Extrarenal Erythropoietin Production by Macrophages

By Ivan N. Rich, Wolfgang Heit, and Bernhard Kubanek

Murine bone marrow and adherence-separated spleen cells cultured on hydrophilic, gas-permeable Teflon foils (Petriperm dishes) can be shown to synthesize and secrete erythropoietin (Epo) and colony-stimulating activity (CSA) simultaneously into the surrounding medium. The Epo activity in the supernatants of primary cultures as measured by the fetal liver erythroid colony-forming technique, from adherent and nonadherent spleen cells, increases over the first 7 days in culture, followed by a plateau until 14 days. Use of the macrophage-specific cytotoxic agent, crystalline silica, as a tool to release residual Epo contained in these cells produces a similar time-Epo activity curve to that found in the primary supernatants. This, together with functional and morphological examination of the cells, indicates that macrophages are responsible for this activity. The total Epo activity released from adherent and nonadherent spleen cells at plateau levels was estimated to be 25 mU/ml culture/day. Weekly subcultivation of bone marrow and adherence-separated spleen cells initiated from primary cultures demonstrated a massive increase in both Epo activity and CSA above that obtained for the primary cultures. Subcultivation could be continued for at least 6 wk. These results, together with the reversible inhibition of Epo and CSA production by cycloheximide, demonstrate that these molecules are synthesized by the macrophage. The evidence supports the hypothesis that the macrophage is involved not only in extrarenal Epo production, but also in the possible short-range regulation of hemopoiesis.

ERYTHROPOIETIN (Epo), the glycoprotein hormone that regulates red cell production, is produced by the kidney in the adult animal. Erythropoietin can also be produced extrarenally; the primary organ implicated in this function has been the liver. In the fetus, the liver is probably the only site of Epo production, since the kidney has not developed this function at this stage of development. In both the fetus and the adult, the cell thought to be responsible for Epo production is the Kupffer cell or liver macrophage. Since macrophages are extremely versatile cells, producing and secreting many substances that affect the functioning of other cells, we initiated a series of studies to investigate whether macrophages derived from sources other than the liver could release Epo.

Crystalline silica is phagocytized by several cell types, but is specifically cytotoxic only for macrophages. We have shown that when macrophages ingest silica, an erythropoietic stimulating factor (ESF) is released into the extracellular fluid. The ESF has been shown to be erythropoietin by its stimulation of erythropoiesis in vivo and in vitro as well as by its neutralization with anti-Epo. The important question as to whether Epo is produced by macrophages was investigated in vitro.

This report describes a culture procedure in which Epo can be shown to be produced, for at least 4 wk, by suspensions of spleen and bone marrow cells. The cells are cultured on gas-permeable, hydrophilic Teflon surfaces. The culture system supports the proliferation and differentiation of early granulocyte/macrophage precursor cells into the macrophage cell line. In addition, cell suspensions from primary cultures subcultured repeatedly, are shown to produce both Epo and colony-stimulating activity (CSA) simultaneously.

MATERIALS AND METHODS

Animals

Virgin female CBA/Ca mice, 10-12 wk of age, were used (except where indicated) for all experiments. Fetal livers were obtained from 12-13-day pregnant CBA/Ca mice. Preparation of spleen, bone marrow, and fetal liver cell suspensions has been described previously. (Rich & Kubanek, 1979).

Experimental Procedure

Preparation of Primary Cultures

Cell suspensions were prepared from 10-15 spleens and at least 30 femora for each experiment. The cell suspensions were first layered with fetal calf serum (FCS) and left to stand for 30 min at 4°C to remove large particles. After withdrawing the cell suspensions, the cells were washed twice in Hank's balanced salt solution (Hank's BSS) containing 3% FCS, centrifuging (400 g, 10 min, 4°C) between each wash. After the washing procedure, the nucleated cell count was determined with a Coulter Counter (Model ZF).

Spleen cells were separated into adherent and nonadherent cell suspensions by incubating the original spleen cell suspensions at a concentration of 300 x 10⁶ cells in 30 ml of Hank's BSS containing 3% FCS in 250 ml (75 sq cm) tissue culture flasks (Costar, Cambridge, Mass.) for at least 2 hr at 37°C in an atmosphere containing 5% CO₂. Thereafter, the nonadherent cells were withdrawn and transferred to 50-ml plastic tubes (Falcon, Oxnard, Cal.).
The adherent cells were removed using a rubber policeman, transferred to plastic tubes, and centrifuged as described above. The cell pellets were resuspended in Iscove's Modified Dulbecco's Medium (IMDM) containing 5% FCS and the nucleated cell count determined. Bone marrow cell suspensions were not subjected to any separation procedure.

The cell suspensions were transferred to hydrophobic Petriperm culture dishes (Hereus, Hanau, W. Germany) at a concentration of 24 x 10⁶ cells in a total of 3 ml, diluted in IMDM containing 5% FCS. When experiments involved treating cells with silica, duplicate cultures were prepared for each time point shown in the results. The cells of one dish were then used for silica treatment (see below), while those of the other were used for granulocyte/macrophage colony assay, subcultivation, morphology, and cell count determinations. The original supernatants from the cultures were withdrawn carefully, transferred to plastic tubes that were centrifuged, and the supernatants collected and stored at −20°C until the experiment was terminated. The Epo and/or CSA was then assayed (see below).

**Removal of Cells From Petriperm Culture Dishes**

Due to the hydrophobic nature of one side of the Teflon foil, the cells settle but do not adhere to the culture surface. The cells were removed as follows. The dishes were swirled and the medium withdrawn into a syringe through a 25-gauge needle. The contents of the syringe were then used to gentle wash the remaining cells from the Teflon surface. The cells were transferred to plastic tubes and centrifuged. The supernatants were collected and stored at −20°C. The cells were resuspended in 1 ml IMDM containing 5% FCS.

**Subcultivation of Primary Petriperm Cultures**

After removal of the cells from the primary cultures or those that were to be further passaged, the cells were transferred to new Petriperm dishes containing fresh IMDM supplemented with 5% FCS. Subcultivation was performed at weekly intervals.

**Treatment of Cells With Silica**

Although this procedure has been described elsewhere, it is summarized here for clarity. After carefully removing the supernatant from the culture dish using a syringe with a 25-gauge needle, the remaining cells were incubated in 2 ml Hank's BSS containing 3% FCS and a final concentration of 10⁻⁴ g/ml crystalline silica (Min-U-Sil, 2–4 μM, Whittacker, Clark & Daniels, Plainfield, N.J.) for 30 min at 37°C in an atmosphere containing 5% CO₂. The cell-silica suspension was then removed, transferred to a plastic tube, and centrifuged at 400 x g for 10 min at 4°C. The silica-treated cell supernatants were then collected and stored at −20°C and assayed for Epo at the end of the experiment.

**Treatment of Cells with Cycloheximide**

These experiments were performed using both C57BL/6J and BDF1 spleen cells and the supernatants assayed using 13–14-day C57BL/6J fetal liver cells. Although the Epo dose response for C57BL/6J fetal liver cells demonstrates higher CFU-E values for all concentrations, a similar pattern of results is obtained compared to the use of CBA/Ca fetal liver cells.

Unseparated spleen cells were preincubated at a cell density of 24 x 10⁶ cells in 3 ml IMDM containing 5% FCS and 10⁻² M reduced glutathione (GSH). Control dishes did not contain cycloheximide. These suspensions were then incubated for 6 hr. Thereafter, the cells were removed as described above and centrifuged. The supernatant was discarded and the cells washed once with IMDM without any additions. The cells were then recultured in a similar manner as in the preincubation for a further 30 min, 24 hr, and 48 hr. The supernatants from the control and cycloheximide-treated cells were collected and assayed.

**Assay for Erythropoietin Using Fetal Liver as a Source of CFU-E Target Cells**

Erythroid colony-forming units (CFU-E) from early (12–13 days of gestation) fetal livers were used as a source of target cells for assaying the Epo activity present in supernatants. Compared to adult bone marrow, CFU-E-derived fetal liver cells are at least 5 times more sensitive to Epo. Data from at least 20 erythropoietin dose–response curves show that Epo concentrations as low as 1.56 mU/ml produce 150 ± 22 CFU-E/10⁵ cells plated, while optimal concentrations of 75 mU/ml produce 3763 ± 101 CFU-E/10⁵ cells. Since spontaneous colony formation amounts to 17.5 ± 2.5 CFU-E/10⁵ cells, colony incidences greater than this are attributed to Epo activity present in the supernatant. The latter was added at a concentration of 10% to a total culture volume of 1.25 ml. Fetal liver cells were plated at a concentration of 0.75 x 10⁵/ml.

The erythroid colony-forming technique has been extensively described elsewhere. Three modifications were made to this technique. The first was the addition of pure mouse transferrin instead of human transferrin added at a final concentration of 1.3 x 10⁻¹² M. Mouse transferrin was prepared by the method of Sawatzki et al. and has been found to be specific for mouse cells at optimal concentrations between 1.3 x 10⁻¹² M and 1.3 x 10⁻¹⁰ M. The second modification was the use of Iscove's Modified Dulbecco's Medium (Gibco, Grand Island, N.Y.) rather than alpha-medium for all dilutions. Finally, cryopreserved as well as fresh fetal liver cell suspensions were used. Cryopreserved fetal liver cells produce similar CFU-E colony incidences and Epo dose–response curve characteristics compared to fresh cell suspensions (unpublished data).

**Assay for Granulocyte/Macrophage Precursor Cells (CFU-C) and Colony-Stimulating Activity**

A modification of the original assay system described by Byrne et al. was employed using 10% heat-inactivated heart conditioned medium as a source of colony-stimulating factor (CSF) derived from 10³ cells were counted after 6 days of incubation at 37°C in a fully humidified atmosphere containing 5% CO₂. The presence of colony stimulating activity (CSA) is the supernatants determined by substituting the supernatant (10% of the final culture volume) in place of CSF added to the above culture system.

**Methods Used to Establish the Presence of Macrophages in Culture**

Phagocytosis was performed by incubating the cells from Petriperm cultures with latex particles (1.25 μm; Polysciences, Warington, Pa.) for 30 min at 37°C in plastic tubes. Aliquots of this suspension, together with controls, were cytotoxic in a fully humidified atmosphere containing 5% CO₂. The presence of colony stimulating activity (CSA) is the supernatants were determined by examining the supernatant (10% of the final culture volume) in place of CSF added to the above culture system.

Electron microscopic studies were performed using a Zeiss EM9S–Z on samples that had been fixed in glutaraldehyde, stained with osmium tetroxide, and contrasted with uranyl acetate.
Statistics

The supernatants were assayed in duplicate (triplicate for CSA) and experiments were performed at least 3 times. Normal Epo and CSF dose-response curves were performed whenever supernatants were assayed. All values are the mean ± SEM. Tests of significance were performed using the Student’s t test and nonparametric U test of Mann and Whitney.

RESULTS

Production of Erythropoietin From Spleen Adherent and Nonadherent Cell Cultures

Separated spleen cells were divided into four groups, namely adherent and nonadherent cells cultured in the presence or absence of 10⁻⁴ M alpha-thioglycerol (Tg). For each of the four groups, Petriperm cultures were terminated at daily intervals for a period of 14 days. The supernatants were harvested and the remaining cells treated with silica. In this manner it was possible to distinguish between Epo activity released freely into the primary supernatants (middle curve in Fig. 1, A and B) and that which was residually present in the cells and released by silica action (lower curve in Fig. 1, A and B). Addition of these two activities assayed by fetal liver CFU-E growth provides an estimate of the total Epo activity produced (upper curve, Fig. 1, A and B).

In comparison with cultures prepared in the absence of Tg, an increased Epo activity for every time point assayed was observed for cultures prepared in the presence of Tg. Figure 1 (A and B) shows the time-Epo activity curves for adherent and nonadherent spleen cells, respectively, cultured in the presence of Tg. All curves, including those for cells treated with silica, show a common feature: there is an increase in Epo activity to day 7 or 8 followed by a plateau phase, indicating that although Epo activity is being released, there is no net increase. Since release of Epo can be detected for at least 14 days, these results imply

![Graph A](image1.png)

![Graph B](image2.png)

Fig. 1. Time-Epo activity curves for supernatants obtained from Petriperm cultures of (A) spleen adherent and (B) spleen nonadherent cells, cultured in IMDM supplemented with 5% FCS and a final concentration of 10⁻⁴ M alpha-thioglycerol (Tg).
production of Epo rather than gradual release from an intracellular storage pool. Based on Epo sensitivity curves in the vitro assay, the total Epo activity derived from these cultures measured from the adherent and nonadherent spleen cells at the plateau phase corresponds to approximately 25 mU/ml culture/day.

Generation of Macrophages From Early Granulocyte/Macrophage Progenitor Cells in Petriperm Cultures

In a second series of experiments, the presence of granulocyte/macrophage progenitor cells was assayed in the four groups described in the previous section. Cell suspensions from these groups were stimulated with CSF_{HCM}. Figure 2A shows the CFU-C content/ml of culture assessed over the 14-day period. With the exception of the group containing nonadherent spleen cells supplemented with Tg, an increase in CFU-C content is observed after days 6 and 7. Nonadherent cells supplemented with Tg show an increase 2 days prior to that seen for the other groups and attain maximum values on day 7, followed thereafter by an apparent plateau. Compared to the 2 adherent cell groups, the nonadherent cell groups both demonstrate a marked potential for proliferation. Pure macrophage colonies were obtained in all cases, as determined by colony appearance and morphological examination of the cells after withdrawal from the culture dish.

Figure 2B shows the nucleated cell counts for all four groups over the 2-wk period. Again, with the exception of the group, nonadherent + Tg, there is an overall continuous decline until about day 7. Cell maintenance then appears to follow until a modest increase in nucleated cell numbers occurs after day 11. After an initial increase, the nonadherent cells supplemented with Tg maintain their numbers after day 4 of culture.

Approximately 70% of the cells present in both the
adherent and nonadherent 'Petriperm' groups demonstrated positive, nonspecific esterase staining. This, together with the fact that adherence and phagocytosis of latex could be demonstrated, indicated that the majority of the cells observed by electron microscopy were monocytes and macrophages. These results indicate that both adherent and nonadherent cell suspensions contain precursor cells capable of proliferating and differentiating into macrophages.

Production of Epo From Primary Bone Marrow Cultures and the Effect of Subculturing Bone Marrow and Adherence-Separated Spleen Cells on the Simultaneous Production of Epo and CSA

The hypothesis that spleen and bone-marrow-derived macrophages can produce Epo in culture was further tested by determining the Epo activity present in the supernatants derived from cells that had been subcultured from primary cultures. The results are shown in Figs. 3A, 4A, and 5A. Referring to Fig. 3A, the supernatant from a primary culture (designated "P") of bone marrow cells that had been incubated for 1 day was withdrawn and the cells removed. After determining the nucleated cell count, the remaining cells were transferred to a new culture dish and incubated with fresh medium for 1 wk. Thereafter, the supernatant was again withdrawn and the cells again transferred to a new dish with fresh medium. This procedure was then repeated for each passage. The Epo activity contained in the day-1 primary culture supernatant is shown as the first column in this figure (P). The Epo activity contained in the supernatant after the first week of subculture is shown in the second column and designated "1." Thus, a total of 6 subcultures from the day-1 primary culture were performed. All the data are represented in this manner.

The main observations from the data on Epo activity are as follows. First, Epo activity derived from bone

![Fig. 3. (A) Assay of Epo activity and (B) ICSA in supernatants from primary (designated "P") and subcultured bone marrow cells. Primary cultures from days 1, 3, 7, 10, and 14 were subcultured at weekly intervals. The number of weekly passages is shown at the bottom of the graph.](image-url)
marrow primary cultures increases from day 1 to day 14. In fact, after 4 wk in primary culture, $886 \pm 51$ CFU-E/10$^5$ cells were obtained and equivalent to about 10 mU Epo/ml culture. At 14 days, electron microscopy showed that between 80% and 90% of the morphologically identifiable cells were monocytes and macrophages (Fig. 6), assessed by their nonspecific esterase-positive reaction, phagocytosis, and adherence. Second, in almost all cases, subcultivation of bone marrow and adherence-separated spleen cells results in a massive increase in Epo activity above that seen in the primary cultures. Third, maximum levels of Epo activity are obtained from subcultures initiated from day-3 bone marrow cells, day-1 primary cultures of adherent cells, and day-10 primary cultures of nonadherent spleen cells. Thereafter, the overall activity in the subcultures decreases, even though activity in the primary cultures may actually increase (e.g., bone marrow). The Epo activity obtained at maximum levels corresponds to between 20 and 25 mU Epo/ml culture. Finally, in most cases, a gradual increase in Epo activity in the supernatants of subcultured cells occurs over the first 2–3 weekly passages. By the fourth passage, Epo activity levels appear to plateau.

Similar points may be noted for CSA released and assayed in the same cultures, although greater variation is seen (Figs. 3B, 4B, and 5B). Minor amounts of CSA are observed in the supernatants of primary bone marrow cultures. This is in contrast to that seen in the spleen. The CSA levels in the supernatant from adherent cells increase up to day 14 and then appear to remain constant. For nonadherent cells, a maximum is reached at day 10 in primary culture, followed by a decrease. Subculture of the primary cultures from all three cell types results in a massive increase in CSA activity.

Fig. 4. (A) Assay of Epo activity and (B) CSA in supernatants from primary (designated “P”) and subcultured spleen adherent cells. Primary cultures from days 1, 3, 7, 10, 14, 17, and 21 were subcultured at weekly intervals. The number of weekly passages is shown at the bottom of the graph.
Although not shown, the nucleated cell counts for bone marrow and adherence-separated spleen cells from primary and subculture cell suspensions demonstrate that maintenance or an increase in cell number is achieved in the primary and subcultured groups.

These data indicate that the culture system can support the growth of cells capable of producing and releasing Epo and CSA simultaneously into the surrounding medium.

The Effect of Cycloheximide on the Production of Epo and CSA From Spleen Cells

A further substantiation of the hypothesis that macrophages can synthesize Epo and CSA was tested by reversibly inhibiting protein synthesis in unseparated spleen cell cultures using cycloheximide. The data are shown in Table 1 for Epo and Table 2 for CSA.

In these experiments, the cells were preincubated for 30 min, 24 hr, and 48 hr prior to the addition of cycloheximide. The Epo activity and CSA in the supernatants could then be ascertained and compared with the activities of the 2 stimulators found in the supernatants at 30 min, 24 hr, and 48 hr after the cycloheximide had been washed out of the cells after a 6-hr incubation.

The important points from the data may be summarized as follows. In almost all cases, the Epo activity and CSA in the preincubated cultures (second column) that would be later treated with cycloheximide are not significantly different from those to be used as controls. Second, the longer the cells are preincubated, the greater the inhibitory effect of the drug. Third, the...
Fig. 6. Electron micrograph of a macrophage surrounded by two other macrophages and two monocytes obtained from bone marrow cells cultured for 14 days in Petriperm dishes in the presence of $10^{-5}M$ Tg (x13,235).

Table 1. The Effect of Cycloheximide on Epo Production From Unseparated Spleen Cell Cultures.

<table>
<thead>
<tr>
<th>Preincubation Culture Time</th>
<th>Epo Activity (CFU-E/10⁶) in Preincubation Culture Supernatant</th>
<th>Time in Post-Cycloheximide-Treated Incubation Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Pre-Ohex</td>
</tr>
<tr>
<td>30 min</td>
<td>537.6 ± 47.8</td>
<td>543.8 ± 60.4†</td>
</tr>
<tr>
<td>24 hr</td>
<td>Control</td>
<td>Pre-Ohex</td>
</tr>
<tr>
<td>48 hr</td>
<td>Control</td>
<td>Pre-Ohex</td>
</tr>
</tbody>
</table>

Ohex refers to cycloheximide treatment. All values represent fetal liver CFU-E/10⁶ cells plated ± SEM.

*Significantly different ($p < 0.05$) using t test but not significantly different ($p > 0.1$) using the U test, compared to other controls.
†Not significantly different ($p > 0.1$) compared to other controls.
‡Significantly different ($p < 0.05$) compared to control.
§Not significantly different ($p > 0.1$) compared to control.
controls during the preincubation phase. These results indicate that Epo and CSA biosynthesis is taking place and the nonadherence of the cells to hydrophobic Teflon surfaces, since Epo activity and CSA is negligible amount. It may also be assumed that the endogenous presence of macrophages, could be totally accounted for by the spontaneous colony formation, which amounts to less than 20 CFU-E/10^5 cells. The lowest Epo concentration added to the cultures was 1.56 mU/ml, producing 150 CFU-E/10^5 cells. Furthermore, reduction in the FCS concentration to 10% does not affect the result normally obtained with 30% FCS. The fact that Epo activity present in silica-treated spleen supernatants can be detected by the in vivo bioassay and can be neutralized by anti-Epo lends further evidence for the use of the fetal liver CFU-E system as a sensitive Epo in vitro assay. Recent data show that concentrated supernatants can stimulate the incorporation of radioiron into rat bone marrow cells (unpublished data), and preliminary results indicate that Epo in supernatants can be detected by radioimmunassay.

Crystalline silica has been used as a tool to selectively release Epo from macrophages. Although longer the cells are preincubated, the greater the recovery of Epo activity and CSA after cycloheximide treatment. Finally, the kinetics of activity release after treatment are different for Epo and CSA; in contrast to CSA, Epo activity at 24 hr and 48 hr is higher than that Epo and CSA biosynthesis is taking place and the two substances are being produced simultaneously.

**DISCUSSION**

The results presented here clearly show that macrophages can continuously produce and release Epo. The culture system allows granulocyte/macrophage progenitor cells to proliferate and differentiate into macrophages, which then produce Epo and CSA simultaneously. This appears to be dependent on the nonadherence of the cells to hydrophobic Teflon surfaces, since Epo activity and CSA is reduced when the cells are allowed to adhere to a surface.

The assay system used to detect Epo activity released into the surrounding medium of bone marrow and spleen cells relies on the stimulation of early fetal liver CFU-E to form colonies in vitro. There are several valid criticisms against using fetal liver as a source of target cells for the detection of Epo activity. One criticism is that the assay could detect a factor that is not Epo. Although certainly a possibility, this is unlikely, since the Epo activity can be detected in the in vivo bioassay using polycythemic mice as well as the in vitro assay, implying that the Epo detected still retains its sialic acid; asialo Epo cannot be detected by the in vivo bioassay.20 Fetal liver cells also contain macrophages that have been shown to release Epo in fetal liver cultures and by treatment of fetal liver cell suspensions with silica.10 The possibility therefore exists that the macrophages present in the fetal liver assay could provide the Epo activity observed. Zucali et al. demonstrated that the Epo released from fetal liver cultures increases from about 150 mU/ml on day 13 to about 500 mU/ml on day 15 of gestation using about 180 x 10^6 cells. Approximately 4 mU/ml are released from 8 x 10^6 fetal liver cells using the silica technique.10,11 Since 0.75 x 10^5 cells/ml were used in the assay, it can be calculated that about 0.1 mU of Epo would be supplied additionally to each assay dish; a negligible amount. It may also be assumed that the 30% FCS used in the assay would also contribute to the effect seen. However, this as well as the endogenous presence of macrophages, could be totally accounted for by the spontaneous colony formation, which amounts to less than 20 CFU-E/10^5 cells. The lowest Epo concentration added to the cultures was 1.56 mU/ml, producing 150 CFU-E/10^5 cells. Furthermore, reduction in the FCS concentration to 10% does not affect the result normally obtained with 30% FCS. The fact that Epo activity present in silica-treated spleen supernatants can be detected by the in vivo bioassay and can be neutralized by anti-Epo lends further evidence for the use of the fetal liver CFU-E system as a sensitive Epo in vitro assay. Recent data show that concentrated supernatants can stimulate the incorporation of radioiron into rat bone marrow cells (unpublished data), and preliminary results indicate that Epo in supernatants can be detected by radioimmunassay.

Crystalline silica has been used as a tool to selectively release Epo from macrophages.10,11 Although

<table>
<thead>
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<th>Time in Post-Cycloheximide-Treated Incubation Phase</th>
<th>Control</th>
<th>Ohex</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 min</td>
<td>Control</td>
<td>Ohex</td>
</tr>
<tr>
<td>19.9 ± 1.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 hr</td>
<td>Control</td>
<td>Ohex</td>
</tr>
<tr>
<td>61.4 ± 2.0</td>
<td>32.9 ± 1.5</td>
<td>38.2 ± 3.3</td>
</tr>
<tr>
<td>48 hr</td>
<td>Control</td>
<td>Ohex</td>
</tr>
<tr>
<td>101.0 ± 10.3</td>
<td>56.0 ± 1.3</td>
<td>60.9 ± 4.1</td>
</tr>
<tr>
<td>Ohex</td>
<td>97.9 ± 9.8*</td>
<td>15.6 ± 1.5*</td>
</tr>
</tbody>
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Ohex refers to cycloheximide treatment. All values represent bone marrow GM-CFU/10^5 cells plated ± SEM.

*Not significantly different (p > 0.1) compared to other controls.
†Not significantly different (p > 0.1) compared to control using both t test and U test.
‡Not significantly different (p > 0.1) compared to control using only t test.
§Significantly different (p < 0.05) compared to control.
silica is ingested by many cell types, it is specifically cytotoxic only for macrophages. The conditions employed in the above and previous reports constitute a delayed silica cytotoxicity, which results in the disruption of lysosomal membrane integrity, probably by phospholipid peroxidation, and release of the lysosomal contents into the extracellular fluid. Since crystalline silica selectively and specifically injures and destroys macrophages, it is reasonable to assume that release of Epo activity reflects the silica action on those cells. It is therefore possible to conclude that the curves obtained for silica-treated adherent and nonadherent cells cultured in Petriperm dishes demonstrate the residual release of Epo activity from macrophages. Since these curves are similar to those obtained for the free release of Epo activity directly into the surrounding medium, it is also possible to infer the involvement of a cell similar to a macrophage in this phenomenon. This has been substantiated by showing that the cells derived from Petriperm cultures exhibit the functions and characteristics normally ascribed to macrophages, i.e., adherence, phagocytosis of latex and silica particles, nonspecific esterase staining, and electron microscopic identification. That Epo activity can be shown to be continuously released from primary and subcultivated spleen nonadherent, adherent, and bone marrow cells, implies biosynthesis of Epo by macrophages rather than release from an intracellular pool, the possible uptake and secretion of Epo originally present in the culture medium or even residually present after removal of the cells from their in vivo site. A similar argument also applies to the CSA detected in these cultures. To substantiate the biosynthesis hypothesis, spleen cells were treated with the reversible protein synthesis inhibitor, cycloheximide. The results clearly demonstrated that the drug reversibly inhibited Epo and CSA production. Erythropoietin production has been shown to be inhibited by actinomycin-D in the lead-poisoned rat, while CSF production by the lung has been shown to be inhibited by both actinomycin-D and puromycin. This is the first report of simultaneous, reversible inhibition of Epo and CSA production in presumably the same population of cells.

The observation that spleen nonadherent as well as adherent cell populations can produce and release similar levels of Epo activity deserves some discussion. It is generally assumed that adherent cells consist primarily of monocytes and macrophages, but separation by adherence does not produce pure populations. However, an explanation for the similarity in Epo activity is possible if it is assumed that the nonadherent cell population consists of granulocyte/macrophage precursor cells that have a high proliferative potential and possibly a certain amount of self-maintenance. Macrophages already present in the cell suspension could conceivably be producing CSA, which in turn could be stimulating these early precursor cells. In addition, the in vitro environment (medium components, culture surface, etc.) in which the cells find themselves appears to preferentially promote differentiation into the macrophage cell lineage. These factors and possibly others could account for maintaining the high numbers of nucleated, nonadherent cells and greater incidence of macrophage colonies than those of the adherent cell suspension (Fig. 2 A and B). The act of subculturing the cells appears to “amplify” the phenomenon found in the primary cultures by applying a proliferation and differentiation pressure on the progenitor cells into the macrophage cell line. Although bone marrow cells were not separated by adherence, a similar argument would apply. Indeed, Epo activity and CSA measured in the supernatants from subcultures initiated from day-3 primary culture are similar to those found in the adherence-separated spleen cell supernatants. However, the potential for Epo and CSA production in bone marrow subcultures wanes prior to both nonadherent and adherent cultures and may imply a functional difference between the bone marrow and splenic macrophage. The fact that bone marrow and splenic macrophages can produce Epo as well as CSA raises the important question as to the role of the macrophage in the regulation of hemopoiesis.

Bradley et al. and Sheridan and Stanley showed that bone marrow and spleen cells produce CSA, which has also been found in numerous other macrophage-containing organs and tissues. Although caution is necessary in extrapolating in vitro results to the in vivo situation, there is a precidence for suggesting that macrophage-produced Epo from different sources has a physiologic role. The main evidence comes from studies on extrarenal Epo production.

The main extrarenal site of Epo production is considered to be the liver. This is not surprising since the fetal liver supports erythropoiesis by Epo produced by the Kupffer cell. Peschle et al. implicated the Kupffer cells as the site of Epo/erythropoietin production, and work by Naughton et al. has substantiated this. However, Rambach et al. demonstrated that an erythropoietic stimulating factor could be released from spleen cell suspensions. In the mouse, the spleen plays an important role in erythropoiesis during ontogeny as well as during pregnancy. It is therefore also not surprising that a remnant of this activity should be continued in the form of Epo production. When perturbated, erythropoiesis (and granulopoiesis) can be transferred to the spleen, which
acts as an emergency organ until hemopoiesis returns to normal. The fact that Epo and CSA can be detected in the spleen and, in particular from splenic macrophages, may account for this organ being so important in hemopoietic recovery. Extrarenal sites of Epo production are responsive to hypoxia in anephric rats, and the increase in plasma Epo titer observed is abolished by actinomycin-D. We have shown that the increase seen after hypoxia during polycythemia. The extrarenal sites of Epo production, may account for this organ being so important in the spleen and, in particular from splenic macrophages, may account for this organ being so important in hemopoietic recovery.

The apparent low but constant ubiquitous supply of Epo from macrophages from different sources has to be reconciled with the release of Epo by the kidney under normal conditions. The macrophages as part of the reticuloendothelial system and the hemopoietic inductive microenvironment may play a greater role in erythropoietic regulation, especially short range and cell-to-cell interactions, than previously thought, and particularly in the bone marrow where erythropoiesis prevails under normal adult conditions. The addition of Epo to the list of biologically active molecules produced and secreted by the macrophage can only increase the importance of this cell in the physiologic control of cellular growth and differentiation.

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Extrarenal erythropoietin production by macrophages

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