A VARIETY OF hematologic abnormalities has been reported in patients with systemic lupus erythematosus (SLE). In SLE patients with thrombocytopenia, the number of bone marrow megakaryocytes, as estimated from bone marrow smears, is frequently increased. However, thrombocytopenia associated with decreased megakaryocyte is uncommon in SLE. We studied a patient with amegakaryocytic thrombocytopenia (AMT) associated with SLE. In this study, we attempted to investigate the underlying pathogenesis of AMT associated with SLE using an in vitro coculture assay of human CFU-M and in vitro culture techniques.

MATERIALS AND METHODS

Patient

A 47-year-old female (S.O.) was diagnosed as having SLE in 1979 on the basis of fever, arthralgia, malar erythema, alopecia, positive antinuclear antibody, and anti-ds DNA antibody. She had been initially treated with 30 mg of prednisolone and continued to make good progress on a lesser amount until three months before admission, when she developed gingival bleeding and ecchymoses. She was found to have thrombocytopenia of 10,000/μL. She was hospitalized and then transferred to Tsukuba University Hospital in November 1981 for evaluation of thrombocytopenia. She has no history of blood transfusion. Physical examination on admission revealed numerous petechiae and ecchymoses on both legs. Blood cell count revealed the following: RBC, 3.78 × 10^12/L; Hb, 12.0 g/dL; Hct, 38%; reticulocytes, 0.8%; platelet count, 8 × 10^9/L; WBC, 8 × 10^9/L with normal differentiation. The ratio of T to T' in T cell fraction was 28.5%/63.5%. Bone marrow aspiration and biopsy showed no megakaryocytes. She was treated with 60 mg of prednisolone daily and a pulse therapy (methylprednisolone 1 g intravenously for three days). The platelet count was elevated to the normal range for three weeks after treatment. Furthermore, the ratio of T to T' changed to 53%/24.5%. The bone marrow aspirate at this time showed normal cellularity with many mature megakaryocytes. Details of serological tests and clinical course have been previously reported.

Control Subjects

Ten normal subjects, five SLE cases with normal platelet counts (SLE group A), and five SLE cases with thrombocytopenia (less than 50 × 10^9/L) and increased megakaryocytes (SLE group B) were used as a control. All SLE cases were studied before treatment. Informed consent was obtained from prospective marrow donors before collection of blood specimens and aspiration of bone marrows required for this study. The research procedures and informed consents for this study were approved by the institutional research review committee.

Preparation and Freezing of T Cells

Peripheral blood mononuclear cells were separated from freshly drawn heparinized blood by Ficoll-Hypaque density sedimentation from the patient (S.O.), ten cases of SLE, and ten normal donors. A T cell-rich fraction was obtained by E rosette centrifugation through Ficoll Hypaque, and the T cells from the patient (S.O.) were frozen using a programmed freezing apparatus (Cryo-Med, Mount Clemens, Mich). Details of the procedure were previously described.

Frozen T cells were stored at ~80 °C in a concentration of 2 × 10^9/mL in RPMI 1640 (GIBCO, Grand Island, NY) supplemented with 15% fetal calf serum (FCS; Flow Laboratories, Rockland, Md) and 10% dimethyl sulfoxide (DMSO; Merck, Darmstadt, FRG). When further experiments were required, the T cells were thawed and washed twice by RPMI 1640 supplemented with 2% FCS and then checked for viability with the trypsin blue dye exclusion test.

T Cell subsets

T cells bearing receptors for the Fc portion of IgG and IgM were detected by rosette formation with ox red blood cells, which were coated with purified rabbit IgG and IgM antioxi red blood cells.

Plasma Clot Megakaryocyte Cultures

Bone marrow cell preparation. Bone marrow mononuclear cells were obtained by density centrifugation with Ficoll-Hypaque. The interface mononuclear cell layer was collected and washed twice by a-medium (Flow) supplemented with 2% FCS. Bone marrow cells were finally resuspended in a-medium supplemented with 10% FCS at a concentration of 5 × 10^6/mL.

Preparation of phytohemagglutinin-leukocyte conditioned medium (PHA-LCM). Mononuclear cells derived from peripheral blood of the normal subjects were cultured in RPMI 1640 supplemented with 10% FCS and 1% PHA (GIBCO) at a concentration of 8.8 (n=10) colony-forming unit-megakaryocytes (CFU-M) in our plasma clot system. When 2 × 10^9 of the patient’s T cells were added to the culture system, the number of CFU-M decreased to only 3.5 ± 0.6/2 × 10^4 bone marrow cells. No evidence of inhibitory effects was found by the addition of the patient’s serum and complement to the culture system. The T cells stored at ~80 °C on admission were also capable of suppressing autologous CFU-M after recovery from AMT. These results indicate that in vitro suppression of CFU-M from allogenic and autologous bone marrow cells by this patient’s T cells provides an explanation for the pathogenesis of AMT associated with SLE.
2 x 10^5/mL. The supernatant was harvested after four days of incubation and stored at -20 °C as PHA-LCM.

Culture system. The following materials were used for the plasma clot system: Bovine serum albumin solution (BSA): bovine serum albumin fraction V (Sigma Chemical Co, St. Louis) was used. An 8% (wt/vol) BSA stock solution was prepared as described by McLeod et al. L-Asparaginase solution: L-Asparaginase (Sigma) was dissolved in α-medium at a concentration of 2 mg/mL, filtered through a 0.45-μm filter, and stored at -20 °C until used. This stock solution was diluted 1:10 with α-medium before being added to the culture system. Bovine embryo extract: Bovine embryo extract (BEE) (Step-III, Connaught Laboratories, Willowdale, Ontario) was diluted to 20 U/mL by α-medium. Supplemental Eagle’s minimum essential medium with Hanks’ balanced salt solution (supplemented HMEM): The composition of supplemented HMEM was Dulbecco’s modified Eagle’s medium (Flow), 0.086 g; Hanks’ balanced salts without sodium bicarbonate (Flow), 0.99 g; sodium pyruvate (Flow), 0.011 g; 1 mL of MEM nonessential amino acids (GIBCO, 10 mmol/L, 100X concentrate); 1 mL of L-glutamine (GIBCO, 100 mmol/L, 100X concentrate); 0.89 mL of 7% (wt/vol) NaHCO₃ solution; 10 mL of heat-inactivated human type AB serum from the patient, normal donors, and ten SLE patients, and 0.1 mL of complement were added to the culture system. To study humoral inhibitors, 0.1 mL of serum from the patient, normal donors, and ten SLE patients, and 0.1 mL of complement were added to the culture system. After the recovery of megakaryopoiesis, 2 x 10^5 of the patient’s T cells (frozen on admission) were cocultured with 2 x 10^5 autologous bone marrow cells for the coculture studies of CFU-C and CFU-E. 2 x 10^5 T cells obtained from the patient before treatment and from ten normal subjects were cocultured with 2 x 10^5 normal allogenic or autologous bone marrow cells to assay CFU-C and CFU-E.

RESULTS

Table 1 shows the control studies from five SLE patients with normal platelet counts (SLE group A), five SLE patients with thrombocytopenia and increased megakaryocytes (SLE group B), and ten normal subjects. Mononuclear bone marrow cells (2 x 10^6) from ten normal donors produced a mean of 33.6 ± 8.8 megakaryocytic colonies by culturing in the presence of PHA-LCM and Epo. Only 2.6 ± 1.3 megakaryocytic colonies were cloned per 2 x 10^5 cells in the absence of PHA-LCM and Epo. The mean of megakaryocytic colonies from five cases of SLE group A was 35.5 ± 7.6/2 x 10^5 cells. However, the mean of megakaryocytic colonies from five cases of SLE group B was significantly increased to 46.8 ± 11.8/2 x 10^5 cells (P < .05, as compared by Student’s t test). The addition of 2 x 10^5 T cells from normal donors, five cases with SLE group A, and five cases with SLE group B caused a significant increase in megakaryocytic colony formation from allogenic normal bone marrows to 63.5 ± 10.3, 59.3 ± 11.4, and 60.7 ± 9.5/2 x 10^5 cells, respectively (P < .001). Also, an addition of autologous T cells caused a similar increase of megakaryocytic colonies in SLE groups A and B as well as in normal subjects. Furthermore, the number of megakaryocytic colonies from normal subjects, SLE group A, and SLE group B were not influenced by the addition of serum and complement.

Table 2 summarizes the effects of T cells and serum from the patient on megakaryocytic colony formation. Only 1.5 ± 0.4 megakaryocytic colonies were cloned per 2 x 10^5 mononuclear bone marrow cells from the patient. The coculture between 2 x 10^5 of the patient’s T cells and 2 x 10^5 mononuclear bone marrow cells from a normal donor caused a marked decrease of megakaryocytic colonies to 3.5 ± 0.6/2 x 10^5 cells.

When the patient recovered from AMT by combination therapy with a high dose of prednisolone and pulse therapy of methylprednisolone, 2 x 10^5 of the patient’s mononuclear bone marrow cells produced 40.3 ± 8.3 megakaryocytic (CFU-E) assay was carried out using the plasma clot method described by Tepperman et al. in the presence of 2 units of erythropoietin (sheep erythropoietin step III; Connaught). Cultures were maintained at 37 °C in humidified air with 5% CO₂ for seven days. Cell aggregates of more than eight benzidin-positive cells were counted as a colony.
Table 1. Effects of T Cells and Serum From Normal Subjects, SLE Group A, and SLE Group B on Megakaryocytic Colony Formation

<table>
<thead>
<tr>
<th>Coculture Studies</th>
<th>Addition to Culture</th>
<th>Source of Bone Marrow</th>
<th>No. of CFU-M/2 × 10⁴ Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal subjects</td>
<td>None</td>
<td>Own</td>
<td>33.6 ± 8.8</td>
</tr>
<tr>
<td>(n = 10)</td>
<td>2 × 10⁵ T cells</td>
<td>Autologous normal</td>
<td>63.5 ± 10.3</td>
</tr>
<tr>
<td></td>
<td>2 × 10⁵ T cells</td>
<td>Autologous normal</td>
<td>62.8 ± 11.6</td>
</tr>
<tr>
<td></td>
<td>0.1 mL of serum</td>
<td>Autologous normal</td>
<td>31.7 ± 6.2</td>
</tr>
<tr>
<td></td>
<td>0.1 mL of serum and complement</td>
<td>Autologous normal</td>
<td>32.3 ± 6.6</td>
</tr>
<tr>
<td>SLE group A</td>
<td>None</td>
<td>Own</td>
<td>35.5 ± 7.6</td>
</tr>
<tr>
<td>(n = 5)</td>
<td>2 × 10⁵ T cells</td>
<td>Autologous normal</td>
<td>59.3 ± 11.4</td>
</tr>
<tr>
<td></td>
<td>2 × 10⁵ T cells</td>
<td>Autologous normal</td>
<td>61.4 ± 9.8</td>
</tr>
<tr>
<td></td>
<td>0.1 mL of serum and complement</td>
<td>Autologous normal</td>
<td>35.7 ± 7.1</td>
</tr>
<tr>
<td></td>
<td>0.1 mL of serum and complement</td>
<td>Autologous normal</td>
<td>36.0 ± 8.2</td>
</tr>
<tr>
<td>SLE group B</td>
<td>None</td>
<td>Own</td>
<td>46.8 ± 11.8</td>
</tr>
<tr>
<td>(n = 5)</td>
<td>2 × 10⁵ T cells</td>
<td>Autologous normal</td>
<td>60.7 ± 9.5</td>
</tr>
<tr>
<td></td>
<td>2 × 10⁵ T cells</td>
<td>Autologous normal</td>
<td>75.6 ± 8.3</td>
</tr>
<tr>
<td></td>
<td>0.1 mL of serum and complement</td>
<td>Autologous normal</td>
<td>40.7 ± 8.5</td>
</tr>
<tr>
<td></td>
<td>0.1 mL of serum and complement</td>
<td>Autologous normal</td>
<td>45.7 ± 10.4</td>
</tr>
</tbody>
</table>

T cells (2 × 10⁵) from normal subjects, SLE group A, and SLE group B were cocultured with 2 × 10⁵ autologous and allogenic normal bone marrows. Further, 0.1 mL of serum from normal subjects, SLE group A, and SLE group B were added to culture system with 0.1 mL of complement (rabbit lyophilized serum diluted by α-medium). Values for normal subjects were constructed from ten normal subjects by separate experiments (mean ± SD, n = 10). Values for SLE group A and SLE group B were constructed from five SLE group A and SLE group B by separate experiments (mean ± SD, n = 5). All allogenic normal bone marrows were obtained from different donors.

To study the humoral inhibitors influencing megakaryocytic colony formation, the patient's serum (0.1 mL) and complement were added to the culture system. The numbers of megakaryocytic colonies from the patient, normal donors, SLE group A, and SLE group B were not suppressed by the addition of serum and complement, suggesting no evidence of inhibitor in the serum. These coculture studies indicated that T cells were responsible for severe megakaryocytopenia associated with SLE.

In addition, to determine whether T cells from the patient exhibited suppressive effects on autologous and allogenic CFU-C or CFU-E growth or not, 2 × 10⁵ T cells from the patient were cocultured with 2 × 10⁵ mononuclear bone marrow cells from autologous or allogenic bone marrows. Mononuclear bone marrow cells (2 × 10⁵) from normal subjects yielded 105.4 ± 14.5 (mean ± SD, n = 5) of CFU-C and 185.6 ± 21.5 (mean ± SD, n = 5) of CFU-E. Similarly, 2 × 10⁵ mononuclear bone marrow cells from the patient during AMT yielded 125.4 ± 15.2 of CFU-C and 198.3 ± 21.9 of CFU-E. When T cells obtained from the patient before treatment were added, the mean values of CFU-C and CFU-E were increased to 142.6 ± 18.5 and 245.3 ± 47.6/2 × 10⁵ cells, respectively, indicating no suppression of autologous CFU-E and CFU-E growth. T cells from normal subjects also enhanced colony formation of CFU-C and CFU-E (data not shown).

**DISCUSSION**

Much work has been done on the immune suppression of hematopoiesis in SLE, characterized by major alteration of both the humoral and the cellular branches of immunity. In a previous study, Duckham et al reported that a factor in the serum from patients with SLE inhibited granulocytic colony formation in an in vitro agar system. Also, Fitchen et al described one patient having SLE and IgG inhibitor of granulopoiesis and erythropoiesis. However, in a recent

**Table 2. Effects of T Cells and Serum From Patient With AMT Associated With SLE on Megakaryocytic Colony Formation**

<table>
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<th>Addition to Culture</th>
<th>Source of Bone Marrow</th>
<th>No. of CFU-M/2 × 10⁴ Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>During AMT</td>
<td>None</td>
<td>Own during AMT</td>
<td>1.5 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>2 × 10⁵ T cells</td>
<td>Allogenic normal</td>
<td>3.5 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>0.1 mL of serum</td>
<td>Allogenic normal</td>
<td>28.6 ± 5.5</td>
</tr>
<tr>
<td></td>
<td>0.1 mL of serum and complement</td>
<td>Allogenic normal</td>
<td>3.5 ± 0.6</td>
</tr>
<tr>
<td>After recovery from AMT</td>
<td>None</td>
<td>Own after recovery from AMT</td>
<td>40.3 ± 8.3</td>
</tr>
<tr>
<td></td>
<td>2 × 10⁵ T cells</td>
<td>Own after recovery from AMT</td>
<td>2.7 ± 0.3</td>
</tr>
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</tbody>
</table>

Each value represents mean ± SD (n = 5). Rabbit lyophilized serum (0.1 mL) diluted by α-medium was used as a source of complement. AMT, amegakaryocytic thrombocytopenia.
Acquired AMT is an uncommon disorder that does not represent a single clinical entity. It has been reported in conjunction with SLE without determination of an underlying pathogenesis. It was suggested by Hoffman et al.\(^9\) that acquired AMT could involve several pathogenetic mechanisms, including (a) failure of terminal megakaryocytic differentiation; (b) an intrinsic cell defect; (c) abnormal interaction between CFU-M and bone marrow regulatory cells; (d) decreased production of a hematopoietic growth factor; and (e) immunologic attachment on megakaryocytic progenitor cells. They also demonstrated, from their analyses of six cases, that acquired AMT could be due to at least one of two alternative mechanisms: either an intrinsic defect at the level of CFU-M or a circulating cytotoxic autoantibody directed against the CFU-M.\(^10\) More recently, Hoffman et al.\(^11\) reported an unusual case of chronic idiopathic thrombocytopenic purpura with amegakaryocytosis due to a humoral antibody cytotoxic to megakaryocyte progenitor cells.

Our control studies showed that the number of CFU-M in SLE group B was significantly higher than those in SLE group A and normal controls, suggesting that the regulatory response to the thrombocytopenic state is not impaired in SLE group B. Also, we failed to suppress the number of CFU-M by the addition of complement and serum from patients in SLE group B as well as from those in group A, indicating no inhibitory humoral antibody to megakaryocyte progenitor cells. These findings suggest that a humoral antibody to megakaryocyte progenitor cells is uncommon in SLE.

T cells may play an important role in the regulation of megakaryopoiesis at the progenitor cell level, although it is well known that T cells stimulate CFU-E and CFU-C growth. The present study indicates that T cells from normal donors, SLE group A, and SLE group B are capable of enhancing the number of CFU-M from allogeneic and autologous bone marrow. We have assumed that T cells may secrete a stimulating factor, like a colony-stimulating factor for megakaryocytes (CSF-Meg). However, we could not find a marked difference in T cells from the three groups for enhancing of CFU-M from allogeneic or autologous bone marrow.

In contrast, the CFU-M were strongly suppressed when T cells from this patient were cocultured with normal allogeneic bone marrow cells. Also, the patient's T cells (frozen on admission) suppressed CFU-M of autologous bone marrow cells after the recovery of megakaryopoiesis. This is a direct demonstration that the patient's T cells are capable of suppressing autologous hematopoietic stem cells. These results indicate that abnormal cell-to-cell interaction between the patient's T cells and megakaryocytic progenitor cells may be responsible for the underlying mechanism of megakaryocytic colony suppression in vitro.

It has recently become possible to quantify subsets of T cells as helper T cells (Th) and suppressor T cells (Ts). However, this analysis of subsets of T cells in SLE remains controversial. Morimoto et al.\(^12\) reported a selective decrease in OKT8\(^+\) cells (suppressor T cells), whereas Bakke et al.\(^13\) demonstrated that one half of 28 SLE patients had markedly depressed percentage and absolute number of OKT4\(^+\) cells (helper T cells).

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This patient developed AMT during an inactive serologic state of SLE. Cline and Golde.\(^15\) described a patient having SLE and an IgG inhibitor of granulopoiesis and erythropoiesis and stated that such inhibitors appear to be relatively infrequent in patients with active disease. However, AMT in the present patient responded to a high dose of prednisolone and pulse therapy of methylprednisolone. The analysis of T cell subsets after the recovery of megakaryopoiesis revealed 53.4% of Th and 24.5% of Ts, suggesting the decrease of Ts cells to be due to combination therapy.

We conclude that T cells from this patient are capable of in vitro suppression of megakaryopoiesis. For some cases, this may explain the association of AMT with SLE.

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Cell-mediated amegakaryocytic thrombocytopenia associated with systemic lupus erythematosus

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