Analysis of the Mechanism of Anagrelide-Induced Thrombocytopenia in Humans

By Eric M. Mazur, Alan G. Rosmarin, Patricia A. Sohl, Julie L. Newton, and Amirthini Narendran

Anagrelide is a new therapeutic compound recently demonstrated to have a rapid and selective thrombocytopenic effect in humans. The effects of anagrelide were evaluated in plasma clot and liquid suspension cultures of optimally stimulated normal human peripheral blood megakaryocyte progenitors in order to determine the mechanism of its thrombocytopenic activity. In plasma clot cultures, at clinically relevant, therapeutic concentrations (5 to 50 ng/mL), anagrelide exerted no significant inhibitory effect on megakaryocyte colony numbers or colony size. Only at anagrelide concentrations of 10 to 500 times therapeutic doses did anagrelide inhibit megakaryocyte colony development: an anagrelide concentration of 5 μg/mL reduced colony numbers by 57% and colony size by 31%. In contrast, lower, therapeutic anagrelide concentrations exerted profound effects in liquid culture on megakaryocyte cytoplasmic maturation, size, and DNA content. When present for the entire 12-day culture duration, anagrelide induced left-shifted megakaryocyte maturation and reduced both megakaryocyte ploidy and megakaryocyte diameter. Anagrelide, at concentrations of 5 to 50 ng/mL, shifted the modal cultured megakaryocyte morphologic stage from III to II, reduced the modal ploidy value from 16N to 8N, and decreased the mean megakaryocyte diameter by up to 22%, from 27.8 μm to 21.6 μm. Megakaryocyte diameter was significantly reduced in most instances, even when analyzed as a function of morphologic stage. When anagrelide was added to the cultures after 6- and 9-day delays (during the final 6 and 3 days, respectively, of culture), similar inhibitory effects on megakaryocyte maturation stage and ploidy distribution were observed. However, the magnitude of the left-shift in ploidy appeared to be less as the duration of anagrelide exposure was reduced. Conversely, megakaryocyte diameter was not significantly affected by the shorter 3- and 6-day anagrelide exposures. These data indicate that therapeutic concentrations of anagrelide influence primarily the postmitotic phase of megakaryocyte development, decreasing platelet production by reducing megakaryocyte size and ploidy, as well as by disrupting full megakaryocyte maturation. Inhibition of megakaryocyte diameter appears to require more prolonged anagrelide exposure than inhibition of maturation stage and ploidy. The molecular mechanisms responsible for the inhibitory effects of anagrelide on megakaryopoiesis remain to be defined.

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while normal in number, were significantly reduced in size when quantitated by computerized image analysis. This effect on megakaryocyte size could be reproduced in vitro, but only at the supratherapeutic anagrelide concentration of 5 μg/mL (Dr L. Solberg, personal communication).

Our investigation was undertaken to further elucidate the mechanism by which anagrelide inhibits platelet production in man. Particular attention was directed at defining those changes in megakaryocyte development that could be induced by anagrelide at concentrations which were clinically therapeutic (ie, ≤50 ng/mL). The effects of anagrelide on human megakaryocytogenesis were analyzed in vitro using two different target assays: plasma clot cultures for CFU-Meg2,9 and a liquid culture system that supports the development of mature, polyploid megakaryocytes from peripheral blood stem cells.10 These two assays permit the examination of complementary developmental components of megakaryocytogenesis. Colony-forming assays require stem cell mobilization and proliferation, and the acquisition of megakaryocyte phenotypic markers by the derivative cells. Liquid cultures permit accurate analyses of megakaryocyte size, ploidy, and extent of cytoplasmic maturation. Our data indicate that therapeutic concentrations of anagrelide reduce platelet production in man by affecting the later phases of megakaryocyte development, inhibiting polyploidization, cytoplasmic size, and maturation.

MATERIALS AND METHODS

Human subjects. Peripheral blood for CFU-Meg cultures and normal AB plasma and serum were obtained by routine venipuncture of consenting healthy adult donors, all of whom provided written informed consent. Frequent donors were chosen as the source of peripheral blood progenitor cells because higher concentrations of circulating CFU-Meg are typically observed in such individuals.

Megakaryocyte progenitor cells. Mononuclear cells were prepared from the peripheral blood of normal volunteer donors using Ficoll 400-sodium diatrizoate (Pharmacia, Piscataway, NJ) density centrifugation. Cells were further depleted of monocytes by two cycles of plastic adherence and plated at 5 X 10^5 cells/mL in normal AB plasma and serum were obtained by routine venipuncture of consenting healthy adult donors, all of whom provided consents. Normal AB plasma and serum were obtained by routine venipuncture of consenting healthy adult donors, all of whom provided consents. Normal AB plasma and serum were obtained by routine venipuncture of consenting healthy adult donors, all of whom provided consents. Normal AB plasma and serum were obtained by routine venipuncture of consenting healthy adult donors, all of whom provided consents.

Megakaryocyte size and maturation stage were determined after Wright–Giemsa staining. Megakaryocytes were identified by their characteristic light microscopic morphology. Diameters were calculated as the average of perpendicular measurements obtained using a calibrated eyepiece micrometer.10 Megakaryocytes were assigned to maturation stages I through IV using the criteria of Levine et al.13,14

For determination of megakaryocyte ploidy, unstained cytospin preparations were labeled using a modified Feulgen technique.23,32 2,5-bis(4'-amino-phenyl)-1'-3,4 oxidazole (1) (Fluka Chemical, Haffauge, NY) was chosen as the quantitative DNA fluorochrome because its relative stability to ultraviolet irradiation minimizes the influence of light exposure duration on measurements of DNA fluorescence.23

Cellular DNA content was quantitated by microfluorometry as we have described.34,35 In every experiment, ploidy distributions were derived from at least 200 megakaryocytes, which were evaluated sequentially on cytospin preparations. Megakaryocytes were identified morphologically based on their characteristic large nuclear size and/or nuclear multilobation. Diploid cell DNA fluorescence was determined on each slide in a ratio of approximately 1:2 with the megakaryocytes.18 Diploid cells consisted of the lymphocytes and macrophages, which contaminated aplastic canine serum at 1 to 2 mg/mL. Suspension cultures were incubated for 12 to 13 days at 37°C in a 100% humidified atmosphere of 5% CO₂ in air.

Megakaryocytes were enriched from the liquid cultures by counterflow centrifugal elutriation.36 Cultured cells were suspended and eluted in Mega-buffer,37 a medium specifically designed to preserve megakaryocyte integrity. Elutriation was performed using a Beckman J2-21 centrifuge (Beckman Instruments, Palo Alto, CA) equipped with a JE-6B elutriation rotor and a Sanderson separation chamber. After introduction of the cultured cells into the elutriation chamber at a rotor speed of 2,500 rpm, eluent flow rates were increased incrementally from 31 mL/min to 45 mL/min. Megakaryocytes were concentrated in the cellular fraction remaining in the elutriation chamber. Megakaryocytes constituted 58% of cells in this fraction and exhibited an average viability of 67%.39 For cytologic analyses, megakaryocytes were spun onto poly-L-lysine-coated glass slides by cytocentrifugation (Shandon Cytospin 2, Sewickley, PA).

Anagrelide. Anagrelide was provided in powdered form (Batch 1708-1) by the Pharmaceutical Research and Development Division, Bristol-Myers Company, Wallingford, CT. A stock solution of anagrelide (5 mg/mL) in dimethylsulfoxide (DMSO) was prepared and stored frozen at −20°C. For use in culture, anagrelide was diluted in a-medium with 1% BSA (wt/vol) and filter-sterilized. At the maximum concentration of anagrelide evaluated (5 μg/mL), DMSO constituted only 0.1% of the culture volume. At this concentration, DMSO alone exhibited no effects on megakaryocyte colony number, colony size, ploidy, and maturation (data not shown).

Cultured megakaryocyte analyses. Plasma clot megakaryocyte colony cultures were fixed and labeled fluorescently using a rabbit polyclonal antiplatelet glycoprotein antiserum as we have reported.7 In each plasma clot experiment, megakaryocyte colony numbers were averaged from duplicate culture plates. Colony size was determined by averaging the numbers of megakaryocytes in all colonies within individual culture plates.

Analyses of megakaryocyte size, maturation stage, and ploidy were performed using liquid culture-derived megakaryocytes mounted on glass slides. Compared with megakaryocytes grown in plasma clot, liquid culture-derived megakaryocytes exhibit much better preserved cytologic detail (Fig 1) and tighter coefficients of variation on ploidy analyses.

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the megakaryocyte-rich elutriation fraction. In these investigations, the coefficients of variation for the 2N fluorescent signals averaged 16.2% ±4.4% (SD). Because of the uncertainty in identifying the low-ploidy megakaryocytes, megakaryocyte ploidy distributions were limited to include only cells with DNA contents greater than or equal to 8N.

**Statistical analysis.** Megakaryocyte diameters, colony numbers, and colony sizes were compared using two-tailed Student’s t-tests. Megakaryocyte stage and ploidy distributions were evaluated statistically using Pearson’s χ² test of independence.

**RESULTS**

Figure 2 illustrates the effects of varying concentrations of anagrelide on human megakaryocyte colony development in vitro. Colony growth was inhibited significantly only at anagrelide concentrations greater than 500 ng/mL. Half-maximal reduction of colony numbers was observed at an anagrelide concentration of approximately 1.1 µg/mL. Anagrelide also inhibited megakaryocyte colony size in culture, although the magnitude of this effect was smaller and the concentrations of anagrelide required for inhibition were higher (Fig 3). A significant reduction in megakaryocyte numbers per colony was obtained only at the relatively high anagrelide concentration of 5 µg/mL. The half-maximal inhibitory concentration of anagrelide on colony size was not reached at the highest anagrelide concentration tested. Similar inhibitory effects of anagrelide on megakaryocyte colony number and size were observed when optimal stimulatory concentrations of interleukin-3 were substituted for the asplenic canine serum fraction as a source of colony-stimulating activity in the plasma clot cultures (data not shown). For both megakaryocyte colony number and size, these experiments demonstrated significant inhibition by anagrelide only at concentrations at least 10-fold higher than the peak therapeutic concentrations achieved in vivo (ie, 50 ng/mL).

In contrast, lower, therapeutic concentrations of anagrelide were found to exert profound effects on maturation stage, size, and ploidy of developing megakaryocytes grown in liquid culture. Three concentrations of anagrelide, 5 ng/mL, 15 ng/mL, and 50 ng/mL, were evaluated and exhibited similar effects. Compared with control cultures, anagrelide-containing cultures exhibited a significant left-shift in morphologic stage distribution in all experiments, at

**Fig 2.** Effect of anagrelide on megakaryocyte colony formation in plasma clot culture. Varying concentrations of anagrelide (0.5 ng/mL to 5 µg/mL) were added at time 0 to cultures optimally stimulated with the active ammonium sulfate fraction of aplastic canine serum. 10-stimulated megakaryocyte colony numbers in the absence of anagrelide (control) averaged 62 ± 17 (SD) (range, 36.5 to 74.0) colonies per 5 × 10⁴ mononuclear cells plated. Data are normalized in each experiment by the number of colonies in the optimally stimulated, anagrelide-free cultures. Data are derived from four separate experiments, each performed in duplicate, and are analyzed statistically by the two-tailed Student’s t-test. *P = .058, **P < .001.

**Fig 3.** Effect of anagrelide on megakaryocyte colony size in plasma clot culture. Varying concentrations of anagrelide (0.5 ng/mL to 5 µg/mL) were added at time 0 to cultures optimally stimulated with the active ammonium sulfate fraction of aplastic canine serum. Stimulated colony size in control cultures averaged 7 ± 3 (SD) (range, 5.2 to 10.5) cells per colony. Data are normalized in each experiment by the colony size in optimally stimulated control cultures. Data are consolidated from three separate experiments and are analyzed statistically by a two-tailed Student’s t-test. *P < .01.
all anagrelide concentrations tested (Table 1). Whereas an average of 43.7% ± 3.4% (SD) of megakaryocytes were stages I and II in the control cultures, 64.4% ± 4.8% were stages I and II when grown in the presence of anagrelide (P < .001 by Student’s t test).

Dose-response effects of anagrelide on cultured megakaryocyte stage were not clearly evident across different experiments (Table 1). However, within experiment 4 (Table 1), the megakaryocyte morphologic stage distribution was significantly further left-shifted by anagrelide at 15 ng/mL compared with 5 ng/mL (P < .05 by Pearson’s χ² test). In that same experiment, the stage distributions at anagrelide concentrations of 15 ng/mL and 50 ng/mL did not differ significantly.

Therapeutic concentrations of anagrelide in culture also exhibited a substantial inhibitory effect on megakaryocyte size (Table 1). Overall, anagrelide reduced mean megakaryocyte diameter by 17% to 22%. Since cell volume is a function of diameter cubed, these changes represent a 43% to 53% reduction in megakaryocyte cytoplasmic volume and thus platelet production capacity.

Because megakaryocyte size is in part dependent on the extent of cytoplasmic maturation,11 mean megakaryocyte diameters were also compared within each maturation stage (Table 1). Even when segregated by stage, in most instances exposure of the developing megakaryocytes to anagrelide resulted in significant reductions in diameter within each stage category.

Across experiments, a dose-response effect of anagrelide on stage-segregated megakaryocyte diameter was not evident (Table 3). However, in experiment 4 (Table 3), stage I and III megakaryocytes exhibited decreasing megakaryocyte diameter with increasing anagrelide concentration. For stage I megakaryocytes (experiment 4), this dose-response effect was statistically significant (P = .027 by linear regression analysis).

Megakaryocyte size is also a function of DNA content.12 Therefore, we determined cultured megakaryocyte ploidy distributions in control and anagrelide-containing liquid suspension cultures. One experiment was performed with anagrelide at 5 ng/mL, and two at 50 ng/mL. Since the results did not differ significantly, data were combined for

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Table 1. Effect of Anagrelide on Liquid Culture-Derived Megakaryocyte Stage Distribution

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Morphologic Stage*</th>
<th>Control</th>
<th>Anagrelide 5 ng/mL</th>
<th>Anagrelide 10 ng/mL</th>
<th>Anagrelide 50 ng/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>II</td>
<td>III</td>
<td>IV</td>
<td>P Value*</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>2.5</td>
<td>11.0</td>
<td>18.0</td>
<td>-.001</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>40.0</td>
<td>44.5</td>
<td>53.0</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>54.5</td>
<td>33.0</td>
<td>27.0</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>2.0</td>
<td>2.5</td>
<td>2.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Numbers represent the mean percentage of total megakaryocytes in each morphologic stage category. The modal stage for each experimental condition is set in boldface.

†Statistical significance is determined in each experiment by comparing megakaryocyte morphologic stage distributions in the anagrelide-containing cultures to the relevant controls. Distributions are compared by Pearson’s χ² test.17

‡Numbers in parentheses reflect the total number of megakaryocytes analyzed in each experiment.
ANAGRELIDE AND HUMAN MEGAKARYOCYTOPOIESIS 1935

Table 4. Effect of Anagrelide on Megakaryocyte Ploidy in Liquid Culture

<table>
<thead>
<tr>
<th>Ploidy Class*</th>
<th>8N</th>
<th>16N</th>
<th>32N</th>
<th>64N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>29.4 ± 16.4</td>
<td>56.1 ± 16.6</td>
<td>11.8 ± 2.4</td>
<td>2.8 ± 1.2</td>
</tr>
<tr>
<td>Anagrelide</td>
<td>50.8 ± 14.4</td>
<td>34.6 ± 6.1</td>
<td>14.2 ± 9.1</td>
<td>0.4 ± 0.6</td>
</tr>
</tbody>
</table>

Data are derived from three separate, paired experiments (experiments 1 to 3, see Table 1) and incorporate ploidy determinations on a total of 483 megakaryocytes ≥ 8N. Ploidy distributions differ significantly (P < .001) by Pearson's χ² test.11

*Numbers represent the mean ± SD percentage of total megakaryocytes in each ploidy class.

Table 5. Megakaryocyte Stage in Liquid Culture; Anagrelide Exposure After Varying Culture Intervals

<table>
<thead>
<tr>
<th>Morphologic Stage*</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control†</td>
<td>4.4 ± 0.6</td>
<td>34.4 ± 2.5</td>
<td>56.9 ± 1.7</td>
<td>4.4 ± 2.1</td>
</tr>
<tr>
<td>Anagrelide</td>
<td>6.8 ± 1.9</td>
<td>50.7 ± 4.3</td>
<td>39.9 ± 4.7</td>
<td>2.6 ± 1.0</td>
</tr>
<tr>
<td>Day 6</td>
<td>6.0 ± 0.9</td>
<td>53.1 ± 3.1</td>
<td>38.5 ± 3.1</td>
<td>2.5 ± 1.3</td>
</tr>
</tbody>
</table>

Data are consolidated from four separate experiments and incorporate the analyses of 2,210 megakaryocytes. Anagrelide day 6 and day 9 morphologic stage distributions each differ significantly from control (P < .001) by Pearson’s χ² test.17

*Numbers represent the mean ± SD percentage of total megakaryocytes in each morphologic stage category. The modal stage for each experimental condition is set in boldface.
†Megakaryocytes derived from control cultures were grown for 12 days in the absence of anagrelide.

Table 6. Megakaryocyte Ploidy in Liquid Culture; Anagrelide Exposure After Varying Culture Intervals

<table>
<thead>
<tr>
<th>Ploidy Class*</th>
<th>8N</th>
<th>16N</th>
<th>32N</th>
<th>64N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>24.4 ± 20.1</td>
<td>52.7 ± 8.8</td>
<td>19.9 ± 11.5</td>
<td>3.1 ± 1.9</td>
</tr>
<tr>
<td>Anagrelide</td>
<td>42.3 ± 11.8</td>
<td>42.9 ± 8.9</td>
<td>13.7 ± 6.6</td>
<td>1.2 ± 0.9</td>
</tr>
<tr>
<td>Day 6</td>
<td>36.8 ± 20.0</td>
<td>45.3 ± 10.0</td>
<td>15.3 ± 8.8</td>
<td>2.7 ± 4.2</td>
</tr>
</tbody>
</table>

Data are derived from four separate experiments and include the analyses of 1,835 megakaryocytes. Anagrelide day 6 and day 9 ploidy distributions each differ significantly from control (P < .001) by Pearson’s χ² test.11

*Numbers represent the mean ± SD percentage of total megakaryocytes in each ploidy class.

DISCUSSION

Anagrelide has been observed clinically to have a potent and specific inhibitory effect on circulating platelet numbers in man. Initial in vitro studies were unsuccessful in defining the mechanism of anagrelide-induced thrombocytopenia.3 While these studies did demonstrate an inhibitory effect of anagrelide on human megakaryocyte colony development.

Table 7. Megakaryocyte Diameter Ploidy in Liquid Culture; Anagrelide Exposure After Varying Culture Intervals

<table>
<thead>
<tr>
<th>Diameter by Morphologic Stage*</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control†</td>
<td>14.4 ± 1.5</td>
<td>22.9 ± 1.3</td>
<td>31.1 ± 6.3</td>
<td>41.3 ± 5.7</td>
</tr>
<tr>
<td>Anagrelide</td>
<td>13.5 ± 2.6</td>
<td>23.1 ± 0.9</td>
<td>27.3 ± 2.1</td>
<td>41.3 ± 1.0</td>
</tr>
<tr>
<td>Day 6</td>
<td>13.6 ± 2.4</td>
<td>23.5 ± 0.9</td>
<td>30.3 ± 3.6</td>
<td>34.4 ± 8.6</td>
</tr>
</tbody>
</table>

Data are consolidated from four separate experiments and incorporate measurements on 2,210 megakaryocytes.

*Numbers represent the mean ±SD megakaryocyte diameter in micrometers.
†Megakaryocytes derived from control cultures were grown for 12 days in the absence of anagrelide.
Development, the effects were demonstrable only at anagrelide plasma concentrations well above therapeutic levels. Our data confirm these earlier observations. Significant inhibition of colony growth was observed at anagrelide concentrations greater than 0.5 μg/mL. Since therapeutic levels of anagrelide are in the range of 5 to 50 ng/mL, it is unlikely that anagrelide reduces platelet production in the clinical setting by inhibition at the level of the megakaryocyte progenitor cell.

Our observations indicate that anagrelide-induced thrombocytopenia also is not due to inhibition of the mitotic expansion of developing early megakaryocyte precursors. Mitotic expansion can be quantitated in vitro by measuring megakaryocyte colony size. In our investigation, megakaryocyte colony size was not reduced except at anagrelide concentrations approximately 100-fold above therapeutic levels. These in vitro data are consistent with the in vivo morphologic evaluations that have been performed on bone marrow specimens obtained from patients receiving anagrelide. Such specimens consistently exhibit no changes in megakaryocyte numbers, suggesting no significant effect of anagrelide on megakaryocyte development from the committed stem cell (CFU-Meg) through the early, morphologically identifiable megakaryocyte.

Our data indicate that therapeutic levels of anagrelide influence primarily the postmitotic phases of megakaryocyte development. We observed inhibitory effects in vitro on megakaryocyte ploidy, size, and cytoplasmic maturation. Any or all of these inhibitory effects may account for the thrombocytopenic activity of anagrelide in vivo. Inhibition of megakaryocyte endoreduplication would result in a left-shifted megakaryocyte ploidy distribution. Since there is believed to be a direct, constant relationship between megakaryocyte DNA content and quantitative platelet production per cell, a primary effect shifting megakaryocyte ploidy leftward would result secondarily in reductions in megakaryocyte size and platelet output. A decrease in cytoplasmic volume independent of ploidy could equally well account for diminished platelet-producing capacity per megakaryocyte. If this latter hypothesis is valid, then the focus of anagrelide’s action would be the reduction of the number of platelets produced per unit of megakaryocyte DNA. Regardless, our data indicating that anagrelide reduces megakaryocyte diameter by an average of 20% in vitro translate into a 50% in vivo reduction in platelet production capacity.

The mechanism whereby an anagrelide-induced leftward shift in megakaryocyte cytoplasmic maturation could lead to diminished platelet production is less clear. A simple delay in the platelet output from a cohort of developing megakaryocytes would not affect the net quantity of platelets produced by that cohort; at steady-state, platelet production would remain unchanged. More likely is that the observed cytoplasmic immaturity of megakaryocytes exposed to anagrelide results from the induction of a component of maturation arrest or ineffective megakaryocytopoiesis. If this postulate is true, fewer megakaryocytes would actually mature through stage IV and thus reach the stage at which platelets are shed. The potential quantitative impact of this effect of anagrelide on platelet production in vivo cannot be determined from our data.

It must also be emphasized that ploidy, size, and maturation stage are not independent parameters in megakaryocyte development. Higher ploidy megakaryocytes are as a group larger and exhibit a greater degree of cytoplasmic maturity. Thus, one need not postulate that anagrelide independently affects multiple separate components of megakaryocyte development. A primary inhibitory activity on a single, critical determinant of nonmitotic megakaryocyte maturation would satisfactorily explain the majority of our in vitro observations.

Our data confirm the previous in vivo and in vitro observations reported by Solberg et al. That group demonstrated that bone marrow megakaryocytes obtained directly from anagrelide-treated patients were present in normal numbers, but were significantly reduced in size. In megakaryocyte liquid suspension cultures containing high, supratherapeutic concentrations of anagrelide (5 μg/mL, personal communication), they also detected a comparable reduction of megakaryocyte size, as well as an inhibitory effect on cultured megakaryocyte cytoplasmic maturation. In Solberg’s investigation megakaryocyte maturation was assessed by cultured cellular glycoprotein IIb/IIIa antibody binding and measurements of cell contour regularity or roundness, rather than morphologic stage assignation.

The proposition that anagrelide induces thrombocytopenia by disrupting megakaryocyte maturation is also supported by clinical observations in treated patients. In phase II studies of more than 420 evaluable patients with thrombocytosis, the onset of action of anagrelide in vivo was found to be relatively rapid. The median time to achieve a 50% reduction in the platelet count was 11 days. In addition, following the discontinuation of anagrelide, platelet counts have been observed to increase rapidly within 4 days. This rapid onset and offset of the clinical activity of anagrelide argues for a locus of action late in megakaryocyte development. If it were affecting megakaryocyte development at the stem cell level, one would expect changes in circulating platelet counts to occur more slowly, comparable to the changes observed with conventional cytotoxic drugs. Thrombocytopenia resulting from cytotoxic drugs typically develops over 2 or more weeks and requires at least 1 week for resolution.

Further evidence that anagrelide acts late in megakaryocyte development can be derived from those experiments in which the addition of anagrelide to liquid suspension culture was delayed by 6 and 9 days. We have previously demonstrated that by day 6 in suspension culture, megakaryocyte precursors have undergone several rounds of mitosis and have acquired surface membrane glycoprotein IIb/IIIa (unpublished data). By day 9, mitotic megakaryocyte precursors disappear and both immature megakaryocytes and earlier, nonmitotic IIb/IIIa-positive precursors are present (unpublished data). Our current data demonstrate that exposure of these day 6 and 9
megakaryocyte precursors to therapeutic concentrations of anagrelide results in inhibitory effects on maturity and ploidy comparable to those induced by anagrelide exposure for the entire 12-day culture duration. Thus, the developmental target of the inhibitory activity of anagrelide can be to a large extent isolated to the nonmitotic, late stage of megakaryocyte development.

Somewhat confounding this interpretation are our data indicating that the inhibition of megakaryocyte diameter requires anagrelide exposure for more than 6 days. Despite decreases in ploidy and maturation stage, no consistent effect on megakaryocyte diameter could be demonstrated in those cultures to which anagrelide addition was delayed (Table 6). Thus, the effect of anagrelide on megakaryocyte size appears to be exerted at a developmental time point proximal to that at which ploidy and maturation stage are affected. This latter observation suggests that more than a single molecular mechanism may be involved in anagrelide-induced thrombocytopenia. However, since our data are primarily descriptive in character, any discussion of potential molecular mechanisms is entirely speculative.

The apparent in vitro lineage specificity of anagrelide demonstrated by Silverstein et al has been substantially reproduced in large-scale, phase II clinical trials. Despite continuous therapy with anagrelide for up to 2 years, no significant decrease in circulating white blood cells has been noted among more than 420 evaluable patients and there have been only small (1 g/dL) changes in hemoglobin concentration. The hematopoietic lineage and developmental stage specificities of anagrelide suggest that it will have great future utility, not only in the clinical setting, but also as a valuable research tool for studying the regulation of human megakaryocytopoiesis and platelet production.

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Analysis of the mechanism of anagrelide-induced thrombocytopenia in humans

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