Human urinary erythropoietin has been highly purified by a combination of conventional purification methods and immunoadsorbent columns packed with hybridoma-produced antibodies against contaminants that seemed difficult to separate from erythropoietin by the usual means. By using the partially purified erythropoietin as an antigen, three hybridoma clones have been obtained that secrete monoclonal antibodies against erythropoietin. One of the clones has been quite stable, with a rapid growth rate and high production of antibody. Western blotting technique with monoclonal antibodies revealed occurrence of two species of erythropoietin. The monoclonal antibody will be useful as a probe for the purification of erythropoietin and for further studies of the hormone and its mechanism of action.

Erythropoietin (Ep) is a sialoglycoprotein that is believed to play an important role in regulating, through stimulation, erythropoiesis. Although isolation of this hormone from human urine has been reported, little is known of this hormone with respect to biochemical aspects, such as structure, mechanism of action, and metabolism. The problems in studying the biochemistry of Ep have been the limited supply of starting material, which is urine of patients with aplastic anemia, and the laborious purification procedures with low yields. Cumbersome and insensitive assay methods have been another problem. Monoclonal antibodies have been shown to be a powerful tool for the purification, characterization, and quantitative analysis of macromolecules. A recently published paper reported an Ep-directed hybridoma that was obtained by the fusion of rat spleen cells and mouse myeloma. In this article, we report the production of congenic mouse hybridomas that secrete monoclonal antibodies against human urinary Ep.

MATERIALS AND METHODS

Materials

Materials were purchased from the indicated sources. Standard Ep (sheep plasma Ep step-III, 4 U/mg of protein, Connaught Medical Research Laboratories, Toronto), radioactive compounds (Amersham International Ltd, Buckinghamshire, England), antibodies (Meiji Seika Co, Ltd, Tokyo); microtiter plates (Nunc, Denmark); flexible microtiter plates (Dynatech, Alexandria, Va); Staphylococcus aureus (Calbiochem, San Diego); Freund's complete adjuvant (Difco, Detroit); polyethylene glycol (BDH Chemica, Poole, England); DEAE-cellulose (DE52, Whatman, Clifton, NJ); Affi-Gel 10 (Bio Rad, Richmond, Calif); nitrocellulose paper (Schleicher and Schuell, Keene, NH); Hydrosylapatinite (Clarkson Chemical Co, Williamsport, Pa); rabbit anti-mouse IgG (Miles, Elkhart, Ind); sulfopropyl-Sephadex C-50 and phenyl-Sepharose CL4B (Pharmacia Fine Chemicals, Piscataway, NJ); RPMI 1640 and fetal calf serum (M.A. Bioproducts, Walkersville, Md); peroxidase-conjugated rabbit anti-mouse IgG, peroxidase-conjugated IgG fractions of goat anti-mouse immunoglobulins, and goat anti-mouse IgG (Cappel Laboratories, Cochranville, Pa); biotinylated horse anti-mouse IgG, avidin, and biotinylated horseradish peroxidase (Vector Labs, Burlingame, Calif).

Assay for Ep

For selection of urine from anemic patients with high Ep titer, the activity was assayed by counting erythroid colonies that were formed Ep-dependently in vitro suspension culture of 13-day-old mouse fetal liver cells. Three other assay methods were used, as indicated in legends of figures and tables. The in vitro assay, utilizing the stimulatory effect of Ep on incorporation of 59Fe into heme or 1H-thymidine into DNA in cultured mouse fetal liver cells, was performed with minor modifications as described previously. Each value shown is the mean of duplicate or triplicate cultures. The erythropoietin activity also was determined in vivo, using the starved rat method (4 rats/sample) according to the general procedures.

Immunization Schedule

In fusion experiments that failed to yield hybridomas producing anti-Ep antibody but did yield those producing antibodies against contaminants, 8-week-old female BALB/c mice were immunized with 200 μg of the partially purified Ep (5,100 U/mg protein) emulsified 1:1 in Freund's complete adjuvant in a total volume of 0.5 mL intraperitoneally. Three to four weeks later, they were boosted with 0.3 mL, containing 100 μg of the antigen in 10 mmol/L phosphate-buffered saline (PBS, pH 7.4, without adjuvant, intraperitoneally.

In the experiments that succeeded in raising Ep-specific hybridomas, the further purified Ep (38,000 U/mg protein) was used as an antigen. Two mice were immunized by intraperitoneal injection of about 20 μg of Ep in 0.5 mL of polyacrylamide gel suspension. Two and three weeks later, they were boosted intraperitoneally with 50 μg of the polyacrylamide gel-free Ep emulsified 1:1 in Freund's complete adjuvant in a total volume of 0.5 mL. Four other mice received first immunization and boost (after three weeks) in the same manner.
as the previously mentioned boost. At two weeks after the boosts, the sera (300 μL) were obtained according to the procedures described without anesthetizing the animals. The sera from two mice immunized with the gel suspension containing Ep gave precipitin lines with the Ouchterlony double-immunodiffusion method, and it was found with the immunoprecipitation method that the sera reduced the Ep activity in the antigen to 13% and 40%, indicating that the animals were immunized with Ep. These mice were boosted again with 44 μg of the gel-free Ep without adjuvant in a total volume of 0.3 mL. At three days after the last boost, their spleen cells were subjected to cell fusion. Four other mice that were negative in terms of Ep-binding sera were not used for fusion.

**Cell Culture and Cell Fusion**

Cells were cultured at 37 °C in 5% CO2/95% air atmosphere at 100% humidity. Spleen cell suspension from mice was prepared in RPMI 1640, 2 mmol/L glutamine, 1 mmol/L pyruvate, penicillin (50 U/mL), streptomycin (50 μg/mL) and 15% (vol/vol) fetal calf serum; this is designated as medium 1. Cells (10⁴) from a single spleen were fused with 5 x 10⁷ mouse myeloma cells (P3-NSI/1-Ag4-1 cells) by the procedures described by Oi and Herzenberg, using polyethylene glycol 1500. The fusion mixture in HT medium (medium 1 plus 100 μmol/L hypoxanthine and 15 μmol/L thymidine) was seeded into 96-well microtiter culture plates (0.1 mL suspension containing 5 x 10⁴ cells/well). Cells in the selective HAT medium (medium 1 plus 100 μmol/L hypoxanthine, 0.4 μmol/L aminopterin, and 15 μmol/L thymidine) were cultured according to the procedures and schedule reported. After three weeks of culture, wells demonstrating cell growth were tested for supernatant antibodies by a solid-phase antibody-binding assay. Antibody-positive cells were transferred into 1 mL cultures in 24-well tissue culture plates with BALB/c thymocytes (10⁵ cells/well) as feeder cells and switched to HT medium by removing half of the medium and replacing with fresh medium every three days for 20 days. Before further expansion of culture, the supernatants were again tested for antibody production. Cultures of positive cells were enlarged in medium I for preparing cells to produce ascites in mice.

**Solid-Phase Antibody-Binding Assay**

A solid-phase assay was used to detect antibody in hybridoma supernatants. Procedures were conducted at room temperature unless otherwise indicated. Flexible round-bottom 96-well microtiter plates were washed twice with ethanol and dried. To each well was added 25 μL of the Ep preparation (used for immunization) containing 200 to 500 ng of protein in PBS. The plates were incubated overnight at 4 °C and washed. Every washing in this experiment was done with 0.2 mL (per well) of 1% bovine serum albumin (BSA) in PBS. Usually, the plates were flicked empty at the fourth washing and then components for the subsequent reaction were added. Aliquots (200 μL) of culture supernatants were added and then the plates were incubated for two hours. After washing, 25 μL of PBS containing 0.78 μg of biotinylated horse anti-mouse IgG was added to each well, and the plates were incubated for 60 minutes. After another wash, 25 μL of avidin (50 μg/mL) in PBS was added. The plates were incubated for ten minutes and then washed. To each well was added 25 μL of biotinylated horseradish peroxidase (10 μg/mL in PBS) and the plates were incubated for ten minutes. After washing, 0.2 mL of peroxidase-substrate mixture (0.004% H₂O₂/4mmol/L tyramine in 100 mmol/L PIPi buffer, pH 7.0) was added and incubated for 20 minutes. The plates were illuminated with an ultraviolet light (254 nm). The presence of antibody in test samples was visually detected with the appearance of fluorescence due to oxidized tyramine.

**Immunoprecipitation Procedure for Testing Antibody Binding to Ep**

Reaction mixtures testing for the presence of antibodies capable of binding with Ep in hybridoma supernatants and mouse sera contained 150 μL of an appropriately diluted sample, 30 μL of the partially purified Ep (5,100 U/mg protein) (2.8 μg/mL) or the further purified Ep (38,000 U/mg protein) (0.2 μg/mL). After incubation of the mixtures at 37 °C for 60 minutes, 20 μL of rabbit anti-mouse IgG (2 mg/mL of S. aureus suspension) adsorbed to 30% (wt/vol) S. aureus suspension was added. After incubation at 37 °C for 60 minutes, the mixtures were centrifuged, and then Ep activity in the supernatants was assayed with the in vitro method using H-thymidine. Controls contained hybridoma culture supernatants against guinea pig transglutaminase (manuscript in preparation) or nonimmune mouse sera instead of test sample.

**Preparation and Purification of Antibodies from Ascitic Fluid**

BALB/c mice received intraperitoneal injections of 0.5 mL of sterile pristane at ten and three days before 0.5 mL (10⁶ cells) of hybridoma cells was given intraperitoneally. Ten to fifteen days after cell injection, 5 to 18 mL of ascitic fluid was obtained from each mouse. After centrifugation of fluid, the supernatant was diluted with PBS to a protein concentration of 12 mg/mL. The antibody was pelleted by addition of 0.9 vol of saturated ammonium sulfate solution and by subsequent centrifugation. The pellet was dissolved in and dialyzed thoroughly against 0.2 mol/L NaHCO₃, pH 8, 0.3 mol/L NaCl (buffer A). The dialysate was used to prepare an immunoadsorbent column.

When immunoglobulin was further purified, the antibody pellet was dissolved in and dialyzed against 20 mmol/L Tris-HCl, pH 7.9/40 mmol/L NaCl (buffer B). After centrifugation of the dialysate, the supernatant (20 mg/mL protein) was applied on a DEAE-cellulose (100 mL/g of sample protein) column (1.8 x 10 cm) equilibrated with buffer B. The antibody appeared in flow-through fractions. An equal volume of saturated ammonium sulfate solution was added to the pooled fractions to precipitate antibody. The precipitate was dissolved in and dialyzed extensively against buffer A for immunoadsorbent or PBS for other experiments.

**Preparation of Immunoadsorbents**

Fixation of antibody on Affi-Gel 10 was performed according to the procedure reported.

**Western Blotting**

Western blotting to identify antigen was carried out according to the method of Burnette, with some modifications. Following sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the proteins were transferred to 0.2μm nitrocellulose paper (15 x 15 cm) at 7 V/cm for five hours. The nitrocellulose paper was immersed in 200 mL of 10 mmol/L Tris-HCl, pH 7.4, 5% BSA/saline (buffer C) for 30 minutes at 37 °C to coat any additional protein binding sites on the paper. Then the paper was dipped into 120 mL of buffer containing monoclonal antibody (20 μg/mL) and incubated overnight at room temperature to form an antigen-antibody complex on the paper. The paper was rinsed in 200 mL of 10 mmol/L Tris-HCl, pH 7.4/saline (buffer D) for ten minutes at room temperature, then in 200 mL of buffer D/0.05% NP-40, and in 200 mL of buffer D. The paper was then immersed in 20 mL of buffer C containing peroxidase-conjugated rabbit anti-mouse IgG
(20 μg/mL) for one hour at 37 °C. After rinsing the paper as described previously, it was soaked in 100 mL of a developing solution, 5:1 (vol/vol) mixture of 50 mmol/L Tris-HCl, pH 7.4, 200 mmol/L NaCl and 3 mg/mL 4-chloro-1-naphthol in methanol, which was brought to 0.01% (vol/vol) H2O2 before use. Antigens on the paper were visualized after soaking for one to ten minutes.

**SDS-Polyacrylamide Gel Electrophoresis and Ep Activity in Extracts of Sliced Gels**

SDS-PAGE was performed by the method of Laemmli and then stained with silver according to the manufacturer's (Bio-Rad) directions. In some cases, Ep activity measurement was performed on extracts from sliced gels. The unstained gel was sliced into 1-mm lengths. The gel pieces were put into test tubes containing 0.5 mL (per sliced gel) of PBS/0.1% BSA and ground with a glass rod. After incubating the suspensions overnight to extract protein, they were centrifuged to obtain the clear supernatants. The activity in the supernatants was measured with the in vitro assay methods.

**Protein Determination**

Protein concentrations were determined by measuring absorbance at 280 nm and assuming E1%280 nm = 10.

**Purification of Antigen, Ep, From Human Urine**

Unless otherwise indicated, all procedures for purification were performed at 0 to 4 °C.

**Urine concentrate.** Urine with high Ep (≥ 0.5 U/mL urine), collected from anemic patients, was filtered under suction and concentrated by ultrafiltration on a hollow-fiber device (Amicon) against distilled water and lyophilized.

**DEAE-cellulose.** The lyophilized material, corresponding to 600 L of urine, was dissolved in 3 L of distilled water and dialyzed extensively against 5 mmol/L Tris-malate buffer, pH 6.8 (buffer E). Half of the dialysate was loaded onto a DEAE-cellulose column (6 x 36 cm) equilibrated with buffer E. The column was washed with 500 mL/h stepwise with 10.5 L of buffer E and 5-L portions of buffer E containing 50 mmol/L, 200 mmol/L, and 500 mmol/L NaCl. The Ep activity was eluted with 200 mmol/L NaCl. The same was repeated with the other half of the dialysate.

**Phenyl-Sepharose.** The collected active fraction (6.4 L) was dialyzed against 10 mmol/L NaPi, pH 6.8/4 mol/L NaCl (buffer F), and the dialysate was applied on a phenyl-Sepharose CL4B column (6 x 36 cm) equilibrated with buffer F. After the column was washed with 6.3 L of buffer F, followed by 4.2 L of 10 mmol/L NaPi, pH 7.1/0.5 mol/L NaCl, the Ep was eluted with 2.2 L of 10 mmol/L NaOH/20% ethylene glycol/4 mol/L guanidine hydrochloride. Flow rate was 250 mL/h. After the eluted fraction was neutralized with glacial acetic acid, it was extensively dialyzed against distilled water, and the dialysate was lyophilized. The effectiveness of phenyl-Sepharose for purifying urinary Ep has been reported by Lee-Huang.

**Hydroxyapatite.** The lyophilized material was dissolved in and dialyzed against 5 mmol/L NaPi buffer, pH 6.9 (buffer G). The dialysate of 174 mL (13.4 mg/mL) was applied on a hydroxyapatite column (6 x 36 cm) equilibrated with buffer G, and the column was washed (150 mL/h) with 3.95 L of buffer G. The Ep was recovered in flow-through fractions. The Ep fraction was extensively dialyzed against distilled water and lyophilized.

**Sephadex G-100.** The dry sample (the third part of the total) was dissolved in and dialyzed against PBS, pH 6.9. The supernatant of 6.38 mL (35 mg/mL) after centrifugation was chromatographed with a Sephadex G-100 column (2 x 130 cm) equilibrated with PBS. Flow rate was 9.5 mL/h, and the volume of one fraction was 2 mL. Fractions 95 to 115, containing the bulk of Ep activity, were collected. The same was repeated twice with the rest of the dry sample.

**Sulfopropyl-Sephadex.** Purification with sulfopropyl-Sephadex was performed as described by Miyake et al, with some modifications. The collected fractions, after Sephadex G-100 chromatography, were dialyzed against 5 mmol/L CaCl2, pH 7.5, and the dialysate was brought to pH 4.5 with 0.1 N HCl. The supernatant of 124 mL (673 μg/mL) after centrifugation was subjected to chromatography with sulfopropyl-Sephardex C-50 (3 x 20 cm) equilibrated with 5 mmol/L CaCl2, pH 4.5. The column was washed with 550 mL of 5 mmol/L calcium acetate, pH 4.5, and developed stepwise with 600 mL of 20 mmol/L calcium acetate, pH 5.5, followed with 400 mL of 100 mmol/L calcium acetate/10 mmol/L Tris-HCl, pH 7.5. Flow rate was 48 mL/h. Most (78%) of the total recovered Ep activity was eluted with 20 mmol/L calcium acetate, and the rest with 100 mmol/L. Both active fractions were extensively dialyzed against distilled water, and the dialysates were lyophilized. The fraction eluted with 20 mmol/L calcium acetate was used hereafter because the specific activity was 2.5-fold higher than that eluted with 100 mmol/L.

Eighty-eight-fold purification with 4.5% recovery of the activity was achieved through all purification procedures. Specific activity of the final preparation was determined to be 5,100 U/mg protein with the in vitro 3H-thymidine incorporation procedure. A similar value was also obtained with the 59Fe incorporation method. The preparation was analyzed with SDS-PAGE (Fig 1). A main band, with mol wt ~32,000, and some other minor bands were detected. Activity measurement of Ep in the extracts from sliced gels revealed that the activity does not reside on the main band but on the faint band with mol wt ~3,000. Purification with conventional methods to prepare the Ep sample free from this main contaminant was unsuccessful.

![Fig 1](https://www.bloodjournal.org) SDS-PAGE of the Ep preparation obtained by sulfopropyl-Sephardex column chromatography. Six micrograms of protein per lane was applied on two lanes of a gel. After electrophoresis, one lane was subjected to silver staining (A) and the other lane was used for activity measurement after extracting protein from sliced gels (B).
RESULTS

Hybridomas Secrete Antibodies to Contaminants in Ep Preparation

One of the biggest advantages of the hybridoma technique is that one can select hybridomas directed for production of specific antibody to the desired antigen even when immunizations were performed with impure antigen preparations. We have prepared hybridomas by using our partially purified Ep preparation (5,100 U/mg protein) as an antigen in order to obtain hybridomas producing antibodies to Ep. As described later, however, this trial was unsuccessful, but it yielded a number of hybridomas that secreted antibodies against contaminated proteins as well as the mol wt 32,000 contaminant.

From the fusion with the NS-1 myeloma cells and spleen cells from six mice immunized with the antigen, 898 of the 1,152 culture wells showed growth of hybridomas. Supernatants in the hybridoma-growing wells were tested for antibody production with a solid-phase antibody-binding assay. Of the 898 cultures, 65 supernatants were found to be positive. However, none of the 65 supernatants was able to bind with the Ep in the antigen preparation when tested with the immunoprecipitation method. It is very likely that these antibodies are directed against non-Ep contaminants. Therefore, we decided to prepare the antibodies in large amounts to purify the Ep antigen further by removing contaminants with immunoadsorbent columns. Each of the 65 cultures (culture Nos. 1 to 65) was expanded in 24-well tissue culture plates. Before further enlargement of cultures, production of antibody was tested again with the supernatants. Nine cultures had lost this ability. The remaining 56 positive cultures were expanded, and cells were injected into mice for ascites production. Analysis of ascitic fluids with SDS-PAGE showed that 45 of 56 cultures produced immunoglobulin (figure not shown). Eighteen of 45 cultures were found to produce constantly large amounts of immunoglobulin when injected into mice. Antigens reacting with these antibodies were identified by using the Western blotting method (Fig 2). There were four major proteins, mol wt 32,000, 37,000, 40,000, and 62,000, that reacted with the antibodies. Eight cultures (Nos. 6, 20, 24, 33, 35, 57, 60, 62) of 18 were found to produce the immunoglobulin directed against the mol wt 32,000 protein. Cultures 36, 43, and 3 produced the antibodies reacting with the proteins of mol wt 37,000, 40,000, and 62,000, respectively. Antigens against immunoglobulins from the other seven cultures (Nos. 2, 13, 34, 38, 41, and 42) were not identified.

Further Purification of Ep

Removal of contaminants with immunoabsorbent columns. Two columns (each 1.2 x 7 cm) contained Affi-Gel 10 to which antibodies from hybridomas directed against contaminants in the Ep were fixed. Ammonium sulfate precipitates of ascitic fluid were used as antibody preparations without further purification. About 5 mg protein/mL Affi-Gel 10 was fixed. One column contained antibodies against three identified contaminants (mol wt 37,000, 40,000, and 62,000) and those produced by seven hybridomas directed against the unidentified antigens. The other column contained the antibody against the main contaminant of mol wt 32,000. Both columns were equilibrated with PBS. The Ep eluted with 20 mmol/L calcium acetate on sulfopropyl-Sephadex chromatography was dissolved in PBS (2.67 mg/mL). The resultant solution (6 mL) was applied on the first column and washed with 30 mL of PBS. It was confirmed that Ep activity was completely recovered in the flow-through fractions. The specific activity based on absorbance at 280 nm was estimated to be 23,000 with the in vitro assay methods using $^{59}$Fe and $^3$H-
thymidine. The active fractions were extensively dialyzed against distilled water and the dialysate was lyophilized. Analysis of the preparation by SDS-PAGE indicated the incomplete but effective removal of the main contaminant (mol wt 32,000 protein) and other contaminants (Fig 3, lane 3). Ep was recognized as a main band with about 35,000 mol wt on electrophoresis. Because some other contaminants were still detected, however, the Ep preparation was further purified with preparative SDS-PAGE.

Preparative SDS-PAGE. The Ep preparation was further purified by preparative SDS-PAGE in which all lanes of a slab gel (12 x 10 cm) received application of the sample. About 4.2 mg of protein dissolved in Laemmli's sample buffer13 was subjected to the electrophoresis. The migration position of Ep protein on the gel was identified by cutting out gel strips from both sides of the gel parallel with the direction of migration, followed by staining protein bands. The identified part (4-mm widths) of the unstained gel was cut out. The gel was sliced with a razor blade and then protein was extracted by grinding the gel pieces in a mortar with a pestle in the presence of 10 mL of PBS. After addition of 5 mL PBS, the suspension was centrifuged. The supernatant was extensively dialyzed against distilled water and the dialysate was lyophilized. The dry material was dissolved in PBS for immunizations and other experiments. About 40% of the Ep activity was recovered and 1 mg of protein was obtained. In some cases, the gel suspension containing Ep was injected into mice as an immunogen.

Purity of Ep

Analysis of the final Ep preparation by SDS-PAGE showed a high degree of purity, but the contaminant with mol wt 32,000 was still detected (Fig 3, lane 4). The distribution of Ep activity measured after extraction of protein from sliced gels was precisely superimposed on the protein band of mol wt 35,000 (Fig 3B). The same distribution pattern of activity was found in both in vitro assay methods using 59Fe and 3H-thymidine. About 650-fold purification was achieved, and 1 mg of protein was obtained from 600 L of urine. The specific activity of the final Ep preparation was about 38,000 U/mg of protein by in vitro 59Fe and 3H-thymidine methods. A similar value was also found by the in vivo assay method.

Ep-Directed Hybridoma

One of six mice immunized with the final Ep preparation (38,000 U/mg protein) developed serum that bound Ep. Fusion of spleen cells from this mouse with NS-1 myeloma cells yielded 258 growing hybridomas in the total 264 wells seeded. Cultures demonstrating cell growth were tested for supernatant antibodies by a solid-phase antibody-binding assay. Seven strongly positive (S1 to S7) and 12 weakly positive (W1 to W12) cultures were found. It was possible that besides these positive cultures there were hybridomas producing other classes of immunoglobulins, but screening of the supernatants by a solid-phase antibody-binding assay using peroxidase-conjugated IgG fractions of goat anti-mouse immunoglobulins excluded this possibility.

All positive cultures were expanded in 24-well tissue culture plates. Rescreening of the supernatants with the binding assay before further enlargement of culture in a T flask (50 mL) showed that all of the S1 to S7 group and four (W2, W6, W8, and W9) of the W1 to W12 group were still positive. The negative cultures were discarded. The supernatants of the positive lines in T flask cultures were concentrated 20-fold by precipitating proteins with ammonium sulfate, followed by dissolving the precipitate in and dialyzing against PBS. The dialysates were tested for binding to Ep with the immunoprecipitation procedure. Each dialysate was incubated with Ep, followed with rabbit anti-mouse IgG absorbed to S. aureus. After centrifugation, Ep activity in the supernatant was assayed. It was...
found with the dialysate from S2 that the activity was decreased to 30% of the control (see Materials and Methods). However, no decrease of the activity was observed when addition of rabbit anti-mouse IgG adsorbed to S. aureus was omitted, suggesting that the Ep–antibody complex is fully active. Some of the other dialysates caused a decrease of Ep activity in the supernatants but the results were uncertain. Therefore, we decided to test immunoglobulins produced by the hybridoma cell lines for their binding to Ep with the immunoadsorbent columns.

Cells from S1 to S7, W2, W6, W8, and W9 were injected into mice for the production of ascites, and each ascitic fluid was examined with respect to immunoglobulin production by SDS-PAGE (not shown). Strong production was found in cultures S2, S3, S7, and W8. Small antibody columns were made containing Affi-Gel 10, on each of which an immunoglobulin purified from the ascitic fluid of S2, S3, S7, or W8 was fixed. The immunoglobulin preparations used here were homogeneous on analysis with SDS-PAGE; two bands were found that corresponded to light and heavy chains of IgG (data not shown). The Ep preparation was applied on the control column and left for two hours at 4 °C to assure sufficient time for any binding of Ep to the antibody. The column was washed with 2.8 mL of PBS (flow-through fraction), another 20 mL of PBS, and 5 mL of 0.15 mol/L NaCl in this order, and was eluted with 4 mL of 0.2 mol/L acetate, pH 2.5/0.15 mol/L NaCl. The eluted fraction was immediately neutralized by adding 50 μL of 3.4 mol/L Tris solution per milliliter of the fraction. Some of both flow-through fraction and eluted fraction were diluted 500-fold with PBS, and the activity of the diluted sample was measured with in vitro 3H-thymidine incorporation. The remaining undiluted parts of both fractions were extensively dialyzed against distilled water and the dialysates were lyophilized. The dry materials were used for analysis with SDS-PAGE (Fig 4).

Each column (1 × 4 cm) contained 1 mL of Affi-Gel 10 to which the antibody from ascites of a hybridoma was fixed. Two hundred microliters of the Ep (38,000 U/mg protein) solution (16.8 μg/mL in PBS) was applied on a column and left for two hours at 4 °C to assure sufficient time for any binding of Ep to the antibody. The column was washed with 2.8 mL of PBS (flow-through fraction), another 20 mL of PBS, and 5 mL of 0.15 mol/L NaCl, in this order, and was eluted with 4 mL of 0.2 mol/L acetate, pH 2.5/0.15 mol/L NaCl. The eluted fraction was immediately neutralized by adding 50 μL of 3.4 mol/L Tris solution per milliliter of the fraction. Some of both flow-through fraction and eluted fraction were diluted 500-fold with PBS, and the activity of the diluted sample was measured with in vitro 3H-thymidine incorporation. The remaining undiluted parts of both fractions were extensively dialyzed against distilled water and the dialysates were lyophilized. The dry materials were used for analysis with SDS-PAGE (Fig 4).

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<td>3.4</td>
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*Total activity applied per column was defined as 100% activity. Little decrease in the activity of Ep was found when it was kept in PBS, while the activity decreased to 78% when it was kept in the pH 2.5 elution buffer for four hours.
†Antibody from ascites of hybridoma raised against guinea pig transglutaminase.
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Characterization of Cloned Ep-Specific Hybridomas

In parallel with the screening and propagation of fused cells, hybridomas (S1 to S7) that were strongly positive with the binding assay were cloned by limiting

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**Table 1. Binding of Ep to Immunoadsorbent Columns**

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Characterization of Cloned Ep-Specific Hybridomas

In parallel with the screening and propagation of fused cells, hybridomas (S1 to S7) that were strongly positive with the binding assay were cloned by limiting
dilution in microtiter plates with mouse thymocyte as the feeder layer. Cloning was started at the time of transfer of cells from the original 96-well cultures into 24-well cultures. Fifty cells were seeded per 96 wells for cloning. When results were obtained with the immunoadsorbent columns, culture of all clones, except Si, S1, S2, and S6, was ceased. The cloning efficiencies (hybridoma-growing wells/single cell-containing wells x 100%) were 95, 98, and 92 for S1, S2, and S6, respectively. Hybridoma-growing wells were tested for Ep antibody production in the culture supernatants with the binding assay, and 100%, 100%, and 12% of the hybridoma-growing wells were found to be positive for S1, S2, and S6, respectively. Hybridoma clones from two positive wells of each cell line were recloned. Two cloned hybridomas of each cell line were propagated in large scale cultures and injected into mice for production of ascitic fluid. Analysis of ascitic fluids with SDS-PAGE revealed that S2 produced large amounts of immunoglobulin, whereas S1 and S6 were very poor producers. One of the S2 clones selected for rapid growth (S2c12) has been quite stable in high antibody production and is used throughout this laboratory.

The subclasses of IgG produced by hybridomas were determined by the Ouchterlony double-immunodiffusion method, using rabbit antiserum raised against each subclass of mouse IgG. It was found that IgGs from cloned hybridomas of S1, S2, and S6 were IgG3, IgG1, and IgG1, respectively. Binding of Ep with the antibodies from these clones was confirmed by performing experiments similar to those in Table 1 and Fig 4 (data not shown).

Detection of Ep protein with the Western blotting technique indicated the occurrence of Ep species exhibiting different behavior on SDS-PAGE (Fig 5). Ep samples were subjected to electrophoresis, and the fractionated proteins were transferred to nitrocellulose paper. The blot was reacted with the Ep-specific monoclonal antibody from the S2 clone, followed by the peroxidase-conjugated anti-mouse IgG. The Ep protein was visualized by incubating the blot with the substrate of peroxidase. The Ep eluted with 100 mmol/L calcium acetate on sulfopropyl-Sephadex
chromatography (see Materials and Methods) was found to migrate faster than that eluted with 20 mmol/L calcium acetate or the final Ep with the highest purity. The preparation after Sephadex G-100 chromatography appears to contain both species of Ep, although the Ep species eluted with 20 mmol/L calcium acetate was the predominant component.

DISCUSSION

One enormous advantage of the hybridoma technique is its ability to generate specific antibodies even with impure antigens. An attempt to raise a human urinary Ep-directed hybridoma with about 10% pure antigen (5,100 U/mg protein), however, was unsuccessful; in an earlier fusion in which we used impure Ep for immunization, none of the 65 hybridomas positive with the solid-phase antibody-binding assay was Ep specific. Ep-directed hybridomas were finally obtained with the further purified Ep (38,000 U/mg protein). Only two of six mice were successfully immunized, even with the highly purified Ep, indicating that Ep is a poor antigen.

The most effective procedure in purification of Ep appears to be removal of contaminants with immunoadsorbent columns; both contaminants of mol wt 32,000 and 37,000, which closely resemble Ep in molecular weight, were substantially removed. It is not known at present whether these contaminants are specific for aplastic anemic patients or are also present in normal urine.

REFERENCES


Our goal is to prepare an Ep-directed hybridoma-secreting antibody suitable for an immunoadsorbent column that makes it possible to rapidly purify Ep from human urine in a high yield. Clone S2c12, obtained here, seems to meet our requirements entirely with its rapid growth rate, high production of antibody, and long continuance of these properties. Furthermore, a significantly high recovery of Ep activity was observed in the experiment with an immunoadsorbent column (Table 1). In fact, purification of Ep in human urine concentrate with S2c12 antibody column was successful.15 Ep purified with the antibody column and a subsequent Sephadex G-100 column had the specific activity of 81,600 U/mg of protein with the in vivo assay.15

Western blotting showed the occurrence of two Ep species with different mobilities on SDS-PAGE (Fig 3). Miyake et al1 reported the existence of two Ep species, α and β, distinguished by different electrophoretic mobilities under nondenaturing conditions. The chemical difference between these two species is not known, and therefore the interrelation of the pairs of species found in both laboratories is unclear.

ACKNOWLEDGMENT

We are indebted to Dr A. Ishimoto (Institute for Virus Research, Kyoto University) for his generous support and advice, and to Dr M. Ueda (Institute for Virus Research, Kyoto University) for his advice.
Hybridomas for production of monoclonal antibodies to human erythropoietin

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