A thrombocytopoiesis-stimulating factor (TSF or thrombopoietin) derived from human embryonic kidney (HEK) cells is known to increase platelet production and to increase the number of morphologically unrecognizable early megakaryocytes, i.e., small acetylcholinesterase-positive (SACHE+) cells in mice. Other recent studies have concluded that interleukin-6 (IL-6) also stimulates murine megakaryocytopoiesis both in vitro and in vivo. Some workers have suggested that IL-6 is thrombopoietin. Therefore, the purpose of this study was to compare the effects of TSF and IL-6 on percent 35S incorporation into platelets, platelet sizes, and the percentages of SACHE+ cells in C3H mice, and to determine if they produce the same or different responses. The results showed that two or four injections of a partially purified TSF (total dose of 2 or 4 units [U] over a 1- or 2-day period) increased percent 35S incorporation into platelets (P < .005) and platelet sizes (P < .005) of both normal and rebound-thrombocytotic mice when compared with values from other mice treated with human serum albumin, the carrier protein for both TSF and IL-6. In eight separate experiments, it was shown that IL-6 (40,000 U, 4 µg), when given to rebound-thrombocytotic mice in four injections over a 2-day period, produced a small but significant (P < .005) increase in percent 35S incorporation into platelets. Additional studies showed that negative results were obtained when similar high doses of IL-6 were administered in two doses over a 1-day period. TSF, but not IL-6, stimulated an increase in platelet sizes of normal mice (P < .005 to .0005); however, IL-6 increased platelet sizes of rebound-thrombocytotic mice when given in two or four injections (P < .05 to .0005). Also, IL-6, but not TSF, caused anemia in normal mice (P < .0005) that were given two injections and tested 3 days later. TSF stimulated an increase (P < .005) in the percentage of SACHE+ cells; whereas IL-6, even at high doses, did not. Because of the observed differences in biologic responses of these two cytokines, we conclude that TSF and IL-6 are separate entities.

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the first injection. Both normal and rebound-thrombocytotic mice were used in these studies and all mice were given the same total dose of test materials. Mice were made thrombocytotic by a single intraperitoneal (IP) injection of rabbit antitherboid platelet serum (RAMPS) 5 days before injection of test substances, ie, human serum albumin (HSA), TSF, or IL-6.

The TSF used in this study was Step II material, a partially purified preparation from HEK cell culture media. A total dose of 2 or 4 units (U) of TSF was given per mouse. A unit of TSF is defined as the amount of material in milligrams of protein that is required to increase the percent $^{35}S$ incorporation into platelets of immunothrombocytotic mice by 50% above baseline. The specific activity of the TSF used in this study was approximately 3.5 U/mg protein. The TSF preparation contained a small amount of IL-6, ie, ~875 U of IL-6/U of TSF as determined by the B9 cell proliferation assay. Because large amounts of HSA (approximately 1.8 mg for each TSF U) were added to stabilize the partially purified TSF, other mice were treated with 7.5 mg of HSA as a control. Previous studies showed that saline and HSA gave similar responses in mice, ie, platelet sizes, percent $^{35}S$ incorporation into platelets, and average polyploid megakaryocyte DNA content were almost identical at 2 and 3 days after the beginning of the treatment. Therefore, in the present studies, HSA was used as the control substance.

Recombinant human IL-6 (rhIL-6) was obtained from Boehringer Mannheim (Indianapolis, IN). This preparation of rhIL-6 was produced utilizing Escherichia coli and purified by standard chromatographic techniques. The material was greater than 98% pure as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and endotoxin (LAL) was less than 10 EU/mg. Specific activity was greater than 1 x 10^7 U/mg as determined by the B9 cell proliferation assay. The material was diluted into HSA (7.5 mg/mouse) before injection.

Percent $^{35}S$ incorporation into platelets and platelet sizes were measured using previously described techniques. Thirty microcuries of Na$^{35}SO_4$ were injected intravenously (IV) 24 hours before killing. Blood for determination of percent $^{35}S$ incorporation into platelets and platelet sizes was taken by cardiac puncture after injection of a heparin-sodium pentobarbital solution. Approximately 0.5 mL of blood was collected into syringes containing 1 mL of 3.8% sodium citrate solution and expressed into plastic tubes. Platelet-rich plasma (PRP) was obtained by centrifugation at 160g for 4.5 minutes at 22°C. The tube containing the PRP was capped to minimize pH changes, which can alter platelet sizes. The platelets were washed and the percent $^{35}S$ incorporation measured as previously described.

Platelet size measurements were made using a Particle Data Instrument (Particle Data Inc, Elmhurst, IL) with a logarithmic scale as previously described. Platelet counts and hematocrits were performed on blood obtained by retroorbital puncture using standard techniques.

After bleeding, the mice were killed and marrow was taken from one of the femurs and dispersed into a single cell suspension by mixing with a plasma expander (Polyvinyl-pyrolidone, 3.5% in saline with 1% EDTA added). Marrow smears were made and stained for the presence of acetylcholinesterase (AChE) activity using the method of Karnovsky and Roots. For staining, marrow smears were rinsed in 0.1 mol/L sodium phosphate, pH 6.0, for 1 minute and then incubated in the acetylthiocholine substrate mixture at room temperature for 3 hours. After staining, the smears were postfixed in absolute methanol for 10 minutes and 50% methanol for 30 seconds. All AChE + cells on each slide were counted. The SAChE + cells stain positively for AChE, are usually round, stain evenly without granulation, and are less than 13 μm in diameter. For each mouse, approximately 500 AChE + cells were examined; the number of SAChE + cells and the total number of cells that stain positively for AChE were recorded and the percentage of SAChE + cells was calculated.

To eliminate the possibility that the small amount of IL-6 present in the TSF preparation was responsible for the stimulation of platelet sizes and percent $^{35}S$ incorporation into platelets found after TSF injection, an additional experiment using a more highly purified preparation of TSF (Step III TSF) was used. This preparation of TSF (specific activity of 15.8 U/mg protein) was found to be free of IL-6 by both Western blotting techniques and by the B9 cell proliferation assay. The more highly purified TSF was injected into immunothrombocytotic assay mice in four doses over a 2-day period and the percent $^{35}S$ incorporation into platelets was determined as previously described.

Student's t-test was used for evaluation of the data.

RESULTS

Figure 1 shows the results of dose-response studies of TSF and IL-6 on $^{35}S$ incorporation into platelets of rebound-thrombocytotic mice. As shown, TSF gave a highly significant linear relationship between the dose of TSF and the percent $^{35}S$ incorporation into platelets ($r^2 = .99$). IL-6 also showed a dose-response relationship ($r^2 = .90$), but re-

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**Fig 1.** Percent $^{35}S$ incorporation into platelets of immunothrombocytotic mice after injection of various doses of TSF (A, one experiment) or IL-6 (B, pool of four separate experiments). The numbers next to the points indicate the number of mice used at each treatment and the vertical lines indicate the standard errors. The saline-treated control values were: 1.790 ± 0.25 x 10^-3 (A) and 2.25 ± 0.12 x 10^-3 (B). Values were significantly elevated over saline-treated control mice: *P < .05; **P < .005.
required 40,000 U/mouse (4 μg) for significant increase of percent 35S incorporation into platelets of assay mice (P < .005).

Figure 2 shows the results of measuring percent 35S incorporation into platelets of mice after two or four injections of either HSA, 4 U of TSF, or 40,000 U of IL-6 in both normal mice and mice in rebound-thrombocytosis (RAMPS-treated mice). The results showed that in both normal mice and RAMPS-treated mice, TSF significantly (P < .005) increased percent 35S incorporation into platelets of mice after two or four injections. In agreement with previous studies, TSF had greater effects when administered to mice in rebound-thrombocytosis than when given to normal mice. IL-6, on the other hand, did not increase percent 35S incorporation into platelets of mice after two injections, but after four injections IL-6 significantly increased platelet production (P < .05) in both normal and rebound-thrombocytotic mice. Although not shown, platelet counts of mice treated with either IL-6 or TSF were not increased when compared with counts of mice injected with HSA.

Platelet size measurements on blood of the same mice as presented in Figure 2 are shown in Figure 3. TSF caused significant (P < .005 to P < .0005) increases in platelet sizes at all injection schedules in both normal mice and rebound-thrombocytotic mice when compared with other mice treated with HSA. IL-6 did not increase platelet sizes of normal mice, but there were significant (P < .05 to P < .0005) increases in sizes of platelets in RAMPS-treated mice after IL-6 treatment. In the present study, TSF and IL-6 did not increase platelet counts of mice when measured 3 days after the initial injection.

Table 1 shows the results of measuring hematocrits of these mice. Normal mice treated with two injections of IL-6 had significantly (P < .0005) lower hematocrits on day 3 than mice treated with HSA. Otherwise, there were no changes in packed cell volumes of treated mice.

Figure 4 shows the results of measuring the percent SAChE+ cells in marrow of mice treated with HSA, 2 U of TSF, or 40,000 U of IL-6. In agreement with previous studies, TSF in small doses increased the percentage of SAChE+ cells (P < .005) compared with the results of other mice treated with HSA. High doses of IL-6 did not increase the percentages of SAChE+ cells of mice. Furthermore, preliminary experiments showed that 2, 4, or 10 x 10^3 U of IL-6 did not stimulate an increase in percent SAChE+ cells, ie, after treatment with 7.5 mg of HSA, 6.7 ± 0.7 (4) percent SAChE+ cells were found in the BM of normal mice. After 2 x 10^3 U of IL-6/mouse, 6.0 ± 1.3 (4) percent SAChE+ cells were counted; 4 x 10^3 U of IL-6/mouse gave 7.1 ± 1.5 (6) percent SAChE+ cells; and 10 x 10^3 U of IL-6/mouse, resulted in 6.9 ± 0.8 (5) percent SAChE+ cells.

Table 2 shows the results of measuring the percent 35S incorporation into platelets of TSF assay mice after injec-

<table>
<thead>
<tr>
<th>Material</th>
<th>Normal Mouse</th>
<th>RAMPs-Treated Mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSA</td>
<td>46.2 ± 0.2 (20)</td>
<td>46.1 ± 0.2 (14)</td>
</tr>
<tr>
<td></td>
<td>46.1 ± 0.2 (14)</td>
<td>46.0 ± 0.3 (15)</td>
</tr>
<tr>
<td>TSF</td>
<td>45.5 ± 0.8 (5)</td>
<td>46.2 ± 0.3 (9)</td>
</tr>
<tr>
<td></td>
<td>45.1 ± 0.6 (10)</td>
<td>46.1 ± 0.3 (10)</td>
</tr>
<tr>
<td>IL-6</td>
<td>44.6 ± 0.3 (15)</td>
<td>46.3 ± 0.4 (10)</td>
</tr>
<tr>
<td></td>
<td>45.6 ± 0.7 (19)</td>
<td>46.2 ± 0.4 (9)</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SE (number of mice/treatment). HSA was injected at 7.5 mg/mouse. TSF was injected for a total dose of 4 U/mouse. IL-6 was injected for a total dose of 40,000 U/mouse.

*Significantly lower than hematocrits of HSA-treated control mice, P < .0005.
the vertical lines represent the standard errors. Mice treated with TSF administered as a single IP injection 8 hours before the mice were killed. The data represent a pool of three separate experiments. Numbers on the bars represent the numbers of mice/treatment and the vertical lines represent the standard errors. Mice treated with TSF had significantly more SACHe+ cells than HSA-treated mice (P < .05).

DISCUSSION

The present study shows that both IL-6 and TSF will increase percent 35S incorporation into platelets of mice if given in four injections (Figs 1 and 2), but only TSF is effective when the material is given in two doses (Fig 2). Unlike TSF, IL-6 did not increase the numbers of SACHe+ cells (Fig 4) or platelet sizes (Fig 3) of normal mice. This study agrees with previous studies showing that TSF increases both platelet production and megakaryocytopoiesis in rodents, ie, percent 35S incorporation into platelets, percent SACHe+ cells, and platelet sizes. Previous experiments showed that TSF will elevate platelet counts, megakaryocyte sizes and numbers, megakaryocyte endomitosis, megakaryocyte maturation, and megakaryocyte DNA content. The stimulatory effects of IL-6 on megakaryocyte endomitosis and maturation have apparently not yet been tested, but in the present study IL-6 did not stimulate percent SACHe+ cell numbers and platelet sizes in normal mice. We present below additional evidence that the two cytokines are probably separate factors.

Previous studies have shown that administration of IL-6 in vivo will increase platelet counts, megakaryocyte size, and 35SeM incorporation into platelets of both mice and monkeys. Also platelet sizes were reported not to be elevated in mice by IL-6 treatment in a preliminary study. However, 16 µg (160,000 U) of IL-6 were reported to increase WBC counts, without significant changes in hematocrits or megakaryocyte numbers of recipient animals. Hill et al showed that 8 µg of IL-6 given to mice produced an increase in megakaryocyte and granulocyte-macrophage colony-forming cells in both the BM and spleen of mice. However, Asano et al showed in primates that injection of 80 µg/kg per day of IL-6 produced a loss of body weight, anemia, and other adverse side effects. It should be mentioned that in all these studies, large doses of rIL-6 (microgram quantities) were required to increase either megakaryocytopoiesis or thrombocytopoiesis.

In vitro studies, Ishibashi et al and Koike et al showed that IL-6 increased megakaryocyte sizes. Megakaryocyte ploidy and acetylcholinesterase content of megakaryocytes in culture were also reported to be increased by IL-6 treatment, and if IL-3 was added to cultures containing IL-6, an increase in megakaryocyte colonies was observed. However, Bruno and Hoffman showed that the addition of TSF or erythropoietin (Epo) to IL-6 decreased megakaryocytic colony formation. It was concluded that the effects of IL-6 are probably on accessory cells for the release or production of other cytokines. Williams et al showed that IL-6 would stimulate growth of immature mouse megakaryocytes. A similar conclusion was reached by other investigators, who showed that IL-6 would increase the acetylcholinesterase content of cells without an increase in colony formation. Two recent reviews summarize this work. All these in vitro studies showed that IL-6 would enhance megakaryocytopoiesis, but the possibility exists that the effects may not be direct.

In the present study we used a partially purified TSF preparation that contained a small amount of IL-6 (~875 U of IL-6/U of TSF). Sufficient amounts of the more highly purified preparation for these studies were not available. Preliminary experiments and work presented herein showed no effects of IL-6 on percent 35S incorporation into platelets (Fig 1) or on the percentage of SACHe+ cells when IL-6 was given in doses that were present in the TSF. Moreover, one experiment (Table 2) using a more highly purified preparation of TSF that was found to be free of IL-6 showed that this TSF preparation would still stimulate platelet production in rebound-thrombocytotic mice. Therefore, we do not believe that the small amount of IL-6 that was present in the TSF was responsible for the thrombocytopoietic stimulating effects seen in this study. It does not seem possible that a combination of IL-6 and other un-

Table 2. Effects of Step III TSF on Percent 35S Incorporation Into Platelets of Immunothrombocythemic Mice

<table>
<thead>
<tr>
<th>Material</th>
<th>Number of Mice</th>
<th>U/Mouse</th>
<th>Percent 35S Incorporation Into Platelets (x 10^3 ± SE)</th>
<th>% of Control*</th>
<th>P &lt; t</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>5</td>
<td></td>
<td>3.00 ± 0.16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Step III TSF</td>
<td>5</td>
<td>0.3</td>
<td>3.73 ± 0.35</td>
<td>124</td>
<td>NS</td>
</tr>
<tr>
<td>Step III TSF</td>
<td>5</td>
<td>0.6</td>
<td>3.94 ± 0.37</td>
<td>131</td>
<td>0.05</td>
</tr>
<tr>
<td>Step III TSF</td>
<td>5</td>
<td>0.9</td>
<td>4.40 ± 0.12</td>
<td>146</td>
<td>.0005</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SE. Step III TSF was found to be free of IL-6 by two Western blotting techniques and by the B9 cell proliferation assay.

*Correlation coefficient of data: r² = .95.

†Significantly greater than saline-injected control mice.
known factors present in the TSF preparation could have caused the stimulation of thrombocytopoiesis observed herein, based upon our previous findings using purified TSF and the data presented in Table 2. A more likely explanation is that a specific thrombocytopoietic factor (TSF) within our preparation caused the increases in thrombocytopoiesis that we have observed. Experiments with rTSF will be necessary to resolve this issue.

The present work agrees with the work of Hill et al showing that high doses of IL-6 will increase platelet production by increasing incorporation of isotopes into platelets. Moreover, the two studies agree that there is a dose-response relationship between isotope incorporation and the amounts of IL-6 injected. However, it should be noted that in both studies positive responses required multiple injections of large doses of IL-6 (in the order of 40,000 to 80,000 U/mouse, 4 to 8 μg) for increase of isotope incorporation into platelets. In our study, two injections of IL-6 administered over a 1-day period did not elevate percent 35S incorporation into platelets of either normal or rebound-thrombocytotic mice. All other studies showing increase of thrombocytopoiesis by IL-6 required large doses over an extended period of time. It should also be mentioned that high levels of another cytokine (rEpo) will also increase 35S incorporation into platelets of assay mice.

Our study agrees also with a preliminary report of Hill et al showing that mean platelet volumes were unaffected by IL-6 administration into normal mice. Although we showed that TSF treatment resulted in significant increases in platelet sizes of both normal and rebound-thrombocytotic mice, IL-6 did not increase platelet sizes of normal mice. However, increased platelet sizes of rebound-thrombocytotic mice were found after IL-6 injections. The reason for altered platelet sizes in rebound-thrombocytotic mice but not in normal mice after IL-6 is unknown. However, the fact that the rebound-thrombocytotic mice are more sensitive to thrombocytopoietin and IL-6 are separate growth factors: (1) The two compounds have different molecular weights, i.e., IL-6 has a molecular weight of about 26 Kd and the molecular weight of TSF is ~15 Kd; (2) Partially purified preparations of TSF (Step III TSF) have been shown to be free of IL-6 but still increase percent 35S incorporation into platelets of immunothrombocytopenic assay mice (Table 2). IL-6 was not found in Step III TSF by Western blotting techniques and by the B9 cell proliferation assay for IL-6. (3) Previously, nanogram quantities of purified TSF stimulated significant increases in percent 35S incorporation into platelets of assay mice, whereas in the present study and elsewhere microgram quantities of pure IL-6 were required for similar stimulation of platelet production. (4) Some kidney cell lines will not produce IL-6, but produce large quantities of TSF (unpublished results). (5) IL-6 is produced by a variety of tissues, whereas TSF appears to be produced in vitro only by kidney cells. (6) Recently, Hill et al reported that plasma IL-6 levels were not increased by induction of thrombocytopenia in mice, indicating that IL-6 is probably not involved in stimulating platelet production in mice rendered acutely thrombocytopenic. (7) Another recent study found that IL-6 levels did not correlate with platelet counts of patients, but IL-6 was elevated in sera of patients in response to inflammation. (8) Many growth factors (including Epo) will stimulate platelet production in mice if given in high enough doses. (9) Unlike thrombopoietin, IL-6 has a broad range of effects in vivo, as well as in vitro. For these reasons, we believe that IL-6 is not thrombopoietin and that thrombopoietin, not IL-6, is the physiologic regulator of thrombocytopoiesis.

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REFERENCES

2. McDonald TP, Jackson CW: Thrombopoietin derived from human embryonic cell cultures stimulates an increase in DNA content of murine megakaryocytes in vivo. Exp Hematol 18:758, 1990
22. Cullen WC, McDonald TP: Comparison of stereologic techniques for the quantification of megakaryocyte size and number. Exp Hematol 14:782, 1986

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Comparative effects of thrombopoietin and interleukin-6 on murine megakaryocytopoiesis and platelet production

TP McDonald, MB Cottrell, CJ Swearingen and RE Clift