The Effect of Etiocholanolone on The Entry of Granulocytes into The Peripheral Blood

By Herman A. Godwin, Theodore S. Zimmerman, Harry R. Kimball, Sheldon M. Wolff and Seymour Perry

 Previous studies of granulocyte kinetics in hematologically normal individuals employing tritiated thymidine (H\textsuperscript{3}TdR) have indicated that there is a 4-5 day period after administration of the radioisotope before labeled granulocytes appear in the peripheral blood.\textsuperscript{1-3} The large mass of non-proliferating granulocytic cells constitutes the marrow granulocyte reserve (MGR) from which the majority of mature granulocytes are released in an orderly fashion.\textsuperscript{4} However, in response to a variety of stimuli such as leukophoresis, bacterial endotoxin, and acute bacterial infection, entry of granulocytes into the peripheral blood from the MGR may be accelerated.\textsuperscript{2,5-8} The bone marrow constitutes the principal reservoir for granulocytes, and it has been established that cells of the myeloid series which leave the vascular tree normally do not return from the tissues.\textsuperscript{6}

Etiocholanolone, a naturally occurring steroid metabolite, has been shown to cause leukocytosis consisting primarily of mature polymorphonuclear neutrophils when injected intramuscularly into man.\textsuperscript{9,10} Following injection, maximum granulocytosis is reached between 14 and 18 hours followed by a slow decline to normal at 24-36 hours.\textsuperscript{10} Based on studies using tritiated diisopropylfluorophosphate labeling of autologous leukocytes, the increment in circulating granulocytes is associated with marked expansion of the total blood granulocyte pool with proportional increases of both the marginal and circulating granulocyte pools. These data give presumptive evidence that the granulocytes mobilized in response to etiocholanolone come from the bone marrow reserve.\textsuperscript{11}

We have studied the effect of etiocholanolone on the appearance of labeled

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<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Sex</th>
<th>Race</th>
<th>Diagnosis</th>
<th>Previous Therapy</th>
<th>Hct</th>
<th>WBC (mm(^3))</th>
<th>Platelets (mm(^3))</th>
<th>Bone Marrow Examination</th>
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<tr>
<td>1</td>
<td>62</td>
<td>F</td>
<td>W</td>
<td>Hodgkin's disease, Stage IV B (12)</td>
<td>Vincristine, HN(_{2}), prednisone, and methylhydrazine 8 months prior to study</td>
<td>38</td>
<td>5100</td>
<td>157,000</td>
<td>Normal</td>
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<tr>
<td>2</td>
<td>60</td>
<td>M</td>
<td>N</td>
<td>Mycosis fungoides, extensive</td>
<td>Radiation therapy 1 year and prednisone 4 months before study</td>
<td>40</td>
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<td>241,000</td>
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<td>3</td>
<td>48</td>
<td>F</td>
<td>W</td>
<td>Malignant melanoma, metastatic</td>
<td>None</td>
<td>39</td>
<td>5100</td>
<td>236,000</td>
<td>Mild erythroid hyperplasia</td>
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<tr>
<td>4</td>
<td>45</td>
<td>F</td>
<td>W</td>
<td>Malignant melanoma, metastatic</td>
<td>None</td>
<td>39</td>
<td>5100</td>
<td>188,000</td>
<td>Normal</td>
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<tr>
<td>5</td>
<td>50</td>
<td>M</td>
<td>W</td>
<td>Carcinoma of stomach, metastatic</td>
<td>None</td>
<td>40</td>
<td>9100</td>
<td>543,000</td>
<td>Normal</td>
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<tr>
<td>6</td>
<td>50</td>
<td>M</td>
<td>W</td>
<td>Carcinoma of mouth, recurrence</td>
<td>Radical neck surgery 4 years prior to study</td>
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<td>7300</td>
<td>247,000</td>
<td>Mild erythroid hyperplasia</td>
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<td>7</td>
<td>48</td>
<td>F</td>
<td>W</td>
<td>Mycosis fungoides, extensive</td>
<td>Electron beam and cytoxan therapy 1 year before study</td>
<td>38</td>
<td>5500</td>
<td>233,000</td>
<td>Mild erythroid hyperplasia</td>
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</table>
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granulocytes in the peripheral blood following administration of H³TdR. The data provide direct evidence for an accelerated mobilization of granulocytes from marrow granulocyte stores and further indicate that marrow granulocytes cannot be released by etiocholanolone stimulation until cellular maturation has taken place.

MATERIALS AND METHODS

Pertinent patient data are summarized in Table 1. The study group consisted of seven hematologically normal individuals (three males and four females) with malignant diseases and shortened life expectancies. Patient No. 1 had received cancer chemotherapy eight months before study and patient No. 2 had been given a short course of prednisone four months prior to investigation. The remaining patients had either received no treatment or at least one year had elapsed since the last therapy. In all cases peripheral blood counts were normal. Bone marrow examinations in three patients showed mild erythroid hyperplasia but were otherwise unremarkable.

Each patient was studied on two separate occasions with H³TdR* (specific activity 1.9 c. per mole), 75 µc. per kg. body weight, injected intravenously in a single dose. Peripheral blood samples of 10–20 ml. with 5 percent EDTA as the anticoagulant were drawn just before injection of the radioisotope and at predetermined intervals thereafter. Sterile, pyrogen-free needles and syringes were used. Leukocytes were isolated by dextran sedimentation and hypotonic lysis as previously described.† The cells were washed in normal saline and an aliquot counted in an automatic cell counter.‡ Cell buttons containing a known number of leukocytes (approximately 1 × 10⁹) were prepared by centrifugation at 2100 g. for 10 min. and then hydrolyzed in 0.5–1.0 ml. of NCS® reagent.§ Following the addition of 15 ml. toluene phosphor scintillation fluid, radioactivity was determined in a liquid scintillation spectrometer.¶ The efficiency for tritium with this system was 30 percent. Correction for quenching was by means of an external standard.

Radioautographs were prepared from each whole blood sample. Smears were made on clear gelatin-coated microscope slides, air dried, and fixed in absolute methanol. Kodak-AR 10 stripping film was applied and the slides were exposed for 4–6 months at 4 C. before being developed, fixed, and stained with Giemsa stain.

During the second study the patients (with the exception of patient No. 1) were given etiocholanolone.‖ 0.3 mg. per kg. body weight, in a single intramuscular injection at varying time intervals prior to or following H³TdR administration as shown in Table 2. Finger stick blood samples were obtained for white blood cell and differential counts prior to injection and at 9, 12, 15, and 18 hours after etiocholanolone. Patients were at bed rest except for bathroom privileges during the period of study. Vital signs were monitored every four hours for 24 hours.

White blood cells were counted in duplicate in the electronic cell counter after saponin lysis of red blood cells, and the average was recorded as the white blood cell count. Differential counts of 100 cells on a Giemsa stained blood smear were performed to determine the baseline granulocyte count and the granulocyte increment (ΔG) associated with etiocholanolone administration. The normal ΔG to this dose of etiocholanolone has previously been established as 2600 granulocytes or more per mm.³ ¹⁰

RESULTS

The peripheral blood leukocyte specific activity curves obtained from base-

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†Coulter Electronics, Hialeah, Florida.
‖Merck, Sharp, and Dohme, West Point, Pennsylvania. Kindly supplied by the Cancer Chemotherapy National Service Center.
Fig. 1.—Patient Nos. 2, 3, and 4 demonstrate that etiocholanolone given before and up to 50 hours after H^3Tdr administration does not affect the peripheral leukocyte kinetic pattern.
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Fig. 2—Patient Nos. 6, 7, and 8. After HDTG administration, the early release of labeled granulocytes was most pronounced in patient No. 7 with the smallest granulocyte increment and least in patient No. 6 who had the largest ΔC.
Table 2.—Effect of Etiocholanolone on the Appearance and Peak Activity of H³TdR in Peripheral Blood

<table>
<thead>
<tr>
<th>Patient</th>
<th>Etiocholanolone Administration in Relation to H³TdR Administration (hrs.)</th>
<th>Maximum Granulocyte Increment (per mm.³)</th>
<th>Onset of Major Radioactivity in Baseline Study (hrs.)</th>
<th>Second Study (hrs.)</th>
<th>Peak Activity in Peripheral Blood Baseline Study (hrs.)</th>
<th>Second Study (hrs.)</th>
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<tbody>
<tr>
<td>2</td>
<td>14 before</td>
<td>8,350</td>
<td>96</td>
<td>93</td>
<td>120</td>
<td>140</td>
</tr>
<tr>
<td>3</td>
<td>26 after</td>
<td>2,800</td>
<td>114</td>
<td>114</td>
<td>163</td>
<td>162</td>
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<tr>
<td>4</td>
<td>50 after</td>
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<td>108</td>
<td>188</td>
<td>180</td>
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<td>5</td>
<td>74 after</td>
<td>8,400</td>
<td>118</td>
<td>92</td>
<td>171</td>
<td>139</td>
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<tr>
<td>6</td>
<td>74 after</td>
<td>14,800</td>
<td>101</td>
<td>89</td>
<td>191</td>
<td>155</td>
</tr>
<tr>
<td>7</td>
<td>74 after</td>
<td>2,050</td>
<td>103</td>
<td>83</td>
<td>150</td>
<td>119</td>
</tr>
</tbody>
</table>
line studies of the seven patients were similar to those previously observed in hematologically normal individuals\(^3\) (Figures 1 and 2). There was an early low level of radioactivity present for 96–118 hours followed by a marked rise with peak radioactivity between 120 and 191 hours (Table 2). Decline in radioactivity was rapid thereafter. By radioautographic analysis, it has previously been shown that the early radioactivity is due predominantly to labeled large lymphocytes in addition to occasional monocytes and small lymphocytes, while the rapid increase in radioactivity is due to the appearance of labeled neutrophilic granulocytes.\(^3\) These observations were confirmed in the present studies.

Patient No. 1 was studied on two separate occasions without etiocholanolone in order to establish reproducibility of the method. Because there was no significant change in the leukocyte specific activity pattern in the two studies, no figure is presented.

In the remaining patients etiocholanolone was given at varying times before and after H\(^3\)TdR administration (Table 2). Compared to the baseline studies, there were no significant changes in the peripheral blood leukocyte specific activity patterns when etiocholanolone was given 14 hours before (patient No. 2, Figure 1) or 26 (patient No. 3, Figure 1) and 50 hours (patient No. 4, Figure 1) after H\(^3\)TdR. Each individual had a normal granulocyte response to etiocholanolone with granulocyte increments of 8350 per mm\(^3\), 2800 per mm\(^3\), and 3000 per mm\(^3\) respectively (Table 2).

By contrast, when etiocholanolone was administered 74 hours following H\(^3\)TdR (patient Nos. 5, 6, and 7), an early appearance and an early peak of radioactivity composed of labeled granulocytes occurred in each case (Figure 2). The initial appearance of radioactivity was 26 (patient No. 5), 12 (patient No. 6), and 20 (patient No. 7) hours earlier than in the baseline studies while the peak activity was 32, 36, and 31 hours earlier, respectively. These differences were significant for both the early onset (\(p < 0.05\)) and peak (\(p < 0.05\), t test) of radioactivity. Maximum granulocyte increments in response to etiocholanolone were 8400 per mm\(^3\) (patient No. 5), 14,800 per mm\(^3\) (patient No. 6), and 2050 per mm\(^3\) (patient No. 7) (Table 2). The \(\Delta G\) in patient No. 7 was abnormal suggesting an inadequate MGR. In patient Nos. 5 and 6, who had normal granulocyte increments to etiocholanolone administration, the appearance of unlabeled granulocytes in the peripheral blood preceded the rise in leukocyte specific activity by 10 and 30 hours. In contrast, patient No. 7 who had an abnormal granulocyte response had granulocytosis occurring almost simultaneously with the rise in peripheral blood radioactivity.

**DISCUSSION**

In hematologically normal individuals, there is a sequential progression in the development of myeloid cells in the bone marrow. There is a self-replicating stem cell pool, a differential proliferating pool (myeloblasts, promyelocytes, myelocytes), a non-proliferating pool (metamyelocytes, bands, segmented neutrophils) where maturation occurs, and circulating in the peripheral blood there is a functional pool (primarily segmented neutrophils).\(^{14}\) The most
mature cell capable of division, and thus of incorporating H^3TdR, is the myelocyte.\textsuperscript{14,15} The minimum time required for a myelocyte to mature to a polymorphonuclear neutrophil is 72 hours.\textsuperscript{14} Once the neutrophil stage is reached, cells usually remain in the bone marrow pool for 24 hours or more before release into the peripheral blood.\textsuperscript{2,4} Thus, these cells constitute the MGR and are available for mobilization to the functional pool.\textsuperscript{4}

Studies of peripheral blood leukocyte kinetics employing single intravenous injections of H^3TdR followed by liquid scintillation counting\textsuperscript{2} and radioautography\textsuperscript{2,3} have defined the normal emergence and labeling patterns of leukocytes. The early low levels of radioactivity are due to labeled mononuclear cells as discussed earlier. After 4–5 days, there is a sudden, rapid rise in leukocyte radioactivity which reaches a peak at 6–8 days and is due to the presence of labeled mature neutrophils.\textsuperscript{2,3} Studies utilizing inorganic radiophosphorous labeling of leukocytes followed by leukocyte DNA extraction have shown similar peripheral blood leukocyte radioactivity patterns in hematologically normal individuals.\textsuperscript{8,16,17}

The baseline leukocyte specific activity patterns of the patients in this study were normal and similar to those previously reported. In the individuals given etiocholanolone before and up to 50 hours after H^3TdR, no significant alterations in the patterns obtained by liquid scintillation counting or in the cells labeled were discernible. However, in the three patients receiving etiocholanolone 74 hours following H^3TdR, there was an early appearance of peripheral blood leukocyte radioactivity due to labeled mature neutrophils.

The results suggest that when the etiocholanolone stimulus occurred up to 50 hours after H^3TdR administration, the mobilization of granulocytes from the mature reserve did not affect the rate of progression of labeled cells through the various compartments sufficiently to alter the leukocyte kinetic pattern. There was no change in the rate of appearance of labeled leukocytes in the peripheral blood indicating that at these time periods mature labeled granulocytes were not present in the mature marrow reserve in numbers adequate to influence the leukocyte radioactivity curve. In addition, the duration of etiocholanolone effect was limited. As Cronkite and Fliedner have reported, the labeled myelocyte requires 72 hours to develop to the mature granulocyte.\textsuperscript{14} If the etiocholanolone stimulus administered at 50 hours had persisted beyond 70 hours, one would have expected an accelerated appearance of labeled granulocytes in the peripheral blood as was seen when the agent was given at 74 hours after H^3TdR. Thus, it was only after a time (72 hours) consistent with progression of the myelocyte to the mature labeled neutrophil that the stimulus of etiocholanolone resulted in the release of labeled myeloid cells from the bone marrow.

In patient No. 7 who had an abnormal granulocyte response to etiocholanolone, the release of granulocytes coincided with the appearance of peripheral blood radioactivity. In the other two patients (Nos. 5 and 6), the granulocytosis in response to etiocholanolone preceded the rise in radioactivity by 10 and 30 hours. Despite normal peripheral blood values and bone marrow morphologic examination, the functional marrow reserve of patient No. 7 was inadequate,
probably because she had previously received considerable cancer chemotherapy. The remaining two patients had never received chemotherapy. It is possible that with a small granulocyte reserve, a potent stimulus such as etiocholanolone will mobilize newly formed granulocytes from marrow reserves as rapidly as they are produced. On the other hand when reserves are larger, this stimulus will first mobilize cells which have been in the reserve pool for a period of time before promoting the release of more recently matured cells. In these three patients, the release of labeled leukocytes in response to etiocholanolone when compared with the baseline studies occurred earliest in the patient with abnormal granulocyte mobilization and latest in the patient with the largest granulocyte increment. Although this observation may be fortuitous, it lends further support to the concept that the timing of mature granulocyte release depends upon the size of marrow reserves.

These studies provide direct evidence that etiocholanolone induces peripheral blood granulocytosis by stimulating the release of mature cells from the bone marrow granulocyte reserves. This evidence supports the concept that etiocholanolone is useful in the estimation of granulocyte reserves in man.

**SUMMARY**

The influence of etiocholanolone on normal granulocyte kinetics was studied in a group of hematologically normal individuals using H³Tdr for cell labeling. Following the establishment of the baseline peripheral blood leukokinetic pattern in each individual, a second study was performed in which etiocholanolone was administered at varying times in relation to H³Tdr. No influence upon the peripheral leukocyte kinetic patterns was detected when etiocholanolone was given prior to 74 hours. When given at that time, an accelerated release of labeled granulocytes from the bone marrow was produced. The degree of acceleration appeared to be related to the size of bone marrow granulocyte reserves.

**SUMMARIO IN INTERLINGUA**

Le influentia de etiocholanolona super le cinetica de granulocytos normal esseva studiate in un gruppo de hematologicamente normal subjectos con le utilisation del methodo a trititiae TdR pro le marcation del cellulas. Post establir pro omne subjecto individual le configuration leucocinetic de base in sanguine peripheric, un secunde studio esseva effec-tuate, munc con le administration de etiocholanolona a varie tempores relative al adminis-tration de trititiae TdR. Nulle influentia de etiocholanolona super le cinetica leucocytic esseva notate quando illo esseva administrate plus que 74 horas ante TdR. Quando le intervallo de tempore esseva 74 horas, un accelerate liberation de granulocytos con marceage ab le medulla ossee esseva producite. Le grado del acceleration pareva esser relateate con le dimensiones del reservas osseo-medullari de granulocytos.

**REFERENCES**


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