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A Monoclonal Anti-platelet Antibody which Recognizes p55 Antigen

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ABSTRACT

A monoclonal antibody (MoAb) H229(IgGl) was obtained by fusion between SP2/0, mouse myeloma cell line and spleen cells from BC_3Fl mice immunized with HEL, a erythroleukemia cell line. MoAb H229 precipitated a 55 kilodalton(KD) molecule in reduced condition. It reacted with platelets and megakaryocytes, but not with monocytes, granulocytes, lymphocytes, thymocytes, red blood cells (RBC), colony-forming units-granulocyte/monocyte (CFU-GM), and burst forming units- erythroid (BFU-E). These findings suggest that MoAb H229 reacts with platelets specifically. However, MoAb H229 did not inhibit platelet aggregation induced by ristocetin, ADP, epinephrine and collagen.

Key words: *Monoclonal antibody, Platelet*

It was reported that about 50 glycoproteins or polypeptides were present on the surface of platelet[1]. Among these, many MoAbs against platelet glycoprotein (Gp) II_b-III_a complex and Gp I_b have been raised[9,10]. Using these MoAb, it was clarified that Gp II_b-III_a and Gp I_b played an important role in the aggregation of platelets.

However, the function of other platelet glycoproteins or polypeptides has not been clarified, since many MoAbs against other platelet glycoproteins or polypeptides have not been produced. We raised MoAb H229 which identified the novel platelet polypeptide with the molecular weight of 55,000. This report describes our characterization of MoAb H229.

MATERIALS AND METHODS

Generation of MoAb

HEL, erythroleukemia line was used as an immunogen. Ten-week-old BC_3Fl female mice were injected i.p. with 2×10^7 cells with 4mg alum as adjuvant. A second immunization was done 3 weeks later with 2×10^7 cells in phosphate buffered saline (PBS) i.p.. Three days later, spleen cells were fused with SP2/0 Ag 14 tumor cells with PEG 1000. Hybridomas were selected in HAT medium:their supernatant were screened by indirect immunofluorescence. The desired hybridomas were cloned on agarose. Details of these procedures have been described[19].

Purification of MoAb

Ascitic fluid rich in MoAb H229 was prepared by intraperitoneal injection of Pristane-pretreated BALB/c mice with $2 \sim 5 \times 10^6$ hybridoma cells that had been washed twice in PBS. Ascitic fluid were precipitated with 50% ammonium sulphate. After dialysis of precipitate against Tris 0.02M pH 8.0 buffer, IgG antibodies were purified by DEAE cellurose column equilibrated with Tris 0.02M pH 8.0 buffer.

Cell preparation

Defibrinated blood from normal volunteers was used as a source of peripheral blood mononuclear cells (PBMC). PBMC were separated as described[16]. Monocytes were separated by Percoll continous gradient centrifugation[18]. Granulocytes were separated by Percoll discontinous gradient[15]. The purity of the granulocyte fraction was more than 97% as determined by Wright-Giemsa staining. Platelets were isolated from platelet-rich plasma and red blood cell (RBC) from the pellets of Ficoll-Hypaque cushion of peripheral blood cells. Thymocytes were obtained from thymus of patients with thymoma, cryopreserved in 10% dimethylsulfoxide in nitrogen tank and recovered before use.

Cell lines

In addition to HEL, ML-1, a myeloid leukemia cell line[13], U937, a monocytoid-histiocytic cell line, KG-1, a myeloblastic leukemia cell line, K562, an erythroid/myeloid line from a patient with chronic myelocytic leukemia (CML) in blastic crisis, and HL-60, a promyelocytic cell line, were used as non-lymphoid lines. For T leukemia cell lines, RPMI 8402, MT-1, Molt-4, Jurkat, and CEM were used.

As B lymphoblastoid cell lines, Raji, Daudi, Josh-7, 8866P, $32a_1$ and SeD were used.

Bone marrow cells and bone marrow cultures

Bone marrow cells were obtained from normal volunteers. They were separated by Ficoll-Hypaque density centrifugation to remove RBC and mature granulocytes. Separated bone marrow cells were subjected to an immune rosette method[5] to obtain cells reactive with MoAb H229. Bone marrow cells were cultured in quadruplicates in Iscove's modified Dulbecco's medium containing 0.9% methylcellulose, 10% conditioned medium from human peripheral blood leukocytes stimulated with phytohemagglutinin, and 30% fetal bovine serum (FBS). This procedure was essentially as described by Messner et al[8]. BFU-E were scored as hemoglobin-containing single or multiple colonies of greater than 64 cells on the 14th day. This culture also allowed CFU-GM colony formation.CFU-GM were scored as colonies of greater than 40 cells on the 14th day.

Remaining bone marow cells which were separated by immune rosette method, were smeared, stained with Wright-Giemsa solution and identified morphologically.

Immunofluorescence studies

Cells (0.05 to 1×10^6) were first incubated with hybridoma culture supernatants for 20 min at 4°C. After three washings with PBS containing 1% bovine serum albumin, fluorescein-labeled $F(ab')_2$ anti-mouse IgG (Tago, Inc., Burlingame, CA, U.S.A.) was added and a 20 min incubation at 4°C was performed. After three washings, cells were analyzed with a flow cytometry (Epics V: Coulter Electronics, Hialeah, FL, U.S.A). Integrated fluorescence of the population gated by forward angle light scatter and right angle light scatter was measured and 10,000 cells were analyzed.

Iodination and immunoprecipitation

Cells were iodinated in suspension by the method of Hubbard and Cohn[6]. In brief, 4×10^7 platelets were incubated with lmCi/ml $Na^{125}I$, 50mU/ml type V glucose oxidase (Sigma Chemical Co., St. Louis, Mo, U.S.A.) and 10μg/ml lactoperoxidase (Calbiochem-Behring Co., San Diego, CA, U.S.A.) for 5 min on ice. The reaction was terminated by aspirating the supernatant and by repeated washings with RPMI 1640. Iodinated cells were solubilized with PBS containing 0.5% NP-40 and 2mM phenylmethyl sulfonyl fluoride (PMSF), and centrifuged at 5000g for 15 min at 4°C. The supernatant were incubated for an hour at 4°C with Sepharose-4B were conjugated with anti-mouse Ig antibody according to Pharmacia's manual, incubated with 0.5ml of hybridoma culture supernatants and washed 3 times. After incubation, Sepharose-4B was washed 5 times with 0.6M NaCl, 0.1%SDS, 0.05%NP-40, 10mM Tris, pH 8.8, suspended with 4%SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.25M Tris, pH 6.8, and heated at 100°C for 3 min. After immunoprecipitation, gel electrophoresis and autoradiography were performed as described[17].

Platelet Aggregation

An aggregometer (Chrono-log Co. AHS Japan Corporation) was used for aggregation studies with platelet rich plasma (PRP) anticoagulated with 0.32% sodium citrate. PRP was obtained from the supernatant after centrifugation of sodium citrate containing peripheral blood at 200g for 15 min at room temperature. PRP was incubated for 20 min with 25 μg/ml of MoAb H229, MoAb NS 8.1 which was kindly provided by Dr. J. Davie of Washington University (St. Louis, Mo, USA) and directed to sheep red blood cells, and MoAb H4 which was produced in our laboratory and directed to platelet Gp II_b-III_a complex. After incubation, stimulants (20 μM of ADP, 20 μg/ml of epinephrine, 5 μg/ml of collagen and 12.5 μg/ml of ristocetin) were mixed with PRP.

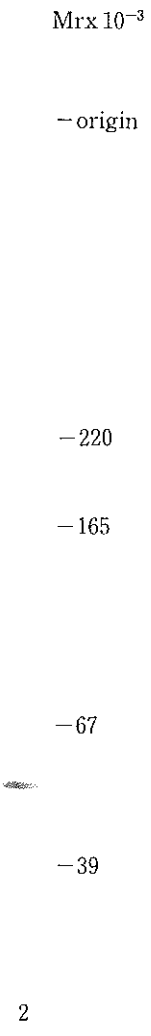

Fig. 1. The 55KD molecule was precipitated by MoAb H229 from ^{125}I-labeled platelets (Lane 2). No band was seen with MoAb HDP-1 (IgG1, anti-DNP) as a control antibody (Lane 1).

Table 1. Reactivity of MoAb H229 with normal hematopoietic cells

Cells		MoAb		
		H229	T.E. (CD2)	Josh524 (HLA-DR)
		(%)	(%)	(%)
Lymphocytes	(6)[a]	<1	87.4 ± 2.9[b]	11.6 ± 3.3
Monocytes	(6)	<1	3.0 ± 1.8	92.6 ± 2.8
Granulocytes	(6)	<1	<1	<1
Platelets	(6)	78.2 ± 5.6	<1	<1
RBC	(6)	<1	<1	<1
Thymocytes	(3)	<1	88.4 ± 8.4	<1

[a]The number of cell samples were analyzed.
[b]This represents the mean and the standard deviation of determinations of samples

Table 2. H5 antigen expression on various human hematopoietic cell lines by indirect immunofluorescence

Cell line	Cell type	%positive cell
ML-1	AML	<1[a]
U937	Monocytic	<1
KG-1	Myelocytic	91.2 ± 3.7
HEL	Erythroleukemia	97.6 ± 1.6
K562	Myeloid/Erythroid	<1
HL-60	Promyelocytic	<1
RPMI 8402	T-lymphoid	<1
MT-1	T-lymphoid	<1
Molt-4	T-lymphoid	<1
Jurkat	T-lymphoid	<1
CEM	T-lymphoid	<1
Raji	B-lymphoid	<1
Daudi	B-lymphoid	<1
Josh-7	B-lymphoid	<1
8866P	B-lymphoid	<1
32a	B-lymphoid	82.2 ± 6.7
SeD	B-lymphoid	<1

[a]Data were obtained from 3 separated experiments for each target cells.

RESULTS

Characterization of MoAb H229

MoAb H229 was established with HEL as an immunogen. By ELISA, MoAb H229 was typed to be IgG1 and it did not fix complements. MoAb H229 precipitated a 55 KD molecule from $^{1\text{-}2\text{-}5}$I-labeled platelets (Fig. 1, lane 2). A similar molecule was precipitated from HEL, the immunogen. This molecule was estimated from 4~11% gradient SDS gel in reduced condition.

Reactivity of MoAb H229 with peripheral blood cells

The cellular distribution of the reactive antigen by H229 was analyzed by immunofluorescence with a flow cytometry. The results are shown in Table 1. MoAb H229 stained most of platelets, but not lymphocytes, monocytes, granulocytes, RBC and thymocytes.

Hematopoietic cell lines

Antigen expression on various human hematopoietic cell lines are shown in Table 2. MoAb H229 reacted with two non-lymphoid leukemia cell line, KG-1 and HEL out of 6 cell lines. It also stained one B-lymphoid cell line, 32a, out of 6 cell lines, but not 5 T-lymphoid leukemia cell lines.

Reactivity of MoAb H229 with bone marrow cells

By our immune rosette method, rosetting and non rosetting cells were examined before lysis. The positive populations contained 90 to 95% desired cells: the non-rosetting population contained less than 5% rosetting cells. As shown in Table 3, $H229^+$ bone marrow cells isolated by an immune rosette method contained less than 11% of BFU-E and GFU-GM. In contrast $H229^-$ cells contained vast majority of BFU-E and CFU-GM. In these experiments, MoAb Josh 524 (anti-HLA-DR) was utilized as a control antibody. In all experiments, CFU-GM and BFU-E progenitors were shown to be $HLA\text{-}DR^+$.

Both $H229^+$ and $H229^-$ populations were smeared and determined morphologically by Wright-Giemsa staining. Megakaryocytes were found in $H229^+$ population.

Effect of MoAb H229 on platelet aggregation

After platelet enriched plasma (PRP) were incubated with MoAbs, ristocetin, ADP, epinephrine and collagen were added, and the platelet aggre-

Table 3. MoAb H229 did not react with bone marrow BFU-E and CFU-GM progenitor cells

Bone marrow cell type	BFU-E/10^5 cells plated		CFU-GM/10^5 cells plates	
	1	2	1	2
Unseparated	358 ± 41[a]	275 ± 16	117 ± 35	77 ± 15
$H229^+$	36 ± 3	23 ± 6	12 ± 3	18 ± 6
$H229^-$	454 ± 29	341 ± 58	132 ± 19	78 ± 19
$HLA\text{-}DR^+$	1,079 ± 62	522 ± 132	343 ± 11	120 ± 7
$HLA\text{-}DR^-$	3 ± 1	1 ± 1	3 ± 2	2 ± 1

[a]Data are presented as the mean ± standard deviation of quadruplicate plates.

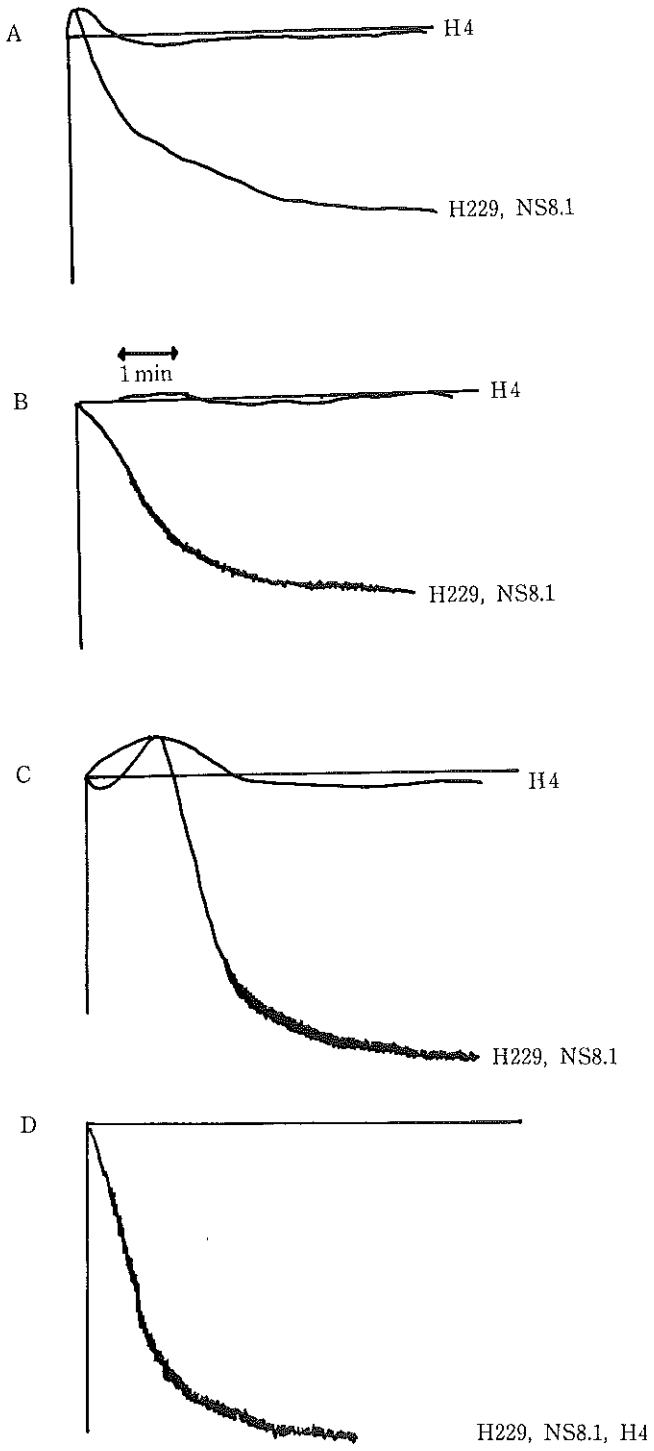

Fig. 2. Effects of MoAbs on platelet aggregations induced by ADP (Panel A), epinephrine (Panel B), collagen (Panel C) and ristocetin (Panel D). AFter preincubation of platelet enriched plasma with MoAbs for 20 min, agonists were added.

gation was determined by an aggregometor. Treatment of PRP with MoAb H229 had no inhibitory effect on the platelet aggregation induced by all these stimulants (Fig.2). Same results were observed when MoAb NS 8.1 (anti-sheep red blood cell mouse MoAb) was used as control antibody. In contrast, MoAb H4 (anti-platelet Gp II_b-III_a complex), inhibited the platelet aggregation induced by all these stimulants except ristocetin.

DISCUSSION

MoAb H229 reacts with platelets and megakaryocytes but not with lymphocytes, monocytes, granulocytes, RBC, thymocytes, CFU-GM and BFU-E. These findings suggest that MoAb H229 reactive with megakaryocyte-platelet lineage specifically. However, it reacted with KG-1 and $32a_1$ cell lines. This reason is not known. But it is also reported that MoAb directed at platelets reacted with a B cell line[14]. It precipitated a 55KD molecule. No other MoAbs which can recognize this platelet polypeptide have been described, although several MoAbs which reacts with platelet glycoproteins or polypeptides, different from Gp II_b-III_a complex and Gp I_b, have been reported[12]. It was reported that glycoproteins of 65, 60~65, 55~60 and 50~52 KD were also present on platelets[11]. There is the possibility that MoAb H229 recognizes one of these glycoproteins. However, it is not known whether MoAb H229 recognizes a glycoprotein or a polypeptide, since carbohydrate-specific or protein-specific labeling techniques were not used in our study.

Several anti-platelet antibodies have been previously described that were raised by immunization with cells other than platelets. Of these, 5F1[2], recognizes a determinant expressed in platelets, monocytes and erythroblasts. CALL-1 identified a 26 KD molecule and reacted with platelets, megakaryocytes and common acute lymphocytic leukemia cells[4]. Another antibody associated with pre-B cell reactivity and 24 KD antigen (DU-ALL1)[7] have been found to react with platelets. MoAb H229 can be distinguished from these MoAbs by the different reactivity or different molecular weight of antigens.

It was reported that MoAb directed at Gp I_b Inhibited the platelet aggregation induced by ristocetin[3] and that MoAb directed at Gp II_b-III_a complex inhibited the platelet aggregation induced by ADP, epinephrine and collagen[9]. Therefore, effect of MoAb H229 on platelet aggregation was investigated, but MoAb H229 had no effect on platelet aggregation induced by ristocetin, ADP, epinephrine and collagen.

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