Effects of Membrane Dialysis and Filtration-Sterilization on Erythropoietin Activity

VINCENT S. GALLICCHIO, Ph.D.,a AND MARTIN J. MURPHY, Jr., Ph.D.

The Bob Hipple Laboratory for Cancer Research, Wright State University School of Medicine, Dayton, Ohio, and a Department of Therapeutic Radiology, Yale University School of Medicine, New Haven, Connecticut

Received November 12, 1980

Most erythropoietin (Ep) preparations contain non-erythropoietin contaminants. The use of such hormone concentrates raises important questions regarding interpretations of results derived from in vivo and especially from in vitro studies. By sterilizing various Ep preparations with Nalgene, Millipore, or Selas silver filtration, or even after conventional membrane dialysis, variable responses were noted when the Ep was assayed with mouse bone marrow cells in vitro (i.e., by stimulating the production of erythroid colonies from CFU-e and BFU-e) and in vivo (i.e., by using the exhyposic, polycythemic mouse bioassay for Ep). The utility and limitations of such preparative procedures are discussed.

INTRODUCTION

Urine from anemic patients and plasma from anemic animals have long served as sources of erythropoietin (Ep), the hormone which specifically regulates erythropoiesis. During the last two decades, simple as well as complex biochemical and biophysical procedures have been employed to purify this glycoprotein. Procedures such as precipitation with ethanol, acetone, tannic acid, and benzoic acid have proven useful in concentrating the hormone. Other techniques such as flash evaporation, ultrafiltration, selective dialysis against drying agents, DEAE-cellulose chromatography, and adsorption and elution from kaolin have also been used [1-3].

Purification of erythropoietin has proven to be a major task because of the lability of the purified moiety and insufficient quantities of initial raw material (e.g., urine from anemic patients or plasma from anemic sheep). The purification of Ep has, however, been achieved by Miyake et al. [4]. This material from urines voided by aplastic anemic patients contained 79,000 units of Ep activity per mg of protein with a molecular weight of 39,000 to 40,000. It produced a single band on gel electrophoresis at pH 6 in the presence of 0.05 percent Triton X-100, and yielded a single band on gel electrophoresis at pH 7 in the presence of sodium dodecylsulfate and mercaptoethanol.

Recent reports have described that contaminated preparations of erythropoietin exist and have discussed their possible effects on hematopoiesis [5]. The following report describes enhancement in erythropoietin activity in vitro after erythropoietin preparations were subjected to sterilization with silver filters, Nalgene filters, or membrane dialysis. Such treatment potentiated the ability of Ep preparations to
stimulate murine progenitor cells to form erythroid colonies in culture, and at the same time did not alter the specific activity of Ep when assayed in the exhypoxic, polycythemic mouse bioassay. On the other hand, Millipore filtration resulted in a loss of Ep specific activity both in vitro by reducing the number of erythroid colony forming cells (CFU-e and BFU-e) from normal mouse marrow and in vivo as measured in the Ep mouse bioassay.

MATERIALS AND METHODS

Mice

BDF₁ male (23–28 gm, Charles River Breeding Labs, Wilmington, MA) were kept in standard plastic cages and fed Purina Lab Chow and water ad libitum. Mice were sacrificed by cervical dislocation. The femurs were excised and the bone marrow cells flushed and suspended in alpha medium (Flow Laboratories, Inc., Rockville, MD). Single cell suspensions were made using a tuberculin syringe with a 23 gauge needle, spun at 2,000 rpm for five minutes and appropriately diluted with alpha medium.

Assay for CFU-e and BFU-e

Culture assays for erythroid precursors were carried out in a methylcellulose system as already described [6,7]. A concentration of 2 × 10⁴ cells per ml was plated in plastic 35 mm tissue culture dishes (Lux Standard, No. 5221-R, Flow). No fewer than eight replicate plates per assay point were used. Media consisted of alpha medium (Flow), 0.8 percent methylcellulose (Fisher Scientific Co., Norcross, GA), 1 percent bovine serum albumin (Calbiochem, San Diego, CA), 30 percent fetal bovine serum (Batch No. 40511240, Flow), 10⁻⁴ M 2-mercaptoethanol in a final volume of one ml. Four different preparations of erythropoietin were used: sheep plasma, Step III Ep [5.88 International Units (IU) per mg; Connaught Laboratories, Willowdale, Ontario, Canada]; WHO-International Reference Preparation (2 IU per mg); and two different human urinary extracts designated “MU-3” (34 IU per mg) and “M-7” (16 IU per mg).¹

The cell cultures were incubated at 37⁰C in a humidified atmosphere flushed with 5 percent CO₂ in air. Colonies were stained and identified as erythroid by the improved benzidine staining method of Gallicchio and Murphy [8] and scored using an inverted microscope (Wild, Heerbrugg, Switzerland) at 40× magnification. For higher resolution light microscopy, individual colonies were plucked from the culture plate and cytocentrifuged (Cytospin, Shandon-Elliot, Sewickley, PA) at 500 rpm for five minutes and examined after Wright/Giemsa counter staining.

Step III, MU-3, and M-7 erythropoietin preparations were individually dissolved in alpha medium and then subjected to continuous dialysis (Spectrophor membrane tubing, cylindrical diameter 14.6 mm, MW cutoff 6,000–8,000, Spectrum Medical Industries, Inc., Los Angeles, CA) against alpha medium for 24 hours at 4⁰C. The dialysis medium (i.e., dialysate) was changed after four hours; the ratio of the dialysate to dialysand was 15:1. Protein determinations before and after analysis were performed using the method of Lowry [9]. Normal murine marrow cells were plated in the presence of varying concentrations of either dialyzed or non-dialyzed erythropoietin (i.e., 0.25 to 2.0 IU per ml). Samples of erythropoietin were assayed for Ep

¹M-7 was collected and concentrated by Centro de Estudios Farmacologicos y de Principios Naturales, Buenos Aires, Argentina, further processed and assayed by Hematology Research Laboratories, Childrens Hospital of Los Angeles, under Research Grant HL-10880 of NHLBI.
activity in the exhypoxic, polycythemic mouse bioassay of Camiscoli and Gordon [10].

The erythropoietin preparations were also sterilized by membrane filtration using Nalgene (0.45 μm pore size, polystyrene nitrocellulose composition, Nalge Corp., Rochester, NY), Millipore [0.45 μm, (type HA) mixed ester of cellulose composition, Ashley, MA] or Selas filters (0.45 μm, silver metal membranes, Selas Corp., Huntington Valley, PA).

P values were determined using a two-tailed Student's t test. P values greater than 0.06 were not considered significant, and these actual values are, therefore, omitted in the tabular data.

RESULTS

The in vitro production of erythroid colonies derived from normal murine marrow cells cultured in the presence of four different concentrations of either non-dialyzed or dialyzed erythropoietin is given in Table 1. Dialyzed erythropoietin preparations produced a greater number of erythroid colonies than non-dialyzed preparations. However, neither dialyzed nor non-dialyzed erythropoietin was able to stimulate as many erythroid colonies as the non-dialyzed WHO-International Reference Preparation. This suggests that the WHO reference material is relatively free from contaminants associated with the other erythropoietin preparations and is, therefore, ideally suited as the standard reference material. There was no substantial protein loss during dialysis (e.g., MU-3 before dialysis = 3.88 mg of protein per ml and after dialysis = 3.86 mg of protein per ml).

Erythropoietin preparations sterilized by passage through Millipore filters had reduced biological activity when compared with Ep preparations sterilized by

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**Table 1**

Effects of Membrane Dialysis on In Vitro Activity of Erythropoietin

| Erythropoietin         | Ep units/plate | CFU-e* | BFU-e* | (P<) | non-D* | D*  | non-D* | D*  |
|------------------------|----------------|--------|--------|------|--------|-----|--------|-----|
| Sheep Plasma, Step III |                |        |        |      |        |     |        |     |
| (5.8 IU/mg)            | 0.25           | 29 ± 6 | 46 ± 6 | 1 ± 1| 7 ± 2  |     |        |     |
|                        | 0.50           | 51 ± 4 | 62 ± 7 | 11 ± 1| 14 ± 5 |     |        |     |
|                        | 1.00           | 63 ± 5 | 73 ± 7 | 16 ± 5| 21 ± 2 |     |        |     |
|                        | 2.00           | 71 ± 4 | 89 ± 5 | 19 ± 1| 24 ± 5 |     |        |     |
| Human Urinary MU-3 (34 IU/mg) |             |        |        |      |        |     |        |     |
|                        | 0.25           | 22 ± 5 | 38 ± 3 | 9 ± 2 | 13 ± 3 |     |        |     |
|                        | 0.50           | 38 ± 4 | 46 ± 5 | 13 ± 3| 16 ± 5 |     |        |     |
|                        | 1.00           | 59 ± 3 | 68 ± 7 | 18 ± 2| 22 ± 2 |     |        |     |
|                        | 2.00           | 86 ± 6 | 105 ± 7| 23 ± 2| 26 ± 5 |     |        |     |
| Human Urinary M-7 (16 IU/mg) |            |        |        |      |        |     |        |     |
|                        | 0.25           | 19 ± 5 | 25 ± 2 | 3 ± 1 | 3 ± 1  |     |        |     |
|                        | 0.50           | 26 ± 5 | 51 ± 3 | 6 ± 2 | 9 ± 1  |     |        |     |
|                        | 1.00           | 61 ± 4 | 84 ± 5 | 17 ± 2| 23 ± 2 |     |        |     |
|                        | 2.00           | 101 ± 5| 129 ± 6| 25 ± 1| 30 ± 4 |     |        |     |
| WHO-Reference Preparation |               |        |        |      |        |     |        |     |
| (2 IU/mg)              | 0.25           | 44 ± 3 |        | 14 ± 5|       |     |        |     |
|                        | 0.50           | 90 ± 7 |        | 18 ± 5|       |     |        |     |
|                        | 1.00           | 111 ± 6|        | 24 ± 2|       |     |        |     |
|                        | 2.00           | 160 ± 8|        | 36 ± 5|       |     |        |     |

*Per 2 × 10⁴ nucleated bone marrow cells/plate; Mean ± S.E. of 8 culture plates/point.

*Non-dialyzed erythropoietin.

*Dialyzed erythropoietin.
Nalgene filtration or Selas silver filtration as determined by the exhypoxic, polycythemic mouse bioassay (Table 2). This in vivo assay was confirmed and extended by the impaired ability of such Millipore sterilized material to induce erythroid colony formation in vitro (Table 3).

**DISCUSSION**

The various contaminants in erythropoietin preparations and the subsequent anomalous effects these moieties may exert on hematopoiesis is of considerable concern. Studies employing Millipore filtration for sterilization of both erythropoietin and colony stimulating activity have reported loss of both factors [11-14]. We now report that Millipore filtered Ep preparations had a reduced capacity to produce CFU-e derived colonies in culture and also a reduced $^{59}$Fe incorporation into red blood cells when measured in the Ep mouse bioassay. Human Ep preparation M-7, treated by whatever means, demonstrated no interference with the development of BFU-e derived bursts. The latter datum is consonate with the proposition that BFU-e are relatively insensitive to Ep but are dependent on an imperfectly defined factor(s), "burst promoting activity" (BPA) [15,16]. CFU-e, on the other hand, are exquisitely sensitive to, and their viability in vitro is reliant upon, Ep [15]. The loss of Ep following Millipore filtration lends support to both of these contentions and indicates that BPA is not removed by Millipore filtration as is Ep.

The treatment of Ep by membrane dialysis prior to its in vitro use indicates that dialysis of Ep can significantly improve the plating efficiency of both CFU-e and BFU-e at 2 IU Ep ($p$ value < 0.01-0.06). Such membrane dialysis treatment removes small molecules which may be inhibitors present in such Ep preparation. Routine membrane dialysis treatment of Ep prior to employment in murine clonal cell cultures has already been recommended by Ogawa [17]. The exact chemical nature of these dialyzable and unwanted moieties remains, however, to be established.

The exact means whereby Millipore filters resulted in a loss of Ep activity is still not clear. It has been suggested that the composition of the filters themselves may

| Test Material | % $^{59}$Fe incorporated in RBC* | Retention of Ep Activity (%) |
|---------------|---------------------------------|-----------------------------|
| 0.20 IU (MU-3) Erythropoietin (Nalgene Filtered) | 15.42 ± 1.31 | 100 |
| 0.20 IU (MU-3) Erythropoietin (Selas Filtered) | 16.50 ± 1.03 | 100 |
| 0.20 IU (MU-3) Erythropoietin (Millipore Filtered) | 9.07 ± 0.55 | 59 |
| 0.20 IU (MU-3) Erythropoietin (Unfiltered) | 12.20 ± 0.38 | 100 |
| Alpha Medium | 1.02 ± 0.37 | - |

*Mean ± S.E. of 10 assay mice per point.*
EP ACTIVITY AFTER DIALYSIS AND STERILIZATION

Comparison of Membrane Filtration-Sterilization on Erythroid Colony Formation

| Ep* Concentration (IU/ml) | CFU-ea Filtration-Sterilization | BFU-ea Filtration-Sterilization |
|-------------------------|---------------------------------|-------------------------------|
|                         | Millipore | Selas | P < | Millipore | Selas |
| 0.25                    | 3 ± 1     | 10 ± 2 |     | 1 ± 1     | 3 ± 1 |
| 0.50                    | 15 ± 2    | 26 ± 4 | (0.01) | 5 ± 2     | 9 ± 2 |
| 1.00                    | 35 ± 4    | 60 ± 6 | (0.01) | 14 ± 3    | 17 ± 3 |
| 2.00                    | 87 ± 7    | 121 ± 8 | (0.01) | 27 ± 4    | 35 ± 4 |

*Ep: M-7 with a specific activity of 16 IU/mg.
*aPer 2 × 10⁴ nucleated bone marrow cells/plate; mean ± S.E. of 8 culture plates/point.

contribute to the resultant loss of Ep activity. Millipore filters used in these studies contained mixed esters of cellulose, whereas Nalgene filters were of nitrocellulose composition. This may explain the observed differences. Other studies [18] have documented the presence of non-ionic detergents, such as Triton X-100, impregnating Millipore filters. Lowy and Keighley [13] have, however, shown that these detergents alone are not solely responsible for the loss of Ep with Millipore filters since washing the filters free of detergents still resulted in a loss of Ep activity. Their work indicates that the high concentration of tryptophan, an amino acid that Ep contains, is selectively bound to the Millipore filters.

Heretofore, all the reported studies which have been performed reflected the inability of Millipore treated Ep preparations to influence ⁵⁹Fe incorporation in diffusion chambers [12] or ⁵⁹Fe incorporation into erythrocytes [13]; however, no documented studies have been reported comparing various filtered preparations using the in vitro clonal erythroid stem cell assay. These present studies suggest that prior treatment of erythropoietin by dialysis followed by either Nalgene or silver filter sterilization reduces contamination by as yet undefined substances without any loss of Ep during filtration. It is suggested that Ep preparations for in vitro clonal stem cell usage be treated in this manner until a supply of pure hormone becomes more readily available.

ACKNOWLEDGMENTS

The authors express their appreciation to Dr. Albert S. Gordon, Ms. C. Gizzi, and Mr. R. Mahar for the erythropoietin bioassays. These studies were supported by NIH grants AM-19741 and AM-07266, and the Bob Hipple Memorial Committee for Cancer Research.

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