Acceleration of Granulopoietic Recovery by Androgenic Steroids in Mice Made Neutropenic by Cytotoxic Drugs

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SUMMARY

Injection of testosterone, nortestosterone, or oxymetholone markedly enhanced granulopoietic recovery in mice made neutropenic by 1,3-bis(2-chloroethyl)-1-nitrosourea, vinblastine, or cyclophosphamide. Blood neutrophils rose to six times normal counts, and the bone marrow showed sequential increases first in dividing granulocytic elements and later in the nondividing reserve. The steroid-induced increase in myeloblasts was preceded by an increase in in vitro colony-forming cells and colony-forming units. Mice whose spleen colony-forming units had been eradicated by busulfan failed to show a granulopoietic response to androgens. The relationship between colony-forming units and -forming cells is discussed, and it is suggested that the granulopoietic effect of steroids results from stimulation of the proliferation of unipotential, granulocytic, colony-forming stem cells.

INTRODUCTION

Androgens and anabolic steroids have been widely used in the therapy of aplastic anemias but with few exceptions (2, 3), their hematopoietic effects were investigated experimentally primarily in regard to the stimulation of erythropoiesis. During a study of the effects of androgens on the erythropoietic recovery in mice given a single dose of BCNU(4), it was noted that nortestosterone (19-ND) markedly increased their suppressed blood neutrophils to several times normal counts (7). In view of the potential importance of this observation in the treatment of drug-induced neutropenias, this study was undertaken to examine the granulopoietic effects of several steroids in mice made neutropenic by a variety of cytotoxic drugs and to delineate, in granulopoietic development, the cell stage at which the steroids exert their enhancing effect. Blood neutrophil counts do not necessarily reflect the rate of granulopoiesis, and the latter was therefore assessed by absolute counts of the total dividing and nondividing granulocytes in the femoral marrow. Myeloblasts, the most immature of the recognizable marrow granulocytes, are most probably derived from unipotential granulocytic stem cells, and accumulating evidence indicates that these stem cells are identical with CFC that give rise to colonies of granulocytes or macrophages in cultures of bone marrow in semisolid agar (6). The granulocytic stem cells, in turn, are derived from pluripotential stem cells (CFU) which, in the mouse, can be assayed by the spleen colony method (5). A stimulation of granulopoiesis thus could be the result of a direct or indirect action of the steroid on any of these cell stages, and measurements of the morphologically unrecognizable progenitor cells were therefore included in the study.

MATERIALS AND METHODS

On Day 0, groups of 6 female (CF1 mice (20 to 25 g) received a single dose of one of the following drugs: BCNU (35 mg/kg) i.p. (kindly supplied by Chemotherapy Evaluation Branch, National Cancer Institute); vinblastine sulfate (3 mg/kg) i.v. (Oncovin; Eli Lilly and Co., Indianapolis, Ind.); cyclophosphamide (200 mg/kg) i.v. (Cytoxan, kindly supplied by Mead Johnson Laboratories, Evansville, Ind.); or busulfan (44 mg/kg) by stomach tube (Myleran; Burroughs Welcome and Co., Research Triangle Park, N. C.). Myleran was dissolved in acetone (11 mg/ml) and diluted with corn oil; the other drugs were diluted in 0.9% NaCl solution.

Daily injections of steroids were given at a dose of 1.25 mg/mouse. 19-nortestosterone decanoate (19-ND, Decadurabol, kindly supplied by Organon, Inc., West Orange, N. J.) or oxymetholone (kindly supplied by Syntex Laboratories, Inc., Palo Alto, Calif.) were dissolved in 0.05 ml propylene glycol and given i.p. Testosterone propionate in oil was given i.m. The 1st steroid injection was given 24 hr after administration of cytotoxic drug, or at a time indicated in "Results." Control mice received daily i.p. injections of 0.05 ml propylene glycol. In experiments presented in Table 1, additional control groups received no injections at all after BCNU administration.

Blood for neutrophil counts was obtained from the orbital sinus, and each sinus was punctured only once in order to avoid local inflammatory reactions. Nucleated cells in the femoral marrow were counted in a Coulter counter after flushing out the marrow with 0.9% NaCl solution and drawing it back and forth through a 24-gauge needle (10). Smears were made from the contralateral femoral marrow for differential counts. The number of CFU in the femoral marrow was determined by the exocolonization assay (5). At various times after drug administration, a femoral marrow suspension was made as described above. Marrows of 4 mice were pooled, and 0.5 ml of the suspension, representing from 1/10 (Myleran mice) to 1/300 (normal mice) of 1 femoral marrow was injected i.v. into each of 24 irradiated mice (850 rad, 60Co
source). Nine days later, their spleens were fixed in Bouin's solution and the surface colonies were counted. Results are expressed as CFU/1 femoral marrow, and no correction was applied for the fraction not lodging in the recipients' spleens. The in vitro CFC were assayed by the method of Bradley and Metcalf (6). A washed suspension of the femoral marrow cells of 4 mice was made in Fischer's medium and plated in triplicate at 3 cell concentrations ranging from 0.5 to 1.0 X 10^6 marrow cells per culture. The same batch of mouse serum, obtained 3 hr after injection of 20 µg endotoxin, was used in all cultures as an inducer (0.05 ml/dish). Cultures of normal marrow served as the standard in each experiment. Colonies consisting of more than 100 cells were counted on Day 7 of culture.

RESULTS

Chart 1 shows the similar pattern of neutropenia induced by 1 injection of either BCNU, vinblastine, or Cytoxan. In each case, spontaneous restoration of normal blood neutrophil counts was seen between Days 5 and 7 after drug administration, and the counts then rose to about twice normal during the following 2 or 3 days. Daily injections of 19-ND accelerated the granulopoietic recovery by 2 days or more and resulted in blood neutrophil counts up to 6 times normal. The elevated counts were maintained for about 5 days. Oxy-metholone had very nearly the same effect as 19-ND (Table 1), whereas identical doses of testosterone induced a somewhat smaller granulopoietic response. Daily injections of the solvent alone (propylene glycol) had no significant effect.

The rise in blood neutrophils in the 19-ND-treated mice was preceded by a significant increase of the myeloblasts-promyelocytes in the femoral marrow on Day 3 after BCNU administration, and the subsequent rise in myelocytes and the accelerated restoration of the nondividing granulocyte marrow reserve (meta-poly) are evidence of the androgen-induced wave of increased granulopoiesis (Chart 2). The rise in myeloblasts was preceded by an increase in marrow CFC which, on Day 2 (that is, 48 hr after BCNU and 24 hr after 1 injection of 19-ND), were nearly twice as numerous as those in mice that received only BCNU. The single dose of BCNU reduced within 48 hr the femoral marrow pluripotential stem cells to around 20% of normal, and CPU levels of 24 and 34% of normal were found 48 hr after injection of vinblastine or cyclophosphamide. Daily injections of 19-ND given to the BCNU mice markedly accelerated the regeneration of their CFU and resulted in nearly normal values on Day 4 after BCNU administration.

The Myleran experiments were designed to test the granulopoietic effects of 19-ND in mice in which CFU were practically absent. As seen in Table 2, Myleran rapidly reduced the femoral CFU to levels below 1% of normal. Injections of 19-ND did not significantly alter this severe suppression, and the CFU data in Table 2 thus very nearly apply to both controls and 19-ND-injected mice. The effect of 19-ND injections on granulopoiesis of these mice was quite different from that seen after treatment with the other cytotoxic drugs. The 1st course of 19-ND injections, given on Days 1 through 5 after Myleran, induced a modest increase in blood neutrophils on Day 5 through 8, but courses of 5-day injections of 19-ND given during the near absence of CFU (Days 5 through 9 or 11 through 15) did not result in increases in blood neutrophils. The lack of granulopoietic stimulation in these mice was confirmed by bone marrow counts of dividing and nondividing granulocytes which were found to show no differences in the mice with or without 19-ND injections. With restoration of measurable CFU levels on Day 18, the mice regained their granulopoietic responsiveness to 19-ND, as evidenced by the increase in blood neutrophils after injections given on Days 18 through 22 after Myleran treatment.

![Chart 1. A, blood neutrophils in mice after a single dose of BCNU (35 mg/kg); B, vinblastine (3 mg/kg); C, Cytoxan (200 mg/kg). O, daily 19-ND injections; o, no 19-ND injections; mean ± S.E. (vertical bars) of 6 mice per group; normal range.](image-url)

Table 1

<table>
<thead>
<tr>
<th>Day after BCNU</th>
<th>No injection</th>
<th>19-ND</th>
<th>Testosterone</th>
<th>Oxymetholone</th>
<th>Solvent</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>244 ± 5</td>
<td>1,669 ± 286^a</td>
<td>501 ± 232</td>
<td>1,745 ± 477^a</td>
<td>344 ± 1,206</td>
</tr>
<tr>
<td>5</td>
<td>810 ± 150</td>
<td>4,009 ± 433^a</td>
<td>2,870 ± 321^a</td>
<td>5,050 ± 603^a</td>
<td>915 ± 401</td>
</tr>
<tr>
<td>6</td>
<td>2,310 ± 327</td>
<td>6,987 ± 699^a</td>
<td>5,990 ± 735^a</td>
<td>6,904 ± 766^a</td>
<td>2,740 ± 883</td>
</tr>
<tr>
<td>8</td>
<td>4,181 ± 935</td>
<td>11,033 ± 1,289^a</td>
<td>6,162 ± 1,216</td>
<td>9,303 ± 1,216^a</td>
<td>6,677 ± 1,685</td>
</tr>
<tr>
<td>11</td>
<td>3,720 ± 389</td>
<td>5,066 ± 755</td>
<td>6,345 ± 907</td>
<td>4,858 ± 1,174</td>
<td>4,270 ± 990</td>
</tr>
</tbody>
</table>

^a Significant difference from control (p < 0.01).
DISCUSSION

The sequential increase in the dividing marrow granulocytes and the rapid, overshooting restoration of the nondividing granulocyte marrow reserve are clear evidence of marked stimulation of granulopoiesis by androgenic or anabolic steroids in the BCNU-suppressed mice. No detailed marrow counts were performed on the vinblastine- or Cytoxan-treated mice, but the close similarity in the pattern of blood neutrophil increases leaves little doubt that the latter also resulted from a true stimulation of granulopoiesis in these mice. The cytotoxic effects of these 3 drugs did not reduce the CFU levels below 20% of normal whereas, in the Myleran mice, the marrow CFU were below 1% of normal. The failure of 19-ND injections to induce granulopoietic stimulation in the latter mice indicates the presence of an adequate stem cell population as a prerequisite of the androgen effect, and this finding may explain the therapeutic failure of androgens in some patients with aplastic anemia (4). Only tentative conclusions can be drawn as to the cell stage affected by the androgen action. Chronologically, the first effects were increases in CFU and CFC, but this would not rule out enhancing effects on later stages, for instance, a stimulation of self-replication of myeloblasts. However, such a mechanism seems less likely in view of the lack of granulopoietic effect in the Myleran-treated mice given 19-ND on Days 5 to 9. The myeloblasts in the control mice were still 35% of normal during that time (10), and some increase in neutrophils would be expected if 19-ND had stimulated the self-replication of myeloblasts. In the BCNU-suppressed mice, the rise and fall of CFC preceded that of myeloblasts, and it seems likely that the myeloblast increase was the direct result of the increase in CFC, in the sense that the presence of a greater number of CFC permitted a greater number to differentiate into myeloblasts. It is known that each myeloblast undergoes a number of successive divisions with concomitant maturation, and hence a greater number of myeloblasts can be expected to result in a greater rate of neutrophil production. Rosenblum and Carbone (8) recently showed that the in vitro addition of testosterone to cultures of human marrow increased the number of colonies, and on cytokinetic grounds they attribute.

Table 2
Blood neutrophils in mice given 5-day courses of 19-ND injections at various intervals after treatment with Myleran (44 mg/kg)

<table>
<thead>
<tr>
<th>Day after Myleran (as % of normal)</th>
<th>CFU (% of normal)</th>
<th>Neutrophils/cu mm blood</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>2,475 ± 311</td>
</tr>
<tr>
<td>1</td>
<td>9.4</td>
<td>2,475 ± 311</td>
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<tr>
<td>2</td>
<td>4.5</td>
<td>1,682 ± 382</td>
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<tr>
<td>3</td>
<td>1.2</td>
<td>1,682 ± 382</td>
</tr>
<tr>
<td>4</td>
<td>&lt;1</td>
<td>1,682 ± 382</td>
</tr>
<tr>
<td>5</td>
<td>258 ± 44</td>
<td>367 ± 98</td>
</tr>
<tr>
<td>6</td>
<td>81 ± 17</td>
<td>3,645 ± 925</td>
</tr>
<tr>
<td>8</td>
<td>178 ± 47</td>
<td>4,062 ± 860</td>
</tr>
<tr>
<td>10</td>
<td>223 ± 115</td>
<td>628 ± 184</td>
</tr>
<tr>
<td>12</td>
<td>1,059 ± 501</td>
<td>4,062 ± 860</td>
</tr>
</tbody>
</table>

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this effect to a direct stimulation of CFC proliferation. Our findings would be compatible with such a mode of action, but other mechanisms need to be considered, in particular, the possible effects of androgens on the level of the colony-stimulating factor. Some workers consider that this factor represents the physiological regulator of granulopoiesis (9), and further work is needed to clarify the granulopoietic effect of steroids in relation to this factor.

The 19-ND injections also caused a significant relative increase in the number of CFU in the femoral marrow of BCNU-suppressed mice. Byron (1) has shown that androgenic steroids in vitro induced an increased proliferation of CFU and resulted in vivo in absolute increases in the number of marrow CFU in normal mice. It seems highly likely, therefore, that the accelerated regeneration of CFU in the BCNU mice was the result of a stimulation of the proliferation of surviving CFU by the injected steroid. However, the relationship between the observed relative increase in CFU and that of CFC and their progeny is not clear at this time. The role of the CFU may well be a permissive one in the sense that a certain CFU population density is required to permit cell inflow into the CFC compartment, and that this inflow is essential in maintaining the CFC population which per se is not self-maintaining. The results of the Myleran experiments would be compatible with such a concept. The granulopoietic effect of 19-ND injected during the 1st few days after Myleran may have been due to its action on the CFC that had survived the drug, but in the absence of CFU the CFC population became rapidly depleted, and later injections of 19-ND had no granulopoietic effect. Although the primary granulopoietic effect of 19-ND thus may result from its stimulation of CFC proliferation, it must be emphasized that the stimulation of CFU regeneration may also be of importance, namely, under conditions in which the accelerated regeneration of CFU is the prerequisite for restoration of cell inflow into the CFC compartment. Further work is obviously necessary to delineate the relationship of the steroid action on these 2 stem cell populations.

REFERENCES

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