Identification of Human Megakaryocyte Coagulation Factor V

By William L. Nichols, Dennis A. Gastineau, Lawrence A. Solberg, Jr, and Kenneth G. Mann

Specific monoclonal and polyclonal antibody reagents and a double antigen indirect immunofluorescence microscopy technique were used to visualize coagulation factor V in human bone marrow. Marrow aspirates were smeared directly on glass slides, or washed and cytospun onto glass slides, or processed and plated into a plasma/methylcellulose cell culture system. Morphologically identifiable colonies of megakaryocytes, erythrocytes, granulocytes, or monocytes/macrophages were removed from 14- to 18-day marrow culture dishes by micropipette and streaked onto glass slides. Smears of marrow cell preparations were air-dried, fixed, washed, and incubated sequentially with primary IgG antibody reagents and with secondary anti-IgG antibody reagents conjugated with either fluorescein or rhodamine. Preparations were examined and photographed through a microscope suitably equipped for two-color fluorescence and phase contrast analysis. Cells of megakaryocytic lineage were identified by their immunofluorescent reactivity with murine monoclonal antibody HP1-1D, specific for human platelet plasma membrane glycoprotein Ib/IIa (GP Ib/IIa), or by their immunofluorescent reactivity with monoclonal or polyclonal antibodies specific for von Willebrand factor (vWF) or for platelet factor 4 (PF4). Coagulation factor V in bone marrow was detected by simultaneous immunofluorescent staining with polyclonal burro anti-human factor V antibody or with a panel of murine monoclonal anti-human factor V antibodies. The double antigen immunofluorescence staining technique, incorporating appropriate controls, revealed that coagulation factor V was principally located in marrow cells simultaneously identified as megakaryocytes by antibodies to GP Ib/IIa, vWF, or PF4. The specific immunofluorescence of factor V in megakaryocytes and platelets was eliminated when excess purified factor V antigen was preincubated with anti-factor V antibody. Our observations establish the presence of human megakaryocyte coagulation factor V, confirm the presence of human platelet factor V, and indicate that human megakaryocyte/platelet coagulation factor V is a lineage-associated protein.

COAGULATION FACTOR V is a single-chain high-molecular-weight protein that, on proteolytic activation, functions as an essential nonenzymatic cofactor for the optimum conversion of prothrombin zymogen to the blood clotting enzyme, thrombin. Activated factor V (factor Va) fulfills its cofactor role by forming the receptor for the serine protease, coagulation factor Xa, on the platelet surface or on an equivalent phospholipid surface. This membrane-bound complex of enzyme (factor Xa) and cofactor (factor Va), together with calcium ion, constitutes the enzymatic complex prothrombinase, the terminal complex in the blood coagulation cascade. The complete prothrombinase complex catalyzes thrombin formation from prothrombin substrate at 300,000 times the rate achieved with factor Xa enzyme alone, while deletion of factor Va cofactor from the complex reduces the rate of thrombin formation by four orders of magnitude.

Blood platelets, by virtue of their capability to interact with damaged vasculature and with coagulation factors Va and Xa, are believed to represent the physiologically relevant surface that provides a vehicle for the discrete localization, assembly, and explosive activity of prothrombinase enzyme, essential for thrombin generation and for normal hemostasis. The importance of coagulation factor V for hemostasis is exemplified in the bleeding disorder manifest by individuals with congenital isolated factor V deficiency, or by some individuals with antibody-induced acquired factor V deficiency. Quantitative radioimmunoassay studies have recently documented that in addition to its presence in blood plasma, coagulation factor V antigen is present in human platelets, which have been determined to contribute from 18% to 25% of the total amount of factor V protein contained in whole blood. Platelet factor V appears to be of hemostatic significance, since individuals with functionally defective platelet factor V, but functionally normal plasma factor V, may have a bleeding diathesis. In addition to its presence in plasma and platelets, factor V is found in and is synthesized by endothelial cells. The distribution of coagulation factor V into plasma, platelet, and endothelial cell compartments is similar to the distribution of von Willebrand factor (vWF) (factor VIII-related antigen), which is also present in and is synthesized by megakaryocytes. Because factor V is present in human platelets and appears to have a tissue compartmentalization similar to that of factor VIII-related antigen, we investigated...
cells expressing factor V in human bone marrow by using specific monoclonal and polyclonal antibody reagents and an indirect immunofluorescence technique. Herein we report the identification of human megakaryocyte coagulation factor V.

**MATERIALS AND METHODS**

**Human Blood and Bone Marrow Cells**

**Blood.** Peripheral venous blood (4 to 10 mL) from consenting normal subjects was anticoagulated either with 0.1 vol 3.8% trisodium citrate solution or with 0.02 vol 7.5% tripotassium ethylene diamine-tetraacetaete (EDTA). Fresh whole blood smears on glass microscope slides were allowed to air-dry and then were fixed and processed for immunofluorescence staining as described later. Blood buffy coat smears were prepared in the same fashion, using the buffy coat isolated after centrifugation of the blood for eight to ten minutes at 150 g. Washed platelet smears were prepared using platelets isolated from EDTA-anticoagulated platelet-rich plasma by centrifugation (2,500 g, ten minutes) and washing three times by centrifugation (1,500 g, ten minutes) in a buffer containing 10 mmol/L TRIS, 150 mmol/L NaCl, and 2.5 mmol/L sodium EDTA, pH 7.4.

**Marrow.** Bone marrow aspirates were obtained from the posterior iliac crest in the conventional manner, and a small portion (0.5 to 2.0 mL) was placed in a glass tube coated with 0.08 mL 7.5% tripotassium EDTA anticoagulant (Venoject, Terumo Medical Corp, Elkhon, Md). After serving their clinical purpose, the residual specimens from subjects with normal bone marrow, or with non-megaloproliferative hematologic disorders (lymphoma, myeloma, or amyloidosis), were used for study. Direct smears of anticoagulated marrow aspirate were air-dried on glass slides.

Alternatively, marrow cytospin smears were prepared after treatment of the marrow cells to remove erythrocytes and plasma components. The supernatant plasma and lipid were removed from anticoagulated marrow aspirate after centrifugation for ten minutes at 1,200 g. Marrow cells were resuspended in approximately 10 vol of erythrocyte-lysing buffer (155 mmol/L NH4Cl, 10 mmol/L KHCO3, and 2.5 mmol/L sodium EDTA, pH 7.3, 20 °C) and immediately centrifuged. After an additional resuspension and centrifugation in a similar volume of erythrocyte-lysing buffer, the marrow cells were resuspended and washed by centrifugation three times in washing buffer containing phosphate-buffered saline (PBS; 150 mmol/L NaCl, 10.5 mmol/L Na2HPO4, and 1.5 mmol/L NaH2PO4), 1% bovine serum albumin (BSA; Sigma A9647, Sigma Chemical Co, St Louis), and 2.5 mmol/L sodium EDTA, pH 7.4.

The washed marrow cells were finally resuspended in approximately 20 vol of the latter buffer, and 0.1-mL aliquots were cytocentrifuged onto glass microscope slides using a Cytospin centrifuge (Shandon Southern, Sewickley, Pa). The direct and washed marrow cell preparations were then fixed and processed for immunofluorescence staining as described later.

**Cultured marrow.** Bone marrow aspirates, obtained as described, were collected into sterile glass tubes containing preservative-free sodium heparin (O’Neal, Jones and Feldman, St Louis). A mono-nuclear marrow cell suspension of density less than 1.077 g/cm³ was isolated, diluted, and plated into a plasma/methylcellulose cell culture system, as previously described. After incubation for 14 to 18 days at 37 °C in a humid atmosphere supplemented by 5% CO2, the plates were examined by inverted microscopy for the presence of morphologically recognizable megakaryocytic colonies and colonies of other lineage. Individual colonies were removed from culture dishes by micropipette, linearly streaked onto glass microscope slides, air-dried, fixed, and processed for immunofluorescence staining as described later.

**Immunofluorescence Staining and Microscopy Technique**

**Fixation.** We investigated the effect of three specimen fixation methods on the indirect immunofluorescent reactivity of each of the primary antibody reagents with platelets in peripheral blood smears and with megakaryocytes in marrow smears or picked from cell culture. After air-drying of the cytologic smear preparations for at least 30 minutes, the cells were fixed by (a) immersion in ice-cold acetone for five minutes, followed by air-drying; or (b) immersion in ice-cold buffered formalin-acetone for 30 seconds, followed by air-drying, three washes in distilled water or PBS, and repeated air-drying; or (c) immersion in ice-cold methanol for 30 minutes, followed by air-drying. Fixed smears were then stained, as outlined later, usually immediately after fixation. Occasionally, fixed smears were stored for one or two days at 4 °C, or for several days at -70 °C, before staining. In the latter case, smears were thawed at room temperature and then rehydrated by incubation in a moist chamber for one hour at 37 °C before staining.

**Staining.** Indirect immunofluorescent staining of fixed peripheral blood and marrow cell smears was performed in a fashion similar to that described by Kastmann et al. After three rinses of specimen slides in PBS, one rinse in PBS + BSA, and two rinses in PBS, 0.2- or 0.1-mL aliquots of one or two appropriately diluted primary antibody reagents (monoclonal ascites or polyclonal sera) were layered onto specimen smears and incubated for 45 minutes at room temperature in a sealed, moist chamber. When a preparation was stained simultaneously with two primary antibodies obtained from different species (ie, mouse monoclonal ascites plus a polyclonal rabbit or burro antiserum), 0.1-mL aliquots of each reagent were mixed after application to the slide, by stirring the surface of the solution with the bottom of a small glass test tube or by tilting the slide. After incubation, the slides were rinsed briefly with a stream of PBS + BSA, washed three times by immersion in PBS, overlaid with 0.2 mL normal goat serum (diluted 1:20 in PBS), and incubated for 30 minutes. After brief rinsing with PBS + BSA, the slides were further exposed for 45 minutes to a similar volume of PBS + BSA that contained one or two appropriately diluted fluorescein-conjugated anti-immunoglobulin reagents. After three washes in PBS, coverslips were affixed over a few drops of freshly prepared mounting fluid (one part glycerol, two parts PBS, 1 mg/mL p-phenylenediamine) and sealed with clear fingernail polish.

**Microscopy and photography.** Stained slides were stored at 4 °C until completion of examination and photographic recording, which were accomplished using a Zeiss Standard 18 microscope suitably equipped for phase-contrast and for two-color (fluorescein and rhodamine) epifluorescence microscopy and photography. Ektachrome and Tri-X Pan 35-mm ASA 400 photographic films (Eastman Kodak, Rochester, NY) were used to record color and moncolor images, respectively. Identical photographic exposure, development, and printing times were used to depict immune and nonimmune (control) immunofluorescence. Films were processed by the Section of Photographic Services, Mayo Clinic. Montage illustrations were prepared by the Section of Medical Graphics, Mayo Clinic.

**Antibody Reagents**

Table 1 summarizes the antibody reagents we used for indirect immunofluorescence staining of human blood and bone marrow cells.
cytes obtained from a BALB/c mouse three days after the second
containing a monoclonal IgG antibody (W1-23), specific for vWF, is
Clinic).
and was obtained from Drs iA. Katzmann and D.N. Fass (Mayo
the antibody described as Group IV, clone 23 by Katzmann et al.6
secreting platelet-specific antibody, appropriately diluted hybri-
platelets. Cell fusion, cloning, and subcloning at limiting dilution
antibody, a monoclonal IgG, is specific for a mycobacterial anti-
containing TB5-2 monoclonal antibody. The former ascites served as
control reactions included pooled ascites obtained from mice inocu-
examined by fluorescence microscopy, after additional incubation
acetone-fixed human peripheral blood smears. The smears were
monthly intraperitoneal immunization with washed normal human
mann (Mayo Clinic).
lated with NS-l mouse myeloma cell line, and pooled ascites
Secondary antibodiest
Primary Antibodies

Table 1.
Antibody Reagents Used for Indirect
Bone Marrow Cells
Polyclonal immunoglobulins
Polyclonal serums
Monoclonal ascites
TB5-2 Mouse Mycobacterium tuberculo-
RaHWF Rabbit Human vWF
Nl-R Rabbit Nonimmune
F1Tc-GaR lgG Goat Rabbit lgG
RITC-GaH lgG Goat Horse lgG
FITC-GaB lgG Goat Burro lgG
lgG Goat Mouse lgG
RITC-GctM
FITC-GaM IgGAM Goat Mouse immunoglobulins
BaHFV Burro Human coagulation factor
MaHFV Mouse Human coagulation factor
HP 1
NS-1 Mouse Nonimmune
V
V
V
glycoprotein I1b/I1la
sis antigen 5
serum containing antibodies specific for human factor VIII (factor
the preliminary characterization of HP1-ID antibody has been
fluorescence or enzyme-linked immunocytochemical staining. HP1-ID

types of hematologic disorders, as determined by indirect immuno-
themia, as determined quantitatively by double antibody radioimmu-

marrow
or

immunoassay, but reacts extensively with normal platelets or with

patients with megakaryocytic leukemia but does not react with

with homogeneous human PF4, and was provided by Dr
rabbits
human platelet factor 4 (PF4) was prepared by immunization of

VIII-related
antigen:vWF) was obtained from Calbiochem-Behring

Mar

I1b/I1la) from human platelets solubilized with Triton X-100 (Sigma),

immunofluorescence

in abstract form.22

presented
in

The preparation and characterization of
Polyclonal serums.


The hybridoma cell line secreting antibody HP1-ID has thus far
been passed five times in mice, and appears to be a stable cell line.

platelets in citrate-anticoagulated plasma. Negative controls used in

or enhance the aggregation or procoagulant functions of

a modification of the double antibody radioimmunoassay technique

conjugated) antibodies were determined empirically by an iterative

ions ranging from 2.8 to 5.4 mg/mL, as determined by radial
process. Ultimately, reagent concentrations were selected so as to achieve optimum immunofluorescence reactivity of blood and marrow cell antigens with a minimum of background (nonspecific) cellular fluorescence.

To detect the simultaneous cellular expression of two antigens, combinations of two primary and two secondary antibody reagents were selected such that significant nonspecific cross-reactivity did not occur. The absence of undesired cross-reactivity of primary and secondary antibody reagents was determined by inclusion of appropriate combinations of immune and nonimmune (control) primary antibody reagents in the panel of antibody reagents used to stain the smear preparations of each blood, marrow, or cultured marrow specimen.

Table 2 summarizes the reagent composition of the antibody panels we used for simultaneous double antigen immunofluorescence. In each experiment a minimum of four primary antibody reagents (immune and nonimmune mouse monoclonal ascites, and immune and nonimmune burro or rabbit polyclonal serums) from one panel were applied in paired combinations (monoclonal plus polyclonal) to specimen slides that were then stained with the designated combination of secondary antibody reagents. For example, a minimum of four specimen slides stained with panel A reagents included the following paired combinations of primary antibody applications, each slide subsequently stained with the secondary antibody combination of FITC-GaM IgG + RITC-GaH IgG: slide 1, TB5-2 + NI-B; slide 2, HP1-1D + NI-B; slide 3, TB5-2 + BrHFV; slide 4, HP1-1D + BrHFV.

**Coagulation Factor V Antigen-Antibody Competition**

Human coagulation factor V protein, isolated and characterized as described,1 was used in antigen—antibody competition studies to establish the specificity of factor V immunofluorescence in human megakaryocytes and platelets. A competition mixture, consisting of 0.5 mL of a 1:100 dilution of burro anti-human factor V serum in PBS + BSA and 0.01 mL of a 70%-saturated ammonium sulfate solution containing 0.8 mg human factor V per milliliter, was incubated 30 minutes at 37 °C before use as a primary antibody reagent for immunofluorescent staining of marrow cells. A control mixture, identical except for omission of human factor V antigen from the ammonium sulfate solution, was similarly used for concomitant staining of companion marrow specimens.

**RESULTS**

**Effect of Fixation Technique on Cellular Morphology and Immunofluorescence**

Each of the investigated cellular fixation techniques resulted in adequate preservation of the immunofluorescent reactivity of the antigens we evaluated. The microscopic appearance of cellular immunofluorescence was somewhat dependent on the fixation technique used, but in each case there was satisfactory immunofluorescent detection of (presumably) predominantly intracellular platelet or megakaryocyte antigens (eg, vWF or PF4), of surface antigen (eg, GP IIb/IIIa), and of factor V antigen. Figure 1 shows typically observed effects of fixation technique on cellular morphology (phase-contrast illumination) and cellular immunofluorescence. Because both phase-contrast morphology and immunofluorescent staining intensity seemed optimal after methanol fixation, this technique was preferred.

**Localization of Factor V in Peripheral Blood Cells**

Using specific antibody reagents, platelets were found to be the principal cellular locus of coagulation factor V in the peripheral blood, as determined by immunofluorescence (Fig 1). The results depicted are representative of those obtained in 20 experiments using acetone, formalin-acetone, or methanol fixation techniques. We observed that factor V was detectable in essentially all of the blood platelets identifiable by their simultaneous reactivity with antibodies specific for GP IIb/IIIa, vWF, or PF4. Similar results were

### Table 2. Antibody Reagent Panels Used for Simultaneous Double Antigen Cellular Immunofluorescence

<table>
<thead>
<tr>
<th>Panel</th>
<th>Monoclonal Mouse Ascites</th>
<th>Polyclonal Serums</th>
<th>Secondary Antibody Reagents</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Immune</td>
<td>Nonimmune</td>
<td>Immune</td>
</tr>
<tr>
<td>Panel A</td>
<td>1:1,000</td>
<td>1:1,000</td>
<td>1:200</td>
</tr>
<tr>
<td>HP1-1D</td>
<td>NS-1</td>
<td>TB5-2</td>
<td>BrHFV</td>
</tr>
<tr>
<td>W1-23</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Panel B</td>
<td>1:1,000</td>
<td>1:1,000</td>
<td>1:200</td>
</tr>
<tr>
<td>HP1-1D</td>
<td>NS-1</td>
<td>TB5-2</td>
<td>BrHFV</td>
</tr>
<tr>
<td>W1-23</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Panel C</td>
<td>1:1,000</td>
<td>1:1,000</td>
<td>1:200</td>
</tr>
<tr>
<td>MaHFVs</td>
<td>NS-1</td>
<td>TB5-2</td>
<td>RoHF4</td>
</tr>
<tr>
<td>HB1-1D</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>W1-23</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Panel D</td>
<td>1:1,000</td>
<td>1:1,000</td>
<td>1:200</td>
</tr>
<tr>
<td>HP1-1D</td>
<td>NS-1</td>
<td>TB5-2</td>
<td>RoHF4</td>
</tr>
<tr>
<td>W1-23</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Reagent abbreviations and specificities are summarized in Table 1 and detailed in Materials and Methods. Each vertical column of antibody reagents is headed by an indication of the final dilution at which the antibodies were used (eg, 1:1,000). For simultaneous double antigen immunofluorescence staining, one monoclonal and one polyclonal primary antibody reagent were applied to a specimen slide that was subsequently stained with the designated combination of fluorochrome-conjugated secondary antibody reagents.
obtained using smears of isolated, washed blood platelets. Appropriate control incubations established the specificity of the immunofluorescence reactions. Peripheral blood erythrocytes and leukocytes did not display significant factor V immunofluorescence or significant reactivity with GP IIb/IIIa, vWF, or PF4 antibodies.

Each of the monoclonal anti-human factor V antibodies from the library described by Foster et al. recognized factor V in peripheral blood platelets, as determined by immunofluorescence (Table 3). We observed that certain monoclonal anti-factor V antibodies produced more intense platelet immunofluorescence than did others. Based on these results, we constructed a polyclonal anti-factor V antibody reagent by combining equal amounts of monoclonal
Table 3. Immunofluorescent Reactivity of Monoclonal Antibodies With Normal Human Blood Platelets

<table>
<thead>
<tr>
<th>Antibody*</th>
<th>Antigen Specificity</th>
<th>No. of Preparations Examined</th>
<th>Relative Platelet Immunofluorescence Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>NS-1</td>
<td>None</td>
<td>11</td>
<td>-</td>
</tr>
<tr>
<td>TB5-2</td>
<td>Mycobacterial</td>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td>HP1-1D</td>
<td>GP IIb/IIIa</td>
<td>11</td>
<td>++ + + +</td>
</tr>
<tr>
<td>W1-23</td>
<td>vWF</td>
<td>4</td>
<td>++ +</td>
</tr>
<tr>
<td>MaHFV-1</td>
<td>Factor V</td>
<td>4</td>
<td>+</td>
</tr>
<tr>
<td>MaHFV-2</td>
<td>Factor V</td>
<td>6</td>
<td>+ / +</td>
</tr>
<tr>
<td>MaHFV-3</td>
<td>Factor V</td>
<td>2</td>
<td>+</td>
</tr>
<tr>
<td>MaHFV-4</td>
<td>Factor V</td>
<td>3</td>
<td>+</td>
</tr>
<tr>
<td>MaHFV-4ac</td>
<td>Factor V</td>
<td>3</td>
<td>+</td>
</tr>
<tr>
<td>MaHFV-5</td>
<td>Factor V</td>
<td>7</td>
<td>+ / +</td>
</tr>
<tr>
<td>MaHFV-6</td>
<td>Factor V</td>
<td>8</td>
<td>+</td>
</tr>
<tr>
<td>MaHFV-7</td>
<td>Factor V</td>
<td>3</td>
<td>+ / +</td>
</tr>
<tr>
<td>MaHFV-8</td>
<td>Factor V</td>
<td>4</td>
<td>+</td>
</tr>
<tr>
<td>MaHFV-9</td>
<td>Factor V</td>
<td>2</td>
<td>+ / +</td>
</tr>
<tr>
<td>MaHFV-10</td>
<td>Factor V</td>
<td>3</td>
<td>+</td>
</tr>
<tr>
<td>MaHFV-3 + 6 + 8 + 9 (MaHFVs)</td>
<td>Factor V</td>
<td>3</td>
<td>+ / + + +</td>
</tr>
</tbody>
</table>

Platelets in acetone-fixed whole blood smears from different donors were analyzed. Grading of the immunofluorescence intensity was subjective. -, absent; + + + +, most intense, +, least intense specific immunofluorescence.

Ascites fluid diluted 1/1000; secondary antibody diluted 1/100 (affinity purified, FITC-conjugated goat IgG specific for mouse immunoglobulins: FITC-GaM IgGAM).

Ascites 3, 6, 8, and 9 (MaHFVs). This reagent enhanced the immunofluorescence of cellular factor V, compared with the immunofluorescence produced by individual monoclonal anti-factor V antibodies, and was used to investigate the localization of factor V in bone marrow.

Localization of Factor V in Bone Marrow Cells

In nonwashed marrow, coagulation factor V immunofluorescence (polyclonal burro anti-factor V antibody) was principally located in megakaryocytes and platelets whose identity was established immunologically by simultaneous reactivity with monoclonal GP IIb/IIIa or vWF antibodies (Fig 2). Polyclonal vWF or PF4 antibodies generally produced more intense megakaryocyte immunofluorescence than did polyclonal factor V antibodies. When bone marrow cells were washed to remove loosely associated plasma components, marrow megakaryocytes and platelets retained immunofluorescent reactivity with antibodies specific for human coagulation factor V (Fig 3). The results depicted in Figs 2 and 3 are representative of those obtained in 17 and four experiments, respectively. In each experiment, appropriate control incubations established the specificity of the immunofluorescence reactions. Marrow cells of nonmegakaryocytic lineage served as negative immunofluorescence controls in each preparation.

Analysis of the immunofluorescence reactivity of cultured marrow cells with polyclonal anti-factor V antibody confirmed the localization of factor V in megakaryocytes identified by colony morphology, by phase-contrast microscopy, and by their simultaneous reactivity with monoclonal antibodies specific for GP IIb/IIIa or vWF (Fig 4). Cells obtained from cultured erythroid, granulocytic, and monocytic colonies did not display factor V immunofluorescence or specific immunofluorescent reactivity with GP IIb/IIIa, vWF, or PF4 antibodies. The results depicted are representative of those obtained in eight experiments.

Monoclonal antibodies specific for human coagulation factor V were found to react positively with marrow cells identified as megakaryocytes by their morphological appearance and their simultaneous immunofluorescent reactivity with polyclonal vWF or PF4 antibodies (Fig 5). Monoclonal GP IIb/IIIa or vWF antibodies (HP1-1D and W1-23) generally produced more intense megakaryocyte immunofluorescence than did polyclonal factor V antibodies (MaHFVs). The results depicted are representative of four experiments.

Preincubation of the polyclonal burro anti-factor V serum with purified human coagulation factor V eliminated the specific immunofluorescence of factor V in marrow megakaryocytes and platelets (Fig 6), as determined in four experiments.

Localization of Other Antigens in Megakaryocytes

Indirect immunofluorescence confirmed the platelet and megakaryocyte localization of vWF and PF4 in cells identified by their morphology and simultaneous immunofluorescent reactivity with monoclonal antibody HP1-1D (eg, Figs 2 and 5). These results corroborate the megakaryocyte and platelet specificity of HP1-1D antibody, and the identification of megakaryocytes as the principal cellular locus of coagulation factor V, vWF, and PF4 in human bone marrow.
Fig 2. Simultaneous double antigen indirect immunofluorescence of factor V and other platelet-related antigens in human bone marrow aspirate (directly smeared EDTA-anticoagulated marrow aspirate, acetone-fixed, stained with panel B or with panel C reagents, reproduction magnification ×285). (A) A1 - GP IIb/IIIa (HP1-1D); A2 - factor V (BoHFV). (B) B1 - GP IIb/IIIa (HP1-1D); B2 - control serum (NI-B). (C) C1 - GP IIb/IIIa (HP1-1D); C2 - vWF (RoHWF). (D) D1 - GP IIb/IIIa (HP1-1D); D2 - PF4 (RoHPF4). (E) E1 - GP IIb/IIIa (HP1-1D); E2 - control serum (NI-R).

Fig 3. Simultaneous double antigen indirect immunofluorescent reactivity of polyclonal anti-factor V and monoclonal anti-GP IIb/IIIa antibodies with washed cells from human bone marrow aspirate (washed cytopsin marrow aspirate, acetone-fixed, stained with panel A reagents, reproduction magnification ×285 [A] and ×180 [B]). (A) A1 - GP IIb/IIIa (HP1-1D); A2 - factor V (BoHFV). (B) B1 - GP IIb/IIIa (HP1-1D); B2 - control serum (NI-B).
Fig 4. Simultaneous double antigen indirect immunofluorescent reactivity of polyclonal anti-factor V and monoclonal anti-GP Iib/IIa antibodies with cultured human megakaryocytes (picked colonies of megakaryocytes cultured from human bone marrow, methanol-fixed, stained with panel A reagents, reproduction magnification x 260). (A) A1 = phase-contrast illumination; A2 = GP Iib/IIa (HP1-1D); A3 = factor V (BaHFV). (B) B1 = phase-contrast illumination; B2 = control ascites (TB5-2); B3 = factor V (BaHFV). (C) C1 = phase-contrast illumination; C2 = GP Iib/IIa (HP1-1D); C3 = control serum (NI-B).

Fig 5. Simultaneous double antigen indirect immunofluorescent reactivity of monoclonal factor V, GP Iib/IIa, or vWF antibodies and polyclonal antibodies with cultured human megakaryocytes (picked colonies of megakaryocytes cultured from human bone marrow, methanol-fixed, stained with panel C reagents, reproduction magnification x 260). (A) A1 = phase-contrast illumination; A2 = vWF (RoHWF); A3 = factor V (MoHFVs). (B) B1 = phase-contrast illumination; B2 = PF4 (RoHPF4); B3 = factor V (MoHFVs). (C) C1 = phase-contrast illumination; C2 = vWF (RoHWF); C3 = GP Iib/IIa (HP1-1D). (D) D1 = phase-contrast illumination; D2 = PF4 (RoHPF4); D3 = vWF (W1-23). (E) E1 = phase-contrast illumination; E2 = vWF (RoHWF); E3 = control ascites (TB5-2). (F) F1 = phase-contrast illumination; F2 = control serum (NI-R); F3 = factor V (MoHFVs).
Fig 6. Simultaneous double antigen indirect immunofluorescent reactivity of cultured megakaryocytes with polyclonal anti-factor V antibodies preincubated with or without excess purified factor V antigen (picked colonies of megakaryocytes cultured from human bone marrow, methanol-fixed, stained with panel A reagents ± preincubation, reproduction magnification ×340). (A) A1 = GP IIb/IIIa (HP1-1D); A2 = factor V (BoHFV preincubated with control solution). (B) B1 = GP IIb/IIIa (HP1-1D); B2 = factor V competition (BoHFV preincubated with excess factor V antigen). (C) C1 = GP IIb/IIIa (HP1-1D); C2 = factor V (BoHFV preincubated with control solution). (D) D1 = GP IIb/IIIa (HP1-1D); D2 = control serum (NI-B). (E) E1 = control ascites (TB5-2); E2 = factor V (BoHFV preincubated with control solution); E3 = Brightfield illumination.

DISCUSSION

Our indirect immunofluorescence studies of human bone marrow and blood, using specific monoclonal and polyclonal antibody reagents and a double antigen labeling technique, clearly demonstrate that in these tissues megakaryocytes and platelets constitute the principal cellular locus of coagulation factor V. The specificity of megakaryocyte and platelet factor V immunofluorescence was established through the incorporation of appropriate control immunologic reagents in the panels of staining reagents we used, and by the use of both monoclonal and polyclonal antibody reagents demonstrated to be specific for human coagu-
lation factor V either by predefined specificity (monoclonal antibodies) or by antigen–antibody competition studies (polyclonal antibodies). We observed specific factor V immunofluorescence of human megakaryocytes that were obtained using three cellular preparative techniques: (1) megakaryocytes contained in direct marrow aspirate smears; (2) megakaryocytes aspirated from marrow and extensively washed to remove loosely associated plasma components; (3) megakaryocytes derived in vitro from the proliferation of committed megakaryocytic progenitor cells. In each of these preparations, marrow cells of nonmegakaryocytic lineage served as negative immunofluorescence controls.

Marrow cells were identified as megakaryocytes by their morphological appearance and by their immunofluorescent reactivity with a panel of antibody reagents specific for platelet- and megakaryocyte-related antigens. The specificity of monoclonal antibody HPI-1D (anti-GP Iib/IIIa) for the identification of megakaryocytes and platelets has been established in previous studies. Platelet plasma membrane GP Iib/IIia antigen appears to be a specific and early-appearing marker of human megakaryocyte differentiation. PF4 and vWF are also distinct antigen markers of the megakaryocytic cell line and are expressed early during the differentiation of these cells. Synthesis of the latter antigens by cultured nonhuman megakaryocytes has been demonstrated. Each of the lineage-related antibody reagents we used (antibodies to GP Iib/IIia, vWF, or PF4) identified marrow or blood cells as megakaryocytes or platelets and, used in conjunction with simultaneous immunofluorescent cell labeling with anti-factor V antibodies, identified human platelet and megakaryocyte localization of coagulation factor V.

The present investigation and previously reported investigations performed in this laboratory establish that the tissue distribution of coagulation factor V includes megakaryocytes, platelets, and endothelial cells, in addition to the presence of factor V in plasma. This tissue distribution of factor V is similar to that of factor VIII-related antigen (vWF). Tracy et al have recently demonstrated that human peripheral blood monocytes and lymphocytes contain factor V antigen and express factor V activity. Monocytes contain approximately eight times more factor V antigen per cell than do platelets, as determined by radioimmunoassay. However, because of the cellular size (volume) difference between monocytes and platelets, the calculated formal concentration of factor V in platelets exceeds that in monocytes by an approximate factor of 50. In our studies we did not consistently observe factor V immunofluorescence of human blood or marrow mononuclear leukocytes. It is possible that the factor V concentration differential between platelets/megakaryocytes and monocytes/lymphocytes accounts for our inability to detect factor V immunofluorescence in the latter cells.

The cellular sources of circulating plasma coagulation factor V have not been clearly defined. Recently reported studies of a transformed human hepatoma cell line document factor V synthesis in cell culture, and suggest that hepatocyte factor V synthesis and secretion may account for the majority of circulating plasma factor V protein. Cultured bovine aortic endothelial cells synthesize and secrete factor V, and may thereby contribute to plasma factor V, at least in this species. Cultured human umbilical vein endothelial cells synthesize factor V but do not appear to secrete factor V. It has not yet been determined whether human arterial endothelial cells synthesize factor V or secrete it into plasma, nor whether human megakaryocytes synthesize the factor V contained in blood platelets. Isolated guinea pig megakaryocytes appear to synthesize factor V antigen, but it has not been reported whether such synthesis accounts quantitatively for megakaryocyte and platelet factor V. Our observations reported here establish that human megakaryocytes contain factor V but do not establish whether the factor V expressed by human megakaryocytes has been synthesized by megakaryocytes.

Human platelet coagulation factor V may provide an essential hemostatic contribution uniquely distinct from the hemostatic contribution of plasma factor V. Human megakaryocyte factor V deserves further investigation to quantitate and correlate its accumulation relative to the expression of other lineage-related antigens during megakaryocyte differentiation; to determine whether synthesis of factor V by megakaryocytes accounts for the presence of megakaryocyte and platelet factor V; and to discover whether structural or functional differences might exist between plasma and megakaryocyte/platelet factor V. Presently, we conclude that human megakaryocyte and platelet coagulation factor V represents a lineage-associated protein.

ACKNOWLEDGMENT

We thank Dr Nick J. Gonchoroff for advice concerning immunofluorescence; Karl J. Oles and Dr Robert B. Jenkins for assistance with marrow cell culture; Scott E. Kaese for assistance with immunofluorescence staining and photography; Marvin L. Ellingsen and Kenneth Mork for preparation of illustrations; Dr Paula B. Tracy for scientific counsel; and Sandra J. Schumann, Marilyn H. Parker, and Lynn Wytaske for secretarial assistance.

REFERENCES

Identification of human megakaryocyte coagulation factor V

WL Nichols, DA Gastineau, LA Solberg Jr and KG Mann