The Effect of Etiocholanolone on Granulocyte Kinetics

By JAMES M. VOGEL, RONALD A. YANKEE, HARRY R. KIMBALL, SHELDON M. WOLFF AND SEYMOUR PERRY

ETIOCHOLANOLONE (3a-hydroxy-5β-androstan-17-one), a metabolite of dehydroepiandrosterone, produces fever and leukocytosis when administered to man. The increment in circulating leukocytes is comprised principally of mature polymorphonuclear granulocytes with a small number of immature cells. In contrast to the response to bacterial endotoxin, early leukopenia, thrombocytopenia, and absolute mononucleopenia have not been observed. Although the immediate origin of the cells mobilized into the peripheral circulation is unknown, it has been suggested that these cells are released directly from the bone marrow rather than the result of a redistribution of granulocytes within the vascular tree.

In the studies to be described, the effects of etiocholanolone on granulocyte kinetics were investigated by use of radioactive cell labeling technics.

MATERIALS AND METHODS

The study group was composed of four males and eight females, all of whom had a diagnosis of neoplastic disease (Tables 1 and 2). Nine of these patients were studied at least 2 years after administration of cytotoxic drugs or radiotherapy and had no evidence of disease at the time of these studies. One patient (C.H.), who was clinically free of disease, was studied 1 month after mid-thigh amputation for osteogenic sarcoma. A patient with Stage IIIa Hodgkin's disease (A.C.) and another with bronchogenic carcinoma (B.B.) were studied prior to treatment. In these latter two patients, bone marrow examination was normal. In all subjects the hemoglobin and white blood cell and differential counts were within the normal range. During the period of investigation, none of the patients received antimumor therapy.

Previous studies in normal volunteers have shown that an intramuscular dose of 0.2 mg/Kg. etiocholanolone (in propylene glycol, 10 mg/ml.) results in a mean maximum granulocyte increase of 9100 cells per cu. mm. with a minimum increment of 2600 cells per cu. mm. Propylene glycol when administered alone had no significant effect on the white blood count. A characteristic response curve, derived from the previous studies in 14 normal individuals, is shown in Figure 1 and illustrates the time relationship between the injection of etiocholanolone and the onset and duration of granulocytosis. Granulocyte counts reach a maximum approximately 12 hours after injection and then remain relatively constant for the subsequent 7 to 8 hours (Fig. 1). All labeling procedures in patients receiving etiocholanolone were performed during this latter period in which steady state kinetics may reasonably be assumed to apply.

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Fig. 1.—The mean change in circulating granulocytes and mononuclear cells over baseline values following a zero time injection of 0.2 mg./Kg. of etiocholanolone in seven male and seven female normal subjects. The decrease in mononuclears is not statistically significant.

Baseline leukocyte kinetic studies, using the labeling procedure described below, were performed in seven patients, including the two with active disease. The studies were repeated within 1 week in the same individuals after the administration of etiocholanolone. The control group included five patients in whom leukocyte labeling procedures were also performed on two separate occasions but without etiocholanolone in order to evaluate the reproducibility of the method.

All leukokinetinc studies were begun between 8 A.M. and 10 A.M. Following a white blood cell count (WBC) and differential count, 400 to 500 ml. of whole blood was collected from each subject into sterile pyrogen-free plastic bags containing 75 ml. ACD (NIH Formula A). Leukocyte labeling was accomplished with tritiated diisopropylfluorophosphosphate (3H-DFP) by a modification of a technic previously described. The contents of a vial containing 250 to 350 μci of 3H-DFP† with a specific activity of 1 curie per millimole were injected directly into the bags, which were then incubated with gentle agitation at room temperature for 45 minutes. A 10 ml. aliquot was obtained for analysis and the remainder was then infused into the donor within 10 to 15 minutes. The exact volume of blood infused was obtained by subtracting the weight of the empty bag from that of the blood-filled bag prior to infusion. Serial 10 ml. blood samples were obtained by venepuncture at 12 minutes and 1, 3, 5, 7%, and 22 to 24 hours after termination of the infusion. Duplicate white blood cell counts were performed using an electronic counter and 200 cell differential counts were done on blood from the infusate and from all serial samples.

The leukocytes from each sample were separated from the red cells and platelets by a method of dextran sedimentation, hypotonic lysis and differential centrifugation. Siliconized glassware was used throughout. Platelets were removed by three centrifugations for 10 minutes at 600 g. The isolated leukocytes were suspended uniformly in a measured volume of saline and counted in duplicate. Red blood cell and platelet counts were performed using an electronic counter.*

*Fenwall Laboratories, Morton Grove, Illinois.
†New England Nuclear Corporation, Boston, Massachusetts.
‡Coulter Counter, Model B, Hialeah, Florida.
Table 1.—Leukokinetic Studies in Patients before and after Etiocholanolone

<table>
<thead>
<tr>
<th>Patient</th>
<th>Diagnosis</th>
<th>Study 1 Baseline</th>
<th>Study 2 Etiocholanolone</th>
<th>Change in TBGP (Study 2 minus Study 1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T 1/2 (hours)</td>
<td>CGP</td>
<td>MGP</td>
<td>TBGP</td>
</tr>
<tr>
<td>B.B.</td>
<td>Carcinoma of lung</td>
<td>3.4</td>
<td>26.0</td>
<td>23.2</td>
</tr>
<tr>
<td>A.C.</td>
<td>Hodgkin's disease</td>
<td>3.6</td>
<td>39.2</td>
<td>120.8</td>
</tr>
<tr>
<td>D.G.</td>
<td>Hodgkin's disease</td>
<td>4.0</td>
<td>18.7</td>
<td>28.5</td>
</tr>
<tr>
<td>D.C.</td>
<td>Hodgkin's disease</td>
<td>4.0</td>
<td>14.7</td>
<td>79.4</td>
</tr>
<tr>
<td>W.V.</td>
<td>Hodgkin's disease</td>
<td>3.7</td>
<td>37.3</td>
<td>64.0</td>
</tr>
<tr>
<td>G.H.</td>
<td>Osteogenic sarcoma</td>
<td>3.2</td>
<td>26.8</td>
<td>40.3</td>
</tr>
<tr>
<td>B.H.</td>
<td>Testicular embryonal</td>
<td>5.5</td>
<td>32.6</td>
<td>78.5</td>
</tr>
<tr>
<td></td>
<td>cell carcinoma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>3.9</td>
<td>27.9</td>
<td>62.1</td>
</tr>
<tr>
<td>Standard error</td>
<td></td>
<td>0.3</td>
<td>3.5</td>
<td>13.0</td>
</tr>
</tbody>
</table>

* Circulating granulocyte pool, 10⁶ granulocytes per Kg.
† Marginal granulocyte pool, 10⁶ granulocytes per Kg.
‡ Total blood granulocyte pool, 10⁶ granulocytes per Kg.
§ Increase in granulocytes (per cu. mm.) over baseline 12 hours after etiocholanolone administration.
formed to determine the degree of contamination by these cells. The suspension contained an average of 60 per cent (range 45 to 70 per cent) of leukocytes, less than 0.01 per cent of the red cells, and less than 2 per cent of the platelets originally present in each 10 ml. sample. There was no significant change in differential counts before and after white blood cell isolation.

The leukocyte suspension was adjusted to contain $1 \times 10^8$ to $8 \times 10^8$ cells and centrifuged for 15 minutes at 1200 g. The sedimented leukocytes were then hydrolyzed in either 5 per cent NaOH or in basic reagent NCS Solubilizer and transferred to a scintillator solution consisting of one part Liquifluor, 10 parts ethylene glycol monomethyl ether, 15 parts toluene, and two parts naphthalene. Samples were counted for 100 minutes in a liquid scintillation counter. Quenching was monitored by a channel ratio method and corrections made accordingly.

In each study counts per minute (c.p.m.) per 10$^8$ cells were plotted against time on semilogarithmic paper. A straight line of best fit connecting the points during the initial 7½ hours was constructed, and extrapolation of this line to the ordinate yielded a value of c.p.m. per 10$^8$ cells at zero time. Calculations of granulocytes in the blood compartments were based on this figure. The reciprocal of the slope of this line was taken as the half-time ($t_{1/2}$) for the disappearance of labeled granulocytes. The circulating granulocyte pool (CGP) was calculated by multiplying the granulocyte count per cu. mm. by the blood volume as determined by the body surface area method of Baker et al.\* 

\[ \text{CGP} = \left( \frac{WBC}{100} \right) \times \text{Blood Volume} \]

The total blood granulocyte pool (TBGP) was calculated from the isotope dilution method of Mauer et al.\* and Athens et al.\* in which the radioactivity of the tagged unit is assumed to be equal to the radioactivity in the blood at zero time.

\[ \text{TBGP} = \frac{\text{(Specific Activity Infused Granulocytes)}}{\text{(Total Granulocytes Infused)}} \times \frac{\text{(Specific Activity Blood Granulocytes)}}{\text{(Specific Activity Blood Granulocytes)}} \]

The marginal granulocyte pool (MGP) was calculated by subtracting the CGP from the TBGP.\*\*

**Results**

Determinations of TBGP, CGP, and MGP prior to and following the administration of etiocholanolone in seven subjects are presented in Table 1. In every instance injection of etiocholanolone was followed by an expansion of all three blood compartments when compared to baseline values. The large mean increases in the sizes of these pools are in contrast to the small mean differences in pool sizes calculated from duplicate studies without etiocholanolone performed in five control subjects (Table 2). Furthermore, the data in Table 2 attest to the reproducibility of the experimental methods employed. The alteration of pool size induced by etiocholanolone is most apparent when the mean changes between individual paired studies are compared (Tables 1 and 2). In the etiocholanolone group a 98 per cent mean increase in TBGP was found in contrast to an 8 per cent mean decrease in the control group. These differences are significant (p < .01, t test).

The numbers of granulocytes present in the circulation immediately after infusion of labeled cells can be expressed as the ratio of the CGP divided by the TBGP. This ratio calculated from the mean pool sizes measured before (0.34) and after (0.36) etiocholanolone shows little variation, indicating that

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†TM Pilot Chemicals, Inc., Watertown, Massachusetts.

‡Tri-Carb Liquid Scintillation Spectrometer, Model 3002, La Grange, Illinois.

§Body surface area in square meters $\times$ 2680.
### Table 2.—Leukokinetic Studies in Control Patients Not Receiving Etocholanolone

<table>
<thead>
<tr>
<th>Patient</th>
<th>Diagnosis</th>
<th>Study 1 T ( \frac{1}{2} ) (hours)</th>
<th>CGP *</th>
<th>MGP †</th>
<th>TBGP ‡</th>
<th>Study 2 T ( \frac{1}{2} ) (hours)</th>
<th>CGP *</th>
<th>MGP †</th>
<th>TBGP ‡</th>
<th>( 10^9 ) Granulocytes/Kg</th>
<th>Per cent Change in TBGP (Study 2 minus Study 1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G.M.</td>
<td>Carcinoma pharynx</td>
<td>2.2</td>
<td>29.0</td>
<td>23.1</td>
<td>52.1</td>
<td>0.56</td>
<td>2.8</td>
<td>33.5</td>
<td>26.0</td>
<td>69.5</td>
<td>+17.4 +33.4</td>
</tr>
<tr>
<td>H.G.</td>
<td>Carcinoma nasopharynx</td>
<td>3.4</td>
<td>23.8</td>
<td>44.1</td>
<td>67.9</td>
<td>0.35</td>
<td>3.8</td>
<td>24.6</td>
<td>30.2</td>
<td>54.8</td>
<td>-13.1 -19.3</td>
</tr>
<tr>
<td>R.B.</td>
<td>Seminoma</td>
<td>3.0</td>
<td>27.6</td>
<td>25.2</td>
<td>52.8</td>
<td>0.52</td>
<td>3.5</td>
<td>24.1</td>
<td>20.3</td>
<td>44.4</td>
<td>-8.4 -15.9</td>
</tr>
<tr>
<td>R.M.</td>
<td>Carcinoma pharynx</td>
<td>4.3</td>
<td>27.0</td>
<td>94.7</td>
<td>121.7</td>
<td>0.22</td>
<td>4.4</td>
<td>26.1</td>
<td>47.0</td>
<td>73.1</td>
<td>-48.6 -39.9</td>
</tr>
<tr>
<td>W.R.</td>
<td>Carcinoma pharynx</td>
<td>3.9</td>
<td>20.0</td>
<td>32.1</td>
<td>52.1</td>
<td>0.38</td>
<td>3.8</td>
<td>21.5</td>
<td>31.2</td>
<td>52.7</td>
<td>+0.6 +1.1</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>3.4</td>
<td>25.5</td>
<td>43.8</td>
<td>69.3</td>
<td>0.40</td>
<td>3.7</td>
<td>26.0</td>
<td>32.9</td>
<td>58.9</td>
<td>-10.4 -8.1</td>
</tr>
<tr>
<td>Standard error</td>
<td></td>
<td>0.4</td>
<td>1.6</td>
<td>13.2</td>
<td>13.4</td>
<td>0.06</td>
<td>0.3</td>
<td>2.0</td>
<td>4.3</td>
<td>5.4</td>
<td>0.03 10.9 12.8</td>
</tr>
</tbody>
</table>

* Circulating granulocyte pool, \( 10^9 \) granulocytes per Kg.
† Marginal granulocyte pool, \( 10^9 \) granulocytes per Kg.
‡ Total blood granulocyte pool, \( 10^9 \) granulocytes per Kg.
Fig. 2.—The relationship between the granulocyte response to etiocholanolone and the per cent change in the total blood granulocyte pool. The solid line is constructed by the method of least squares. The broken lines indicate the 95 per cent confidence limits.

both the CGP and TBGP are increased in the same proportion after granulocyte mobilization (Table 1). Moreover, the per cent change of the TBGP after the administration of etiocholanolone is proportional to the number of granulocytes mobilized by this agent (Fig. 2). The per cent change in the TBGP in two patients of this group was similar to that recorded in the control studies (+11 per cent and +39 per cent, respectively). It is of interest that these two patients (Table 1) had impaired responses to etiocholanolone when compared to normal subjects.

In all studies a semilogarithmic graph of counts per minute per 10\(^7\) cells versus time was linear over the first 7½ hours, permitting calculation of the 50 per cent survival time (T%) of the circulating cells. The mean T% of the control group (Table 2) and the mean T% of the group studied prior to and following etiocholanolone stimulation (Table 1) were similar. In both groups extrapolation of the line to 22 or 24 hours resulted in a projected specific activity which was lower than the measured specific activity. This difference was large in the patients responsive to etiocholanolone and was smaller in the other. As illustrated in Figure 3, patient B.B. mobilized only 950 granulocytes per cu. mm. following etiocholanolone stimulation, and the granulocyte survival curve was virtually identical with that of the baseline study. Patient D.G., however, mobilized 5050 granulocytes per cu. mm. after injection of this substance, resulting in increased specific activity over baseline at 24 hours. Thus, the granulocyte specific activity at 22 or 24 hours was related to the granulocytic response after etiocholanolone.

**DISCUSSION**

In these studies, the effect of etiocholanolone on granulocyte kinetics and
Fig. 3.—Survival curves of $^3$H-DFP-labeled granulocytes before and after etiocholanolone. Counts per minute per $10^7$ cells have been adjusted to a similar scale. Patient B.B. had an increase of only 850 granulocytes per cu. mm. after etiocholanolone injection, and granulocyte survival was similar to that of the baseline study. Patient D.G. had an increase of 5050 granulocytes in response to etiocholanolone and showed a markedly higher leukocyte specific activity than the baseline study at 24 hours.

Intravascular pool sizes has been determined. In order to assess the reproducibility of the methods employed, five control subjects who received no etiocholanolone were studied on two separate occasions. The mean total blood granulocyte pools were $69.3 \pm 13.4 \times 10^7$ and $58.9 \pm 5.4 \times 10^7$ granulocytes/kg. These values agree with those reported for normal male volunteers ($65.0 \pm 22.4 \times 10^7$ and $68.0 \pm 19.3 \times 10^7$ granulocytes/kg.) by Athens et al.\textsuperscript{12} and Alexanian and Donahue,\textsuperscript{13} respectively. The mean TBGP in the group studied before etiocholanolone (Table 1) was larger than the mean pool size of the control group (Table 2) but is within the same range and reflects an abnormally high value in one patient (A.C.) with active Hodgkin's disease. It is of interest that in active Hodgkin's disease an expanded TBGP may be present without an elevation of circulating granulocytes.\textsuperscript{14}

The administration of etiocholanolone was followed in 12 hours by an increase in CGP, MGP, and TBGP. The CGP/TBGP ratio before and after etiocholanolone changed only slightly, suggesting that the mobilized granulocytes were distributed into the circulating and marginated pools in the same proportion. The large increase observed in the TBGP (98 per cent), compared to baseline after etiocholanolone, must largely be due to an influx of cells from
the bone marrow. Expansion of the TBGP can theoretically result from the
mobilization of leukocytes from the bone marrow or from some tissue pool.
However, the evidence is quite conclusive that the marrow reserve is the chief
source of leukocytes entering the vascular tree in response to a stimulus\(^{15,17}\)
and that if there is another source of cells, it must be quite small.

It has been suggested that circulating white blood cells are trapped in the
spleen and lungs.\(^{16,19}\) Patients with splenomegaly with impaired granulocyte
mobilization following endotoxin administration may have a normal response
after splenectomy.\(^{19,20}\) In two subjects (B.B. and A.C.) a diminished granulo-
cyte response was associated with only a slight increase in the TBGP. Both
patients had extensive metastatic disease but in neither was hepatospleno-
megaly present. No apparent explanation for the depressed granulocyte
response was found, but conceivably marrow involvement by the neoplastic
process could be contributory.\(^{20}\)

The mean T% in the control (Table 2) and baseline (Table 1) studies
were 3.4 ± 0.4, 3.7 ± 0.3, and 3.9 ± 0.3 hours, which agree closely with the
mean T% of 3.8 ± 0.9 hours reported by Alexanian and Donahue.\(^{13}\) These
times are less than the T% of 6.6 ± 1.16 obtained by Athens et al.\(^{12}\) The reasons
for these differences are not clear. It does not seem to be related to the type
of anticoagulant employed, as has been suggested since EDTA was used by
Alexanian and Donahue,\(^{13}\) while ACD was used by Athens et al.\(^{12}\) and in the
present study. The rapid decline in specific activity during the first few hours,
followed by a more gradual decline, might be explained in several ways. It is
conceivable that a portion of the labeled cells may have been damaged and
that their removal is responsible for the initial rapid decline in specific activity.
It is also possible that there are two “populations” of granulocytes, one with a
short T% and one with a longer T%. However, the most likely explanation is
that the granulocytes equilibrate rapidly with a readily accessible pool which
in turn equilibrates more slowly with a larger compartment. The latter hy-
thesis has been suggested previously.\(^{13}\)

The specific activity curves for both the control and baseline studies are
linear for the first 7% hours so that a T% can be calculated accurately only
for this period. If these lines were to be extended, they would fall below the
specific activity actually observed at 22 or 24 hours. Others have made similar
observations,\(^{13}\) suggesting that this was due to reappearance of approximately
2 per cent of the tagged cells at 24 hours. A steady state is a requisite for
linearity in survival curves, but peripheral leukocyte counts are higher in the
evening hours compared to those in the morning.\(^{21}\) The present studies were
so designed that the 22- or 24-hour determination was made during the
morning hours. If the rate of entry of unlabeled cells from the bone marrow is
decreased from midnight to early a.m., the observed specific activity at 22 or
24 hours will be higher. It does not, therefore, appear necessary to postulate
the reappearance of labeled cells.

During the first 7% hours, the T% of circulating labeled cells following
etiocholanolone injection has been shown to be identical with that obtained
in the baseline studies. Beyond this time the specific activity of labeled cells in
patients responsive to etiocholanolone is proportionally greater than that ob-

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served in the baseline studies. The high specific activity at 22 or 24 hours can be explained by a decreased inflow into the TBGP of nonlabeled cells presumably from the bone marrow.

The level of the peripheral blood granulocyte count depends upon the presence of an adequate bone marrow reserve of granulocytes and a normal leukocyte release mechanism. By the use of isotopic cell labeling technics, it has been shown that the granulocytes in the vascular tree are distributed equally between two pools, the CGP and the MGP, which together comprise the TBGP. This distribution is altered in response to various agents. Epinephrine increases the size of the CGP at the expense of the MGP, but the TBGP remains unchanged. In contrast, bacterial endotoxin and prednisone enlarge the TBGP by mobilization of granulocytes presumably from the bone marrow. Based on these observations, bacterial endotoxin has been used to assess bone marrow granulocyte reserve. The present studies would suggest that etiocholanolone has effects similar to those of endotoxin on granulocyte kinetics and may be useful as an agent in the estimation of granulocyte reserves in man.

SUMMARY

The effect of etiocholanolone on granulocyte kinetics in 12 hematologically normal patients has been investigated using the technic of 3H-DFP labeling of autologous blood in vitro.

Baseline determinations of the total blood granulocyte pool (TBGP), the circulating pool (CGP), and the marginated pool (MGP) were performed. The values for the total blood granulocyte pools were similar to those previously reported. Following the administration of etiocholanolone, there was a 98 per cent increase in the TBGP, which was considered to be due to mobilization of granulocytes from the bone marrow reserve. There was no change in the ratio of CGP to MGP.

These studies suggest that etiocholanolone may be a useful agent for the estimation of bone marrow reserve.

SUMMARIO IN INTERLINGUA

Esseva utilisate le technica del marcacion in vitro de sanguine autologe con diisopropylfluorophosphato a tritium in le studio del effecto de etiocholanolona super le kinetica del granulocytos de 12 hematologicamente normal patientes.

Esseva effectuate determinaciones basal (i.e., de controlo) del total pool sanguinee de granulocytos (TPSG), del pool circulante de granulocytos (PCG), e del pool marginate de granulocytos (PMG). Le valores obtenite pro le TPSG esseva simile a illos previemente reportate in le litteratura. Post le administration de etiocholanolona, il occurreva un aumento per 98 procento in le TPSG, reguardate como efecto del mobilisation de granulocytos ab le reservas del medulla ossee. Esseva constatare nulle alteration del proportion de PCG a PMG.

Iste studios suggestiona que etiocholanolona es possiblemente un utile agente in le estimation del reservas del medulla ossee.

ACKNOWLEDGMENTS

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EFFECT OF ETIOCHOLANOLONE

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