecific binding). The values of specific binding are given.

Determination of CRC64 antigen was performed using immunoprecipitation. CRC64 precipitated two proteins with molecular weights of 130 and 90 kD under nonreducing and of 120 and 100 kD under reducing conditions (Fig. 3) from ^{125}I-labeled platelet lysate, which corresponds to the electrophoretic characteristics of GP IIb and IIIa. Treating platelets with ethylenediamine tetraacetate (EDTA), which causes dissociation of the GP IIb-IIIa complex, prevents MAB binding and target protein precipitation, testifying to a complex-specific nature of CRC64 antigen.

Binding of ^{125}I-labeled CRC64 with platelets was studied in a medium containing Ca^{2+}/Mg^{2+} or EDTA. As is shown in Fig.4, treatment of platelets with EDTA prevents antibody binding, which corresponds to the immunoprecipitation data and points once again to the complex-specific nature of the CRC64 epitope. In the presence of Ca^{2+}/Mg^{2+} the binding curve reached a plateau at a ^{125}I-CRC64 concentration of 1 μg/ml, which corresponds to the MAB concentration at which inhibition of platelet aggregation occurred. In a saturating concentration around $40\text{-}50\times10^3$ molecules of ^{125}I-CRC64 bound with one platelet, which corresponds to the number of GP IIb-IIIa copies on the platelet surface [6,10]. The nature of the curve with an abrupt plateau did not make it possible to construct interpretable plots in Skatchard coor-

dinates or to determine the K_d and B_{max} values accurately.

CRC64 MAB are directed against the GP IIb-IIIa complex and possess the ability to inhibit completely fibrinogen-dependent aggregation of platelets induced by ADP and thrombin. $F(ab')_2$ fragments of CRC64 possess the same property. Like the majority of described inhibitory antibodies [2,4,5], they are complex-specific and do not interact with dissociated proteins. Evidently, like other MAB, CRC64 blocks platelet aggregation, preventing fibrinogen (and, possibly other adhesion protein) binding with its receptor on the surface of activated platelets, the GP IIb-IIIa complex. Since the fibrinogen-dependent mechanism is common to the majority of agonists, and not only to ADP and thrombin, it may be assumed that CRC64 MAB will also block aggregation caused by collagen, epinephrine, and other inductors.

Some inhibitory MAB were used previously as antithrombic preparations in experiments on animals and in clinical trials [3,7,11]. The comparison of CRC64 properties with reported MAB attests that the MAB obtained and characterized by us are not only not inferior to them in the capacity to inhibit aggregation, but are even superior to them (Table 1).

Thus, the CRC64 MAB obtained by us may be recommended for further testing as an antithrombic agent and for prospective use in clinical practice.

TABLE 1. Characterization of MCA Inhibiting Platelet Aggregation

MCA	Inhibiting concentrations of MCA, μg/ml		Reference
	ADP–aggregation	Thrombin–aggregation	
A2A9	50	75	[2]
10E57	–	[5]	
7E3	10	10	[4]
CRC64	3	5	

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