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Characterization of the Novel Murine Monoclonal Anti-von Willebrand Factor (vWf) Antibody GUR76-23 Which Inhibits vWf Interaction with $\alpha_{IIb}\beta_3$ but Not $\alpha_v\beta_3$ Integrin

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von Willebrand factor (vWf) is known to interact with the two β_3 integrins, $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$, in an RGD-dependent manner. We characterized a novel murine monoclonal antibody to human vWf, GUR76-23, which recognized a site within the carboxy-terminal half of the molecule containing the RGD sequence. This antibody inhibited high shear-induced platelet aggregation and blocked adhesion of ADP plus epinephrine-stimulated platelets to vWf, indicating that it interferes with the interaction with $\alpha_{IIb}\beta_3$. Unlike antibodies against the RGD site, however, the antibody was without effect on adhesion of cultured human umbilical vein endothelial cells to vWf, a phenomenon known to involve the interaction with $\alpha_v\beta_3$. GUR76-23 binding was not displaced by anti-RGD antibodies. These results suggest that the adhesive interaction of vWf with these two β_3 integrins may be differentially modulated by a site(s) other than the common RGD module. © 1997 Academic Press

vWf plays a primary role in physiological hemostasis under flow conditions by supporting platelet adhesion to the subendothelium and subsequent platelet-platelet interactions (1, 2). The GPIb-IX complex (3, 4, 5,) and the GPIIb-IIIa (2, 6) complex ($\alpha_{IIb}\beta_3$ integrin) on platelets function in a coordinated and sequential fashion in this adhesive phenomenon. The binding site for the GPIb-IX complex has been clearly demonstrated to reside within a limited area of the amino-terminal half of the molecule called the A1 loop domain (3, 4, 5, 6), while that for the $\alpha_{IIb}\beta_3$ receptor has been localized to an area containing an Arg-Gly-Asp (RGD) sequence in the carboxy portion of the ligand (6, 7, 8, 9, 10). vWf is also known to serve as a substrate for the other β_3 integrin ($\alpha_v\beta_3$) on vascular endothelial cells (11, 12) which, previously recognized as a vitronectin receptor, has been shown to have an important role in angiogenesis or vascular wall remodeling (13). The binding site for $\alpha_v\beta_3$ has also been localized to the region containing the RGD sequence on the carboxy half of vWf. However, it remains unknown whether exactly the same RGD site is responsible for receptor binding of the two β_3 integrins (10, 14, 15) or a second, non-RGD site(s) is actually involved in differential recognition by the receptors. To examine this issue, we characterized a novel murine monoclonal antibody to human vWf, GUR76-23, which was found to interfere with the interaction between vWf and $\alpha_{IIb}\beta_3$, but had no effect on cultured human umbilical vein endothelial cell (HUVEC) adhesion to vWf mediated by $\alpha_v\beta_3$.

MATERIALS AND METHODS

Antibodies. A murine monoclonal anti-vWf antibody (GUR76-23, IgG_1) was produced by immunizing BALB/c mice with intraperitoneal injections of a water-soluble, wheat-germ agglutinin-bound fraction of sonicated, washed human platelets as described previously (16). Antibodies (IgG) were purified from murine ascitic fluid using a protein A sepharose 4B gel (Pharmacia LKB, Uppsala, Sweden). Fab fragments were produced by limited proteolysis with solid-phase papain using a commercially-available kit (Pierce, Rockford, IL). The following are reference anti-vWf murine monoclonal antibodies: AVW1 (anti-carboxy-terminal region, provided by Dr. R.R. Montgomery, the Blood Center of Southeastern Wisconsin, Milwaukee, USA)

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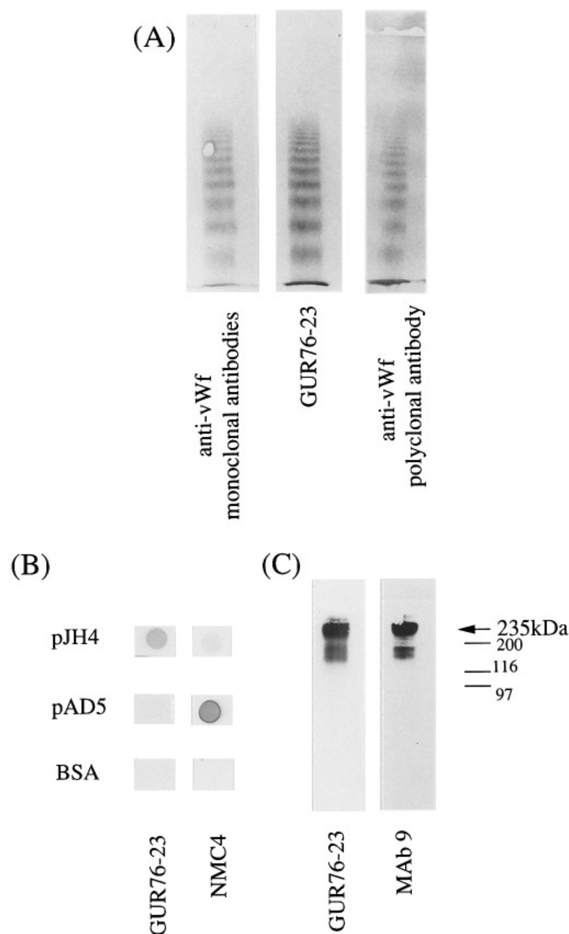

FIG. 1. Immunoreactivity of GUR76-23 with human vWf multimer (A), recombinant vWf (B), and vWf SPII fragment (C). (A) Purified human vWf was electrophoresed on SDS-agarose gel and transblotted onto a nitrocellulose membrane, then stained with GUR76-23, an anti-vWf monoclonal antibody cocktail containing AVW1, VW1-2, VW40-1 and VW53-2, or anti-vWf polyclonal antibody. (B) pJH4, pAD5, and BSA were dotted onto a nitrocellulose membrane, then stained with GUR76-23 or NMC4. (C) The vWf SPII fragment was electrophoresed on 7.5% SDS gel and transblotted onto a nitrocellulose membrane, then stained with GUR76-23, or MAb 9. The arrows indicate the vWf SPII fragment (−235kDa).

(17), NMC4 (anti-A1 domain, provided by Dr. A. Yoshioka, Department of Pediatrics, Nara Medical University, Kashihara, Nara, Japan) (18), 152B6 (anti-RGD like peptide, from Dr. Z.M. Ruggeri, The Scripps Research Institute, La Jolla, California, USA) (8), MAb 9 (anti-RGD region, from Dr. D. Meyer, INSERUM, U.143, Hospital de Bicetre, Paris, France) (9, 19, 20), VW1-2 (anti-RGD region), VW40-1 (anti-Staphylococcus V-8 protease (SP) fragment II which has been assigned to the carboxy half of vWf) (21), and VW53-2 (anti-amino - terminal region) (all produced by Takara-Shuzo). Anti-vWf rabbit polyclonal antibody was purchased from Sigma. Anti-GPIIb-IIIa murine monoclonal antibody (C4G1) was a generous gift from Dr. S. Yano (Institute for Drug Discovery Research, Yamanouchi Pharmaceutical Co., Ltd., Tsukuba, Ibaraki, Japan) (22).

vWf. vWf was isolated from fresh frozen plasma according to the method described by Thorell *et al* (23). Alternatively, purified vWf generously provided by Dr. Z.M. Ruggeri was used in some experiments. Proteolytic fragmentation of vWf with SP and subsequent separation of SP fragment II were carried out as described previously (24). A recombinant human vWf fragment (Arg^{441}-Asn^{730}) which contains the A1 loop (pAD5) (25) and full length vWf (pJH4) were generously provided by Dr. H. Azuma of Tokusima University.

Platelet aggregation. Using citrated platelet rich plasma (PRP), agonist-induced platelet aggregation was measured in an aggregometer (Hema Tracer TM Model 601, Niko Bioscience, Tokyo, Japan) with continuous stirring. The measurement of shear-induced platelet aggregation (SIPA) was performed using a modified cone-plate viscometer as previously described (26).

Platelet adhesion assay. Platelet adhesion assay was carried out as previously described (27). In essence, ^{51}Cr-labeled platelets were washed twice with 10 ml of the RCD solution (pH 6.5, 36 mM citric acid, 5 mM glucose, 1 mM $MgCl_2$, 5 mM KCl, 103 mM NaCl, 57 nM PGE_1) and finally resuspended in TRIS/BSA (pH 7.4, 50 mM Tris, 140 mM NaCl, 5 mM glucose, 2 mM $MgCl_2$, 0.5%BSA). A 96-well microtiter plate was precoated with 10 μg/ml of vWf or BSA. Washed platelets (1×10^7/well), which had been stimulated with ADP (10 μM) and epinephrine (20 μg/ml) for 15 min, were added to the wells. Non-adherent platelets were removed and adherent platelets were solubilized with 5% SDS and radioactivity was measured with a gamma counter (ARC600, Aloka, Tokyo, Japan).

HUVEC adhesion to vWf. Adhesion of HUVEC to vWf was examined according to previously described methods with modification (11, 14, 15). In short, a 96-well microtiter plate was precoated with 10 μg/ml of vWf. Monoclonal antibodies or peptides were added to the wells, then 2×10^5 HUVEC were added to each well. Non-adherent cells were removed and adherent cells were fixed with 100% methanol, stained with Giemsa solution and qualitatively analyzed microscopically.

Competitive antibody binding to vWf. A 96-well microtiter plate was coated with 5 μg/ml of vWf or 5% skim milk solution. Then,100 μg/ml of competing antibodies were allowed to bind to the immobilized protein on the wells, followed by the addition of 1 μg/ml of ^{125}I-labeled antibody (GUR76-23 or 152B6) was added to the wells. After removal and washing of the unbound antibody, bound antibody was solubilized with 5% SDS and radioactivity was measured.

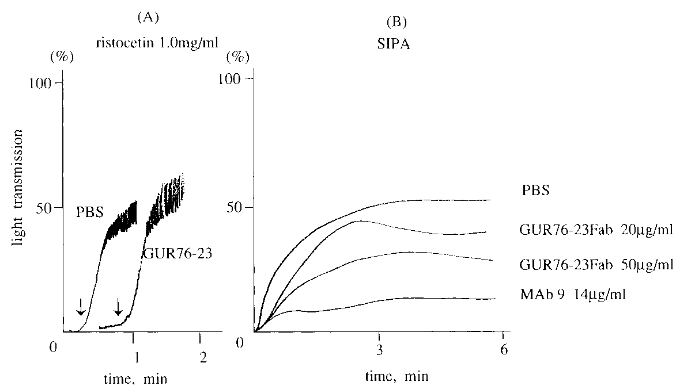

FIG. 2. Effects of GUR76-23 on platelet aggregation induced by ristocetin (A) or SIPA (B). (A) PRP was incubated with 200 μg/ml GUR76-23 or PBS for 10 min, then ristocetin was added (indicated by the arrow) and platelet aggregation was measured using an aggregometer. (B) PRP was preincubated with GUR76-23 (Fab fragments, 20 or 50 μg/ml), MAb 9 (IgG, 14μg/ml) or PBS for10 min, then exposed to high shear-stress (108 dyn/cm^2) for 5 min 30 sec. Shear-induced platelet aggregation was monitored with a modified cone-plate viscometer.

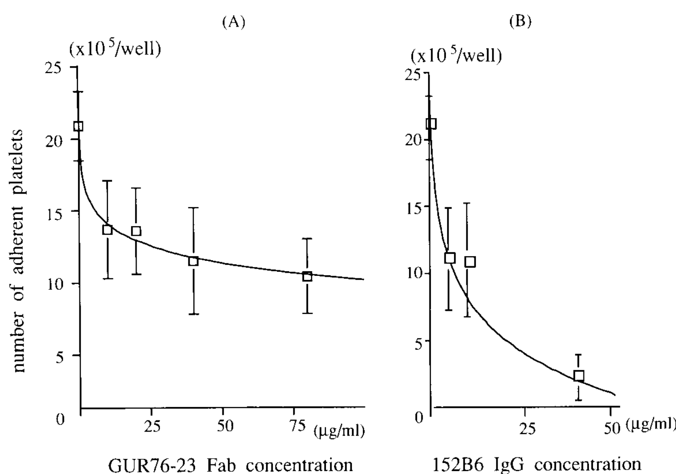

FIG. 3. Effects of GUR76-23 Fab fragments on adhesion of ^{51}Cr-labeled platelets to vWf immobilized on microtiter plates. Wells precoated with vWf (10 μg/ml) or BSA were incubated with various concentrations of GUR76-23 Fab fragments (A) or 152B6 IgG (B) for 15 min. ^{51}Cr-labeled platelets, that had been preactivated with ADP (10μM/ml) plus epinephrine (20 μg/ml) for 15 min, were then added to the well followed by a 90 min incubation at 37°C. Each incubation mixture (200 μl/well) contained 10^7 platelets. After washing, adherent platelets were soubilized with 1% SDS. Radioactivity was then measured to count the number of adherent platelets. Specific adhesion to vWf was calculated by subtraction of the number of platelets adherent to BSA-coated wells. Each experiment was performed in triplicate and the data obtained are expressed as the mean ± standard deviation (SD) from 3 independent experiments.

RESULTS AND DISCUSSION

Murine Monoclonal Antibody GUR76-23 Recognized an Epitope as Residing on the SPII vWf Fragment

A murine hybridoma clone, GUR76-23 (IgG_1), was originally selected on the basis of its reaction with washed platelets immobilized on microtiter plates. Antibody binding was increased when thrombin-stimulated platelets were used. After an extensive search for the antigen, it was finally found that the antibody reacted with purified vWf by dot blotting under non-denaturing conditions (data not shown). In fact, the antibody recognized the multimeric structure of vWf transblotted onto nitrocellulose after separation with SDS-agarose gel electrophoresis (FIG.1A). Disulfide reduction completely destroyed antigenecity suggesting that a disulfide-linked conformational structure may be essential for maintaining the antibody epitope (data not shown). To localize the antibody's epitope on the vWf molecule, antibody binding to vWf fragments was examined by dot blotting. GUR76-23 reacted with recombinant full length vWf (pJH4: Ser^{1}-Lys^{2050}) but not with the recombinant amino-terminal vWf fragment (pAD5: Arg^{441}-Asn^{730}) (25), while NMC4 (18) the epitope of which has been localized between Val ^{449}and Lys^{728} reacted with both products (FIG.1B). On Western blotting, this antibody, like MAb 9 the epitope of which has been shown to reside on Gln^{1704}-Asp^{1746} (9, 19, 20), reacted with the carboxy terminal SPII vWf fragment (Glu^{1366}-Lys^{2050}) containing the RGD site (residues1744-1746) (FIG.1C). These results indicate that the recognition site of GUR76-23 on vWf resides in the carboxy-terminal half of the molecule (residuesGlu^{1366}-Lys^{2050}).

The Antibody Inhibited Interaction between vWf and Platelet $\alpha_{IIb}\beta_3$ Integrin

This antibody (IgG and Fab fragments), even at cocentrations as high as 200 μg/ml, was without effect on ristocetin (1 mg/ml)-induced platelet aggregation (FIG.2A). In contrast, although never fully, Fab fragments of the antibody dose-dependently inhibited shear-induced platelet aggregation (SIPA) occurring under high shear stress conditions of 108 dyn/cm^2 (FIG.2B). The optimal concentration of antibody for obtaining maximal inhibition (55± 16 %, mean ± SD, n=6) was approximately 50 μg/ml. SIPA has been shown to be mediated by interaction of vWf with both GPIb-IX and $\alpha_{IIb}\beta_3$ complexes on platelets (26). Since the antibody was demonstrated to be without effect on ristocetin-induced platelet aggregation caused by the vWf-GPIb-IX interaction, the inhibitory effect of the antibody on SIPA under high shear force conditions may be due to its involvement in the vWf-$\alpha_{IIb}\beta_3$ complex interaction. In fact, the antibody MAb 9 (14 μg/ml), which recognizes the RGD site on vWf (19, 20), inhibited SIPA completely (FIG.2B). To further test this hypothesis, platelet adhesion to vWf insolubilized on plastic was carried out in the presence of antibodies. ADP (10 μM) and epinephrine (20 μg/ml)-stimulated platelets readily adhered to solid-phase vWf in a manner dependent upon the $\alpha_{IIb}\beta_3$ complex, since this adhesion was completely inhibited by either synthetic RGD peptide (0.1 mM) or a functional anti-$\alpha_{IIb}\beta_3$ antibody (C4G1, 10 μg/ml) (22) but not by a functional anti-GPIb-IX antibody (GUR83-35, 10 μg/ml) (data not shown). Fab fragments of GUR76-23 did dose-dependently inhibit adhesion, by up to 62% (50± 11 %, the mean ± SD, n=9) at 80 μg/ml (FIG. 3), while a reference antibody, 152B6 (40 μg/ml), which recognizes the RGD site on vWf (8), inhibited adhesion almost completely (90± 7 %, the mean ± SD, n=9). These observations indicate that the antibody's binding epitope may be responsible for or closely related to the vWf-$\alpha_{IIb}\beta_3$ complex interaction.

The Antibody Did Not Interfere with HUVEC Adhesion to vWf Mediated by $\alpha v\beta 3$ Integrin

vWf is also known to be one of the target ligands for $\alpha_v\beta_3$ integrin on vascular endothelial cells (11). This

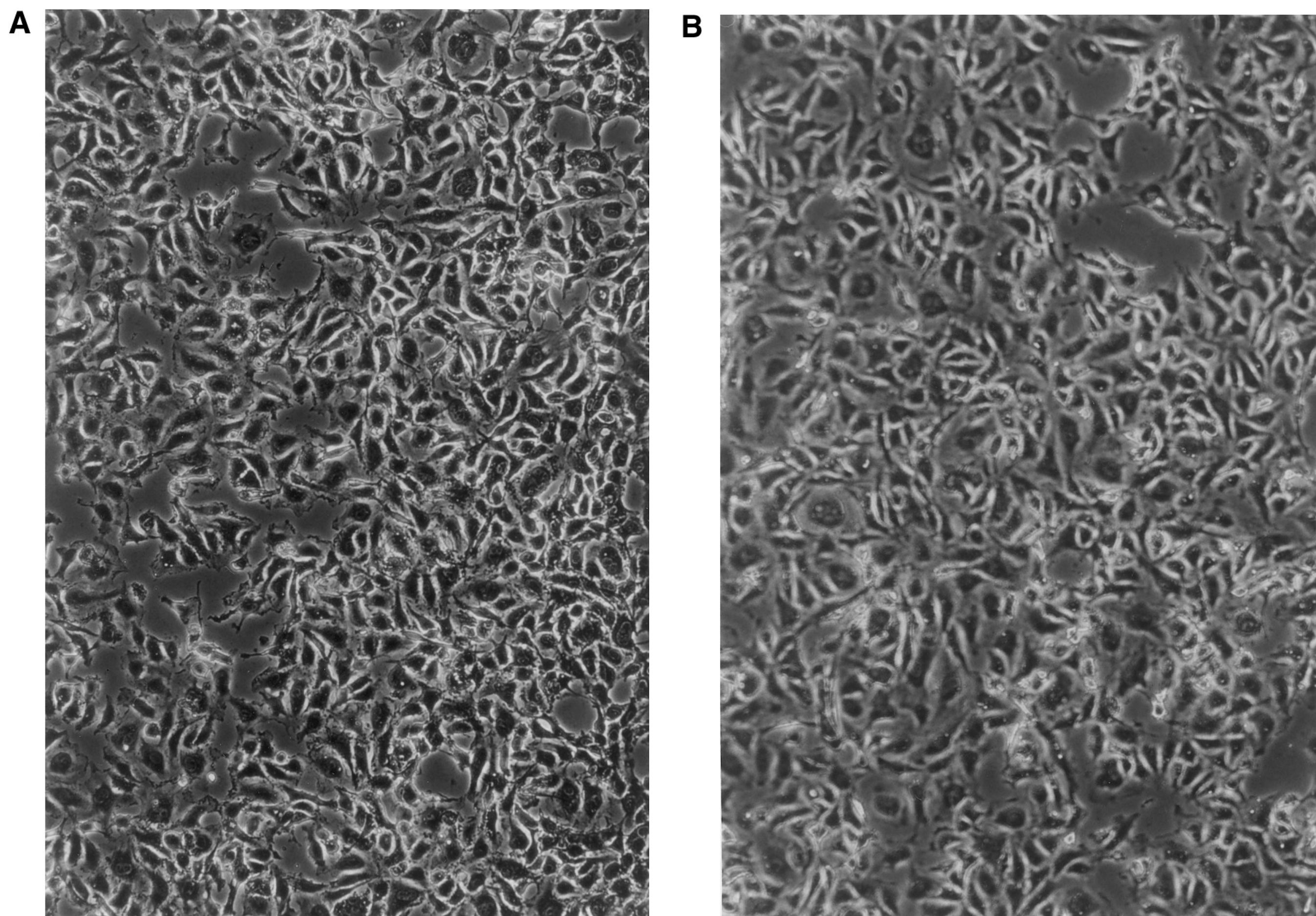

FIG. 4. Effect of GUR76-23 on HUVEC adhesion to vWf. Wells precoated with vWf (10 μg/ml) or BSA were incubated with VW53-2 (A, 50 μg/ml), GUR76-23 (B, 50 μg/ml), MAb 9 (C, 50 μg/ml) or RGD peptide (D, 0.1 mM/ml) for 30 min. at 37°C. After washing, the wells were further incubated with HUVEC (2×10^5 cells/well) for 3 hrs at 37°C. After removing non-adherent cell by washing with the incubation buffer, the adherent cells were stained with Giemsa solution and photographed under microscopy at an original magnification of 100 $\times$.

adhesive interaction has been shown to be mediated by a region on vWf containing the RGD sequence (10, 14). To examine whether the antibody's epitope is involved in the vWf-$\alpha_v\beta_3$ interaction, HUVEC adhesion to vWf immobilized on plastic was carried out (FIG.4). On microscopic examination, a synthetic RGD peptide (0.1 mM) completely inhibited cell adhesion, while GUR76-23 (IgG or Fab fragments, 50 μg/ml) had no effect on adhesion and subsequent spreading. Pretreatment of the vWf-coated surface with MAb 9 (50 μg/ml) resulted in a marked loss of cell adhesion confirming the previously reported characterization (15). These observations suggest that the epitope of the antibody may not be involved in the vWf-$\alpha_v\beta_3$ interaction. To date, all reported anti-vWf antibodies (152B6 and MAb 9), the epitopes of which reside at or near to the RGD site, have been shown to equally abrogate interactions with $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$. Therefore, it is reasonable to speculate that the antibody recognizes a non-RGD site topologically discrete from the RGD site, which mediates an interaction only with $\alpha_{IIb}\beta_3$.

The Epitope of GUR76-23 Did Not Compete with Previously Described Functional Anti-SPII Antibodies

To test the aforementioned hypothesis, labeled antibody binding to solid-phase vWf was carried out in the presence of competing antibodies including 152B6 (Glu^{1737}-Ser^{1750}) (8) and MAb 9 (Gln^{1704}-Asp^{1746}) (19, 20) which have been shown to recognize a limited region containing the RGD site (residues1744-1746). The binding of ^{125}I-labeled GUR76-23 IgG to surface-bound vWf was not inhibited by any of the unlabeled anti-vWf SPII fragment antibodies tested. In contrast, the binding of ^{125}I-labeled 152B6 to vWf was almost completely inhibited by unlabeled MAb 9 and partially inhibited by VW1-2, but by neither GUR76-23 nor VW40-

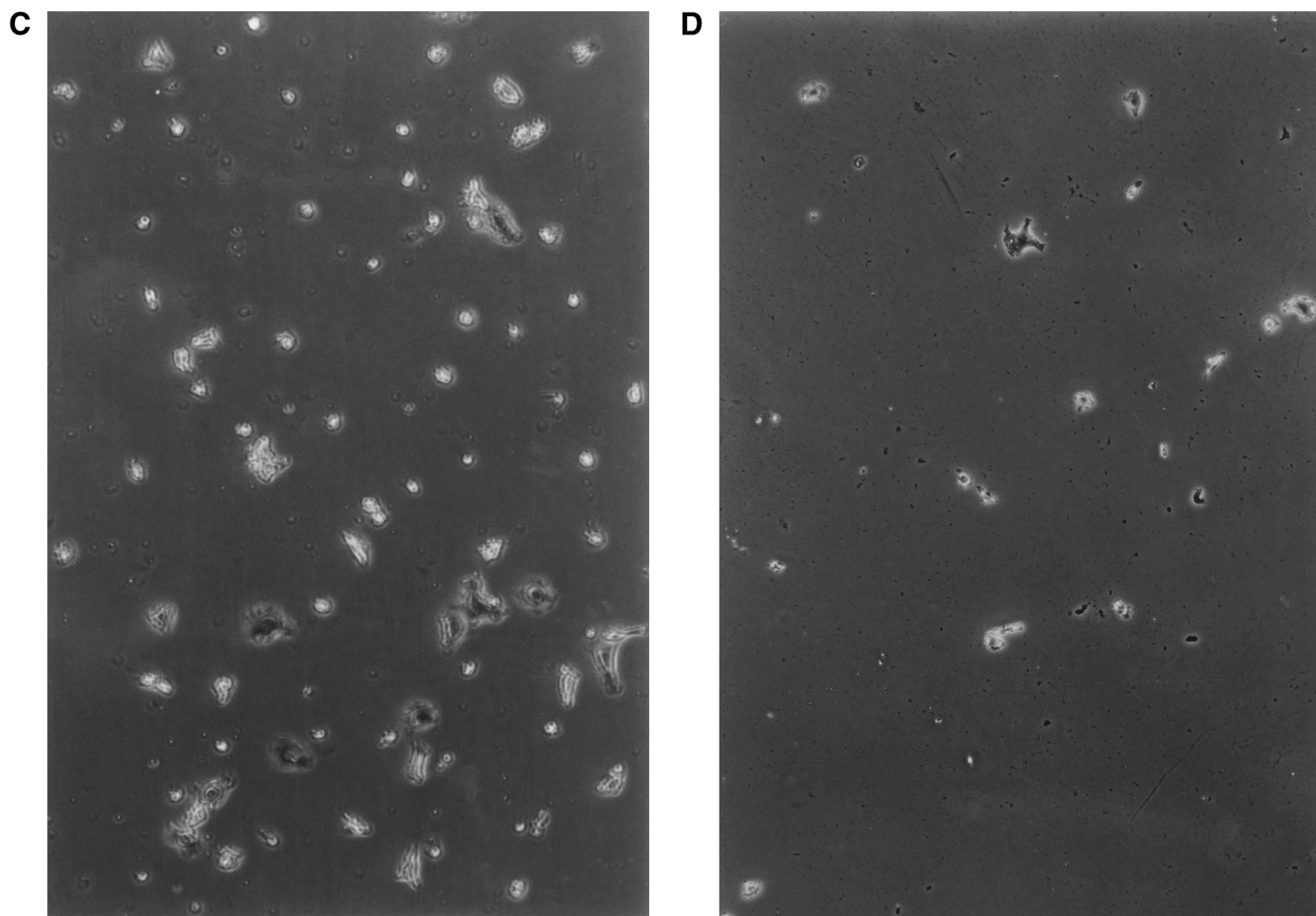

FIG. 4—*Continued*

1 (FIG.5). VW1-2 is an antibody known to inhibit interactions between vWf and endothelial cells which are mediated by $\alpha_v\beta_3$, and VW40-1 is a non-functional anti-vWf SPII fragment antibody the binding epitope of which has been localized within the region carboxy-terminal to Cys^{1786} (21). Thus, these results suggest that the binding epitope of GUR76-23 on immobilized vWf is topologically distinct or far from the RGD sequence region recognized by 152B6 or MAb 9, which has been shown to mediate interactions between vWf and, either platelet $\alpha_{IIb}\beta_3$ (8, 9) or endothelial $\alpha_v\beta_3$ (14, 15). The binding of ^{125}I-labeled GUR76-23 was almost completely inhibited by a 100-fold excess of unlabeled GUR76-23, whereas the binding of ^{125}I-labeled 152B6 was only partially displaced in the presence of a 100-fold excess of unlabeled 152B6. This might be related to the difference in binding affinities of unlabeled and labeled antibodies for solid-phase vWf.

In this communication, a novel anti-vWf SPII fragment antibody, GUR76-23, has been demonstrated to have an inhibitory effect on the vWf-platelet interaction mediated by $\alpha_{IIb}\beta_3$ but not on the vWf-endothelial cell interaction mediated by $\alpha_v\beta_3$. To the best of our knowledge, this is the first evidence of the structure-function relationship between vWf and $\alpha_{IIb}\beta_3$ integrin being clearly segregated from that of vWf and $\alpha_v\beta_3$ integrin, suggesting the possible existence of a second, non-RGD binding site on the vWf molecule selectively interacting with $\alpha_{IIb}\beta_3$, which is located at or near to the epitope recognized by GUR76-23. In fact, as has already been demonstrated with other RGD-bearing ligands for $\alpha_{IIb}\beta_3$ such as fibrinogen and fibronectin, it is tempting to speculate that a second, RGD-independent interaction also may occur between vWf and the platelet integrin. A region containing a dodecasequence (residues 400-411) derived from the carboxy-terminus of the fibrinogen γ-chain selectively interacts with the α_{IIb} subunit (28) and mediates an interaction with $\alpha_{IIb}\beta_3$ alone (29, 30). Likewise, a limited sequence (residues 1373-1383) amino-terminal to the primary cell adhesion site (containing the RGD site) on fibronectin has been shown to be involved in a selective interaction with $\alpha_{IIb}\beta_3$ which involves binding to the α_{IIb} subunit (31). Neither non-RGD site interacts with $\alpha_v\beta_3$, while

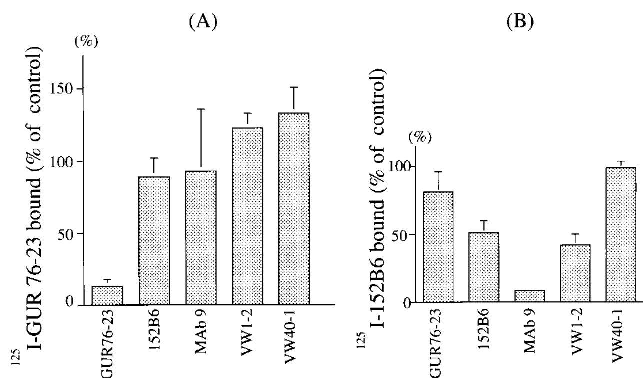

FIG. 5. Epitope competition of ^{125}I-labeled GUR76-23 IgG binding to vWf coated on plastic wells with anti-vWf SPII fragment antibodies. Wells precoated with vWf (5 μg/ml) or skim milk were incubated with 100 μg/ml of competing unlabeled antibodies (IgG) including GUR76-23, 152B6, MAb 9, VW1-2 and VW40-1 for 30 min. at 37°C. Then,1 μg/ml of ^{125}I-labeled GUR76-23 (A) or152B6 (B) was added to the well followed by a 1 h incubation at 37°C. Following removal of unbound IgG, labeled antibody was eluted by 1% SDS and radioactivity was measured with a gamma counter. Each experiment was performed in triplicate. Specific antibody binding was calculated by subtraction of antibody binding to skim milk-coated wells and the data obtained are expressed as a percentage of control binding determined in the absence of competing antibody (the mean ± SD, n=5).

the RGD sites of both ligands bind to the common $\beta 3$ subunit interacting similarly with either $\alpha_{IIb}\beta_3$ or $\alpha_v\beta_3$ (12, 29, 32).

Thus, detailed epitope mapping of the antibody should be conducted to further test the above hypothesis.

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