Incomplete Antigenic Cross-Reactivity Between Platelets and Megakaryocytes: Relevance to ITP

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Immune thrombocytopenias are usually associated with normal or increased numbers of megakaryocytes in the marrow. Therefore, the mechanism(s) responsible for the destruction of circulating platelets may not affect megakaryocytes in the same way. One of the possibilities which could account for the differential effect on the cells would be the development of antibodies to components of platelet membranes which are not exposed on the surface of all megakaryocytes. To investigate this possibility, a rabbit antisera specific for mouse platelets was tested against fresh and cultured mouse megakaryocytes by indirect immunofluorescence. This antiserum cross-reacted with 48% of fresh murine megakaryocytes and 54% of cultured megakaryocytes. Phase-contrast microscopy revealed the reacting megakaryocytes to be fully granulated with irregular contours and in the process of releasing platelets. Nonreactive megakaryocytes demonstrated smooth contours and lacked morphological evidence of thrombocytopoiesis. Electron microscopy showed that only in megakaryocytes (MK) with an irregular contour had the demarcation membrane system (DMS) reached continuity with the plasma membrane. Ultrastructural analysis of megakaryocytes from patients with ITP showed ~25% to 50% of megakaryocytes without evidence of injury, whereas 50% to 75% had extensive damage. In undamaged cells, platelet territories had not yet reached the peripheral zone. The DMS of damaged megakaryocytes opened to the exterior elaborating platelets. The observations suggested that some platelet antibodies react only with megakaryocytes which have reached the stage of thrombocytopoiesis. Relevant target antigens may not be exposed on all megakaryocytes before cytoplasmic fragmentation occurs.

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with 20% horse serum (Flow Laboratories), and 1 μg/L Solucortef (Upjohn, Kalamazoo, Mich). Fresh murine marrow megakaryocytes (MK) represented 0.01% to 2.9% of nucleated cells. Cultures were incubated at 37 °C in 5% CO₂ and maintained up to 20 weeks. Each week, half of the medium and nonadherent cells were removed and replaced with an equal volume of fresh medium with 20% horse serum. Removed cells were assayed for total cell count, number of megakaryocytes, and number of platelets. The percentage of megakaryocytes ranged from 1% to 13.9%. However, when all cells were counted, this variability was found to be due to changes in the numbers of neutrophils and monocytes during the 2 to 20 weeks of culture. The absolute number of MK remained constant with a mean of 3.64 ± 2.97 × 10⁶/mL (n = 11) at weekly time points representing from three to seven flasks each. Similarly, weekly supernatant platelet counts were constant, 1.70 ± 0.86 × 10⁶/mL (n = 11). These relatively constant numbers of megakaryocytes and platelets confirmed their continuous production, as did the finding on electron microscopy of numerous free platelets as well as megakaryocytes actively releasing platelets throughout the culture period. Because the absolute number of MK and the pool of platelet-producing MK remained stable throughout the culture period, MK cultures of different ages were often combined for these studies to obtain the largest number of cells. No differences in the fluorescence or ultrastructural characteristics of MK obtained from cultures of different ages were found. Cells were harvested for fluorescence and ultrastructural studies between 2 and 20 weeks after initiation of cultures. Cell culture suspensions containing nonadherent cells were collected by centrifugation of the supernatant at 1,000 g for ten minutes. Both the megakaryocyte and platelet containing pellets were resuspended, combined, and washed three times.

**Antiplatelet serum.** Rabbit anti-mouse platelet serum (RAMPS) was produced as previously described. Mouse platelets were harvested from ~200 male C3H mice by differential centrifugation, washed three times in 1% ammonium oxalate and twice in saline, resuspended into water (5% suspension), and frozen; the purified platelet homogenate was thawed and injected subcutaneously at multiple sites twice each week for three weeks. Seven days after the last injection, the rabbits were bled. The antisera was obtained by heating at 56 °C for 30 minutes and absorbed three times with mouse RBC from equal volumes of defibrinated blood to remove mouse antibodies not specific for mouse platelets. RAMPS has previously been shown to cause in vitro platelet aggregation and, in vivo, to cause a marked thrombocytopenia without anemia or granulocytopenia. Thus, there was no detectable cross-reactivity with any other hematopoietic cells. Preparation of isolated F(ab')2 fragments of the mouse antiplatelet antibody was not felt necessary, since mouse megakaryocytes and platelets are known to be devoid of Fc receptors.

**Fluorescent antibody staining.** Fluorescent antibody staining was done by the indirect technique. Cell suspensions were kept on ice. Each specimen was divided in half and stained in parallel with RAMPS and control preimmune serum. Cell suspensions were incubated sequentially with 0.02 to 0.04 mL RAMPS or 0.02 to 0.04 normal rabbit serum for 30 minutes and 0.04 mL of fluorescein-conjugated goat antirabbit globulin (Meloy, Springfield, Va) for 30 minutes. Between steps, the cells were washed three times with phosphate-buffered saline (PBS). After the final wash, the cells were fixed in 2% paraformaldehyde with 0.1% glutaraldehyde for six minutes to preserve cell integrity and to prevent nonspecific interiorization of the label by living cells. On some occasions, the cells were fixed first and then stained with antibody. The same results were obtained if living cells were fixed following staining. All cells, including megakaryocytes and platelets, were identified initially by phase microscopy and then by fluorescence microscopy. A Leitz Orthoplan microscope with ultraviolet UV optics using epifluorescence and 63x or 100x oil-immersion objectives was used.

**Electron microscopy.** The specimens intended for ultrastructural analysis were placed in glutaraldehyde and postfixed with osmium tetroxide for two hours followed by en bloc staining with 0.5% uranyl acetate in saline for one hour. Dehydration and embedding in Poly/Bed 812 was carried out as described elsewhere. An LKB ultrotome was used for sectioning, and a Siemens Elmiskop I electron microscope was the instrument used for all studies.

**RESULTS**

**Fluorescence and electron-microscopic analyses.** Cell suspensions of fresh as well as cultured murine bone marrow incubated with RAMPS followed by fluorescein-conjugated anti-rabbit Ig showed fluorescent and nonfluorescent MK in almost equal numbers. Megakaryocytes were identified primarily on the basis of their size and irregularly-shaped nuclei. In fresh bone marrow specimens obtained from eight different mice, 53.8% megakaryocytes identified by phase-contrast microscopy did not show any fluorescent staining with RAMPS. Likewise, cells from three preparations of cultured MK showed 46.2% nonreactive megakaryocytes. Platelet clumps were always strongly positive in fresh and cultured marrow specimens, whereas no other hematopoietic cells reacted with the antiserum. In fresh bone marrow suspensions, which always contained large numbers of myeloid and erythroid cells, no fluorescence was observed in cells other than platelets or megakaryocytes. Neither was nonspecific fluorescence seen in suspensions of marrow cultures which have been shown to contain large numbers of monocytes and myeloid cells. No fluorescent cells were seen in identical specimens stained with control serum.

The pattern of fluorescent MK staining was also noteworthy. Although it is well recognized that the fluorescent antibody technique when used on unfixed living cells or on cells fixed in suspension with aldehydes will only detect antigenic determinants on their surface, the majority of reacting MK had a diffusely stained or mottled cytoplasm (Fig 1a). Phase-contrast microscopy of fluorescent and nonfluorescent cells and electron microscopy carried out on similar specimens were used to elucidate this phenomenon and to characterize the type of MK which reacted with the antiserum. On phase-contrast microscopy (Fig 1b), the positive cells were seen to have an irregular contour, as is typical for MK which have reached the stage of thrombopoiesis. The ultrastructure of a cell comparable to this state of maturation is shown in Fig 1c to illustrate this point. Some MK were entirely nonreactive, whereas others showed fluorescence only on a portion of the rim and adjacent cytoplasm (Fig 2a). Phase-contrast microscopy of the latter cells indicated that the fluorescent areas corresponded to regions where platelets were being released (Fig 2b). The nonstaining areas of the surface membrane were smooth, which is presumed to be a reflection of the lack of continuity of the demarcation membrane system with the surface, as exemplified ultrastructurally in Fig 2c. Occasionally, a positively stained MK with an irregular contour and a negative MK with a smooth periphery were seen side by side (Fig 3). In such situations, the “smooth” MK probably had an unattached peripheral zone like the cells whose ultrastructure is illustrated in Figs 2 and 4. The observations indicate that the platelet antibody did not cross-react with all megakaryo-
Fig 1. (A) Mouse megakaryocyte (MK) treated in suspension before fixation with RAMPS followed by fluorescein-conjugated goat anti-rabbit globulin. The cytoplasm stained diffusely without clear delineation of a surface membrane. N, nucleus. Apparent indentations are caused by surrounding and overlying nonreactive marrow cells (see B). (B) Phase-contrast photomicrograph of the same cell as depicted in A shows fully granulated cytoplasm and irregular contour characteristic of cells releasing platelets. Arrows indicate other hemopoietic cells which partially cover the MK. (C) Ultrastructure of a mouse megakaryocyte considered to represent the same stage of maturation as the cells shown in A and B. Platelet territories are fully developed (P). The contour is irregular. Antiserum would have access to platelet fields located in the interior without prior fixation of the cell (arrows). Magnification ×3,700.

Fig 2. (A) Mouse MK from the same specimen as the one described in the legend to Fig 1A does not fluoresce. With the exception of the small area near the rim (bottom), this cell did not stain with the antiserum. In contrast, the platelet clump at the top is brightly fluorescent (arrow). (B) Phase-contrast photomicrograph of the nonfluorescent cell depicted in A. The contour is smooth except for a small portion at the bottom which corresponds to the fluorescent area in A. Arrow indicates platelet aggregate seen to fluoresce in A. Also shown is the specificity of the antiserum for platelets. The surrounding bone marrow cells are negative. (C) Detail of a mouse MK showing an intact peripheral zone (PZ) illustrating that platelet territories need not reach the surface of the cell. Magnification ×12,000.
Fig 3. Mouse megakaryocytes treated as described in the legend to Fig 1A. A strongly fluorescent clump of platelets is seen at the top. An MK with diffusely stained cytoplasm and with an irregular contour is seen at the bottom. The arrow indicates a second platelet clump which may represent platelets in the process of being released. Between the fluorescent platelet clump and the fluorescent MK, the outline of a negative MK can be discerned.

Fig 4. Human megakaryocyte obtained from a patient with ITP. The cell shows no evidence of damage. Platelet territories are fully developed within the cytoplasm. The peripheral zone (PZ) is intact. Magnification ×2,700.

Fig 5. Mouse MK in the process of thrombopoiesis. Arrows indicate sites where antiserum could gain access to fully developed platelet territories located in the interior of the cell. The platelets at the bottom are likely to have been released by this MK. Magnification ×17,500.
cytes, but primarily with those releasing platelets, ie, when the membranes delineating platelet territories had established continuity with the surface. In Fig 5, this is illustrated more clearly. When the MK cytoplasm was in the process of fragmenting into platelets, the extracellular medium had access to the interior of the cell. Therefore, in this situation, antisera could reach internal structures without the need for agents that enhance plasma membrane permeability.

Ultrastructure of ITP megakaryocytes. In light of the observations described above and the recognition that patients with severe immune thrombocytopenia may have normal or even increased numbers of MK, we studied the MK of four patients with ITP. Contrary to reports by others,\textsuperscript{16,17} we found that about 25% to 50% of the MK of patients with ITP showed no morphological evidence of injury (Fig 4). Normal undamaged cells were observed which had an intact, smooth peripheral zone, devoid of organelles and demarcation membranes, but with a cytoplasm often exhibiting fully developed platelet territories (Fig 4). Such cells were ultrastructurally indistinguishable from the smooth contoured mouse MK (Fig 2c). Approximately 50% to 75% of the ITP MK in well-preserved specimens showed extensive damage. This consisted primarily of a markedly distended demarcation membrane system that made the cells appear vacuolated on light microscopy, as reported by others.\textsuperscript{16,17} The surface membrane of injured MK could usually be seen in continuity with the DMS, whereas pieces of MK cytoplasm and/or abnormal platelets had partially or completely detached from the main body of the cell (Fig 6A and B). Pathologic MK were often emperipolesed by mononuclear leukocytes and neutrophils (not shown). Frequently, damaged cells showed attached monocytes, which appeared to phagocytose fragments of megakaryocytes (Fig 7). In specimens examined by us to date, no morphological alterations have been seen in ITP megakaryocytes that had an intact peripheral zone.

DISCUSSION

The studies reported in this communication have shown that a rabbit polyvalent antiserum made specific for mouse platelets cross-reacted with only approximately one-half of the mouse megakaryocytes examined. Subsequent examination of the fluorescing megakaryocytes with phase optics revealed them to be fully granulated, to have irregular contours, and to be in the process of releasing platelets. In contrast, nonreactive megakaryocytes had smooth contours and did not seem to be undergoing thrombopoiesis. Ordinarily, immunohistochemical staining of intracellular structures requires permeabilization of the surface mem-
brane by drying or lipid solvents. It is well recognized that antibodies do not enter living or glutaraldehyde-fixed cells unless they have large openings or invaginations in continuity with the extracellular space, which is the case in fragmenting megakaryocytes. In all likelihood, the smooth, negatively staining cells correspond to those which have been shown ultrastructurally to have an intact peripheral zone (Fig 2c). Conversely, cells with an irregular contour and reactivity with RAMPS represented MK with a DMS extending through the peripheral zone. Electron-microscopic analysis of ITP megakaryocytes seemed to support our hypothesis because only cells that had reached the stage of thrombocytoptosis appeared damaged. Damaged ITP megakaryocytes had irregular contours and many extensions of the DMS to the periphery (Figs 6 and 7) in continuity with the extracellular medium. ITP megakaryocytes with an intact peripheral zone, in which platelet fields had not yet involved the surface, showed no obvious damage (Fig 4).

Previous investigators, using heterologous anti-platelet antisera and human anti-platelet antibodies conjugated with fluorescein demonstrated antigenic cross-reactivity between platelets and megakaryocytes. Our observations are not at variance with these reports. However, by identifying megakaryocytes on phase microscopy before exposing them to UV light, we noted megakaryocytes that were not reacting with the platelet antibody. In the earlier studies cited above, the emphasis was placed on the identification of positively stained cells. More recently, antisera directed against specific platelet proteins as well as monoclonal antiplatelet antibodies have been prepared and tested against MK. Thus, it has been shown that antisera to platelet membrane glycoproteins Ib, IIb, and IIIa, platelet factor VIII:Ag, and
fibronectin are reactive with >90% of megakaryocytes analyzed.\textsuperscript{2,19} Others, using a monoclonal antibody recognizing an antigen on the platelet membrane glycoprotein IIb/IIIa complex have confirmed these data.\textsuperscript{22,23,24} However, when a monoclonal antibody specific for platelet glycoprotein Ib was used, there was much weaker binding of the antibody to MK, and some recognizable megakaryocytes failed to react altogether.\textsuperscript{22} These studies leave no doubt that MK and platelets share important antigens, but they also suggest that the antigenic cross-reactivity is not complete. This statement may be particularly relevant to the immune process operative in chronic ITP. Evaluating autoantibody specificity in patients with this condition, Woods et al have found antibodies directed against the platelet glycoprotein IIb/IIIa complex in only 5 of 56 patients,\textsuperscript{25} and against glycoprotein Ib in only 3 of 106 patients.\textsuperscript{26} Thus, the autoantibodies in this disorder are frequently directed against platelet antigens other than these well-characterized glycoproteins. To what extent any of these autoantibodies react with megakaryocytes is currently unknown. Previous studies evaluating megakaryocyte reactivity with ITP sera have been reported.\textsuperscript{27,28} McMillan et al\textsuperscript{28} showed increased megakaryocyte binding of purified radiolabeled IgG from serum and cultured splenic cells in two patients with ITP. Using sera preabsorbed with platelets, McKenna and Pisciotta demonstrated cross-reactivity in some patients with ITP but not in others.\textsuperscript{27} Various ultrastructural abnormalities in ITP megakaryocytes have been reported before.\textsuperscript{16,17} These consisted of vacuoles and a distended demarcation membrane system and the intussusception of other marrow cells.\textsuperscript{16,17,29,30} In essence, such studies only confirmed light-microscopic observations\textsuperscript{31,32} and phase-contrast data published earlier.\textsuperscript{33} Unlike reports which claimed that some patients with ITP had only normal megakaryocytes whereas others had damaged cells,\textsuperscript{16} the present study demonstrated heavily injured megakaryocytes alongside entirely normal megakaryocytes in the same specimens. The normal-appearing megakaryocytes had not reached the stage of thrombocytopenia, whereas significantly damaged cells were releasing platelets. That the damage had indeed occurred in vivo is further supported by the finding of bone marrow reticuloendothelial sequestration of heavily damaged megakaryocytes. This is not seen in normal bone marrow aspirates obtained for diagnostic purposes.

Taken together, the observation recorded here should be of interest from two points of view. One has to do with the normal physiology of thrombocytopenia, the other with the mechanism of immune thrombocytopenic disease. The steps which should be taken to build on the data related to the first subject seem clear. Immunohistochemical studies on the ultrastructural level will be necessary to determine whether some antibodies specific for platelets react only with DMS and/or other internal membranes without affecting the surface membrane of megakaryocytes and at what stage of differentiation such antigens arise. The second area is more complicated. Although it does not seem farfetched to postulate that such antibodies may develop to normal or aberrant platelet membrane components which are not exposed on the surface of megakaryocytes or at least not at every stage of differentiation, the possible heterogeneity of such naturally evolving antibodies may be very great. Such heterogeneity must, however, be taken into account when new modalities for the management of immune thrombocytopenias are contemplated.

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