

Characteristics of platelet aggregation induced by the monoclonal antibody ALB₆ (acute lymphoblastic leukemia antigen p 24)

Inhibition of aggregation by ALB₆Fab

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The leukemia-associated cell surface antigen p 24 is found on normal platelets as well as on Bernard Soulier syndrome and thrombasthenia type I platelets. ALB₆ IgG (a monoclonal antibody against p 24) induces the aggregation of platelets from normal donors but not from thrombasthenia. In contrast, ALB₆ Fab inhibits platelet aggregation induced by collagen, ADP, thrombin, ionophore A 23187 and ALB₆ IgG. The results suggest that ALB₆ interferes with a mechanism common to all aggregation pathways; the possible mechanisms are discussed.

Platelet aggregation

Monoclonal antibody

Leukemia

Calcium

1. INTRODUCTION

A set of monoclonal antibodies against common acute lymphoblastic leukemia has been produced in our laboratory [1,2]. Among these antibodies one of them, called ALB₆, showed a pattern of reactivity against various tissues similar to monoclonal antibody Ba₂ in [3,4]. These reagents react with surface components of many hematopoietic and non-hematopoietic cells as shown by immunofluorescence studies. These cells are either malignant or normal and include a large percentage of acute lymphoblastic leukemia and neuroblastoma or retinoblastoma cell lines; chronic lymphocytic leukemia and acute myeloid leukemia cells are also slightly labelled in some cases. On frozen sections these antibodies have been shown to react with distal tubules of the

kidney and the wall of lymph node vessels. Biochemical studies have shown that in leukemic cells a membrane protein of M_r 24 000 (p 24) is immunoprecipitated by Ba₂ [3] and ALB₆ (in preparation). Interestingly these antibodies also react with a platelet surface component. We therefore studied the effect of ALB₆ IgG and ALB₆ Fab on platelet functions and determined the M_r value of the platelet molecule identified by ALB₆.

2. MATERIALS AND METHODS

2.1. Antibody production and purification

Techniques used for the production of monoclonal antibodies have been reported. ALB₆ is an IgG₁ which has been produced by fusing spleen cells of Balb/c mice, immunized with non-T non-B acute lymphoblastic leukemic cells, with NS₁ myeloma cells [1,2]. Here, we used either cell culture supernatants, ascitic fluids previously

Abbreviation: SDS-PAGE; Sodium dodecyl sulfate polyacrylamide gel electrophoresis

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heated at 55°C for 30 min, or purified antibody fractions. Controls were performed using ALB₁ (anti-common acute lymphoblastic leukemia antigen) and B₄ F₂ (anti-HLA monomorphic determinant).

IgG was purified from ascitic fluid by precipitation in 40% ammonium sulfate. The precipitate was dissolved in phosphate buffer (pH 8, 10 mM) and desalted on a GFO₅ column (Industrie Biologique Française) followed by ion-exchange chromatography on DEAE-trisacryl (IBF) equilibrated in phosphate buffer (pH 8, 10 mM). Elution pattern with a molarity gradient (0–0.3 M NaCl) showed 3 major peaks. All the immunological activity was found in the first peak. This final preparation contained 96% globulins and 3% albumin as assessed by cellulose acetate electrophoresis.

After reprecipitation with ammonium sulfate ALB₆ IgG were dissolved and dialysed against 0.1 M phosphate buffer (pH 7) containing 10 mM cysteine and 2 mM EDTA to determine the optimal conditions for Fab obtention, a cleavage kinetic with mercuripapain (Sigma, St Louis MO) at a 2% ratio of papain/protein was performed. The reaction was stopped in an ice bath with 5% 1 M iodoacetic acid (Sigma) in 0.05 M Tris (pH 8). The fractions were immediately tested by immunofluorescence on platelets and SDS-PAGE for assessment of activity and degradation ratio. At 6 h the degradation was partial (60%) but the preservation of activity was satisfactory.

Therefore, 3 mg ALB₆ were cleaved in these conditions and undigested IgG was immediately separated by Sephadex G-75 gel filtration. Tris, 10 mM (pH 8) was used for elution and the second peak containing a mixture of Fc and Fab fragments was applied to an ion-exchange DE-52 (Pharmacia Uppsala) column equilibrated in 10 mM Tris (pH 8). Fab fragments were easily released by a NaCl gradient (15–50 mM). The solution of Fab fragments at 0.4 mg/ml was adjusted to 0.15 M (pH 7.4) with phosphate buffer.

2.2. Preparation of platelets and platelet aggregation

2.2.1. Preparation of platelet-rich plasma (PRP)

Blood was collected into 1/9 vol. of 0.13 M sodium citrate. PRP was obtained by centrifugation at 18°C at 120 × g for 10 min.

2.2.2. Preparation of washed platelets

Venous blood from healthy volunteers and patients was anticoagulated with ACD [0.8% (w/v) citric acid, 2.2% (w/v) trisodium citrate, 2.25% (w/v) dextrose] at a 4:5 ratio of blood/anticoagulant. Immediately prior to blood collection, apyrase (grade 1, Sigma) and PGE₁ (Sigma) were added to the anticoagulant to a final concentration of 25 ng/ml and 20 nM, respectively. PRP was obtained by centrifugation at 120 × g for 10 min at 18°C and then the PRP was recentrifuged at 1200 × g for 10 min. The pellet was resuspended in washing buffer (36 mM citric acid, 5 mM glucose, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 103 mM NaCl, 20 mM PEG₁, 25 ng apyrase/ml and 3.5 mg BSA/ml, adjusted to pH 6.5). After two washings carried out in plastic tubes at room temperature, the platelets were resuspended in tyrode buffer (pH 7.35, 137 mM NaCl, 2 mM KCl, 12 mM NaHCO₃, 0.3 mM PO₄H₂Na, 2.9 mM CaCl₂, 1 mM MgCl₂, 5.5 mM glucose and 3.5 mg BSA/ml).

2.2.3. Preparation of fixed platelets

Washed platelets were resuspended in tyrode buffer without albumin in the presence of 1% formaldehyde for 18 h at room temperature. After 5 washings, the platelets were resuspended in tyrode buffer without BSA.

2.2.4. Platelet aggregation

Platelet aggregation was measured as in [5]. Platelet aggregation was followed by recording light transmission through a stirred platelet suspension (3×10^8 /ml) in an aggregometer cuvette (Chronolog Coultronic) at 37°C in a final volume of 0.4 ml.

The apparatus was calibrated so that platelet suspension (or PRP) and buffer solution (or PPP) produced 90 and 0 absorbance units (AU), respectively. Lag phase before platelet aggregation was measured and the extent of platelet aggregation was evaluated when aggregation was complete and expressed in AU. ADP, collagen, (from bovine achille tendon, dissolved in glycine buffer, pH 4), arachidonic acid, thrombin, ionophore A23187 and ristocetin were used as aggregating agents (the concentration was noted for each result). The absence of lysis was assessed by assaying lactate dehydrogenase in the supernatant (LDH optimized UV test, Merck, Darmstadt).

2.2.5. ALB₆ IgG-induced platelet aggregation

Various amounts of culture supernatants, ascitic fluid or purified IgG were tested both on PRP and washed platelets from normal donors and from thrombasthenia and Bernard Soulier syndrome patients. ALB₆ Fab was also tested.

2.2.6. Inhibition of ALB₆ platelet aggregation

Various amounts of EDTA, PGE, aspirin (lysine salt), ticlopidin, chlorpromazin and diltiazem diluted in 0.15 M NaCl were added to 0.3 ml platelet suspensions, before adding 0.1 ml ALB₆ IgG and aggregation was measured. The same amount of solvent was used as control.

2.2.7. Effect of ALB₆ Fab fragments on platelet aggregation induced by aggregating agent

ALB₆ Fab fragments (0.1 ml 400 µg/ml) were incubated with either 0.3 ml PRP (0.3 ml washed platelets) for 4 min at 37°C with continuous stirring (in the aggregometer). Then, the aggregating agent was added and the aggregation curves were recorded. Controls with buffer alone were always performed.

2.3. Immunofluorescence

Washed platelets were used for immunofluorescence. Samples of 3×10^6 platelets in 20 µl Tyrode buffer were incubated at 4°C for 30 min with appropriate dilutions of ALB₆, washed 3-times and incubated with FITC-labelled goat antimouse Ig. Examination of the preparation was performed on a Zeiss 2 microscope equipped with epifluorescence. Controls were performed using other monoclonal antibodies B₄ F₂ and ALB₁.

2.4. Effect of ALB₆ Fab on fibrinogen-binding to platelets

Binding of [¹²⁵I]fibrinogen to ADP-treated platelets was performed as in [6]. The binding was measured on platelets in the presence or absence of ALB₆ Fab (10 min incubation with 60 µg/ml ALB₆ Fab) before ADP addition. The results were expressed in percentages of fibrinogen bound to platelets.

2.5. Molecular mass determination of the antigen recognized by ALB₆ ¹²⁵I-labelling of platelets

Platelets (3×10^8) in 100 ml Tyrode buffer were labelled with 400 µCi ¹²⁵I by the addition of 100 µg

chloramine T. The reaction was stopped after 1 min incubation by dilution and rapid washing in ice-cold Tyrode's buffer. The cells were lysed by resuspension in ice-cold extraction buffer NET NP 40 (NaCl 30 mM, Tris 30 mM, EDTA 1 mM, NP 40 1%, PMSF 2 mM). The extract was centrifuged at $1000 \times g$ for 10 min and the supernatants aliquoted and kept at -70°C.

2.6. Immunoadsorption and SDS-PAGE

After ultracentrifugation for 10 min in an air-fuge (Beckman) to eliminate residual cell membranes, the extracts were passed through microcolumns (5 µl) of immunoadsorbents (glutaraldehyde-activated ultrogels coated with ALB₁ IgG, ALB₆ IgG or non-specific mouse IgG) as in [7]. After washing, the immunoadsorbent was directly plunged into 50 µl 0.0625 M Tris-HCl buffer (pH 6.8) containing 5% (v/v) 2 mercaptoethanol, 10% (v/v) glycerol, 2% SDS and 0.001% (w/v) bromophenol blue. After dissociation for 2 min in boiling water, the samples were analyzed by SDS-PAGE gradient gel electrophoresis as in [8] except that the running gel was 5-20% (w/v) acrylamide. After drying of the gels, labelled proteins were detected by autoradiography.

3. RESULTS

3.1. Induction of platelet aggregation by ALB₆

As shown in fig. 1 addition of ALB₆ IgG to PRP led to a typical aggregation pattern preceded by a lag phase. (Just before aggregation a slight increase in absorbance was seen.) The same was observed with washed platelets without addition of fibrinogen (not shown). This aggregation could be obtained with heated supernatants or ascites as well as with purified IgG. The lag phase increased with increasing dilutions of ALB₆ IgG (fig. 1). However, the intensity of maximal aggregation was independent of the quantity of ALB₆ IgG. Between 2-32 µg/ml there is a linear relationship between the inverse of the concentration of ALB₆ IgG and the logarithm of the lag phase (not shown). The minimal concentration after 30 min was observed at 1 g/ml. None of the monoclonal antibodies used as controls ALB₁ or B₄ F₂ induced aggregation. In order to eliminate the action of proteolytic enzymes contained in supernatants or

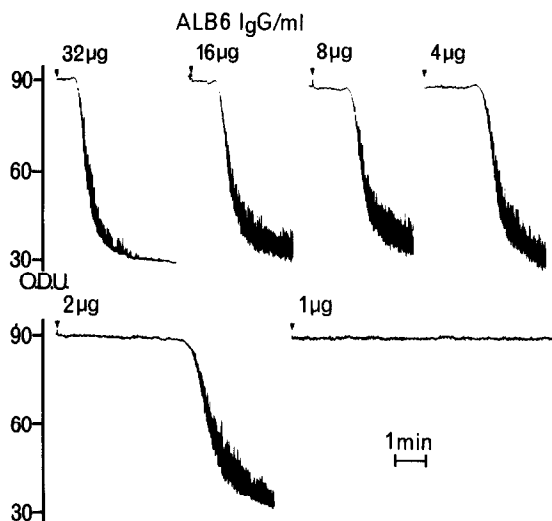


Fig. 1. Platelet aggregation induced by ALB₆ IgG. There is an increase in the lag phase with decreasing concentrations of ALB₆ IgG without a change in the intensity of maximal aggregation. The lag phase observed at 64 and 128 µg/ml was identical to 32 µg/ml (O.D.U. absorbance units).

ascitic fluids we checked that neither iniprol (10^3 units/ml) nor heparin antithrombin III (to inhibit thrombin) inhibited ALB₆ IgG-induced platelet aggregation. ALB₆ IgG induced to the same extent platelet aggregation from normal PRP and from PRP of Bernard Soulier syndrome. No aggregation however occurred when ALB₆ was added to PRP of a thrombasthenia type I and 2 thrombasthenia type II patients, only a change in shape was observed. ALB₆ IgG even at high concentrations did not induce agglutination of formaldehyde-fixed platelets. The level of LDH in PRP was not increased after ALB₆-induced platelet aggregation.

3.2. Effect of ALB₆ Fab fragments

No aggregation was observed with purified ALB₆ Fab fragments up to 100 µg/ml. Conversely Fab fragments inhibited ALB₆-induced platelet aggregation. When Fab fragments and purified IgG (used at 100 µg/ml and 4 µg/ml, respectively) were added simultaneously in PRP 50% inhibition of ALB₆-induced aggregation was observed (velocity and maximal intensity). When ALB₆ Fab was preincubated with the PRP (5 min at 37°C with stir-

ring) the inhibition was total. In these conditions 100 µg/ml and 75 µg/ml ALB₆ Fab completely inhibited ALB₆ IgG platelet aggregation whereas 50 µg/ml ALB₆ Fab led to slightly delayed aggregation.

Preincubation for 5 min of PRP with ALB₆ Fab (at 100 µg/ml) totally inhibited platelet aggregation induced by collagen (0.4 µg/ml), arachidonic acid (0.6 µM), ADP (2.5 µM) and on washed platelets by thrombin (0.1 units/ml). ALB₆ Fab also inhibited aggregation by ionophore A 23187 but this effect was with low doses (7.5 µM) as ionophore induces only partial aggregation. At higher doses (15 µM) Fab fragments partially inhibited the aggregation which was followed by disaggregation (fig. 2).

3.3. Effect of antiaggregating substances on ALB₆ IgG-induced platelet aggregation (ALB₆ IgG at 4 µg/ml)

With aspirin up to 100 µg/ml only a slight inhibition was observed. No inhibition was observed with ticlopidin (up to 2.5×10^{-3} M). Total inhibition was observed with chlorpromazin (100 µg/ml), diltiazem (400 µg/ml), verapamil (400 µg/ml), EDTA (12.5 µM) and PGE₁ (1.25 µM). The concentrations used were chosen after establishing controls with the usual aggregating drugs (ADP, collagen, thrombin).

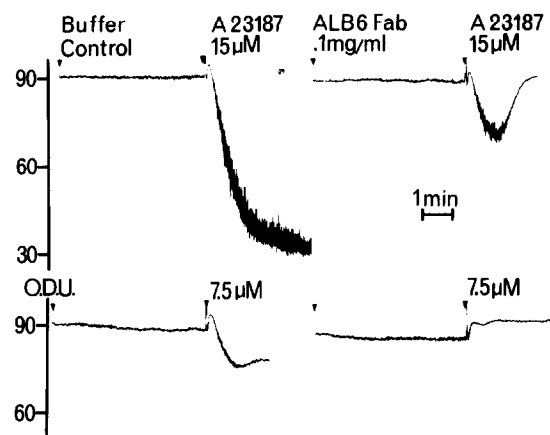


Fig. 2. Effect of ALB₆ Fab fragments on platelet aggregation induced by ionophore A 23187. The inhibition is partial at 15 µM A 23187 which induces a characteristic aggregation after preincubation for 5 min with the buffer control. The inhibition is total at 7.5 µM A 23187 but this concentration leads only to partial platelet aggregation in the control curve.

3.4. Immunofluorescence

Platelets from 8 different donors were tested and a bright membrane fluorescence was observed in each case. The platelets of one patient with Bernard Soulier syndrome and one patient with type I thrombasthenia were also tested and the same fluorescence was observed.

3.5. [125 I]Fibrinogen-binding to platelets in the presence of ALB₆ Fab

Without ALB₆ Fab, ADP-treated platelets bound 7% [125 I]fibrinogen. In the presence of ALB₆ Fab, the binding was reduced to 3.5%. In the absence of ADP and ALB₆ Fab the non-specific binding was 1%.

3.6. M_r determination of the platelet component recognized by ALB₆

The antigen defined by the monoclonal antibody ALB₆ in platelets has an app. M_r of 24000. No other band appeared in the ALB₆ IgG-immunoadsorbed 125 I-labelled platelet extract run under reducing conditions (fig. 3).

4. DISCUSSION

The monoclonal antibody ALB₆ prepared against all cells recognizes a membrane protein of M_r 24000 (p 24). The epitope recognized by this antibody is widely distributed in the body tissues including membrane platelets.

Affinity chromatography of 125 I-labelled NP 40 membrane extracts followed by SDS-PAGE has shown that the molecule recognized by ALB₆ on platelet membrane has the same M_r -value. Preliminary experiments using electrofocusing and immunoblotting indicate that the isoelectric point of the p 24 is identical in platelets and leukemic cells ($pI \approx 7.6$, not shown). In leukemic cells this molecule has been largely studied and it appears as a single polypeptide chain which is located outside the cell membrane and it does not form an intimate association with the lipid matrix [4]. Positive reactions in immunofluorescence using platelets from type I thrombasthenia and Bernard Soulier syndrome indicate that the epitope is not on the major platelet membrane glycoproteins GP I, GP V; and the complex GP IIB-III A; therefore ALB₆ is different from the antibodies AN 51 [9] and 6 D1 [10] which recognize GP I as well as B 59.2 [11], which recognizes the complex GP II B-III A.

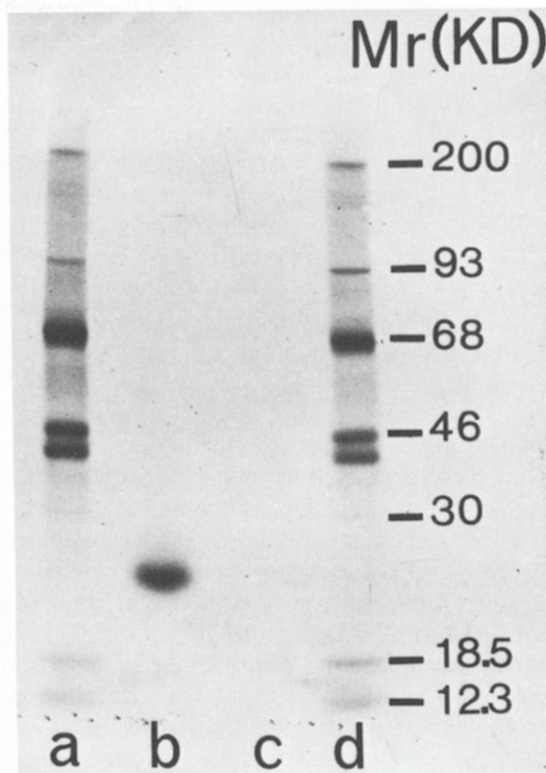


Fig. 3. M_r determination of the antigen recognized by ALB₆ on platelets was performed using microcolumns of immunoadsorbents linked to monoclonal antibodies followed by SDS-PAGE electrophoresis of the bound material. Platelets were previously labelled with 125 I and extracted with NET-NP40 (see text): (a,d) 14 C-labelled protein markers, in order of decreasing M_r -values: myosin, phosphorylase b, BSA, ovalbumin (partially degraded), carbonic anhydrase, lactoglobulin A, cytochrome c; (b) eluate of the ALB₆ IgG microcolumn (anti-CALLA gp 100 which is not expressed on platelets). Fluorography for 14 C-labelled proteins and an intensifying screen for 125 I-labelled platelet extracts were combined to obtain this autoradiography.

To analyze the possible role of the p 24 in the platelet membrane, the effect of ALB₆ was tested on platelet functions. As shown in these experiments the McAb ALB₆ acts as an aggregating agent. This effect meets all necessary criteria for an aggregation: absence of lysis demonstrated by absence of LDH release, inhibition by various substances which could not inhibit an immunological agglutination. ALB₆ IgG-induced platelet aggregation occurred with normal platelets

and platelets from patients with Bernard Soulier syndrome, but no aggregation occurred with platelets from thrombasthenia type I and type II. The first point is the requirement of bivalence of ALB₆ for the induction of aggregation since the Fab fragments do not induce the effect. Moreover, the Fab fragments inhibit aggregation induced by ADP, collagen, thrombin, ALB₆ IgG and ionophore A 23187, but do not inhibit ristocetin-induced agglutination. This indicates that the p 24 in platelets is involved in aggregation of platelets induced by a variety of stimuli such as ADP, collagen, thrombin and A 23187. Therefore, it is assumed that the biological effect of this antibody infers a common metabolic requirement for all aggregation pathways. A direct blocking effect of the fibrinogen receptor, essential to platelet aggregation, is not likely to be concerned since thrombasthenia type I platelets with < 1% GP II B-III A which bears the receptor for fibrinogen [11] are identically labelled by ALB₆. Thus the defect in fibrinogen-binding induced by pre-incubation of platelets with ALB₆ Fab before adding ADP was due to an inhibitory effect on the mechanism which involves the exposure of the platelet fibrinogen binding site. On the other hand, EDTA and all anti-calcium drugs inhibit ALB₆ IgG-induced aggregation. These substances, like ALB₆ Fab, have a relatively non-specific effect [12,13] since Ca²⁺ is involved crucially in the process of aggregation [14,15] and some of these drugs are known as calcium channel blockers [16,17] since the antibody does not penetrate into the platelet, ALB₆ platelet interaction could involve either a constituent of the putative calcium channel or a membrane protein which plays a role in the level of free cytoplasmic Ca²⁺ flux. It must be noted that it has been shown [18] that a M_r 23000 protein regulates the calcium efflux from isolated membrane platelet vesicles; therefore, further studies will be undertaken to compare the p 24 and this phosphoprotein.

In conclusion, the aggregating properties of this antibody could be of interest in two different fields: on the one hand by helping to understand some aspects of platelet aggregation; on the other hand the functional effect observed on platelets could lead to explanations for the restricted expression and the role of this molecule, originally described as a differentiation antigen.

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