Acquired Amegakaryocytic Thrombocytopenic Purpura: A Syndrome of Diverse Etiologies

by Ronald Hoffman, Edward Bruno, Joyce Elwell, Eric Mazur, Alan M. Gewirtz, Paul Dekker, and Alex E. Denes

The possible pathogenetic mechanisms responsible for the production of acquired amegakaryocytic thrombocytopenic purpura (AATP) were investigated in a group of patients with this disorder. Absence of megakaryocytes and small platelet glycoprotein-bearing mononuclear cells, as determined by immunocytochemical staining of patient marrow with antisera to platelet glycoproteins, suggested that the defect in AATP occurs in an early progenitor cell of the megakaryocytic lineage. Using an in vitro clonal assay system for megakaryocytic progenitor cells or megakaryocyte colony-forming units (CFU-M), the proliferative capacity of AATP marrow cells was then assessed. Bone marrow cells from three of four patients formed virtually no megakaryocyte colonies, suggesting that in these individuals the AATP was due to an intrinsic defect in the CFU-M. Bone marrow cells from an additional patient, however, formed 12% of the normal numbers of colonies, providing evidence for at least partial integrity of the CFU-M compartment in this patient. Serum specimens from all six patients were screened for their capacity to alter in vitro megakaryocyte colony formation. Five of six sera enhanced colony formation in a stepwise fashion, demonstrating appropriately elevated levels of megakaryocyte colony-stimulating activity. The serum of the patient with partial integrity of the CFU-M compartment, however, stimulated colony formation only at low concentrations. At higher concentrations, this patient's serum actually inhibited the number of colonies cloned, suggesting the presence of a humoral inhibitor to CFU-M. Serum samples from all patients were further screened for such humoral inhibitors of megakaryocyte colony formation using a cytotoxicity assay. The patient whose serum was inhibitory to CFU-M at high concentrations was indeed found to have a complement-dependent serum IgG inhibitor that was cytotoxic to allogeneic and autologous marrow CFU-M but did not alter erythroid colony formation. These studies suggest that AATP can be due to at least two mechanisms: either an intrinsic defect at the level of the CFU-M or a circulating cytotoxic autoantibody directed against the CFU-M.

Materials and Methods

Patients

The clinical features of the six patients with AATP that comprise the subject of this study are presented in Table 1 (patients D and E have been previously reported). Patients A, B, C, and F were anemic at presentation due to gastrointestinal bleeding, but their erythron was otherwise intact. The only morphological abnormality of note was macrocytosis in five of the six patients studied. In no case did bone marrow examination reveal sideroblasts or other dysplastic features. Granulopoiesis at the time of study was normal in all patients as assessed by peripheral blood and bone marrow morphology. The duration of the disorder prior to the time of study ranged from 0.5 to 34.0 months. In striking contrast to the erythroid and myeloid elements, examination of the bone marrow aspirates and biopsies obtained from patients A, C, and F revealed a total absence of megakaryocytic elements, while extremely rare megakaryocytes were noted after examination of the bone marrows obtained from patients B, D, and E. The hematologic status of both patients C and D progressed over a period of 6 months and 1 year, respectively, to a syndrome of full-blown aplastic anemia characterized by pancytopenia and hypocellular or acellular bone marrows. Patients B and F died of spontaneous intracranial hemorrhages. Patient A had only a transitory response to 6-mercaptopurine therapy and then relapsed while receiving that medication. Patients A, B, D, and F had, at some time during their course, all received and failed to respond to corticosteroids, androgens, and vincristine. Patient A was splenectomized with no improvement in her platelet count.

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HOFFMAN ET AL. -

- air-dried, and stored at a concentration of 1:1 with alpha-medium minus nucleosides (a slides. Specimens for use in immunofluorescent staining were fixed as aspirates were collected in EDTA and smeared directly onto glass.

- normal volunteers were obtained by density centrifugation with normal volunteers, and patients with normal hematologic parameters undergoing bone marrow study for a number of reasons. The marrow
gas dioxide in sodium pyruvate, 0.2 mmole/ml. Culture dishes were incubated for 25 mm at 4°C in a Beckman Model J-6B centrifuge. The interface

- distilled water, and then drying in air. Plasma clots were stored in acetone:methanol (9:1)

- supportive serum for fetal calf and

- culture contained the following supplements: MEM nonessential amino acids, aspirate C 1.5 x 10² 4.8 6,600

- F 44 F 11.5 10² 1.8 3.200

- serum was added to the culture growth mediums, they replaced an activity greater than may be present in normal AB activity,

- Plasma Clot Megakaryocyte Cultures

- samples were added to the culture growth mediums, they replaced an activity greater than may be present in normal AB activity,

- MC

- a potent source of Meg-CSA) to these cultures

- anemia serum (a

- Patient

- Sex (g/cM)

- Hb MCV Count (% Neutrophils/ Platelets

- Table 1

- Hb MCV Count (% Neutrophils/ Platelets

- E 62 M 14.9 91 0.6 8,100

- F 44 F 11.5 10² 1.8 3.200

- summaries of the results of the study of the effect of sera of aplastic anemia patients on megakaryocyte colonies in plasma clot culture system for megakaryocyte colonies in the

- CFU-E- derived colonies in this modified plasma clot assay system was seen at erythropoietin

- BFU-E (burst-forming unit-erythroid), were harvested after 6 and 12-14 days of incubation, respectively. Cul-

- or patient bone marrow cells were suspended in 2% heat-inactivated

- attempt to define serum inhibitors of megakaryocytopoiesis. Control

- plasma clot technique of McLeod et al.5 was modified by the

- selected mixtures in the form of 0.1 ml fresh rabbit serum or human

- complement was added to

- to identify inhibitors of myelopoiesis, was modified and utilized in an

- duration of the incubation, the cells were washed and plated in the

- presence of 20% aplastic anemia serum. The addition of aplastic

- same marrow cells.

- tested against at least three different control marrows. A reduction

- in inhibitory activity of the various sera was determined by also

- factor of the number of CFU-M-derived colonies formed by at least 50%

- factors were stained with 1% benzidine and hematoxylin as previously

- factor that had no other possible source of Meg-CSA. Each serum was tested

- x 106 cells/ml To 0.2 ml of cells was added 0.1 ml of patient or

- factor of patients' sera and that of two normal controls

- IgG

- Preparation of

- An IgG fraction of patients' sera and that of two normal controls

- sulfite precipitation and diethylaminoethyl cellulose column chro-

- was extracted and purified by the standard techniques of ammonium

- IgG

- Preparations of

- An IgG fraction of patients' sera and that of two normal controls

- An IgG fraction of patients' sera and that of two normal controls
phosphate (pH 7.3) buffer and adjusted to the desired protein concentration with RPMI 1640 medium. Purity of the initial protein peak as IgG was established by immunoelctrophoresis. The concentration of IgG was measured by means of the Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond, Calif.). These IgG fractions were then assayed for inhibitory activity against CFU-M using the cytotoxicity assay system.

**Immunofluorescent Staining and Scoring**

Whole rabbit antiplatelet glycoprotein (PGP) antiserum was prepared and specificity demonstrated as previously described. The antiserum was diluted in PBS (1/200) and layered over the specimens to be studied. Human bone marrow smears and plasma clots were incubated with the antiserum for 30 and 60 min, respectively, at 37°C in 5% CO2, 100% humidified air. After washing three times with PBS, the specimens were reincubated with fluorescein-conjugated goat anti-rabbit IgG (Meloy, Springfield, Va.) diluted in PBS, final concentration 0.36 mg protein/ml, for an equivalent period of time. After washing again with PBS, the specimens were counterstained with 0.125% Evan’s blue and coverslips were wet mounted with isotonic barbital buffer, pH 8.6, in glycerol (1:3).

Bone marrow megakaryocytes were identified by their bright, homogeneous, apple-green fluorescence, large size, and nuclear multinucleation. Small megakaryocytic precursor cells were identified by qualitatively identical staining, an estimated diameter of 7–14 μm, and nuclei that were visible by either fluorescent or phase microscopy. Between 150 and 250 total fluorescein-positive cells were counted on each normal bone marrow smear. Results were derived from studying 8 normal individuals and patients A, B, C, and F. Only megakaryocytes and rare mononuclear cells exhibited fluorescence under these conditions. No cellular elements were labeled when bone marrow preparations were incubated with fluorescein-conjugated goat anti-rabbit IgG alone.

In vitro plasma clot cultures were scored in situ. The 35-mm Petri dishes were inverted, completely scanned, and fluorescein-positive colonies enumerated using fluorescent microscopy at 100X (Zeiss standard microscope 18 with 1VFL. vertical fluorescent illuminator). A megakaryocyte colony was defined as a cluster of three or more intensely fluorescent cells.

**Antithymocyte Globulin Treatment**

The method of Ascensao and coworkers was utilized. Briefly, 10⁶ cells from patient F were suspended in RPMI medium and 2% fetal calf serum and incubated for 60 min at 37°C in 5% CO2 with or without antithymocyte globulin (Upjohn, lot 16, 138-15B) at a concentration of 1/1000. To this mixture, 0.1 ml of fresh human serum was added as a source of complement. The cells were washed twice and then assayed in the plasma clot assay system for megakaryocytic colonies in the presence of 20% aplastic anemia serum.

**RESULTS**

When normal bone marrow smears were examined using indirect immunofluorescent labeling, only platelets, megakaryocytes, and an infrequent population of lymphoid-like cells fluoresced. Bone marrows obtained from patients A, B, C, and F were examined in a similar fashion. Rare fluorescein-positive cells that morphologically resembled megakaryocytes were observed only in patient B’s marrow cells, while no fluorescein-positive lymphoid-like cells or megakaryocytes were noted in the other specimens examined.

Control marrow smears stained on the same day showed frequent megakaryocytes and occasional small mononuclear cells that displayed immunofluorescence.

The ability of amegakaryocytic thrombocytopenic bone marrow cells to form megakaryocyte colonies in the presence of a source of Meg-CSA is shown in Fig. 1. Patients A–C formed rare megakaryocyte colonies, while patient F’s bone marrow cells generated approximately 12% of the number of colonies formed by normal bone marrow cells. By contrast, when amegakaryocytic bone marrow cells were cloned in the presence of erythropoietin, normal numbers of CFU-E-derived colonies were enumerated. However, erythropoietic burst formation was reduced in the two patients studied (Table 2). Treatment of AATP marrow cells with antithymocyte globulin in the presence or absence of complement did not alter CFU-M-derived colony formation.

Serum samples from each of the six patients studied were assayed for megakaryocytic colony-stimulating activity by substituting normal human AB serum with an equivalent amount of test serum. Concentrations of 5%–30% of the patient sera were tested in this manner. As can be seen in Fig. 2, increasing concentrations of sera from patients A–E resulted in increasing numbers of megakaryocyte colonies cloned. Patient F’s serum behaved in a different fashion. Although low concentrations of patient F’s serum resulted in a 57% increase in cloning efficiency, increasing concentrations of this serum resulted in actual inhibition of colony formation. The inhibitory effect of high concentrations of
Table 2. Erythroid Colony Formation by Acquired Amegakaryocytic Thrombocytopenic Bone Marrow Cells

<table>
<thead>
<tr>
<th>Patient</th>
<th>A</th>
<th>C</th>
<th>Control (5)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>BFU-E-derived colonies/5 x 10⁶ cells plated</td>
<td>42 ± 6</td>
<td>26 ± 2</td>
<td>80 ± 32</td>
</tr>
<tr>
<td>CFU-E-derived colonies/5 x 10⁶ cells plated</td>
<td>512 ± 23</td>
<td>420 ± 6</td>
<td>420 ± 38</td>
</tr>
</tbody>
</table>

Each value represents mean ± 1 SEM of results. Each study was carried out in duplicate.

*The control data were obtained by assaying bone marrow cells from 5 normal control subjects.

patient F's serum could be reversed by heat inactivation of the serum at 56°C for 30 min. High concentrations of heat-inactivated patient F serum (30%) actually augmented CFU-M-derived colony formation by 200%. As can be seen in Table 2, serum obtained from patients A–E augmented megakaryocyte colony formation when the patient serum constituted 30% of the culture mix. No other serum source of Meg-CSA was present in these cultures.

Using a cytotoxicity assay system to further search for humoral inhibitors of megakaryocytic colony formation, neither control serum nor serum of patients A–E in the presence or absence of a source of complement altered colony formation by normal human marrow cells (Table 3). By contrast, patient F's serum in the presence of complement almost completely abolished CFU-M-derived colony formation. The inhibitory effect of patient F's serum was also demonstrable against autologous marrow cells. As can be seen in Table 4, patient F's serum in the presence of complement virtually abolished autologous megakaryocyte colony formation.

In order to localize the inhibitory activity, IgG fractions from patients A, B, and F (Table 5) were isolated and assayed for inhibitory activity against allogeneic CFU-M. Neither control IgG nor that of patients A or B inhibited megakaryocyte colony formation in the presence or absence of a source of complement. By contrast, patient F's IgG fraction in the presence of complement almost completely abolished CFU-M-derived colony formation. To verify the selectivity of this inhibitory activity, the cytotoxicity of patient F's IgG against CFU-E- and BFU-E-derived colonies was also tested. Neither patient F's IgG nor control IgG in the presence or absence of complement altered either CFU-E- or BFU-E-derived colony formation (Table 6).

DISCUSSION

AATP is a rare disorder which in all likelihood does not represent a single entity. The cause of the deficiency of bone marrow megakaryocytes with relative sparing of other marrow elements is unknown. One might hypothesize that this disorder could involve a number of pathogenetic mechanisms including: (1) failure of terminal megakaryocytic differentiation; (2) an intrinsic stem cell defect; (3) abnormal interaction between CFU-M and bone marrow regulatory cells (lymphocytes); (4) decreased production of a hematopoietic growth factor (thrombopoietin or megakaryocytic colony-stimulating activity); or (5) immunologic attack on megakaryocytes or megakaryocytic progenitor cells.

When bone marrow smears from four of our patients were immunochemically labeled with anti-PGP anti-
AMEGAKARYOCYTIC THROMBOCYTOPENIA

Table 4. Effect of Patient F's Serum on Megakaryocytic Colony Formation by Autologous Marrow Cells

<table>
<thead>
<tr>
<th>Prior Treatment</th>
<th>CFU-M-Derived Colonies/5 x 10^6 Cells Plated</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>10.5 ± 0.5</td>
</tr>
<tr>
<td>Control</td>
<td>13.0 ± 1.4</td>
</tr>
<tr>
<td>Complement</td>
<td>11.5 ± 2.5</td>
</tr>
<tr>
<td>Control + complement</td>
<td>12.5 ± 0.5</td>
</tr>
<tr>
<td>Patient</td>
<td>11.5 ± 0.7</td>
</tr>
<tr>
<td>Patient F + complement</td>
<td>0.5 ± 0.5</td>
</tr>
</tbody>
</table>

Values are the mean ± standard error of the mean of experiments performed in duplicate in the presence of 20% aplastic anemia serum.

Table 5. Inhibitory Activity of IgG Fraction of Patient Serum and Control Serum on Normal Marrow Cells

<table>
<thead>
<tr>
<th>Prior Treatment</th>
<th>Percent Inhibition of Megakaryocyte Colony-Forming Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control IgG</td>
<td>0</td>
</tr>
<tr>
<td>Control IgG + complement</td>
<td>7</td>
</tr>
<tr>
<td>Patient A and patient B IgG</td>
<td>0</td>
</tr>
<tr>
<td>Patient A and patient B IgG + complement</td>
<td>0</td>
</tr>
<tr>
<td>Patient F IgG</td>
<td>34</td>
</tr>
<tr>
<td>Patient F IgG + complement</td>
<td>88</td>
</tr>
</tbody>
</table>

Table 6. Effect of Patient F's IgG Fraction on Erythroid Colony Formation of Normal Human Marrow Cells

<table>
<thead>
<tr>
<th>Treatment of Bone Marrow Cells</th>
<th>Percent Inhibition of CFU-E-Derived Colonies</th>
<th>Percent Inhibition of BFU-E-Derived Colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complement alone</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Patient F IgG</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Patient F IgG + complement</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Control IgG</td>
<td>12</td>
<td>13</td>
</tr>
<tr>
<td>Control IgG + complement</td>
<td>3</td>
<td>9</td>
</tr>
</tbody>
</table>

The absence of these cells from our patients' bone marrows, together with the absence of morphologically identifiable megakaryocytes, suggests that the cellular defect in AATP is not due to a failure of terminal megakaryocyte differentiation. These findings suggest instead that the defect in AATP occurs at a more primitive level of megakaryocyte differentiation that precedes the acquisition of platelet glycoproteins.

Cloning of AATP bone marrow cells in vitro revealed dramatic abnormalities in megakaryocyte colony formation. Three of the four patients formed virtually no colonies in response to varying concentrations of megakaryocyte colony-stimulating activity. These findings suggest that the defect in these patients is probably due either to a quantitative deficiency of CFU-M or a qualitative CFU-M abnormality (e.g., abnormal or absent receptors to megakaryocyte colony-stimulating activity). The fact that normal numbers of CFU-E-derived colonies were generated by sera, fluorescein-positive cells that morphologically resembled mature megakaryocytes were virtually absent. In addition, small platelet glycoprotein-bearing mononuclear cells were also absent in these preparations. These small cells likely represent the human counterpart of the small acetylcholinesterase-positive cells found in rodents, which are thought to represent the immediate precursors of mature megakaryocytes. In humans, these small cells are encountered only 20% as frequently as the morphologically identifiable megakaryocytes, but are easily identified by fluorescein labeling. The absence of these cells from our patients' bone marrows, together with the absence of morphologically identifiable megakaryocytes, suggests that the pathogenetic mechanism underlying AATP might eventually involve other cell lines. In this regard, patient A also developed aplastic anemia.

Recent in vitro studies have suggested that interaction between lymphocytes and hematopoietic stem cells might be important in the production of bone marrow failure states. An alternate explanation for the poor cloning efficiency of AATP marrow cells could be that such an interaction between bone marrow accessory cells and CFU-M might be occurring. Augmentation of colony formation by prior incubation with antithymocyte globulin and complement has been used as a means of detecting such a relationship. Similar treatment of the marrow cells of our patients did not alter megakaryocytic colony formation. These results suggest that such cellular phenomena were not operating in the production of the selective deficiency of bone marrow megakaryocytes.

We have recently demonstrated that serum from aplastic anemia patients with thrombocytopenia and decreased numbers of marrow megakaryocytes contains a factor that markedly enhances megakaryocyte colony growth. We have termed this activity megakaryocytic colony-stimulating activity (Meg-CSA). In order to determine if a deficiency of megakaryocytic colony-stimulating activity could be a cause of AATP, sera from each of the six patients studied were assayed for this activity. Serum specimens from patients A–E repeatedly augmented megakaryocytic colony formation, suggesting that a deficiency of functional megakaryocyte colony-stimulating activity would be an unlikely pathogenetic mechanism leading to amega-karyocytic thrombocytopenia. The behavior of patient F's serum, however, was unique. Low concentrations of serum stimulated, while higher concentrations actually
inhibited CFU-M-derived colony formation by allogeneic marrow cells. Interestingly, the inhibitory activity of high serum concentrations could be reversed by heat inactivation. We interpreted these findings as suggesting that patient F’s serum actually contained not only increased levels of megakaryocyte colony-stimulating activity but also an inhibitor to the CFU-M. The reversibility of the inhibitory effect by heat inactivation implies that a heat-labile factor, such as complement, is necessary for its action.

The cloning efficiency of patient F’s CFU-M is extremely reminiscent of the cloning efficiency of erythroid progenitor cells in acquired pure red cell aplasia. Isolated bone marrow cells from a subset of patients with acquired pure red cell aplasia have been shown to proliferate near normally in vitro in response to erythropoietin. Many of these patients have subsequently been shown to have IgG inhibitors of erythropoiesis.

We further searched for humoral inhibitors of megakaryocytopoiesis using the cytotoxicity assay of Fitchen and Cline. Only whole serum as well as an IgG fraction of patient F’s serum was cytotoxic in the presence of complement to both autologous and allogeneic CFU-M. However, this inhibitory activity was not directed against erythroid committed stems, demonstrating the specificity of its action. Since all the patients studied were equally multiply transfused, it seems unlikely that the inhibitory activity was the consequence of chronic transfusion therapy.

Our studies demonstrate that acquired amegakaryocytic thrombocytopenia is a syndrome due to at least two pathogenetic mechanisms. In the majority of patients this syndrome appears to be a consequence of an intrinsic defect at the level of the CFU-M. However, an additional mechanism also appears to be capable of resulting in AATP. In one of our patients we were able to demonstrate a cytotoxic IgG whose activity was specifically directed against CFU-M. Therapy with immunosuppressive agents was initiated in this patient, but unfortunately she died 4 days after their institution, a time period too short to judge their efficacy. These in vitro studies hopefully will provide the basis for more rational therapeutic approaches to patients with AATP. However, additional studies of greater numbers of patients with this disorder will be required to define the true incidence of each of these pathogenetic mechanisms.

ACKNOWLEDGMENT

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