Biochemical and Biophysical Research Communications **262,** 167–173 (1999)
Article ID bbrc.1999.1172, available online at http://www.idealibrary.com on IDEAL®

Preparation of Monoclonal Antibodies to Murine Platelet Glycoprotein IIb/IIIa (αIIbβ3) and Other Proteins from Hamster–Mouse Interspecies Hybridomas

Stephan Lengweiler,[1] Susan S. Smyth,[1] Marketa Jirouskova, Lesley E. Scudder, Helen Park, Thomas Moran, and Barry S. Coller[2]
Department of Medicine, Mount Sinai School of Medicine, New York, New York 10029

Received July 14, 1999

To obtain mouse-specific monoclonal antibodies (mAbs) against platelet proteins, an Armenian hamster was immunized with washed mouse platelets. Immune splenocytes were then fused with a nonsecreting murine myeloma cell line, and the resulting heterohybridomas were screened for antibody production utilizing an ELISA in which the target antigen was mouse platelets adsorbed onto microtiter plates in the presence of thrombin. Secondary screening assays included ELISA tests using murine fibrinogen or platelets from β3-integrin knockout mice, flow cytometry, immunoblotting, immunoprecipitation, and a functional assay to identify antibodies that inhibit platelet–fibrinogen interactions. Hybridoma cells producing hamster mAbs against murine glycoprotein (GP) IIb/IIIa, fibrinogen, CD9, and other platelet integrins were identified. Two hybridomas (1B5 and 9C2) producing antibodies that react with the GPIIb/IIIa complex in immunoprecipitation analysis were subcloned twice. Functional analyses by means of aggregation and adhesion assays revealed that 1B5 completely inhibits platelet–fibrinogen interactions, whereas 9C2 does not affect platelet aggregation or platelet adhesion.

Animal models based on wild-type mice and genetically manipulated mice have significantly furthered our understanding of the physiology and pathophysiology of the hemostatic system (for reviews, see 1, 2). A number of hemostasis-related proteins have been targets for transgenic engineering or gene disruption, and many more are likely to be investigated by these techniques in the future. Such experimental approaches are often hampered, however, by the limited availability of appropriate monoclonal antibodies (mAbs) that react with mouse proteins. mAbs to mouse antigens have previously been generated by utilizing rats or hamsters as host animals [3]. Syrian and Armenian hamsters develop good immune responses to a variety of mouse immunogens, and their splenocytes are excellent fusion partners with mouse myeloma cells [4, 5]. The resulting hybridomas grow well in tissue culture [6, 7] or as ascites tumors in nude or immunosuppressed rodents [8, 9], and the antibodies can be isolated by standard purification techniques.

Our laboratory has focused on the biochemistry and physiology of human blood platelets using murine mAb directed at platelet surface glycoprotein receptors to study platelet function. One of the antibodies we developed to the major platelet receptor mediating platelet aggregation, the integrin GPIIb/IIIa (αIIbβ3; CD41/CD61 [10, 11]), was redesigned as a chimeric Fab and is now used widely as an antiplatelet agent to prevent ischemic complications of unstable angina and percutaneous coronary interventions [12]. This antibody does not, however, react with murine platelets and thus cannot be used to assess the impact of inhibition of murine GPIIb/IIIa in murine models of disease. Our goal, therefore, was to produce at least one antibody that inhibits murine platelet GPIIb/IIIa function and another antibody to murine GPIIb/IIIa that does not inhibit platelet function that could serve as a control.

Since the supply of antibodies to mouse glycoprotein receptors is quite limited, and to our knowledge, there are no published reports of using mouse platelets to immunize hamsters for the purpose of making monoclonal antibodies, we investigated the ability of an Armenian hamster to make antibodies to murine platelets. We report on the screening strategy we developed and the specificities of the antibodies produced.

[1] These authors contributed equally to this study.
[2] To whom correspondence should be addressed at Department of Medicine, Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, NY 10029. Fax: 212 876 5844. E-mail: bcoller@smtplink.mssm.edu.

0006-291X/99 $30.00

MATERIALS AND METHODS

Isolation of platelets. Mice from different wild-type lineages (C57Bl/6, BALB/c; Jackson Laboratory, Bar Harbor, ME) as well as homozygous integrin β3-knockout strains with a mixed 129 and C57Bl/6 background [13] were anesthetized by placing them in a covered glass beaker containing evaporated methoxyflurane (Metofane; Pitman-Moore, Mundelein, IL) and bled (~0.5 ml) by puncture of the retrobulbar venous plexus with a glass capillary [14]. For immunizations, the blood was collected into EDTA-containing microtubes (10 mM final concentration) and centrifuged at 100*g* for 7 min at 22°C. The resulting platelet-rich plasma (PRP) was harvested, while the erythrocyte pellet was resuspended in an equal volume of 10 mM Tris/HCl, 150 mM NaCl, 10 mM EDTA, pH 7.4 (TBS/EDTA), and centrifuged at 100*g* for 3 min. The platelet-rich buffer (PRB) thus obtained was pooled together with the PRP, and the red cell fraction subjected to a third extraction with the above buffer in order to yield an additional volume of PRB. Thus, PRP/PRB pools of ~0.6 ml (~2×10^8 platelets/ml) were routinely obtained per animal. Platelet counts were obtained using a particle counter (Coulter Counter Z1, Beckman-Coulter Inc., Miami, Florida; settings 1.43–4.83 μm).

For other studies, mouse blood was anticoagulated with 0.1 vol of 3.8% sodium citrate and the PRP/PRB prepared by using modified Tyrode's buffer [138 mM NaCl, 2.7 mM KCl, 0.4 mM NaH_2PO_4, 12 mM $NaHCO_3$, 10 mM 4-(2-hydroxyethyl)-1-piperazine-2-ethanesulfonic acid (Hepes), 0.2% bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, MO), 0.1% glucose, pH 7.4] instead of TBS/EDTA. PRP from human blood was prepared as previously described [15].

Production of monoclonal antibodies. Mouse PRP/PRB was washed three times in TBS/EDTA by repetitive centrifugations at 2000*g* for 8 min at 22°C. After the final centrifugation the pelleted platelets were resuspended in 10 mM sodium phosphate, 150 mM NaCl, pH 7.4 (PBS) to a count of ~4×10^9/ml and mixed with an equal volume of Freund's adjuvant (Sigma Chemical Co.) [15]. A volume of 0.3 ml of this suspension was then injected intraperitoneally into a 4 month old male Armenian hamster (Cytogen Inc., Cambridge, MA) under methoxyflurane anesthesia. Another 3 similarly prepared platelet preparations were given at one week intervals, followed by 2 injections at 20 day intervals. The serum of the animal was subsequently screened for antibodies to platelet antigens by the screening assays described below. Six months after the last intraperitoneal injection and three days before the fusion, a final boost of 2.4×10^8 platelets without adjuvant was given intravenously into the jugular vein of the anesthetized hamster [5].

The hamster–mouse hybridomas were prepared as we previously prepared murine hybridomas, with some modifications [5, 15–17]. Hamster splenocytes were fused to murine SP2/0-Ag14 myeloma cells (American Type Culture Collection, Rockville, MD) at a 1:4 myeloma to spleen cell ratio by drop-wise addition of 50% polyethylene glycol (PEG; EM Science, Germany) to the cell pellet. The PEG was washed away with serum-free DMEM, and the fused cells were directly resuspended in selective Iscove's modified Dulbecco's medium (GIBCO-BRL Life Technologies, Gaithersburg, MD) containing 10% fetal calf serum (FCS) and supplements (penicillin, streptomycin, L-glutamine, sodium pyruvate, and hypoxanthine/aminopterin/thymidine) according to standard protocols, and distributed into 10 96-well plates. The supernatants from hybridoma-containing wells were collected, assayed, and selected for subcloning twice by limiting dilution.

ELISA screening. Primary screening was performed with an ELISA adapted from published protocols [18, 19]. PRP/PRB, collected from C57Bl/6 mice as described above, or human PRP, was centrifuged once in the presence of 0.16 volume of acid-citrate dextrose at 2000*g* for 10 min at 22°C. The pelleted platelets were resuspended to a count of 4×10^7/ml in modified Tyrode's buffer containing 2 mM $MgCl_2$ and 50-μl aliquots were added to flat-bottomed microtiter plate wells (Immuno StarWell modules; Nunc Inc., Naperville, IL) containing 10 μl of 12 U/ml human thrombin (Sigma Chemical Co.). Platelets were deposited onto the bottom surface of the wells by centrifugation at 900*g* for 10 min at 22°C and the plates were stored overnight or until use within 10 days at 4°C. After blocking the wells with 100 μl of TBS (150 mM NaCl, 10 mM Tris/HCl, pH 7.4) containing 0.05% Tween and 3% BSA for 1 h at 22°C and three successive washings with 100 μl TBS/0.05% Tween, 50 μl of supernatant culture medium, serum, or control mAb (see below) were added and incubated for at least 1 h. Wells were incubated with a mixture of two biotin-labeled mouse anti-hamster secondary mAbs (PharMingen, San Diego, CA; anti-hamster IgG cocktail clones G70-240 and G94-56; 50 μl at 2.5 μg/ml in TBS) followed by an alkaline phosphatase-conjugated goat anti-biotin tertiary antibody (Vector, Burlingame, CA; 1:500). Immunoreactivity was detected with a chromogenic substrate (BCIP/NBT; Sigma Chemical Co.) according to the manufacturer's instructions with absorbance measured at 405 nm after a 30-min incubation. A sample was considered positive if the absorbance was $\geq$0.8 optical density units. Secondary screening employed essentially the same ELISA protocol, but β3-null platelets, human platelets, mouse fibrinogen (Sigma Chemical Co.), or human fibrinogen (Enzyme Research Laboratories, South Bend, IN) were used instead of wild-type mouse platelets. Fibrinogen was coated at a concentration of 10 μg/ml in 50 mM Tris, 100 mM NaCl, pH 7.4, and the plates were developed 1 h later as outlined above.

Serum from the immunized hamster (serially diluted from 1:5,000 to 1:80,000 in culture medium) as well as a commercially available hamster anti-mouse integrin-β3 mAb (PharMingen clone 2C9.G2; 20 μg/ml in culture medium) were used as positive controls, whereas serum from a nonimmune hamster (1:5,000 in culture medium), and a hamster anti-trinitrophenol IgG isotype control mAb (PharMingen clone A19-3; 20 μg/ml in culture medium) served as negative controls.

Flow cytometry. Murine PRP/PRB or human PRP was diluted to 0.5–1 $\times 10^8$/ml with modified Tyrode's buffer containing 2 mM $MgCl_2$. Aliquots of 50 μl were incubated with 25 μl of culture supernatant or hamster mAbs (at a final concentration of 2 μg/ml) for 1 h at 22°C. An FITC-conjugated mouse anti-hamster secondary mAb (PharMingen) was then added (5 μg/ml final concentration) and the mixture incubated for another hour at 22°C. Finally, the samples were diluted by addition of 1 ml of modified Tyrode's buffer before analyzing in a Coulter flow cytometer.

Immunoblot analysis. Native or reduced murine fibrinogen solubilized in electrophoresis sample buffer [60 mM Tris/HCl, pH 6.8, containing 10% glycerol, 2% sodium dodecyl sulfate (SDS), 0.05% bromophenol blue] at 0.3 mg/ml, or SDS-solubilized mouse platelet lysates prepared as previously described [20], were separated by SDS–polyacrylamide gel electrophoresis (PAGE), electroblotted onto polyvinylidene difluoride membranes (PVDF; Millipore, Burlington, MA), and subjected to immunoblot analysis using serum from the immunized hamster, a nonimmune control hamster (1:1,000 in TBS/0.1% BSA), or supernatant culture medium (1:5 in TBS/0.1% BSA).

Immunoprecipitation. Platelets were labeled with biotin in order to detect surface-exposed proteins containing primary amines [21, 22] by incubation (2×10^9 platelet/ml) with sulfo-*N*-hydroxysuccinimide biotin (sulfo-NHS–biotin; Pierce Chemical Co. (Rockford, IL); 5 mM final concentration) for 30 min at 22°C. After three washes with TBS/EDTA containing 5 mM glycine (Fisher Scientific, Pittsburgh, PA), the platelets were lysed at a count of 1×10^8/ml in 4°C TBS/EDTA containing 1% Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride, 1% aprotinin, 6 mM *N*-ethylmaleimide (NEM) and 100 μg/ml leupeptin (all compounds from Sigma Chemical Co.) for 30 min. Biotinylated platelet lysates were precleared with a mixture of protein A and G-Sepharose beads (Pharmacia, Piscataway, NJ). The resulting supernatant was added in equal volumes (50 μl) to hybridoma culture supernatants, hamster anti-mouse β3 integrin mAb, rat

anti-mouse CD9 mAb (PharMingen, clone KMC8), or control mAb (1 μg/ml in culture medium) and then incubated for at least 2 hours at 4°C. The immune complexes were isolated with protein A/G-coated Sepharose beads and dissociated by boiling in SDS; the proteins were then separated by SDS–PAGE, electroblotted onto PVDF membranes, and visualized by means of enzyme-conjugated streptavidin.

Antibody purification. Purification of the antibodies was performed on a Poros Protein G perfusion chromatography column in a BioCAD Sprint 250 liquid chromatograph (PerSeptive Biosystems, Framingham, MA) according to the manufacturer's procedure. In brief, the column was first equilibrated with 150 mM NaCl, 50 mM sodium phosphate, pH 8.0, and then directly loaded with tissue culture supernatant. After extensive washing with equilibration buffer, the antibodies were eluted with 150 mM NaCl, 0.1% HCl. Alternatively, antibodies were purified by subjecting culture supernatant to 50% $(NH_4)_2SO_4$ precipitation, followed by chromatography of the dialyzed precipitates on a nProtein A AvidGel F column (Unisyn; Tustin, CA). The bound proteins were eluted by lowering the pH in a stepwise fashion [15].

Determination of mAb isotype. Purified mAbs were characterized by an ELISA [17] involving capture with mAbs specific for anti-hamster light chain κ or λ and detection with mAbs specific for Armenian hamster IgG_1 (clone G94-56), IgG_1 and IgG_2 (clone G94-90.5), IgG_2, IgG_3, and IgG_4 (clone G192-1), or IgG_1, IgG_2, and IgG_3 (hamster IgG cocktail) from PharMingen.

Platelet aggregation. Mouse PRP/PRB (adjusted to $\sim 1.4 \times 10^8$ platelets/ml with modified Tyrode's buffer containing 1 mM $MgCl_2$) was incubated with varying quantities of purified antibody (0–20 μg/ml final concentration) for 15 min, after which aggregation was initiated by the addition of 10 μM ADP (Chronolog, Havertown, PA).

Platelet adhesion. Isolated mouse platelets (3×10^8/ml) were incubated with $Na_2{}^{51}Cr\ O_4$ (ICN, Costa Mesa, CA) in normal saline for 30 min at 37°C (50 μl ^{51}Cr per 1 ml platelet suspension). Following the addition of 0.5 μM prostaglandin E_1 (Sigma Chemical Co.), the platelets were centrifuged at 2000*g* for 12 min and resuspended to a count of $1–3 \times 10^8$/ml in modified Tyrode's buffer containing 0.35% BSA and 1 mM $MgCl_2$. Microtiter plate wells (Polysorp, Nunc) were coated with 10 μg/ml of mouse fibrinogen for 1 h at 22°C, and blocked with modified Tyrode's buffer containing 0.35% BSA. Fibrinogen was >95% pure as judged by SDS–PAGE analysis, and ≥95% clottable. To measure adhesion, 50-μl aliquots of platelet suspension were added to the wells and incubated for 2 h at 22°C. In some instances, platelets were pretreated with increasing quantities of mAb 1B5 (0–10 μg/ml final concentration) for 30 min at 22°C. Unbound platelets were removed by inverting the plates and washing three times with 100 μl of modified Tyrode's buffer containing 0.35% BSA and 1 mM $MgCl_2$. Platelet adhesion was quantitated by measuring ^{51}Cr bound per well in a gamma spectrometer (Packard Instrument Co, Meriden, CO). Results are expressed as mean ± SD of triplicate samples.

RESULTS

Identification of antigen specificities. Serum from the immunized Armenian hamster immunoblotted wild-type nonreduced platelet proteins with molecular masses of ≥300, 210, 115, 90, and 70 kDa (data not shown). The nonimmune control serum did not react with any of these proteins. When using platelet lysate from integrin-β3 knockout mice instead of wild-type mice, only bands at 210 and 115 kDa could be detected. Since platelets from β3-null mice lack surface-expressed GPIIb and GPIIIa, and contain only small amounts of fibrinogen [13], we suspected that GPIIIa

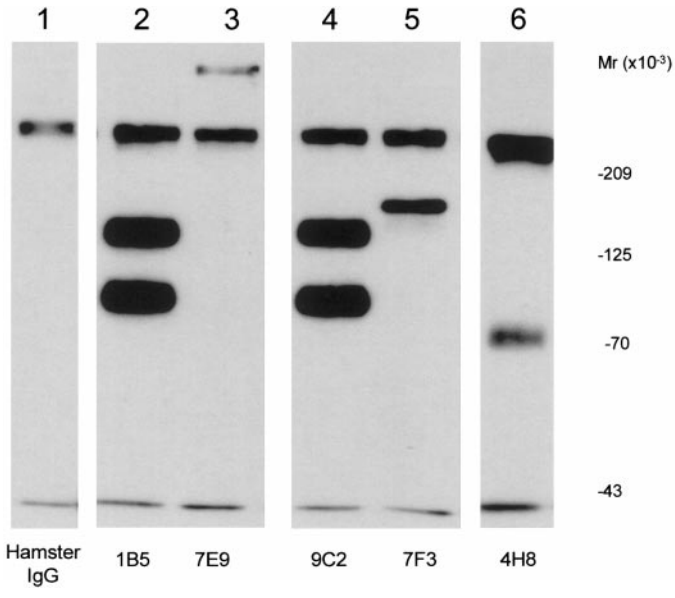

FIG. 1. Immunoprecipitation of surface-biotinylated platelet proteins by selected hybridoma culture supernatants. Murine wild-type platelets labeled with sulfo-NHS-biotin were solubilized and incubated with hamster isotype control (lane 1) or supernatant from wells 1B5 (lane 2), 7E9 (lane 3), 9C2 (lane 4), 7F3 (lane 5), or 4H8 (lane 6). The immunoprecipitated proteins were separated by SDS–PAGE and the bands identified by streptavidin–HPR. The protein of M_r 210,000 appear to be contaminating mouse IgG that most likely was present on the surface of the platelets during the biotinylation reaction.

(~90 kDa) and fibrinogen (340 kDa) were the predominant immunogens. The identity of the 70-kDa protein is not known.

Fusion of the immune hamster splenocytes to murine myeloma cells resulted in the growth of hybridoma cells in all 960 wells. Ten days after the fusion ~85% of the culture supernatants were positive in the ELISA against wild-type platelets. After additional testing with ELISAs employing murine fibrinogen and integrin-β3 null mouse platelets, the cells in 210 (22%) wells were expanded and frozen for storage.

Immunoblot and immunoprecipitation analyses were used to further elucidate the antibody specificities. Based on the results of these assays, 43 clones appeared to produce antibodies that reacted with fibrinogen, 2 with a 250-kDa protein, 5 with a 210-kDa protein, 8 with GPIIb/IIIa, 4 with a 150-kDa protein, 3 with proteins of 70–85 kDa, and 16 with a doublet of proteins <40 kDa. Results of a representative immunoprecipitations of surface-biotinylated mouse platelet proteins using different clones is illustrated in Fig. 1. The clones whose supernatants gave particularly high signals in the screening assays are summarized Table 1.

Hamster mAb to mouse GPIIb/IIIa. The culture supernatants from wells 1B5 and 9C2 reacted strongly with mouse wild-type platelets in the ELISA, but did not react with β3-deficient platelets or fibrinogen. Im-

TABLE 1

Relative Reactivities and Inferred Antigen Specificities of Generated Hamster–Mouse Hybridomas

	ELISA					Flow cytometry			Immunoblotting		Immunoprecipitation		
	Platelets			Fibrinogen		Platelets			M_r		M_r		
Clone	WT	β_3-Null	Human	Murine	Human	WT	β_3-Null	Human	Nonreduced	Reduced	Nonreduced	Reduced	Specificity
1B5	+++	−	−	−	−	+++	−	−	−	−	135,000 90,000	124,000 103,000	GPIIb/IIIa
9C2	+++	−	−	−	−	+++	−	nd	−	−	135,000 90,000	124,000 103,000	GPIIb/IIIa
7E9	+++	+++	+	+++	+	nd	nd	nd	340,000	48,000	340,000	nd	Fibrinogen
2E7	+++	+	−	+++	−	nd	nd	nd	340,000	−	nd	nd	Fibrinogen
7A1	+++	+	−	+++	−	nd	nd	nd	340,000	60,000	nd	nd	Fibrinogen
10C6	+++	+++	−	−	−	+++	+++	−	25,500	25,500	25,500	25,500	CD9
1D11	+++	+++	−	−	−	+++	+++	−	nd	nd	25,500	25,500	CD9
7F3	++	++	+	−	−	++	++	−	−	−	160,000 140,000	nd	β1 integrin
4H8	+++	+	+	−	−	nd	nd		nd	nd	70,000	nd	70 kDa

Note. WT, wild type; nd, not done.

munoprecipitation analysis demonstrated that the supernatants reacted with two major bands of M_r 135,000 and 92,000 when separated under non-reducing conditions (Fig. 1) and M_r of 120,000 and 108,000 when separated under reducing conditions (data not shown). The migration of these bands is characteristic of human and mouse GPIIb/IIIa [2, 24]. Further support for the identification of GPIIb/IIIa as the immunoprecipitated material came from the similarity to bands immunoprecipitated by the commercially available hamster anti-mouse integrin-β3 mAb. Neither 1B5 nor 9C2 exhibited binding to human platelets in the ELISA or in flow cytometry, nor did they react with mouse platelets in immunoblots. The latter observation suggests that the epitopes are complex-specific and/or are altered by SDS-induced conformational changes.

Since one of our major goals was to develop antibodies to murine GPIIb/IIIa, 1B5 and 9C2 were subcloned twice by limiting dilution, and the secreted antibodies isolated by affinity chromatography. ELISA-based subtype analysis revealed that mAb 1B5 is of the IgG_3 κ class and mAb 9C2 is of the IgG_1 λ class. Flow cytometric analysis confirmed that purified mAb 1B5 reacts with surface proteins present on wild-type but not β3-integrin-deficient mouse platelets (Fig. 2). Purified mAb 1B5 inhibits ADP-induced platelet aggregation in a dose dependent fashion, with complete inhibition achieved at ~10 μg/ml (Fig. 3). The platelets, however, are still able to undergo their characteristic shape change, as revealed by the transient increase in optical density after ADP is added. This pattern is identical to that observed when β3-integrin-deficient mouse platelets are stimulated with ADP, when antibodies to human GPIIb/IIIa are added to normal human platelets, or when platelets from patients with Glanzmann thrombasthenia, which lack GPIIb/IIIa on an inherited basis, are tested [13, 24]. 1B5 (10 μg/ml) inhibited ^{51}Cr-labeled murine platelet adhesion to immobilized murine fibrinogen by 85% ($1045 \pm 10 \times 10^3$ adherent platelets/well in the absence versus $158 \pm 16 \times 10^3$ adherent platelets/well in the presence of 1B5; $n = 3$), which was essentially identical to inhibition by 5 mM EDTA (86%). In contrast, 9C2 did not interfere with either platelet aggregation or platelet adhesion (data not shown).

Hamster mAb to mouse fibrinogen. The culture supernatants from wells 7E9, 2E7, and 7A1 all reacted strongly with mouse platelets and mouse fibrinogen in the ELISAs. 7E9 also bound to human fibrinogen in

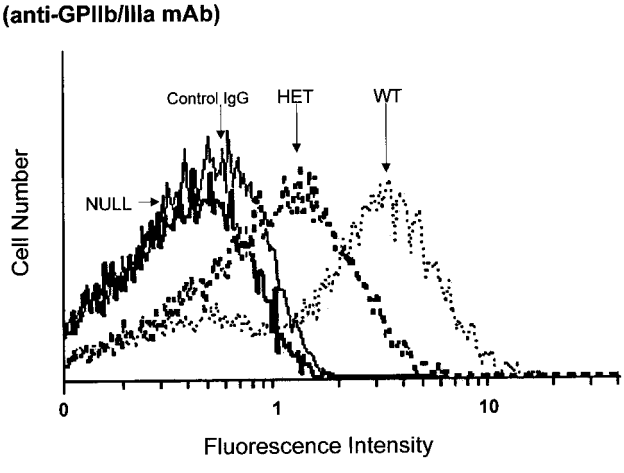

FIG. 2. Flow cytometric analysis of purified mAb 1B5. PRP/PRB (1×10^8/ml) from wild-type (WT; small dashed line), β3-heterozygous (HET; medium dashed line), and β_3-null (NULL; thick solid line) mice was incubated with 5 μg/ml 1B5 or an isotype control mAb (control IgG; thin solid line), followed by an FITC-mouse anti-hamster secondary mAb.

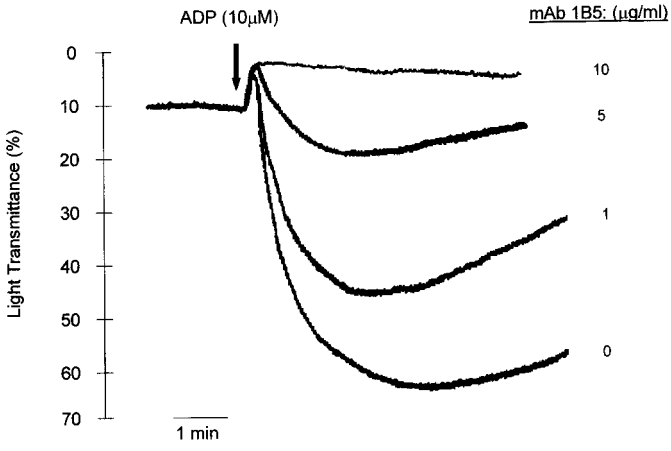

FIG. 3. Inhibition of ADP-induced platelet aggregation by mAb 1B5. Aliquots of fresh mouse PRP/PRB ($\sim 1.4 \times 10^8$/ml) were incubated with increasing quantities of purified mAb 1B5 (0-10 μg/ml final concentration) for 15 min at 37°C. ADP (10 μM final concentration) was added, and the changes in light transmission recorded by an optical aggregometer.

ELISAs, whereas 2E7 and 7A1 did not. Each supernatant immunoblotted non-reduced murine fibrinogen (M_r 340,000) and the results with 2E7 are shown in Fig. 4A. When tested with reduced fibrinogen, 7E9 selectively reacted with the γ chain (M_r 48,000), 2E7 did not show any reactivity, and 7A1 selectively recognized the α chain (M_r 60,000) (Fig. 4B). Of the other 40 clones that recognized fibrinogen, 16 were tested for reactivity against reduced murine fibrinogen by immunoblotting; 14 reacted with all three chains of fibrinogen.

Hamster mAb to mouse CD9. The culture supernatants from wells 10C6 and 1D11 were positive in ELISAs and flow cytometric analysis utilizing both wild-type and β3-deficient murine platelets, but negative with human platelets. Immunoprecipitation analysis with 10C6 and 1D11 yielded a band at M_r 25,500 (reduced and nonreduced; Fig. 5A). A band at the same M_r was immunoprecipitated by a commercially available rat anti-mouse CD9. Moreover, 10C6 immunoblotted a M_r 25,500 protein in platelet lysates and in the immunoprecipitate formed by incubating the lysate with a known anti-CD9 mAb (Fig. 5B). We therefore conclude that 10C6 and 1D11 produce antibodies against murine CD9. Fourteen other antibodies reacted by immunoprecipitation or immunoblot analysis with protein(s) of $M_r < 40{,}000$ (see Fig. 1; 4H4) and some of these may also be anti-CD9 antibodies.

Hamster mAb to murine platelet β1 integrins. The culture medium obtained from well 7F3 reacted with wild-type and β3-integrin-null platelets but not human platelets in both ELISAs and by flow cytometry. Immunoprecipitation gave a major band at M_r ~155,000 and a minor band at M_r ~135,000 (Fig. 1). In previous studies designed to prepare monoclonal antibodies to human platelet surface receptors, we obtained a similar pattern for a GPIa/IIa-specific antibody [25]. These observations suggest that the 7F3 hybridoma produces

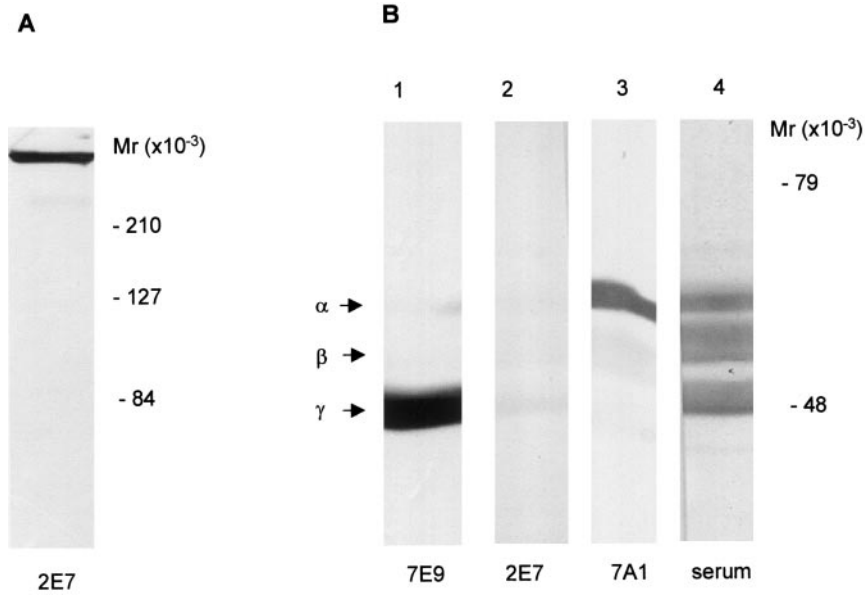

FIG. 4. Immunoblot analysis with culture supernatants from wells 7E9, 2E7, and 7A1. Non-reduced (A) and reduced (B) murine fibrinogen were separated by SDS–PAGE, and then electroblotted onto PVDF membranes. The lanes were reacted with the diluted supernatants or immunized hamster serum (1:1000) as indicated and then developed by sequential reactions with an alkaline phosphatase-conjugated goat anti-hamster antibody.

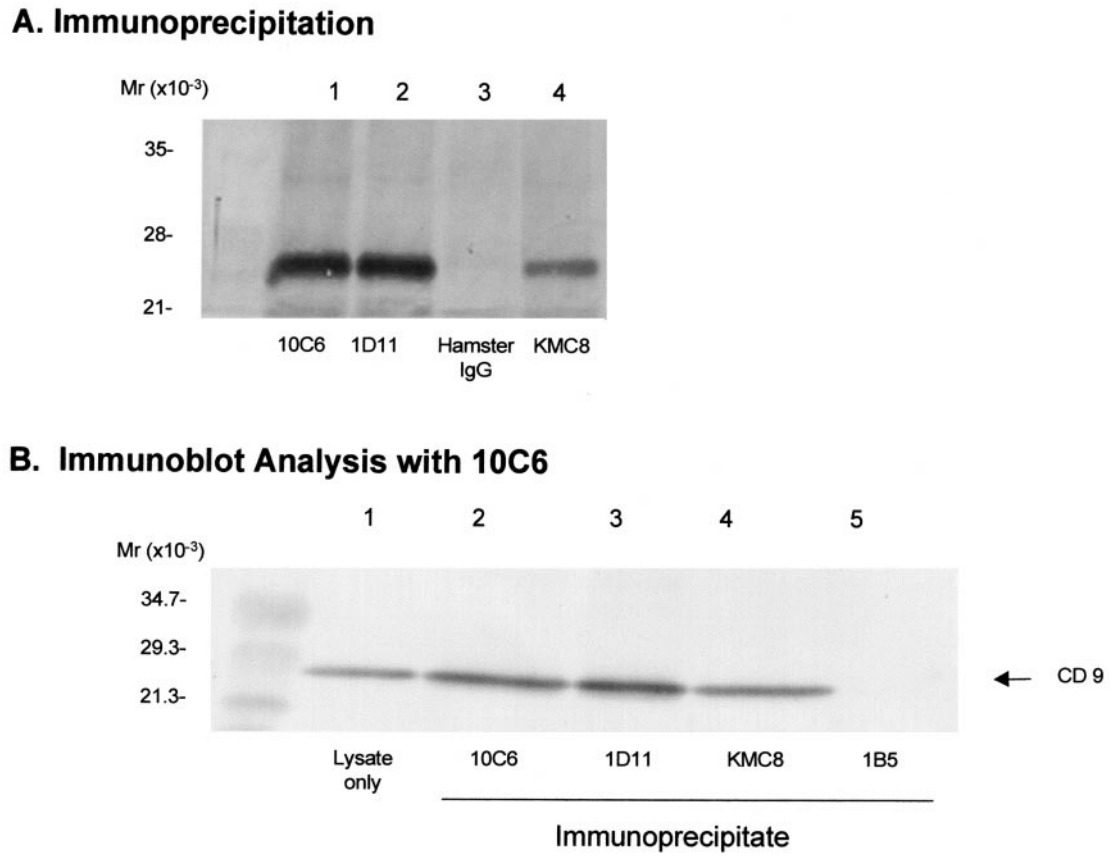

FIG. 5. Immunoprecipitation analysis with purified mAb 10C6 and 1D11. (A). Sulfo-NHS-biotin labeled-murine platelets were solubilized and incubated with purified mAb 10C6 (lane 1), 1D11 (lane 2), hamster IgG control (lane 3), or a known rat anti-mouse CD9 mAb (KMC8) (lane 4). The immunoprecipitated proteins were separated by SDS–PAGE and the bands identified by alkaline phophatase-conjugated streptavidin. Immunoblot analysis with purified mAb 10C6 (B). Platelet lysate (lane 1) or the immunoprecipitates formed by incubating platelet lysate with mAb 10C6 (lane 2), mAb 1D11 (lane 3), a known rat anti-mouse CD9 mAb (KMC8) (lane 4), or mAb 1B5 (lane 5) were separated by SDS–PAGE, transferred to PVDF membranes, and immunoblotted with mAb 10C6 (5 μg/ml). The reaction was visualized by incubating with an alkaline phosphatase conjugated goat anti-hamster antibody (1:5000) and BCIP/NBT.

an antibody that recognizes murine GPIa/IIa ($\alpha 2\beta 1$; CD49b/CD29) or another murine platelet integrin.

DISCUSSION

Our studies demonstrate that antibodies to murine platelets, including antibodies that inhibit murine platelet GPIIb/IIIa function, can be generated by immunizing an Armenian hamster with murine platelets and fusing the splenocytes to a nonsecretory mouse myeloma cell line. Compared to our previous experience with murine monoclonal antibodies using the P3X63-Ag8.653 myeloma cell line for fusion, the high fusion frequency and high frequency of antiplatelet production were remarkable. The immunization schedule employed and/or the choice of the SP2/0-Ag14 myeloma cell line may be responsible for these results. Bright *et al.* made similar observations when using SP2/0 cells as fusion partner for hamster splenocytes [16]. However, in our study a fairly high number (~60%) of initially positive hybridomas proved to be unstable, or required repeated cycles of cloning and expansion to be maintained.

The hybridomas generated were subsequently screened with an ELISA utilizing platelets attached to microtiter plates in the presence of thrombin. Thrombin stimulation ensured maximum expression of GPIIb/IIIa on the surface of the adsorbed platelets and also resulted in the surface expression of glycoproteins that are ordinarily selectively contained in granule membranes, such as P-selectin. The availability of platelets from mice deficient in β_3 helped us to rapidly make a preliminary antibody-specificity assignment, since these platelets have no GPIIIa, virtually no GPIIb, and little fibrinogen.

Based on the binding patterns yielded by the different ELISAs, the cultures could be classified into three categories as follows: (1) anti-GPIIb/IIIa antibodies, which gave high signals in the ELISA using wild-type platelets but no binding with either β3-integrin-

deficient platelets or fibrinogen; (2) anti-fibrinogen antibodies, which were positive in the ELISA using wild-type platelets and fibrinogen, but only weakly positive with β3-integrin-null platelets; and (3) antibodies to other platelet surface components, which were positive in ELISAs using both wild-type and β3-deficient platelets and negative with fibrinogen.

Immunoblot and immunoprecipitation analyses demonstrated antibodies of at least four specificities (GPIIb/IIIa, fibrinogen, CD9, and most likely a β1 integrin) as well as antibodies to less well characterized antigens. Of major importance, were the antibodies that recognized GPIIb/IIIa. mAb 1B5 is able to inhibit ADP-induced aggregation of mouse platelets with a potency comparable to that of the earlier reported human-specific mouse mAb 10E5 [24], indicating that murine GPIIb/IIIa, like human GPIIb/IIIa, plays a dominant role in platelet function. In addition, we were able to produce a GPIIb/IIIa-specific mAb (9C2) that is devoid of any inhibitory activity, thus providing a useful control for studies to assess the effects of 1B5. The large number of hybridomas producing antibodies to murine fibrinogen suggest that there are significant differences between the murine and hamster proteins. These antibodies should be of value in assessing the correlation between murine fibrinogen structure and function, as well as providing insights into the evolution of the protein.

In conclusion, this paper demonstrates the utility of Armenian hamsters to produce monoclonal antibodies to murine platelet proteins. These antibodies may be valuable reagents to study murine platelet function and to assess the role of murine GPIIb/IIIa in murine disease models.

ACKNOWLEDGMENTS

This work was supported by Grant 19278 from the National Heart, Lung, and Blood Institute to B. S. Coller. S. Lengweiler was supported in part by a Swiss National Science Foundation Grant for Future Researchers.

REFERENCES

1. Carmeliet, P., Moons, L., and Collen, D. (1998) *Cardiovasc. Res.* **39,** 8–33.
2. Tsakiris, D. A., Scudder, L. E., Hodivala-Dilke, K., Hynes, R. O., and Coller, B. S. (1999) *Thromb. Haemost.* **81,** 177–188.
3. Galfre, G., and Milstein, C. (1981) *Methods Enzymol.* **73,** 3–46.
4. Sanchez-Madrid, F., Szkult, P., and Springer, T. A. (1983) *J. Immunol.* **130,** 309–312.
5. Sanchez-Madrid, F., and Springer, T. A. (1986) *Methods Enzymol.* **12,** 239–244.
6. Schreiber, R. D., Hicks, L. J., Celada, A., Buchmeier, N. A., and Gray, P. W. (1985) *J. Immunol.* **134,** 1609–1618.
7. Croix, D. A., Yeh, H. Y., Sedlacek, J., Luftig, R. B., and Gottlieb, P. D. (1993) *J. Acq. Immune Defic. Syndr.* **6,** 558–566.
8. Fujioka, N., Mukaida, N., Harada, A., Akiyama, M., Kasahara, T., Kuno, K., Ooi, A., Mai, M., and Matsushima, K. (1995) *J. Leukocyte Biol.* **58,** 90–98.
9. Kayano, T., Motoda, R., Usui, M., Ando, S., Matuhasi, T., and Kurimoto, M. (1990) *J. Immunol. Methods* **130,** 25–31.
10. Naik, U. P., and Parise, L. V. (1997) *Curr. Opin. Hematol.* **4,** 317–322.
11. Lefkovits, J., Plow, E. F., and Topol, E. J. (1995) *N. Engl. J. Med.* **332,** 1553–1559.
12. Coller, B. S. (1995) *Circulation* **92,** 2373–2380.
13. Hodivala-Dilke, K. M., McHugh, K. P., Tsakiris, D. A., Rayburn, H., Crowley, D., Ullman-Culleré, M., Ross, F. P., Coller, B. S., Teitelbaum, S., and Hynes, R. O. (1999) *J. Clin. Invest.* **103,** 229–238.
14. Frenette, P. S., Johnson, R. C., Hynes, R. O., and Wagner, D. D. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 7450–7454.
15. Scudder, L. E., Kalomiris, E. L., and Coller, B. S. (1992) *Methods Enzymol.* **215,** 295–311.
16. Bright, S. W., Chen, T.-Y., Flebbe, L. M., Lei, M.-G., and Morrison, D. C. (1990) *J. Immunol.* **145,** 1–7.
17. Harlow, E., and Lane, D. (1988) Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
18. Stockelberg, D., Hou, M., Jacobsson, S., Kutti, J., and Wadenvik, H. (1996) *Eur. J Haematol.* **56,** 72–77.
19. Whiss, P. A., Andersson, R. G. G, and Srinivas, U. (1997) *J. Immunol. Methods* **200,** 135–143.
20. Beer, J., and Coller, B. S. (1989) *J. Biol. Chem.* **264,** 17564–17573.
21. Smith, J. W., Hayward, C. P. M., Warkentin, T. E., Horsewood, P., and Kelton, J. G. (1993) *J. Immunol. Methods* **158,** 77–85.
22. Fabris, F., Cordiano, I., Mazzuccato, M., Pradella, P., Randi, M. L., and Girolami, A. (1992) *Thromb. Res.* **66,** 409–419.
23. Coller, B. S. (1980) *Blood* **55,** 841–847.
24. Coller, B. S., Peerschke, E. I., Scudder, L. E., and Sullivan, C. A. (1983) *J. Clin. Invest.* **72,** 325–338.
25. Coller, B. S., Beer, J. H., Scudder, L. E., and Steinberg, M. H. (1989) *Blood* **74,** 182–192.