Characterization of Erythropoietin Produced by 1W32 Murine Erythroleukemia Cells

By J. Choppin, C. Lacombe, N. Casadevall, O. Muller, P. Tambourin, and B. Varet

1W32 is a recently described murine erythroleukemia cell line that produces an erythropoietic factor similar to erythropoietin by in vivo and in vitro bioassays and without species specificity. Biochemical characteristics of 1W32 erythropoietic factor and sheep or mouse plasma erythropoietins were compared. Murine colonies derived from erythroid colony-forming units (CFU-E) in plasma clot culture were used as the bioassay system. Both 1W32 erythropoietic factor and sheep plasma erythropoietin were stable in the pH range of 3 to 10, after exposure to denaturing agents (8 mol/L urea, 4 mol/L guanidine hydrochloride, 1% sodium dodecyl sulfate), to a reducing agent (0.1 mol/L 2-mercaptoethanol) and to an oxidizing agent (5 mmol/L sodium metaperiodate). Only the combination of 0.1 mol/L 2-mercaptoethanol and 1% sodium dodecyl sulfate resulted in a significant loss of activity. 1W32 erythropoietic factor and murine plasma erythropoietin were similarly precipitated by ethanol and ammonium sulfate. 1W32 erythropoietic factor eluted as a single major peak after gel exclusion chromatography, with an estimated molecular weight of 45,000 daltons. Results were identical using supernatants from cultures in the presence of and absence of fetal calf serum. The supernatant of 1W32 cells cultured without serum induced erythroid colonies after seven days on normal human bone marrow nonadherent mononuclear cells cultured in serum-free conditions. All these results made it very likely that 1W32 cells produce an authentic erythropoietin. This cell line would be very useful for the study of murine erythropoietin.

Erythropoietin is the glycoprotein hormone that plays a major role in the development of erythrocytes from undifferentiated erythroid progenitors. Usual sources of erythropoietin are urine of anemic human patients or plasma of anemic sheep. The supply of this material is quantitatively limited; therefore, the availability of a cell line producing large amounts of erythropoietin would be very useful. Tumor cells have been used to produce large amounts of growth factors in other systems. Several cell lines have been recently established from human patients with renal2-5 or hepatic6 carcinomas and have been shown to produce some erythropoietin in the culture medium. We have recently described a murine erythroleukemia cell line, 1W32, which in vitro produces large amounts of an erythropoietic factor.6-7 This cell line was derived from a leukemia induced in an ICFW mouse by a helper of the Friend virus that was biologically cloned in our laboratory. The observation that this transplantable leukemia gave rise to polycytemia in syngeneic recipients led to this discovery.6 This factor appeared biologically similar to erythropoietin according to the following criteria: (1) the ability to increase in vivo erythrocyte production in polycythemic mice; (2) the ability to induce in vitro the terminal erythroid differentiation of colony-forming units (CFU-E) without species specificity. Preliminary comparative studies between a standard erythropoietin (Step III, Connaught Laboratories, Toronto) and the 1W32 erythropoietic factor showed that both activities were highly heat resistant and that trypsin sensitivity was only observed at high enzyme concentrations.7

In this article, the 1W32 erythropoietic factor is further analyzed. We demonstrate that some biochemical characteristics of the 1W32 erythropoietic factor are identical to those of sheep or mouse erythropoietin. These results are compatible with the hypothesis that 1W32 produces an authentic erythropoietin.

MATERIALS AND METHODS

Cultivation of 1W32 Cells

The 1W32 cell line has been maintained in continuous culture for 14 months. Cells at a density of 10⁶ cells/mL were cultured in 25 cm² culture flasks (Corning, Medfield, Mass) with 10 mL alpha medium supplemented with 5% FCS and 200 mmol/L glutamine. Cells were then split at a ratio of 1/20 two times a week.

Preparation of 1W32 Conditioned Media

Two different conditioned media were prepared. 1. 5% FCS, referred to as 1W32-CM (FCS). Culture supernatant was collected when cell density reached 10⁶ to 1.5 x 10⁶ cells/mL. Cells were removed by centrifugation, and conditioned medium was filtered through 0.2-μm filters (Millipore, France) and stored at −20 °C.

2. FCS-free conditioned medium, referred to as 1W32-CM (BSA). When exponential growth was achieved, cells were centrifuged, washed, and resuspended in alpha medium containing 1.7 mg/mL bovine serum albumin (BSA, Sigma, St Louis), detoxified according to McLeod et al.5 5 μg/mL transferrin (Sigma), 5 μg/mL insulin (Sigma), and several trace elements as described by Honma.

From the Laboratoire d’Immunologie et de Virologie des Tumeurs, INSERM U 152, Hôpital Cochin, Paris; and INSERM U 22, Faculté des Sciences, Orsay, France.

Supported in part by grants DGRST 81 L 07 29 and INSERM CRL 82 20 35.

Submitted Sept 22, 1983; accepted March 1, 1984.

Address reprint requests to Dr Jeaninne Choppin, Laboratoire d’Immunologie et de Virologie des Tumeurs, INSERM U 152, Hôpital Cochin, 27 rue du Faubourg Saint-Jacques, 75674 Paris Cedex 14, France.

© 1984 by Grune & Stratton, Inc.

0006-4971/84/6402-0003$03.00/0
et al. and allowed to grow for 48 hours before collecting the supernatant.

Sources of Erythropoietin
Sheep plasma erythropoietin, Step III (Connaught) from the same batch No. 3077 was used in all experiments. Its specific activity was 3.3 IU/mg protein. Mouse erythropoietin was prepared from the supernatant.

Bioassay for Erythroid Progenitors (CFU-E Assay)

CFU-E assay was performed in plasma clot cultures according to McLeod et al. with a few modifications. Cultures were set up with normal BALB/c mouse bone marrow cells. Assays were performed in 35 x 10 mm Petri dishes (Falcon 3001) containing 0.2 mL FCS, 0.1 mL bovine serum albumin (10%), detoxified according to McLeod et al., 0.1 mL L-asparagine (Calbiochem, San Diego, Calif) at 1.3 mmol/L in alpha medium plus CaCl_2 (2.5 mmol/L), 0.1 mL cell suspension, 0.2 IU erythropoietin Step III (Connaught) in 0.1 mL alpha medium, and 0.3 mL alpha medium. To test the IWF32-conditioned media, erythropoietin was omitted, and 0.3 mL of conditioned media was added instead of alpha medium. Duplicate cultures were allowed to clot after addition of 0.1 mL of bovine-citrated plasma (Flobio) and were then incubated at 37 °C in 5% CO_2 humidified atmosphere. After a 60-hour incubation, clots were fixed with glutaraldehyde, stained with benzidine-hematoxilin, and scored for erythroid colonies.

Characterization of IWF32 Erythropoietic Factor

Chemical Treatments
Crude IWF32-conditioned medium, IWF32-CM (FCS), was diluted 1:1 in Tris-buffered saline (TBS), pH 7.4, before treatments. Freeze-dried sheep erythropoietin Step III was dissolved at the concentration of 0.2 IU/mL in alpha medium containing 5% FCS and TBS, pH 7.4 (vol 1:1).

Each treatment described in the Results section was performed by one-hour incubation at 20 °C of 1-mL samples. Different reagents were used: 0.1 mol/L 2-mercaptoethanol (2-ME), 8 mol/L urea, 8 mol/L urea plus 2-ME, 4 mol/L guanidine hydrochloride (Gu-HCl), 4 mol/L Gu-HCl plus 0.1 mol/L 2-ME, 1% sodium dodecyl sulfate (SDS), 1% SDS plus 0.1 mol/L 2-ME, 1 mol/L acetic acid, 10 mmol/L sodium hydroxide, 5 mmol/L sodium metaperiodate. Reaction with sodium metaperiodate was stopped by addition of 50 mmol/L sucrose. The majority of SDS was eliminated by flocculation at 0 °C. Samples were dialyzed (see Preparations of Samples Before Assay) and adjusted to 1.5 mL in alpha medium.

Ammonium Sulfate Fractionation
This fractionation procedure was performed on plasma of anemic mice, IWF32-CM (FCS), IWF32-CM (BSA), and FCS alone. Mouse plasma and FCS were diluted 1:20 in TBS, pH 7.4. Proteins were precipitated at 0 °C with ammonium sulfate (Normapur, Prolabo, France). The powder was added slowly with constant stirring to achieve 50%, 60%, 70%, 80%, or 90% saturation for IWF32-CM (FCS) and plasma of anemic mice and to achieve 50%, 70%, and 90% saturation for IWF32-CM (BSA) and FCS. The successive precipitates were collected after centrifugation at 35,000 g for 30 minutes, dissolved in TBS, pH 7.4, and dialyzed (see Preparations of Samples Before Assay).

Ethanol Precipitation
One volume of plasma of anemic mice or IWF32-CM (FCS), containing 3 mol/L NaCl, was mixed successively with 1, 2, 3, and 4 vol of absolute ethanol (Ultrapur, Prolabo, France) with stirring at room temperature. After the flocculent precipitates had been allowed to settle, they were removed by centrifugation at 100 g for eight minutes. Precipitates were diluted in TBS (pH 7.4) and dialyzed.

Preparation of Samples Before Assay

After all chemical treatments and protein fractionations, 1-mL samples were dialyzed at 4 °C for three days against TBS (pH 7.4) and for one day against alpha medium using a microdialysis system (model 1200 MD, Bethesda Research Laboratories Inc, Rockville, Md) and membranes with a molecular weight exclusion range of 6,000 to 8,000. After dialysis, sample volumes were adjusted to 1.5 mL, and 0.3-mL fractions were tested in the CFU-E assay.

RESULTS

Effects of Chemical Reagents on IWF32 Erythropoietic Factor and on Sheep Plasma Erythropoietin

In vitro, erythropoietic activities were compared by murine CFU-E bioassay after various treatments of IWF32-CM (FCS) and Step III sheep plasma erythropoietin. As shown in Fig 1, treatment with the reducing agent, 0.1 mol/L 2-ME, and with the two denaturing agents, 8 mol/L urea and 4 mol/L Gu-HCl with and without 2-ME, did not significantly decrease the activity of IWF32-CM (FCS) factor (Fig 1A). Full recovery of activity was also observed after 1% SDS, acidic (1 mol/L acetic acid, pH 3) or oxidizing (5 mmol/L sodium metaperiodate) treatments. Significant increase of activity appeared after action of 10 mmol/L NaOH (pH 10). In contrast, a significant decrease of activity was observed after treatment with SDS and 2-ME. The activity of sheep erythropoietin was very stable and even appeared to be increased after most treatments (Fig 1B). Significant decrease of activity also appeared after combination of SDS and 2-ME.
Fig 1. Action of chemical treatments on activity of IW32-CM erythropoietic factor (A) and of sheep plasma erythropoietin (B). One-milliliter samples of IW32-CM (FCS) were diluted 1:1 in TBS, pH 7.4. One-milliliter samples of sheep erythropoietin diluted in alpha medium 5% FCS plus TBS, pH 7.4 (1:1) were adjusted to the concentration of 0.2 IU/mL. Chemical reagents were added at the concentration mentioned in Materials and Methods, and samples were kept at 20°C for one hour. After a dialysis at 4°C for four days, samples were adjusted to 1.5 mL in alpha medium. Three hundred microliters were tested in vitro in the murine CFU-E assay. Results were expressed as percent activity of controls (C). Each value ± SEM was calculated from results of three different experiments done in duplicate. Statistical analysis made by the Student's t test gave P < 0.001 after the SDS + 2-ME treatment (**) for IW32-CM factor. Most treatments gave significant increases (P < 0.01) of sheep plasma erythropoietin activity. The activities of controls were, respectively, 600 ± 110 CFU-E-derived colonies/5 x 10⁶ bone marrow cells for IW32-CM and 450 ± 75 CFU-E-derived colonies/5 x 10⁶ bone marrow cells for sheep plasma erythropoietin.

Fractionation of IW32-CM and Mouse Plasma Erythropoietin

Proteins of different samples were fractionated using ammonium sulfate (Figs 2 and 3). IW32-CM (FCS) erythropoietic activity was recovered in proteins precipitating between 50% and 80% saturation (Fig 2A). The erythropoietin equivalent activity was estimated in this experiment to 30 IU of erythropoietin in the crude medium (100 mL). About 50% (16 IU) was recovered after ammonium sulfate precipitation. Murine plasma erythropoietin was also precipitated by ammonium sulfate between 50% and 80% saturation (Fig 2B), and its activity (13 IU) was comparable to the activity of crude plasma (12 IU). Erythropoietic activity in IW32-CM (BSA) was precipitated by ammonium sulfate between 50% and 90% saturation (Fig 3A). The majority of active proteins was in the 50% to 70% fraction. The recovery (19 IU, about 60% of the activity of the crude supernatant) was similar to that of IW32-CM (FCS) factor. FCS alone had very low activity (10 ± 5 CFU-E/5 x 10⁶ bone marrow cells) when FCS was diluted 1:20 in alpha medium. After precipitation by ammonium sulfate, all fractions showed very low or no activity (Fig 3B).

When proteins of IW32-CM (FCS) and of plasma of anemic mice were fractionated by 3 mol/L NaCl-ethanol precipitation (Fig 4, A and B), the activity was recovered in the pool of proteins isolated after addition of 3 vol of ethanol. Recovery was approximately 60% of the activity of crude IW32-CM (FCS) and 70% of the initial activity of mouse plasma.

Fig 2. Fractionation of proteins of IW32-CM (FCS) and of plasma of anemic mice with ammonium sulfate. (A) Fractionation of 100 mL IW32-CM (FCS) with an erythropoietin equivalent activity of 30 IU. (B) Fractionation of 2.5 mL mouse plasma diluted 1:20 in TBS, pH 7.4. Activity of crude plasma was estimated to 5 IU/mL. Activity was tested in the murine CFU-E assay, and results were obtained by comparison with a standard titration curve using sheep plasma erythropoietin Step III. The total amount of protein per precipitate is expressed as total OD (280 nm) per fraction. ■ = Units of EP activity.

Fig 3. Fractionation of proteins of IW32-CM (BSA) (A) and FCS (B). One hundred milliliters of IW32-CM (BSA) and 2.5 mL of FCS, diluted 1:20 in TBS, pH 7.4, were fractionated. Activity was tested in the murine CFU-E assay, and results were obtained by comparison with a standard titration curve using erythropoietin Step III. Activity of crude IW32-CM (BSA) was 0.3 IU/mL, and activity of FCS was not detectable (< 0.01 IU/mL). The total amount of proteins per precipitate is expressed as total OD (280 nm) per fraction. ■ = Units of EP activity.
Fig 4. Fractionation of proteins of IW32-CM (FCS) (A) and of mouse plasma (B) by 3 mol/L NaCl-ethanol. A 20-mL sample of IW32-CM (FCS) with an erythropoietic activity of 0.6 IU/mL and 2.5-mL samples of crude plasma of anemic mice (activity of 5 IU/mL) diluted in TBS, pH 7.4 (50 mL), were fractionated. Activity was tested in the murine CFU-E assay, and results were calculated from a titration curve obtained with standard sheep plasma erythropoietin. The total amount of proteins per precipitate \( \equiv \) is expressed as total OD (280 nm) per fraction. \( \equiv \) = Units of EP activity.

When subjected to gel exclusion chromatography with ultrogel AcA 44 equilibrated in TBS, pH 7.4, IW32-CM (FCS), and IW32-CM (BSA) erythropoietic activities eluted as a single major peak (Fig 5 A and B). The active molecules were excluded slightly before ovalbumin in a volume corresponding to an approximate molecular weight of 45,000. Sheep plasma erythropoietin also eluted as a single peak, but slightly after ovalbumin in a volume corresponding to a mean molecular weight of 40,000 (Fig 6). Murine plasma erythropoietin eluted for a small proportion close to albumin, but the major peak was excluded in a volume corresponding to a molecular weight of 50,000 (Fig 7). The proteins of FCS were also submitted to gel filtration on ultrogel AcA 44, and all the fractions were inactive (data not shown).

Culture of Human CFU-E in Serum-Free Conditions in the Presence of IW32-CM (BSA)

In order to rule out the hypothesis that IW32 cells produced an erythropoietin-enhancing activity able to enhance the activity of the trace amounts of erythropoietin present in normal fetal calf serum, we tested the erythropoietic activity of IW32 cell supernatant in complete serum-free conditions. IW32-CM (BSA) was added, instead of erythropoietin, to nonadherent human bone marrow mononuclear cells cultured without serum. The experiment was carried out twice and in duplicate. The addition of 0.35 mL of IW32-CM (BSA) to \( 2 \times 10^5 \) cells plated in a volume of 1 mL induced 10 ± 5 reddish erythroid colonies per plate after eight days in culture. The erythroid nature of these colonies was confirmed by micropipetting and benzidine staining of individual colonies. No erythroid colonies were observed in control cultures without addition of erythropoietin or IW32-CM.

DISCUSSION

The recently described IW32 cell line is a murine erythroleukemia cell line induced after in vivo inoculation of a biologically cloned helper of Friend virus in ICFW mice. IW32 cells were shown to induce polycythemia after in vivo transplantation in syngeneic recipients. As previously described, the cell line supernatant was able to induce the terminal erythroid differentiation in vivo and in vitro. The erythropoietic activity was stable at 100 °C for three minutes, which ruled out the hypothesis of a polycythemia-inducing virus being
ERYTHROLEUKEMIA PRODUCING ERYTHROPOIETIN

345

alpha medium. Samples of 300 µL were tested in the CFU-E assay. (- -) Number of colonies/5 × 10⁶ marrow cells. Molecular weight markers: bovine serum albumin, ovalbumin, chymotrypsinogen A. Absorbance at 280 nm; (A—A) number of colonies/5 × 10⁶ marrow cells. Molecular weight markers: bovine serum albumin, ovalbumin, chymotrypsinogen A.

responsible for this erythropoietic activity. The erythropoietin equivalent activity of IW32 supernatant was measured against standard sheep plasma erythropoietin Step III and was estimated up to 0.3 IU/mL by in vivo assay and 1 IU/mL by in vitro bioassay. Such a discrepancy between in vivo and in vitro assays was recently observed in the supernatant of cultured human renal carcinoma cells. The aim of the present article is to determine whether IW32 erythropoietic factor is biochemically related to erythropoietin. In our previous paper, we reported that both IW32 factor and sheep plasma erythropoietin were heat stable and exhibited the same sensitivity to trypsin digestion. In this report, we showed that IW32 factor, as well as sheep plasma erythropoietin, were stable to denaturing agents, such as 8 mol/L urea or 4 mol/L guanidine hydrochloride or 1% SDS, and to a reducing agent, 0.1 mol/L 2-ME. These results suggest either that tertiary structure is not important for erythropoietic activity or that this structure can be easily reconstituted or that no conformational change occurs in the tertiary structure under strong denaturing conditions. Moreover, the observation that the erythropoietin activity could be increased after sodium hydroxide was added to the IW32 factor and to sheep plasma erythropoietin suggests that some inactive forms exist. Miyake et al have previously reported the tendency for erythropoietin to aggregate and the appearance of a monomeric form in the presence of both SDS and the reducing agent dithiothreitol. Sytkowski also demonstrated that human urinary erythropoietin could exist in biologically inactive complexes and that an active hormone could be slowly dissociated from the complexed form. On the other hand, we observed that treatment of plasma sheep erythropoietin and of IW32 erythropoietic factor with SDS in the presence of 2-mercaptoethanol resulted in a significant decrease of activity. This suggests that the reduction of one or more disulfide bonds critical for biologic activity is possible only in the presence of SDS. Similar data were reported by Sytkowski with human urinary erythropoietin after treatment with 2-ME plus SDS plus urea.

In order to further compare biochemical characteristics of IW32 factor and erythropoietin and to establish a protocol for purification, some fractionation procedures were analyzed. A method currently used in the first or the second step of purification for many growth factors is ammonium sulfate fractionation. IW32 factor was precipitated between 50% and 80% saturation. Results of fractionation were similar using supernatant of IW32 cells cultured with and without serum. Moreover, no significant erythropoietic activity could be obtained after fractionation of fetal calf serum. These results ruled out the hypothesis that the erythropoietic activity of IW32 supernatant resulted from proteins of FCS or from an interaction between
an erythropoietic activity induced by IW32 cells on the erythropoietin of fetal calf serum. In the purification of human erythropoietin, an ethanol fractionation was described with an excellent recovery of activity. For IW32 factor and plasma murine erythropoietin, the active proteins could also be isolated using this method. The two factors have the same precipitation characteristics using the two fractionation procedures. The better recovery of plasma murine erythropoietin than of IW32-CM erythropoietic factor after ammonium sulfate precipitation might be due to differences in the structures of both proteins, but it could be simply explained by the role played by environmental proteins on this type of precipitation.

After gel filtration, the molecular weight of IW32 erythropoietic factor was estimated at 45,000. This mean value ranges between the value of sheep plasma erythropoietin and the value of murine plasma erythropoietin in our experimental conditions. Previously reported molecular weight of erythropoietin appears to vary according to the method of separation used: for the sheep hormone, a molecular weight of 46,000 for the native form and of 41,000 for the asialo molecule was determined with polyacrylamide gel electrophoresis in SDS. For human erythropoietin, a molecular weight of 43,000 had been described after extraction from the sera of patients with aplastic anemia and chromatography on a Sephadex G100 column. Using polyacrylamide gel electrophoresis in SDS, lower molecular weights had been reported: 39,000 and 25,000 in denaturing conditions. Our data showed differences of migration among sheep plasma erythropoietin, murine plasma erythropoietin, and IW32 factor. However, erythropoietin is a glycosylated molecule, and the values we have determined may be influenced by the degree and amount of carbohydrate on the molecule. Further experiments are being carried out after deglycosylations and electrophoresis.

The biologic properties of IW32 erythropoietic factor are those of erythropoietin: it induces in vivo and in vitro erythroid differentiation without species specificity. One could argue that this factor is an enhancer of erythropoietin, but this hypothesis can be ruled out because the supernatant of IW32 cells cultured without serum induced erythroid colonies in serum-free culture. To set up this experiment, we used the technique for culturing human CFU-E in serum-free conditions that we have recently described. Together with these biologic properties, the biochemical characteristics described in this paper (resistance of activity after exposure to different denaturant conditions, fractionation of factor with ammonium sulfate and ethanol, molecular weight value compatible with that of erythropoietin) make it very likely that the IW32 erythropoietic factor is an authentic erythropoietin. This does not rule out the possibility that minor structural differences might exist between IW32 erythropoietin and other erythropoietins. This would explain the differences between in vivo and in vitro erythropoietic activities of IW32-CM and some slight differences in the biochemical properties of IW32-CM and sheep or murine erythropoietin. The amount of erythropoietin produced by IW32 cells is relatively large—up to 1 IU/mL by in vitro bioassay. This is a much higher activity than those reported for some recently described human cell lines with erythropoietin activities ranging from 10 mU to 300 mU/mL. The IW32-conditioned medium in serum-free culture had a specific activity of 0.5 IU/mg protein by in vitro bioassay. Therefore, the IW32 cell line might be very useful for the production and the purification of murine erythropoietin. Most likely, it will make the study of the erythropoietin gene(s) easier.

ACKNOWLEDGMENT
We thank J.J. Metzger and J.P. Levy for helpful discussion and comments, and H.R. Gralnick for reviewing the manuscript.

NOTE ADDED IN PROOF
Using an antibody that recognizes human, rat, and mouse erythropoietin, E. Goldwasser found an erythropoietin concentration of 245 mU/mL in IW32 (FCS) supernatant (< 20 mU/mL in control culture medium with 5% FCS). This further confirms the production of erythropoietin by IW32 cells.

REFERENCES
7. Tambourin P, Casadevall N, Choppin J, Lacombe C, Heard...
ERYTHROLEUKEMIA PRODUCING ERYTHROPOIETIN

347


From www.bloodjournal.org by guest on October 22, 2017. For personal use only.
Characterization of erythropoietin produced by IW32 murine erythroleukemia cells

J Choppin, C Lacombe, N Casadevall, O Muller, P Tambourin and B Varet

Updated information and services can be found at: http://www.bloodjournal.org/content/64/2/341.full.html
Articles on similar topics can be found in the following Blood collections

Information about reproducing this article in parts or in its entirety may be found online at: http://www.bloodjournal.org/site/misc/rights.xhtml#repub_requests

Information about ordering reprints may be found online at: http://www.bloodjournal.org/site/misc/rights.xhtml#reprints

Information about subscriptions and ASH membership may be found online at: http://www.bloodjournal.org/site/subscriptions/index.xhtml