Characterization of Erythropoietin Produced by IW32 Murine Erythroleukemia Cells

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

IW32 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 IW32 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 IW32 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. IW32 erythropoietic factor and murine plasma erythropoietin were similarly precipitated by ethanol and ammonium sulfate. IW32 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 IW32 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 IW32 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 renal or hepatic carcinomas and have been shown to produce some erythropoietin in the culture medium. We have recently described a murine erythroleukemia cell line, IW32, which in vitro produces large amounts of an erythropoietic factor. 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 polycythemia in syngeneic recipients led to this discovery. 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 IW32 erythropoietic factor showed that both activities were highly heat resistant and that trypsin sensitivity was only observed at high enzyme concentrations.

In this article, the IW32 erythropoietic factor is further analyzed. We demonstrate that some biochemical characteristics of the IW32 erythropoietic factor are identical to those of sheep or mouse erythropoietin. These results are compatible with the hypothesis that IW32 produces an authentic erythropoietin.

MATERIALS AND METHODS

Cultivation of IW32 Cells

The IW32 cell line has been maintained in continuous culture for 14 months. Cells at a density of 10^6 cells/mL were cultured in 25 cm^2 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 IW32 Conditioned Media

Two different conditioned media were prepared.

1. 5% FCS, referred to as IW32-CM (FCS). Culture supernatant was collected when cell density reached 10^7 to 1.5 x 10^8 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 IW32-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 μg/mL transferrin (Sigma), 5 μg/mL insulin (Sigma), and several trace elements as described by Honma.
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 normal BALB/c mouse bone marrow cells. Assays were performed in 35 × 10 mm Petri dishes (Falcon 3001) containing 0.2 mL FCS, 0.1 mL bovine serum albumin (10%), detached according to McLeod et al.10, 0.1 mL L-asparagine (Calbiochem, San Diego, Calif.) at 1.3 mmol/L in alpha medium plus CaCl₂ (2.5 mmol/L), 0.1 mL cell suspension, 0.2 IU erythropoietin Step III (Connaught) on 0.1 mL alpha medium, and 0.3 mL alpha medium. To test the 1W32-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% CO₂ humidified atmosphere. After a 60-hour incubation, clots were fixed with glutaraldehyde, stained with benzidine-hematoxylin, and scored for erythroid colonies.

Culture of human CFU-E in methylcellulose and in serum-free conditions was done as previously described using 1.5 × 10⁻⁵ mol/L cholesterol (Sigma) and 1.5 × 10⁻⁵ linoleic acid (Serva) instead of liposomes.

**Characterization of 1W32 Erythropoietic Factor**

**Chemical Treatments**

Crude 1W32-conditioned medium, 1W32-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, 1W32-CM (FCS), 1W32-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 1W32-CM (FCS) and plasma of anemic mice and to achieve 50%, 70%, and 90% saturation for 1W32-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 1W32-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.

**Gel Filtration**

A column (0.9 × 66 cm) was packed with Ultrogel AcA 44 (IBF, France) and equilibrated with TBS (pH 7.4). Proteins of 1W32-CM, with and without serum, mouse plasma, and FCS were concentrated by ammonium sulfate precipitation, then dialyzed in TBS (pH 7.4). Sheep erythropoietin Step III (41U) was added to 3.5 mg bovine serum albumin (Sigma) and diluted in alpha medium. Samples of 0.6 mL were applied to the column, and proteins were eluted at a flow rate of 7 mL/h. Sixty fractions (0.6 mL each) were collected. Absorbance at 280 nm was monitored with an LKB Uvicord S II. The fractions were dialyzed at 4 °C for one day against alpha medium.

**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 1W32 Erythropoietic Factor and on Sheep Plasma Erythropoietin

In vitro, erythropoietic activities were compared by murine CFU-E bioassay after various treatments of 1W32-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 1W32-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.
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 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 10⁵ 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 precipitated 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 protein per precipitate is expressed as total OD (280 nm) per fraction. □ = 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 5A 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 x 10⁵ 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

against alpha medium. Samples of 300 assaying.

albumin, ovalbumin, chymotrypsinogen A. 

TBS, pH 7.4. Fractions of 0.6 ml were collected and dialyzed

The sample was applied to the AcA 44 column, equilibrated in

alpha medium (0.6 ml) containing 3.5 mg bovine serum albumin.

5 x 10^7 marrow cells. Molecular weight markers: bovine serum 

albumin, ovalbumin, chymotrypsinogen A.

The discrepancy between in vivo and in vitro assays was

vivo assay and 1 IU/mL by in vitro bioassay. Such a 
systematic frequency of increases after various treatments for

erythropoietin probably reflects slight differences in the structures of sheep plasma erythropoietin and 1W32 factor as well as differences in their respective ability to be complexed and inactivated. 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 1W32 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 1W32 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. 1W32 factor was precipitated between 50% and 80% saturation. Results of fractionation were similar using supernatant of 1W32 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 1W32 supernatant resulted from proteins of FCS or from an interaction between

Fig 6. Gel filtration of sheep plasma erythropoietin on Ultrogel AcA 44 column. Sheep plasma erythropoietin (4 IU) was diluted in alpha medium (0.6 mL) containing 3.5 mg bovine serum albumin. Sheep plasma erythropoietin was 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 reconstructed 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 1W32 factor and to sheep plasma erythropoietin suggests that some inactive forms exist. The greater frequency of increases after various treatments for sheep erythropoietin probably reflects slight differences in the structures of sheep plasma erythropoietin and 1W32 factor as well as differences in their respective ability to be complexed and inactivated. 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 1W32 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 1W32 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. 1W32 factor was precipitated between 50% and 80% saturation. Results of fractionation were similar using supernatant of 1W32 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 1W32 supernatant resulted from proteins of FCS or from an interaction between

Fig 7. Gel filtration of mouse plasma erythropoietin on Ultrogel AcA 44 column. Plasma of anemic mice was fractionated by ammonium sulfate precipitation. Precipitates of 60% to 80% were suspended in TBS and dialyzed against TBS, pH 7.4. The equivalent of 0.25 mL of crude plasma was applied to the column in a volume of 0.6 mL. Fractions of 0.6 mL were collected and dialyzed against alpha medium, and 300-μL samples were tested in the CFU-E assay. (---) Absorbance at 280 nm; (A——A) number of colonies/5 x 10^6 marrow cells. Molecular weight markers: bovine serum albumin, ovalbumin, chymotrypsinogen A.
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

6. Nicola NA, Metcalf D: Biochemical properties of differentia-

Characterization of erythropoietin produced by IW32 murine erythroleukemia cells

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