Isolation and Characterization of an Anti-peptide Monoclonal Antibody to Human Erythropoietin*

(Received for publication, June 6, 1985)

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A site-specific monoclonal antibody to human erythropoietin has been developed. It is secreted by a hybridoma cell line derived from the fusion of murine myeloma cells with the splenocytes of a mouse that had been immunized with a 26-residue synthetic peptide antigen homologous to the amino-terminal sequence of the hormone. The antibody binds specifically to peptide, 125I-erythropoietin, and biologically active erythropoietin. The equilibrium dissociation constants of the antibody-erythropoietin and the antibody-peptide interactions are identical, $K_d = 6.7 \times 10^{-9}$ M, suggesting strong conformational similarity or identity of the epitope as expressed on the peptide and the hormone. Immune complexes formed between the antibody and either human or rat erythropoietin exhibit full biologic activity. However, the antibody does not recognize the baboon, sheep, or canine hormones, indicating antigenic differences or structural variation among these erythropoietins. These results indicate that the aminoterminal region of erythropoietin is not involved in receptor binding. Furthermore, they form a basis for the study of the structure and function of the hormone using anti-peptide antibodies.

The development of red blood cells from undifferentiated progenitor cells in mammalian and avian bone marrow requires the regulatory influence of the glycoprotein hormone erythropoietin. Although the isolation and characterization of genomic and cDNA clones of human erythropoietin have been described recently (1, 2), information on the physicochemical properties of the protein remains incomplete (3–8), and studies of its structure and function using site-specific probes, chemical modification, in vitro mutagenesis, and suitable spectroscopic methods have not been reported. Many functional studies have shown that the erythropoietins of various mammalian species are biologically active when tested against erythroid cells of other species, suggesting a strong similarity or identity among the receptor binding domains. However, a structural basis for this conclusion has yet to be described.

We reported previously the production of site-specific anti-erythropoietin antibodies (9). These antibodies were obtained by immunizing rabbits with a synthetic peptide homologous to a proposed amino-terminal sequence of the hormone (10).

Analyses of this sequence using the methods of Hopp and Woods (11) and of Kyte and Doolittle (12) demonstrated two regions within this sequence, centered at segments 8–15 and 19–24, that were likely to be on the surface of the molecule. We have shown that one of these regions, 8–15, is accessible to antibody, confirming its predicted topological localization (13). Due to their spatial orientation, such surface regions are presumably more likely to participate in the hormone's action, e.g., receptor recognition.

The present study describes the isolation and characterization of a site-specific monoclonal antibody (AE7A5) to the NH$_2$-terminal region of human erythropoietin obtained using this same 26-amino acid synthetic peptide as immunogen. The antibody binds specifically to peptide, 125I-erythropoietin, and biologically active erythropoietin. Its binding properties strongly suggest that it recognizes an epitope on the peptide that is structurally similar or identical to that found on the hormone. We used this monoclonal antibody to compare the erythropoietins of various mammalian species and to determine whether their NH$_2$ termini might play a role in the hormone's action. The data show that the monoclonal antibody-human erythropoietin immune complex retains full biologic activity and that there is antigenic diversity among the NH$_2$ termini of other mammalian erythropoietins.

**MATERIALS AND METHODS AND RESULTS**

In the present study, we have isolated and characterized a site-specific, reagent monoclonal antibody to human erythropoietin using an NH$_2$-terminal synthetic peptide as the immunogen. In so doing, we have obviated the need of erythropoietin antigen in both the immunization and initial screening phases of the study. This approach is of relevance to studies of other proteins available in severely restricted quantities as well as to the preparation of anti-peptide antibodies to protein sequences deduced from DNA sequences (21).

Two other monoclonal antibodies to human erythropoietin

**DISCUSSION**

1 This sequence, reported by Goldwasser (10), differs from that of Yanagawa et al. (8) who reported a Leu and unknowns at residues 3 and 24 and from Jacobs et al. (2) who reported Cys and Asn at the DNA sequence.

2 Portions of this paper (including "Materials and Methods," Results," Figs. 7–4, and Tables I and II) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9660 Rockville Pike, Bethesda, MD 20814. Request Document No. 85M-1860, cite the authors, and include a check or money order for $6.00 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
have been described in some detail. The first, reported by Weiss et al. (22), was prepared from a xenogeneic fusion between murine myeloma cells and the splenocytes of a rat immunized with intact, native erythropoietin. This antibody reportedly binds to 125I-erythropoietin and to biologically active erythropoietin and is non-neutralizing. The second monoclonal antibody, reported by Yanagawa et al. (8), was prepared by immunizing mice with human erythropoietin that was extracted from an acrylamide gel after sodium dodecyl sulfate gel electrophoresis (8). This antibody binds to human erythropoietin only in the presence of sodium dodecyl sulfate and does not recognize the native hormone. The regions of the hormone recognized by these two monoclonal antibodies have not been described.

Antibodies are being employed with increasing frequency in the study of protein structure and function. Earlier work showed that site-specific antibodies could be prepared by immunizing animals with peptides that were cleaved from the intact protein. These antibodies were then affinity-purified against the immobilized peptides (23). The precision and power of this site-specific antibody approach were enhanced greatly by the demonstration that synthetic peptides homologous to specified regions of a protein could be employed as immunogens (21). The use of two algorithms predictive of regions of the protein’s sequence likely to be accessible to antibody (11, 12) has increased the use of these anti-peptide methods. Finally, the application of monoclonal antibody technology (16) to this area, as exemplified by this study, has allowed the preparation of monospecific, reagent antibodies directed to selected domains of proteins.

The binding of monoclonal antibody AE7A5 to the peptide antigen and to human erythropoietin were essentially identical. This is consistent with the interpretation that the epitope that the antibody recognizes on erythropoietin is represented with great fidelity in the peptide in solution. Interestingly, the antibody does not bind to the peptide when the peptide is conjugated to bovine serum albumin (data not shown), indicating that the epitope is rather sensitive to the steric restriction caused by the glutaraldehyde conjugation method. Further efforts to define the epitope structure using synthetic peptides demonstrated that the antibody does not recognize either the 1–15 or the 15–26 peptides (Table II), suggesting that amino acids 15 and 16 are part of the epitope or that the antibody recognizes a conformational property that is not present in either of the smaller peptides. Since the hydrophilicity analysis (11) and the hydropathy analysis (12) both suggest that the 15–16 region lies at a hydrophobic trough and, thus, is more likely to be buried, the recognition of a conformational property within the 1–26 peptide seems the more likely explanation of this observation. It is relevant that the amino acid sequence of erythropoietin based upon the cDNA sequence (2) differs from that obtained by protein sequence analysis (8, 10) at residues 7 and 24. Assuming the deduced sequence (2) to be correct, then monoclonal antibody AE7A5 probably binds between these residues of the hormone.

Our studies of the erythropoietins of several species using affinity-purified monoclonal antibody AE7A5 indicate that the antibody-erythropoietin immune complex with the human or the rat hormone is biologically active (Table I, before precipitation), implying that the antibody-erythropoietin complex can bind to the cell surface receptor. The recent work of Krantz and Goldwasser (24) has established the presence of such erythropoietin-binding sites on erythroid cells. It is not known if erythropoietin enters the cell after binding to its receptor (via receptor-mediated endocytosis). Since erythropoietin apparently can bind to the receptor with the antibody still attached, it is unlikely that the amino-terminal region of the hormone, or at least that domain representing the antibody-binding site, plays a role in receptor recognition. This conclusion is also supported by our observations that monoclonal antibody AE7A5 does not bind to the baboon, canine, or sheep hormones, indicating antigenic differences or other structural variation in this region. In view of the evolutionary relationship of these species, it would, at first, seem unlikely that the antibody would exhibit such a specificity. However, it must be taken into account that any monoclonal antibody recognizes a very small portion of a protein’s overall structure. Indeed, examples of short stretches of amino acid sequence that are identical within homologous proteins across relatively divergent species are readily found. It is conceivable that the receptor may be less sensitive to such structural variations than is the antigen-binding site of the monoclonal antibody AE7A5. If so, it would provide some protection from lethal mutation within the erythropoietin-receptor system. However, this possibility would also reduce the specificity of the hormone-receptor interaction and could result in a loss of regulatory control of erythropoiesis in vivo.

We have demonstrated the usefulness of monoclonal antibody AE7A5 in these initial studies of erythropoietin’s structure and function. This monoclonal antibody should prove suitable for studies of the cellular localization of erythropoietin’s biosynthesis and of its processing after interaction with receptor. It will also be useful in the immunoaffinity purification of the hormone (25). Since the complete DNA sequence and the deduced amino acid sequence have been described (2), the development of additional sequence-specific probes for erythropoietin and the continued elucidation of its structural and functional properties should follow.

Acknowledgments—We thank Sarah Cosgriff and Jesse Brookins for valuable technical assistance, Stuart Orkin and Robert Lange for erythropoietin samples, and David Nathan for his encouragement.

REFERENCES


* For example, human and rat insulin A chains are identical at residues 1-12, whereas the sheep hormone differs at residues 8-10 (26).
Monoclonal Antibody to Human Erythropoietin


Supplementary Material for

ISOLATION AND CHARACTERIZATION OF AN ANTI-PEPTIDE MONOCLONAL ANTIBODY TO HUMAN ERYTHROPOIETIN

Arthur J. Bythikin and James W. Fisher

Materials and Methods

Animal Immunization, Cell Culture and Cell Fusion-Six to twelve week old BALB/c mice were immunized intraperitoneally with 30-100 μg of peptide-bovine serum albumin or peptide-keyhole limpet hemocyanin conjugates in complete Freund's adjuvant. The conjugates were prepared using glutaraldehyde (17) and the peptide-carrier stoichiometries determined with 125I-labeled peptide (18). The 25 residue peptide NH2-Val-Asp-Pro-Arg-Leu-Ile-Asn-Arg-Arg-Val-Leu-Arg-Glu-Arg-Leu-Glu-Leu-Arg-Glu-Leu-Val-Leu-Arg-Glu-Val-Leu-Arg (referred to as COOH) was purchased from Bachem Fine Chemicals. After repeated immunizations over a period of three to six months, blood samples were obtained and the sera were screened for anti-peptide antibodies by enzyme linked immunosorbent assay and for anti-erythropoietin antibodies by immunoprecipitation of 125I-labeled erythropoietin (14) using goat anti-mouse IgG as second antibody. Highly purified human urinary erythropoietin (70,400,000 Da, provided by the Division of Blood Research and Resources, NHLBI, NIH) was used in the initial phases of this study. For more detailed experiments, purified erythropoietin was obtained from Toyo Soda (8). 125I-labeled erythropoietin was prepared with carrier-free Na125I in the presence of Chloramine-T (10). Of the thirty animals tested, virtually all exhibited high anti-peptide titers, and six of these exhibited anti-erythropoietin antibodies. One animal was selected for fusion, and four days after an intravenous boost, its splenocytes were harvested and fused with 5 x 10⁷ P3X63Ag8.653 myeloma cells (Institute for Medical Research, Camden, N. J.) in the presence of polyethylene glycol 1500 (American Type Culture Collection, Rockville, MD) (14). The cell suspension was plated into seven 96-well dishes (Costar) over Hause peritoned macrophage feeder layers in the presence of HAT selective medium (9).

Sources for Antibodies to Peptide and to Erythropoietin-Antiserum sera and culture supernatants were screened for anti-peptide antibodies by enzyme linked immunosorbent assay against unconjugated peptide coated on polystyrene wells (9). The ability of the anti-peptide antibodies in the sera and culture supernatants to recognize erythropoietin was assayed by immunoprecipitation of 125I-erythropoietin or biologically active erythropoietin with 9, 10, and 12 islets or with immobilized goat anti-mouse IgG antibodies (Cooper Biomedical). In some experiments, specified concentrations of peptide or erythropoietin were used to competitively inhibit the antibody binding and immunoprecipitation (see results).

Characterization of Monoclonal Antibody AE7A5-The subclass of the antibody was determined by enzymelinked immunosorbent assay against peptide using subclass-specific anti-mouse antibodies (Bio-Rad). The ability of the antibody to bind to Protein A was demonstrated by the capacity of formalin-

fixed S. aureus (Coourse Center, Boston MA) to immunoprecipitate the antibody-125I-erythropoietin immune complexes and by the binding of horseradish peroxidase conjugated Protein A (Cooper Biomedical, Malvern, PA) to bind to the antibody in an anti-peptide enzyme linked immunosorbent assay. The epitope recognized by the antibody was characterized further by assessing its binding to 125I-labeled peptide fragments (11-15) (Bachem) and fragment (11-26) (Peptide Synthesis Facility, Children's Hospital, Boston, MA.)

Affinity Purification of Monoclonal Antibody AE7A5-The antibody was purified from culture supernatant by affinity chromatography on immobilized peptide. The gel consisted of 0.5 mg of peptide immobilized on 0.5 ml of Affi- 100G-10 (Bio-Rad). The gel was equilibrated with 10 mM Tris-Cl, 0.1 M NaCl, pH 7.5 at 25°C. Thirty five ml of supernatant were recirculated for sixteen hours with the aid of a peristaltic pump. The gel was washed with 10 ml of equilibrating buffer followed by 5 ml of 10 mM Tris-Cl, 6 M NaCl, pH 7.5 and 5 ml of equilibrating buffer. The monoclonal antibody was eluted with 15 ml of 10 mM Tris-Cl, 0.1 M NaCl, 3.0 M MgCl2, pH 7.5. Subsequent elutions with 0.25 M imidazole acetate, pH 2.5 and with 0.5 M guanidine-HCl revealed that essentially all of the antibody was eluted with the 3.5 M MgCl2.

Erythropoietin Biassay-The biological activity of the erythropoietin samples before and after incubation with antibody and immunoprecipitation was determined by in vitro by the method of Krystal (19). Prior to bioassay, all samples were dialyzed against Dulbecco's modified Eagle medium (GIBCO). Determinations were made in triplicate and had standard errors of 2.1%.

RESULTS

Two to three weeks after the fusion, macroscopic colonies were visible in over 300 microtiter plates. Forty-three of the supernatants contained anti-peptide antibodies as assayed by enzyme linked immunosorbent assay. The cells from the supernatant microtiter wells with the highest anti-peptide levels were expanded into milliter scale wells and, after the cells had grown, the supernatants were assessed again for anti-peptide activity. All were positive. Five-fold concentrates of the supernatants were assessed for anti-125I-erythropoietin activity by immunoprecipitation. Ten of these supernatants immunoprecipitated significant amounts of 125I-erythropoietin and four of them were inhibited by excess peptide antigen, indicating specificity of the monoclonal antibody-125I-erythropoietin reaction. The highest titer of these, designated AE7A5, was cloned and cryopreserved and will be the only hybridoma discussed further. Monoclonal antibodies secreted by AE7A5 (Abb AE7A5) were of the IgM class and bound Protein A (see Materials and Methods).

The specific binding of MAb AE7A5 to the NH2-terminal peptide immunogen was demonstrated by an enzymelinked immunosorbent assay of the cell supernatant. Figure 1A) shows the specific titration of supernatant exhibited concentration-dependent binding to the peptide coated on polystyrene wells. In contrast, no binding of MAb AE7A5 was detected in the absence of coated peptide or in the absence or presence of another coated peptide or protein, e.g., bovine serum albumin. The specificity of MAb AE7A5 for erythropoietin was shown by immunoprecipitation of 125I-erythropoietin with decreasing concentrations of supernatant (Figure 1B). The finding that a 1:6000 dilution of supernatant could still immunoprecipitate 125I-erythropoietin suggested that the MAb AE7A5 was present at a relatively high concentration in the medium and not reflected its affinity for erythropoietin.
Monoclonal Antibody to Human Erythropoietin

The monoclonal antibody was purified easily from supernatant or from ascites by affinity chromatography on immobilized peptide (Figure 3). Thirty-five milliliters of supernatant were recirculated over the immobilized peptide affinity gel, and the monoclonal antibody was eluted readily with 3.5 M MgCl₂. This single step resulted in virtually homogeneous MAb AE7A5, determined by SDS gel electrophoresis (20), with 75% recovery of antibody activity. We recovered 1.9 mg of monoclonal antibody protein from 35 ml of medium.

Inoculation of AE7A5 cells into the peritoneal cavities of Pristane-primed mice resulted in ascites fluid containing 50-200 fold greater concentrations of monoclonal antibody.

Both peptide and biologically active erythropoietin inhibited the immunoprecipitation of ¹²⁵I-erythropoietin in a concentration-dependent manner, demonstrating the cross-reactivity of MAb AE7A5 with the peptide immunogen and the hormone (Figure 2A, B). The apparent equilibrium dissociation constants for the interaction of the monoclonal antibody with peptide and with erythropoietin calculated from these data were identical, K₅ = 6.7 × 10⁻⁹ M, suggesting a strong similarity or virtual identity between the epitope structure as expressed on the peptide and on erythropoietin. These values were supported by the results of direct measurement of ¹²⁵I-erythropoietin binding to affinity purified MAb AE7A5 (see below, Figure 4).

The binding of affinity purified antibody to ¹²⁵I-erythropoietin was assessed by immunoprecipitation of the hormone after incubation with specified, decreasing concentrations of the antibody (Figure 4). These data yielded a K₅ of 2.2 × 10⁻⁹ M closely similar to that obtained in the inhibition experiments (see above, Figure 2).

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**Figure 1.** Binding of MAb AE7A5 to peptide immunogen (B) and to ¹²⁵I-erythropoietin (B). Panel A: Enzyme-linked immunosorbent assay of dilutions of AE7A5 cell supernatant against 2.5 ng/well of peptide coated on polystyrene wells. Panel B: Immunoprecipitation of ¹²⁵I-erythropoietin by dilutions of AE7A5 cell supernatant. Results are expressed as percent of maximal precipitable counts and are the means of duplicate determinations.

**Figure 3.** Purification of MAb AE7A5 from cell culture supernatant by affinity chromatography on immobilized peptide. The gel was washed with 10 ml of equilibrating buffer followed by 5 ml each of 10 mM Tris-HCl, 4 M NaCl, pH 7.5 (arrow A) and equilibrating buffer. The monoclonal antibody was eluted with 15 ml of 10 mM Tris-HCl, 0.1 M NaCl, 3.5 M MgCl₂, pH 7.5 (arrow B). Subsequent elutions with 0.25 M sodium acetate, pH 2.5 (arrow C) and with 4 M guanidine-HCl (arrow D) revealed that essentially all of the antibody had been eluted with the MgCl₂. Closed circles (©), open circles (©), anti-peptide activity determined by enzyme-linked immunosorbent assay.

**Figure 4.** Immunoprecipitation of ¹²⁵I-erythropoietin by affinity purified MAb AE7A5.

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Affinity-purified MAb AE7A5 was used to compare the avidity of erythropoietin from various species (Table 1). Biological samples containing erythropoietin were incubated in the absence or presence of $1 \times 10^{-7}$ M monoclonal antibody. This concentration of antibody was $1-2$ orders of magnitude greater than the $K_r$ of the monoclonal antibody-erythropoietin interaction and was employed so that approximately $75-90\%$ of the hormone would be bound to monoclonal antibody. That portion of each sample incubated in the absence of antibody was bioassayed to determine the level of erythropoietin activity. The portion incubated in the presence of excess antibody was divided in two. One-half was subjected first to immunoprecipitation. Then the erythropoietin activity remaining in the supernatant was determined by bioassay. This experiment was designed to determine if the antibody bound to the erythropoietin of the particular species. The other half of the sample with excess antibody was bioassayed directly to test for biological activity of the erythropoietin-antibody immune complexes. This was designed to determine if the binding of antibody to the amino terminus neutralized the activity of this erythropoietin. A finding of neutralization would indicate that the antibody bound at or near a region of the erythropoietin molecule that is essential for receptor recognition and perhaps for some additional step in the hormone's biological activity. All samples contained erythropoietin activity that was easily detected in the absence of antibody. Incubation of human and rat erythropoietin with antibody followed by immunoprecipitation resulted in a depletion of erythropoietin activity from solution to 11 and 20X, respectively. This result indicates that the antibody recognizes and was bound to the amino termini of both these species and implies a structural similarity between these epitopes. The 20X of activity remaining in the rat sample after immunoprecipitation may reflect a difference in the affinity of the antibody for the rat hormone or may be due to the presence of more than one type of erythropoietin activity in the rat serum (analogue to rat insulin 1 and 2). Whatever, bioassay of the replicate samples that had not been immunoprecipitated (Table 1, "Before Precip.") revealed that the immune complexes of both human and rat erythropoietin with antibody were biologically active. Thus, the antibody did not neutralize the biological activity. In contrast to the findings with human and rat erythropoietin, the antibody did not recognize the baboon, canine or sheep hormones.

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<tr>
<th>Table 1</th>
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<tr>
<td>Binding of AE7A5 Monoclonal Antibody to Erythropoietin of Various Species</td>
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<table>
<thead>
<tr>
<th>Erythropoietin sample</th>
<th>Biological activity, $\times$</th>
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<tbody>
<tr>
<td>No MAb</td>
<td>With excess MAb</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Human urine</td>
<td>100</td>
</tr>
<tr>
<td>Rat serum</td>
<td>100</td>
</tr>
<tr>
<td>Baboon plasma</td>
<td>100</td>
</tr>
<tr>
<td>Canine serum</td>
<td>100</td>
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<tr>
<td>Sheep serum</td>
<td>100</td>
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* The activities of each species' erythropoietin have been normalized to that found for the "No MAb" sample (100%) to facilitate comparison. The actual activities (mU/ml) of the "No MAb" samples were as follows: human, 900; rat, 300; baboon, 250; canine, 100; sheep, 200. Erythropoietin samples were incubated with $1 \times 10^{-7}$ M affinity purified MAb AE7A5 for 16 hrs at 4°C.

The epitope recognized by the antibody was characterized further by examining the ability of AE7A5 to bind to $^{125}$I-labeled peptide fragments (1-15) and (15-26). As shown in Table II, AE7A5 readily bound to $^{125}$I-peptide(1-15), the original immunogen, but did not bind to $^{125}$I-peptide(1-15) or to $^{125}$I-peptide(15-26). In contrast, polyclonal rabbit antiserum (9) recognized both halves of the immunogen as well as the intact $^{125}$I-peptide(1-26).

<table>
<thead>
<tr>
<th>Table II</th>
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<tr>
<td>Further Characterization of AE7A5 Monoclonal Antibody Epitope With Synthetic Peptides</td>
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<tr>
<th>Antibody Sample</th>
<th>$^{125}$I-Peptide Precipitated, %</th>
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<tr>
<td>1-26</td>
<td>1-15</td>
</tr>
<tr>
<td>MAb AE7A5</td>
<td>100</td>
</tr>
<tr>
<td>Rabbit anti-peptide(1-26) serum</td>
<td>100</td>
</tr>
<tr>
<td>Rabbit pre-immune serum</td>
<td>&lt;1</td>
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* Each $^{125}$I-labeled peptide (100 µl) was incubated with 10 µl of antibody sample for 16 h at 4°C. The resulting immune complexes were precipitated with 5% TCA.