The Effect of α-Interferon on Bone Marrow Megakaryocytes and Platelet Production Rate in Essential Thrombocythemia

By Hans Wadenvik, Jack Kutti, Börje Ridell, Peter Revesz, Stefan Jacobsson, Bengt Magnusson, Jan Westin, and Lars Vilen

In 10 patients with previously untreated essential thrombocythemia (ET), by using \(^{111}\)In-labeled platelets and megakaryocyte morphometry, the relation between platelet production rate and bone marrow megakaryocytes was evaluated before and during α-2b-interferon (IFN) therapy. A highly significant decrease in platelet count occurred during IFN therapy: the platelet counts, at baseline and after 2 and 6 months of IFN therapy, were 1,102 ± 345 \times 10^9/L, 524 ± 169 \times 10^9/L (P < .0001), and 476 ± 139 \times 10^9/L (P < .0001), respectively. The decrement in platelet count was mainly a result of diminished platelet production rate, which at baseline and after 2 and 6 months of IFN therapy was 89 ± 30 \times 10^9 platelets/d, 53 ± 18 \times 10^9 platelets/d (P = .0033), and 45 ± 20 \times 10^9 platelets/d (P < .0001), respectively. Also, a slight shortening of platelet mean life-span (MLS) was observed in response to IFN treatment; platelet MLS was 7.96 ± 0.69 days at baseline and 6.68 ± 1.30 days (P = .012) after 6 months of IFN therapy. IFN induced a significant decrease in bone marrow megakaryocyte volume; both megakaryocyte nuclear and cytoplasmatic volumes were affected. The mean megakaryocyte volume was 372 ± 126 \times 10^9 \mu L at baseline and 278 ± 147 \times 10^9 \mu L (P = .049) after 6 months of IFN therapy. However, the number of megakaryocytes did not show any significant change in response to IFN. It is concluded that α-IFN reduces platelet production rate and the peripheral platelet count in ET mainly through an anti-proliferative action on the megakaryocytes and to a considerably lesser degree by a shortening of platelet MLS.

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formed written consent was obtained from all subjects participating in the study.

Induction therapy consisted of daily doses of $5 \times 10^{10}$ IU $\alpha$-2b-IFN (Intron a: Schering-Plough Int., USA) and was administered subcutaneously (SC) over 4 weeks. Thereafter, maintenance therapy with IFN was administered three times weekly at a dose of $5 \times 10^{10}$ IU SC. The treatment with IFN was initiated while the patients were admitted to hospital over 2 to 4 days. Thereafter, they were managed on an out-patient basis.

The venous blood cell counts were determined weekly during the first month of therapy and subsequently with 2- to 4-week intervals. Platelet kinetics were determined immediately before the start of IFN treatment and the platelet production rate was calculated. The platelet kinetic studies were repeated after 2 and 6 months of IFN treatment. The spleen size was measured and megakaryocyte morphology of BM biopsies was performed before and after 6 months of IFN therapy.

$^{111}$In-labeling. The method for $^{111}$In-labeling of autologous platelets and calculation of platelet MLS has been described in detail elsewhere. Briefly, 17 mL of venous blood were anticoagulated with 3 mL ACD-A and spun at 260g for 15 minutes. The supernatant platelet-rich plasma (PRP) was removed, acidified to pH 6.5 by addition of ACD-A, and then centrifuged at 800g for 10 minutes to obtain a platelet button. The platelet-poor plasma (PPP) was saved in a sterile syringe and the platelet button and tube walls were carefully washed with 5 mL Modified Tyrode’s Solution. The platelet button was resuspended in 1 mL of the same solution and the required amount of $^{111}$In-oxine (Amersham Radioschemical Centre, UK) added. After incubation for 2 minutes at 37°C, 2 mL of PPP were added and the suspension centrifuged at 800g for 10 minutes. The supernatant plasma was removed and the labeled platelet button was resuspended in 5 to 6 mL PPP. The radioactivity associated with the supernatant and platelet button was measured in a dose calibrator and the labeling efficiency calculated. Finally, to remove contaminating red blood cells (RBCs), the labeled platelet suspension was spun at 100g for 3 minutes and then aspirated into a disposable syringe. The labeled platelet suspension was injected to the donor through a 19-gauge butterfly infusion set. The injection and the injected weight was divided by 1,027 to obtain the injected volume. Standards consisted of duplicate aliquots of the final labeled platelet suspension and were prepared as follows: (1) 100 μL platelet suspension, (2) 100 μL platelet suspension incubated with 2 mL 0.9% NaCl for 10 minutes, centrifuged at 200g for 20 minutes, supernatant discarded, and the platelet button saved, (3) 100 μL platelet suspension incubated with 2 mL 1% ammonium oxalate for 10 minutes, centrifuged at 2,000g for 20 minutes, supernatant discarded, and the platelet button saved, (4) 100 μL platelet suspension incubated with 2 mL 1% ammonium oxalate for 10 minutes, centrifuged at 2,000g for 20 minutes, supernatant discarded, the platelet button resuspended in 3 mL water. The difference between the unwashed and NaCl-washed standards was taken as the plasma-bound radioactivity, and the difference between the NaCl-washed and ammonium oxalate-washed standards was taken as the RBC-bound radioactivity. The ammonium oxalate-washed standards were used for calculation of in vivo recovery of platelet-bound radioactivity, and the total blood volume was calculated after measurement of body weight and height.

Intrasplenic platelet kinetics. For measurements of intrasplenic platelet kinetics, a compartmental analysis of the initial distribution of platelets between systemic blood and splenic pool was used. For this purpose a large field of view gamma camera (MaxiCamere II; General Electronics), fitted with a medium energy parallel hole collimator (max 370 keV) and interfaced to a computer (PDP 11/34; Digital Equipment) with a nuclear imaging system (Gamma-11; Digital Equipment), was used. The patient was placed in the supine position under the gamma camera so that the liver, spleen, and cardiac blood pool could be monitored. Both gamma photons of $^{111}$In (173 and 240 keV) were collected with a 15% energy window setting. After 15 minutes of rest in this position, the $^{111}$In-labeled platelet suspension was injected through a 19-gauge butterfly infusion set and the radioactivity over liver, spleen, and cardiac blood pool was followed for 30 minutes using a frame time of 30 seconds. The injected dose was 6 MBq. The images were stored in the computer using a 64 x 64 matrix system. Time activity curves for different regions of interest (liver, spleen, and heart) were constructed with the computer. Splenic blood flow, intrasplenic platelet transit time, and the size of the exchangeable splenic platelet pool were calculated after fitting monoeponential functions to the experimental data of the spleen and cardiac blood pool time activity curves.

Platelet survival. Venous blood (30 mL) was collected into 0.34 mol/L K$_2$-EDTA at 2, 3, and 4 hours after infusion of radiolabeled platelets, and subsequently at 24-hour intervals over 7 consecutive days. All blood samples were drawn after 15 minutes of rest in the supine position and avoiding venous occlusion. The packed RBC volume was determined in an impedance cell counter (Coulter Counter Model S-Plus VI; Coulter Electronics, UK). Determination of the platelet count was performed by phase contrast microscopy. For determination of radioactivity, duplicate samples of 3 mL whole blood, lysed with saponin, and 3 mL PPP (obtained by centrifugation at 2,000g for 20 minutes) were prepared in association with each sampling. Also, two platelet buttons were obtained from duplicate aliquots of 5 mL whole blood by differential centrifugation. Whole blood (5 mL) was mixed with 2.5 mL 0.9% NaCl, centrifuged at 1,500g for 1 minute, and the supernatant PRP was transferred to a counting tube; after the addition of 5 mL 0.9% NaCl to the packed cells the latter procedure was repeated. The two harvests of PRP were pooled, centrifuged at 2,000g for 20 minutes, the supernatant discarded, and the platelet buttons were saved for counts. By using this technique 90% of the platelets were extracted from the anticoagulated whole blood.

The standards, whole blood samples, PPP, and the platelet buttons were counted in a hole-type crystal scintillation counter (Packard Model PGD PRIAS; Packard Instrument Company, Inc, USA) to a counting error of 1% or less, and with a spectrometer setting including both photo peaks and the summation peak of $^{111}$In.

The platelet MLS was calculated by least square computer fitting of a multiple hit gamma function model to the experimental data. The 2-hour value constituted the first experimental point used for calculation of platelet MLS, and the initial recovery of platelet-bound radioactivity was obtained by extrapolating the gamma function fitted survival curve to time zero. The computer program for these calculations was obtained from the office of ICSH. The platelet MLS was calculated by using the platelet buttons as well as the whole blood samples corrected for plasma-bound radioactivity.

Platelet production rate (platelet destruction rate in steady state conditions) was estimated by dividing the circulating platelet mass by platelet MLS and correcting for platelets residing in the splenic platelet pool (SPP), calculated from compartmental analysis: 

\[ \text{PLC} \times \text{TBV} \times \text{MLS}^{-1} \times (1 - \text{SPP})^{-1}. \]

In the formula PLC denotes the platelet count and TBV the total blood volume. The platelet MLS obtained using whole blood samples was used for this calculation.

Spleen size. The spleen size was determined using a gamma camera fitted with a high resolution parallel hole collimator (max 200 keV), and interfaced to a computer with a nuclear imaging system. Ten minutes after intravenous injection of 200 MBq.


\[ ^{99m} \text{Tc-labeled stannous colloid (Tinn-kolloid; IFE, Norway)}, 10^6 \text{ counts were collected with the gamma camera in the left lateral and posterior projections, and the images were stored in the computer using a } 64 \times 64 \text{ matrix system. The spleen areas in the lateral and posterior projections were calculated after weighted background subtraction and edge detection with an automatic computer program. The measured area in number of pixels was converted to square centimeters with the help of an enlargement factor. In our laboratory the reference value for spleen area, in both projections, is } 57 \pm 12 \text{ (SD) cm}^2. \]

**Megakaryocyte morphometry.** A Jamshidi needle was used to obtain BM. After local anesthesia, the biopsy needle was introduced into the posterior iliac crest and the biopsy was taken. The specimens were fixed in 3% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer at pH 7.4 for 24 hours, dehydrated in alcohol, and embedded in hydroxymethacrylat (Historesin) without decalcification. Sections of a thickness of 2 μm were cut and stained with May-Grünwald-Giemsa, hematoxylin-eosin, and PAS-reaction, according to Luna.\(^z6\) In the PAS-stained sections the number of megakaryocytes and their nuclei (\(A_n\)) was determined by the point counting method\(^z7\) and their mean areas were determined (\(A_n\) and \(A_m\), respectively). The standard error of a single determination was calculated from 10 duplicate determinations according to Luna.\(^z6\) In a similar fashion, the volume fraction (\(V_m\)) occupied by nuclei in the megakaryocytes was calculated as \(A_n/A_m \times V_m\) and the volume fraction of cytoplasm (\(V_c\)) was calculated as \(V_m - V_c\). These volumes are expressed as picoliter per microliter BM. The number of megakaryocytes per microliter BM (\(N_m\)) was calculated as described by others\(^z2^z8\) as

\[ N_m = K/\beta \times N_A^2/N_N \text{ thereby considering the cells to have a spherical form (ie, } \beta = 1.382) \text{ and a size variation coefficient of } 20\% \text{ (ie, } K = 1.06). \text{ From the data on volume and number of megakaryocytes per microliter BM the average cell and nuclear volumes were determined as } V_c/N_m \text{ and } V_n/N_m \text{ respectively.} \]

Standard statistical methods were used. Unless otherwise stated, mean values ± SD are given. The differences between means were tested with the Student’s t-test for paired data, and a \(P\) value <.05 was considered statistically significant.

The present study was approved by the local ethical committee and by the Swedish National Board of Health and Welfare.

**RESULTS**

Before start of IFN treatment, the mean platelet count was 1,102 ± 345 \times 10^9/L. A highly significant decrease in platelet count occurred during IFN therapy (Fig 1); the average platelet counts after 2 and 6 months of IFN therapy were 524 ± 169 (\(P < .0001\)) and 476 ± 139 \times 10^9/L (\(P < .0001\)), respectively. A concomitant reduction in white blood cell count and hemoglobin concentration was also observed. The hemoglobin concentration was reduced from a pretreatment value of 145 ± 19 to 132 ± 18 (\(P = .019\)) and 134 ± 18 g/L (\(P = .056\)) after 2 and 6 months of IFN therapy, respectively. Likewise, there was a statistically significant but clinically negligible lowering of the granulocyte and leukocyte counts.

Table 1 gives the results for the platelet kinetics. IFN induced a shortening of the platelet MLS. The mean pretreatment platelet MLS was 7.96 ± 0.69 days, using whole blood samples, and decreased to 6.95 ± 1.25 days (\(P = .035\)) and 6.68 ± 1.30 days (\(P = .012\)) after 2 and 6 months of IFN therapy, respectively. There was no change as to the SPP size, irrespective of whether it was measured indirectly, using the in vivo recovery of “platelet-bound radioactivity,” or directly, using compartmental analysis. However, a statistically significant increase in splenic blood flow was present after 2 months of IFN therapy, as compared with the pretreatment value (7.3 ± 2.0 v 5.7 ± 2.3 \%TBV/min; \(P = .041\)). The intrasplenic platelet transit time decreased slightly but not significantly so. Furthermore, the calculated platelet production rate showed a

\[ *P < .05. \]
\[ **P < .01. \]
\[ ***P < .0001. \]

\[ \text{Table 1. Results for the Platelet Kinetics and Spleen Size in 10 ET Patients Treated With } \alpha-2b-\text{IFN for Six Months (mean ± SD)} \]

<table>
<thead>
<tr>
<th>Platelet count ((\times 10^9/L))</th>
<th>Pre-treatment</th>
<th>2 mo</th>
<th>6 mo</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,102 ± 345</td>
<td>524 ± 169*</td>
<td>476 ± 139*</td>
<td></td>
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</table>

<table>
<thead>
<tr>
<th>Platelet MLS (whole blood) (d)</th>
<th>Initial recovery of platelet-bound radioactivity (whole blood) (%)</th>
<th>48 ± 8</th>
<th>45 ± 7</th>
<th>49 ± 9</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.69 ± 0.69 95% ± 1.25* 6.68 ± 1.30*</td>
<td>48 ± 8</td>
<td>45 ± 7</td>
<td>49 ± 9</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Platelet MLS (platelet button) (d)</th>
<th>Initial recovery of platelet-bound radioactivity (platelet button) (%)</th>
<th>53 ± 11</th>
<th>48 ± 5</th>
<th>52 ± 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.84 ± 0.88 6.89 ± 1.17* 6.56 ± 1.22*</td>
<td>53 ± 11</td>
<td>48 ± 5</td>
<td>52 ± 10</td>
<td></td>
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</tbody>
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<table>
<thead>
<tr>
<th>SPP size (%)</th>
<th>36 ± 8</th>
<th>41 ± 7</th>
<th>37 ± 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Splenic blood flow (% TBV/min)</td>
<td>5.7 ± 2.3</td>
<td>7.3 ± 2.0*</td>
<td>6.5 ± 2.1</td>
</tr>
<tr>
<td>Intrasplenic platelet transit time (min)</td>
<td>10.4 ± 1.7</td>
<td>9.9 ± 1.8</td>
<td>9.8 ± 1.6</td>
</tr>
<tr>
<td>Platelet production rate ((\times 10^9/d))</td>
<td>88.9 ± 29.8 53.1 ± 17.5* 45.4 ± 20.5*</td>
<td>76 ± 21</td>
<td>74 ± 21</td>
</tr>
</tbody>
</table>

\[ \text{Table 1. Results for the Platelet Kinetics and Spleen Size in 10 ET Patients Treated With } \alpha-2b-\text{IFN for Six Months (mean ± SD)} \]
marked decline following IFN treatment. The pretreatment platelet production rate, $89 \pm 30 \times 10^9$ platelets/d, diminished to $53 \pm 18 \times 10^9$ platelets/d after 2 months ($P = .0033$), and was $45 \pm 20 \times 10^9$ platelets/d after 6 months ($P < .0001$) of IFN therapy (Fig 2).

The mean labeling efficiency was $87 \pm 4\%$. The radioactivity bound to plasma constituted $3.4\% \pm 1.8\%$ of the total radioactivity in the final labeled platelet suspension, and $1.0\% \pm 1.2\%$ was bound to the erythrocytes.

The spleen area showed no statistically significant changes in response to IFN treatment in either the left lateral or in the posterior projections (Table 1).

Due to technical reasons, in two of the study subjects BM specimens were not satisfactorily evaluable on one of the two biopsy occasions. The mean BM cellularity showed no changes in response to IFN. The pretreatment mean BM cellularity was $71\% \pm 10\%$ as compared with $74\% \pm 8\%$ after 6 months of IFN treatment. There was a nonsignificant decrease of the number of megakaryocytes per microliter BM; the megakaryocyte numbers at baseline and after 6 months of IFN therapy were $1,324 \pm 554$ and $1,227 \pm 703$ ($P = .486$), respectively. The pretreatment average megakaryocyte volume was $372 \pm 126 \times 10^5 \muL/BM$ as compared with $278 \pm 147 \times 10^5 \muL/BM$ at 6 months ($P = .049$). This decline in BM megakaryocyte volume was a result of reduced megakaryocyte cytoplasmatic as well as reduced megakaryocyte nuclear volumes. The mean megakaryocyte cytoplasmatic and nuclear volumes, at baseline and at 6 months, were $320 \pm 110$ versus $239 \pm 127 \times 10^5 \muL/BM$ ($P = .058$), and $54 \pm 19$ versus $39 \pm 21 \times 10^5 \muL/BM$ ($P = .007$), respectively (Fig 3). The calculated average cell and nuclear volumes were $29.4 \pm 7.9$ versus $23.1 \pm 4.0 \muL$ ($P = .026$) and $4.2 \pm 0.7$ versus $3.2 \pm 0.6 \muL$ ($P = .001$), respectively, at start and after 6 months of IFN treatment.

IFN therapy, as administered in the present work, was well tolerated and caused a minimum of side effects. Mild flu-like symptoms over the very first days of treatment were easily controlled by the intake of 500 mg paracetamol approximately 30 minutes before the injections; further, most patients experienced mild hair loss, and over the first few months there were complaints of slight fatigue and loss of appetite.

**DISCUSSION**

The present study describes a homogeneous group of previously untreated ET patients in all of whom recombinant human $\alpha$-2b-IFN given over 6 months was shown to dramatically lower the peripheral platelet count; the predominant therapeutic response occurred within the first weeks of treatment.

In 1983, Talpaz et al reported that partly purified human leukocyte IFN reduced the thrombocytosis associated with Philadelphia chromosome positive chronic granulocytic leukemia. These results were confirmed by other investigators, and the capacity of $\alpha$-IFN to reduce the thrombocytosis was found to be valid for all chronic MPD.

The mechanisms by which $\alpha$-IFN exerts this effect are still unresolved. However, several studies have ascribed human IFNs, derived from different sources, antiproliferative activity against normal hematopoietic progenitor cells; suppression of in vitro colony formation has been seen in human and mouse multipotential (CFU-GEMM, CFU-spleen), erythroid (erythroid burst-forming unit [BFU-E]) and mouse megakaryocyte (CFU-MK) progenitor cells. Moreover, IFN has been shown to exert an antiproliferative activity against chronic granulocytic leukemia progenitor cells, and to inhibit the growth, size, and number of CFU-MK from patients with ET. Indeed, anti-proliferative effect of $\alpha$-IFN in ET patients is clearly demonstrated by the present results; both the platelet production rate and the BM megakaryocyte volume were
significantly reduced in our ET patients treated with α-IFN. Other workers have reported a decrease in the number of BM megakaryocytes in response to IFN treatment of ET.6,7,20

In accordance with the present results in a recent study comprising two patients with ET, a decrease of the megakaryocyte area and the megakaryocyte nuclear area was observed after IFN therapy.38 By using various methods, several investigators have demonstrated a significant correlation between nuclear as well as cell size and DNA content of megakaryocytes.6,41 The present finding of significant decrease of nuclear and cell volumes in response to IFN treatment would therefore imply a decrease of the average ploidyization and size of megakaryocytes. Such a decrease might explain the decreased megakaryocyte volume and would ultimately explain the observed reduction in platelet production rate.

An IFN-induced reduction of the platelet half-life was suggested as the mechanism by which IFN decreases the peripheral platelet count.42-44 However, our results do not support this view; the pretreatment mean platelet MLS was 7.97 days and diminished to 6.68 after 6 months of IFN therapy. Thus, platelet MLS was reduced by only 16%, whereas the mean peripheral platelet count decreased by 57%. Hence, the reduction of platelet MLS can only account for about 28% of the peripheral platelet count decrement. Moreover, because the exchangeable SPP size was unaffected by IFN therapy, the IFN-induced change in platelet production rate is responsible for more than 70% of the reduction in venous platelet concentration. However, it could be argued that this shortening in platelet MLS may to some extent explain the initial and rather instant lowering of platelet counts.29

As described by others,6,46 we also found that α-IFN caused a slight decrease in hemoglobin concentration and leukocyte count. In our study, these changes did lack clinical relevance.

The results for the determination of SPP size and spleen size did not show any significant changes in response to IFN treatment. According to the closed two-compartmental model,18,22 the size of the SPP is dependent on only two variables, ie, the splenic blood flow and the intrasplenic platelet transit time. The SPP size and the splenic blood flow are known to increase concomitantly with increasing spleen size, and the splenic blood flow can be modulated by adrenaline and isoprenaline infusions.47 The intrasplenic platelet transit time is a variable that until recently had not been investigated.21 Unlike the splenic blood flow, the intrasplenic platelet transit time appears to be unrelated to spleen size but shows an inverse relationship to splenic perfusion.44,46 Other factors that are expected to influence platelet transit time through the spleen include platelet size, platelet antibody coating, intrinsic platelet abnormalities, and splenic macrophage function.50,62 In the present study, IFN treatment was followed by a slight increase in splenic blood flow, which was shown to be statistically significant at 2 months after start of IFN therapy. This increase in splenic blood flow was counteracted by a slight shortening of the intrasplenic platelet transit time, leaving the SPP size mainly unchanged.

It can be concluded that the present results clearly demonstrate that α-IFN reduces the platelet count in ET mainly through an anti-proliferative action on the BM megakaryocytes and to a considerably lesser extent by inducing a shortened platelet MLS.

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