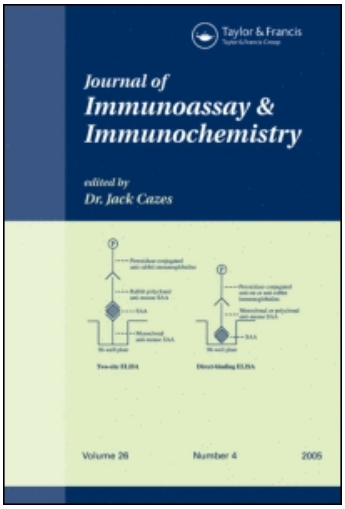

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Abstract: Erythropoietin (EPO) is a growth factor, regulating the proliferation and differentiation of erythroid progenitor cells. In this study, we generated five monoclonal antibodies (mAbs) that reacted specifically with recombinant human EPO (rhEPO). Epitope exclusion and other experiments showed that the mAbs obtained were divided into two groups, differing in recognition sites for rhEPO: group 1 mAbs recognize the N-terminal region of rhEPO, whereas group 2 mAbs seem to recognize a conformation-dependent epitope. Although most of the previously reported anti-EPO antibodies that recognized the N-terminal region of EPO lacked the EPO-neutralizing activity, the group 1 mAbs obtained here had the rhEPO-neutralizing activity. Therefore, the group 1 mAbs may be useful for future study on structure-function relationship of EPO. One of the group 2 mAbs, 5D11A, showed the highest affinity for rhEPO with K_D value 0.52 nM and had the highest rhEPO-neutralizing activity. Using this mAb, we developed a reproducible and sensitive enzyme-linked immunosorbent assay for the quantification of bioactive rhEPO.

Keywords: Recombinant human erythropoietin, Monoclonal antibodies, Conformation-dependent epitope, Enzyme-linked immunosorbent assay, Validation, Cell-based potency assay

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INTRODUCTION

Erythropoietin (EPO) is a heavily glycosylated hematopoietic growth factor that regulates the proliferation of erythroid progenitor cells and induces their differentiation.[1,2] The human EPO gene has been cloned, and biologically active recombinant human EPO (rhEPO) has been produced in mammalian cells.[3,4] This rhEPO product has been used therapeutically to stimulate red blood cell development in patients with anemia due to kidney failure.[5]

Monoclonal antibodies (mAbs) that specifically bind rhEPO have been used in immunoaffinity columns for rapid purification of EPO from human urine or cell culture supernatants[6–8] and in immunochemical detection systems, such as enzyme-linked immunosorbent assay (ELISA), to quantify EPO levels in clinical samples.[7,9–12] On the other hand, mAbs and polyclonal antibodies (pAbs) against EPO peptides or intact rhEPO are especially valuable for investigating the immunochemical and biological properties of EPO.[13–21] For example, epitope mapping studies using anti-rhEPO mAbs and immunochemical studies using pAbs against site-specific peptides of human EPO that could neutralize its bioactivity have contributed to identification of the active sites involved in the interaction with its target receptor.[13–20]

In order to use anti-rhEPO antibodies as a tool for characterization and immunochemical detection of EPO, their properties, including the association and dissociation rate constants, the target recognition sites, and the ability to neutralize EPO activity, must be well characterized. Although several groups have reported the establishment of anti-rhEPO mAbs,[6–8,13,15,17–21] such properties have been poorly documented in most cases.

Here, we describe generation and characterization of five new anti-rhEPO mAbs having high affinity to rhEPO. They can be classified into two groups with respect to their epitope recognition properties: group 1 recognizes the N-terminal region of rhEPO, whereas group 2 is likely to recognize a conformation-dependent epitope. All the mAbs were shown to be capable of neutralizing the biological activity of EPO *in vitro*. Using one of the group 2 mAbs, we developed an ELISA for rhEPO. On the basis of the results obtained, we discuss usefulness of the mAbs and ELISA established in this study in quantification of bioactive EPO and in future studies on structure-function relationship and biological properties of EPO.

EXPERIMENTAL

Production and Biotinylation of Anti-rhEPO pAbs

An anti-rhEPO serum was raised in rabbits by subcutaneous injections with 500 μg of rhEPO (Kirin Brewery, Gunma, Japan), which had been emulsified

with Freund's complete adjuvant (FCA). Three or four subsequent immunizations with 500 μg of rhEPO were given with Freund's incomplete adjuvant (FIA) at intervals of 2 weeks. Two weeks after the last immunization, the serum was collected from the immunized rabbits, and IgG fraction was obtained from the serum by affinity chromatography using a protein G-Sepharose (AmershamPharmacia BiotechAB, Uppsala, Sweden). The anti-rhEPO pAbs were labeled with biotin using the Sulfo-NHS-LC-Biotinylation Kit (Pierce, Rockford, IL) according to the manufacturer's instructions.

Production of Anti-rhEPO mAbs

Balb/c mice were immunized by subcutaneous injections of 50 μg of rhEPO in FCA and boosted three times at 7 day intervals with the same amount of rhEPO in FIA. More than 10 days after the last injection, the mice were boosted intravenously with 50 μg of rhEPO. Four days after the intravenous injection, splenocytes were harvested from spleen of the immunized mice and fused with a myeloma cell line, X63-Ag-8.6.5.3, using polyethylene glycol according to the standard procedures.[6,22] Hybridoma clones were screened by examining their culture supernatants for production of antibodies with the ability to bind to rhEPO directly coated onto plates (Direct ELISA) or captured on plates previously coated with anti-rhEPO pAb (Capture ELISA). Antibodies bound to rhEPO were detected with anti-mouse IgG Fab Horseradish Peroxidase (HRP) conjugate (Immuno-Biological Laboratories, Gunma, Japan) by monitoring color development of *o*-phenylenediamine at 490 nm. Immunoglobulin isotypes were determined by Mouse Monoclonal Antibody Isotyping Kit (AmershamPharmacia BiotechAB).

Surface Plasmon Resonance (SPR) Analysis

Biosensor kinetic analysis was performed with a Biacore 3000 instrument (Biacore AB, Uppsala, Sweden) using an HBS-EP buffer (Biacore AB) as a running buffer. Each anti-rhEPO mAb was captured by immobilized anti-mouse IgG, and various concentrations of rhEPO were used to estimate the kinetic rate constants. Anti-mouse IgG (Biacore AB) was covalently immobilized on research-grade CM5 sensor chips (Biacore AB) at a concentration of 30 μg/mL in 10 mM sodium acetate (pH 5.0) using an amine coupling kit (Biacore AB). Carboxymethylated dextran surface was activated with a 7 min injection of a solution containing 50 mM *N*-hydroxysuccinimide and 200 mM *N*-ethyl-*N*′-(dimethylaminopropyl) carbodiimide. Anti-mouse IgG was then injected for 7 min at a flow rate of 10 μL/min. Unreacted groups were blocked by a 7 min injection of ethanolamine, and any non-covalently bound molecules remaining were washed away with a regeneration buffer (10 mM glycine-HCl, pH 1.7) at a flow rate of 30 μL/min. Typically,

10,000 resonance units (RU) of anti-mouse IgG were coupled in this procedure (one RU represents approximately 1 pg of protein per mm^2 of the sensor chip surface).[23] Next, each anti-rhEPO mAb was injected for 3 min at a flow rate of 10 μL/min. A sensorgram showed that binding of anti-rhEPO mAb onto the immobilized anti-mouse IgG on the surface of the CM5 chip set resulted in an increase in the baseline signal to 1,000 to 1,250 RU. Solutions containing six or seven different concentrations (5.3 to 333 nM) of rhEPO were then injected at a flow rate of 20 μL/min. Association and dissociation phases were monitored for 2 min each. Association and dissociation rate constants were calculated by a 1:1 binding fitting of the primary sensorgram data using a Biaevaluation 2.2.4 software (Biacore AB). The sensor chip surfaces were regenerated with the regeneration buffer at a flow rate of 60 μL/min for 3.5 min.

Epitope Exclusion ELISA

Epitopes recognized by the anti-rhEPO mAbs were analyzed by a competitive binding assay. The anti-rhEPO mAbs were labeled with biotin as described above for labeling the anti-rhEPO pAbs. Each well of microtiter plate (MaxiSorp, Nunc, Wiesbaden-Biebrich, Germany) was treated overnight at 4°C with 100 μL of anti-rhEPO pAb (20 μg/mL) in phosphate-buffered saline (PBS). The plate contents were then removed and a blocking buffer (PBS containing 0.2% I-Block (TROPIX, Bedford, MA)) was added to prevent non-specific binding. After washing twice with a wash buffer (PBS containing 0.1% Tween 80), 100 μL of rhEPO (10 ng/mL) were added to each well and incubated for 1 h at room temperature (RT) to allow formation of anti-rhEPO pAb/rhEPO complexes. Equal volumes of serially diluted unlabeled anti-rhEPO mAbs (0.064 to 1,000 ng/mL) and competing biotinylated anti-rhEPO mAb (500 ng/mL) were added to each well and incubated for 1 h at RT. After washing, HRP-conjugated avidin (Pierce) was added to each well and incubated for 1 h. After washing four times with the wash buffer, bound conjugates were detected using the 3,3',5,5'-tetramethylbenzidine (TMB) Microwell Peroxidase Substrate System (KPL, Gaithersburg, MD). After incubation for 15 min, the reaction was quenched with a TMB Stop Solution (KPL) and absorbance at 450 nm was measured using a plate reader (SPECTRAmax M2; Molecular Devices, Sunnyvale, CA).

Preparation of Digested rhEPO Peptides

To obtain rhEPO peptides, rhEPO was digested with an endoproteinase, endolysine C (Lys-C) (Roche Diagnostics GmbH, Penzberg, Germany), according to the manufacturer's recommendation. The amount of the enzyme was 1/50 (w/w) of the rhEPO protein. After incubation for 6 h at 37°C, digested

samples were reduced with dithiothreitol, separated, and recovered by high pressure liquid chromatography. The amino-acid sequences of individual Lys-C-digested fragments are listed in Table 1. The obtained peptides remained glycosylated in the same positions as the original rhEPO.

Epitope Mapping

Two types of ELISAs, i.e., peptide immobilization ELISA and peptide competition ELISA, were used to map the epitopes for anti-rhEPO mAbs. For the peptide immobilization ELISA, each microtiter plate well was treated overnight at 4°C with 50 μL of a carbonate buffer (50 mM, pH 9.6) containing each of the Lys-C-digested rhEPO fragments at three different concentrations (10, 1, and 0.1 μg/mL). After blocking, 100 μL of anti-rhEPO mAb (1 μg/mL) were added and incubated for 3 h at RT. The well was then washed with the wash buffer and incubated for 1 h with a goat anti-mouse IgG (H + L) HRP conjugate (Bio-Rad Laboratories, Hercules, CA). After washing four times, bound conjugates were detected as described above for Epitope Exclusion ELISA. For the peptide competition ELISA, 1 μg/mL rhEPO was incubated overnight at 4°C in microtiter wells. Serially diluted solutions of each Lys-C-digested rhEPO fragment (0 to 5,000 ng/mL) and anti-rhEPO mAb (10 μg/mL) were mixed in a well and incubated for 3 h at RT. After washing, bound mAbs were detected as above.

Neutralization Assay and Cell-Based Potency Assay

An EPO-dependent cell line UT-7/EPO was used in both the neutralization assay and the potency assay of rhEPO. UT-7/EPO cells are committed to the erythroid lineage and show rhEPO dose-dependent proliferation

Table 1. Amino-acid sequences of Lys-C-digested rhEPO fragments

Amino-acid residues	Length (a.a.)	Sequence
1–20	20	NH_2-APPRLICDSRVLERYLLEAK
21–45	25	NH_2-EAENITTGCAEHCSLNENITVPDTK
46–52	7	NH_2-VNFYAWK
53–97	45	NH_2-RMEVGQQAVEVWQGLALLSEAVLRG QALLVNSSQPWEPLQLHVDK
98–116	19	NH_2-AVSGLRSLTTLLRALGAQK
117–140	24	NH_2-EAISPPDAASAAPLRTITADTFRK
141–152	12	NH_2-LFRVYSNFLRGK
155–165	11	NH_2- LYTGEACRTGD

activities.[24] To test the neutralization activity of the anti-rhEPO mAbs, 100 μL of rhEPO solution (300 pg/mL) were mixed with 50 μL of a culture medium (RPMI1640 medium containing 4% fetal calf serum and 10 mM glutamine) containing increasing amounts of mAbs (0 to 100 μg/mL). Aliquots of 50 μL of culture medium containing about 5,000 cells were then added, and the plates were incubated for 48 h at 37°C under a humidified condition with 5% CO_2. Alamar Blue (Trek Diagnostic Systems, Westlake, OH) was added to each well and the incubation was continued for further 24 h. Fluorescence of excitation at 545 nm and emission at 590 nm produced by cellular reduction of the Alamar Blue dye was then measured using a fluorescence plate reader (Spectra Fluor; Tecan, Gröding Austria). Percent cell proliferative activity was defined as the activity remaining in each experiment relative to a control sample incubated without antibody. The potency of rhEPO sample was estimated using parallel line assay. Briefly, rhEPO standards and samples were diluted with culture medium to obtain several different concentrations. Aliquots of 100 μL of diluted rhEPO standards and samples were mixed with an equal volume of prewashed cell suspension (5.0×10^5 cells per mL). After incubation for 48 h at 37°C, Alamar Blue was added to each well and incubated for 24 h. Fluorescence was detected as described above.

EPO ELISA Procedure

Microtiter plate was coated by incubating overnight at 4°C with 100 μL of the mAb solution at 2 μg/mL in PBS. After blocking with the blocking buffer, 100 μL of rhEPO standards (2.5, 5.0, 10, 20, 40, and 80 mIU/mL) and samples diluted with the blocking buffer were added into each well and incubated for 2 h at RT. After washing four times with the wash buffer, the biotinylated anti-rhEPO pAb solution was added to each well and incubated for 1 h at RT. The conditions for washing, incubation with HRP-conjugated avidin, and development reaction were the same as described above for Epitope Exclusion ELISA.

RESULTS

Isolation and Characterization of mAbs Specific for rhEPO

Hybridoma clones were screened for production of antibodies against rhEPO by examining their culture supernatants with the Direct and Capture ELISAs, and five stable hybridoma clones, designated as 1G12A, 5D11A, 4H10C, 2A2B, and 4G3C, were selected. Isotype analysis indicated that all the mAbs are in the IgG_1 subclass with kappa light chains (data not shown). IgG molecules were purified from the culture supernatant of each hybridoma by protein A-Sepharose chromatography.

These anti-rhEPO mAbs were then tested for their rhEPO binding affinity by SPR analysis. In this analysis, each of the anti-rhEPO mAbs was immobilized using anti-mouse IgG covalently attached to the surface of the carboxymethylated dextran-coated gold chip to allow efficient exposure of the anti-rhEPO mAb to rhEPO. Table 2 summarizes the association and dissociation rate constants (k_{ass} and k_{diss}) obtained in the SPR analysis as well as the affinity constants calculated from these two rate constants. Among the five mAbs obtained in this study, 5D11A was found to show the strongest affinity to rhEPO.

Epitope Mapping

The epitope exclusion ELISA was employed to determine whether the five mAbs could recognize different rhEPO epitopes. Because we found that rhEPO directly coupled to the solid phase often showed a weak ability to bind antibodies (data not shown), rhEPO captured by the anti-rhEPO pAb was used in this assay. The binding of biotin-labeled 1G12A to rhEPO was inhibited by increasing amounts of unlabeled 1G12A, 2A2B, and 4G3C, whereas neither 5D11A nor 4H10C inhibited 1G12A binding even at the highest concentration used (Fig. 1A). The experiment with labeled 4G3C gave essentially the same result as that with labeled 1G12A (Fig. 1B). These results indicate that 1G12A, 2A2B, and 4G3C bind to the same region or closely adjacent regions of rhEPO. Conversely, in the experiment with labeled 4H10C (Fig. 1C), its binding was inhibited by unlabeled 4H10C and 5D11A and not by unlabeled 1G12A, 2A2B, or 4G3C, indicating that 4H10C and 5D11A bind to the same region or closely adjacent regions distinct from those recognized by 1G12A, 2A2B, and 4G3C. However, 4H10C was not able to compete with labeled 5D11A for rhEPO binding (Fig. 1D). This result suggests that the binding activity of 4H10C is significantly weaker than that of 5D11A, which is consistent with the result shown in Table 2. On the basis of these results, we conclude that the five mAbs obtained in this study can be classified into two groups, which recognize distinct epitopes. The mAbs 1G12A and 5D11A were used as representatives of the respective groups for further study.

Table 2. Rate and affinity constants of anti-rhEPO mAb/rhEPO complex formation

mAb	k_{ass} (1/Ms)	k_{diss} (1/s)	K_D^a (M)
1G12A	1.25×10^5	1.03×10^{-3}	8.26×10^{-9}
2A2B	1.24×10^5	8.79×10^{-4}	7.11×10^{-9}
4G3C	3.50×10^5	2.63×10^{-3}	7.52×10^{-9}
4H10C	3.26×10^5	1.06×10^{-3}	3.25×10^{-9}
5D11A	3.08×10^5	1.59×10^{-4}	5.16×10^{-10}

[a]Affinity constant (K_D) was calculated as k_{diss}/k_{ass}.

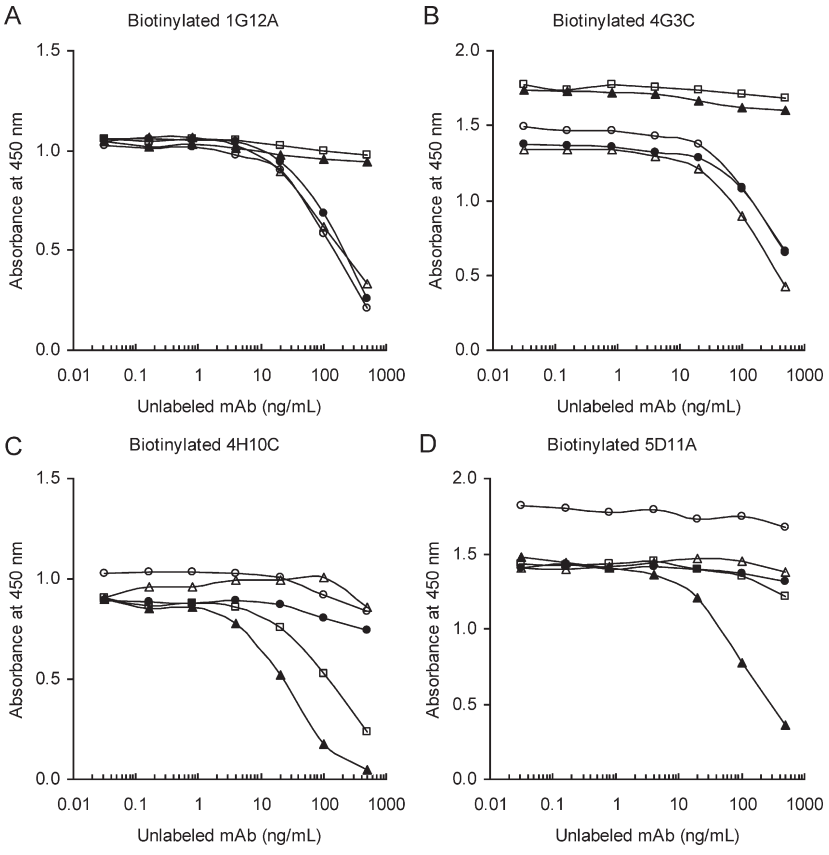

Figure 1. Epitope exclusion ELISA. Biotinylated anti-rhEPO mAbs (1G12A (A), 4G3C (B), 4H10C (C), and 5D11A (D)) and five-fold serial dilutions of the unlabeled mAbs 1G12A (○), 2A2B (●), 4G3C (Δ), 5D11A (▲), or 4H10C (□) were added to microtiter plates previously coated with rhEPO captured by anti-rhEPO pAb. The final concentration of each unlabeled mAb is indicated on the *x*-axis.

To determine the location of the epitopes recognized by the two classes of mAbs, eight Lys-C-digested fragments of rhEPO were used in binding assays. Each fragment was directly immobilized onto a plastic plate, and 1G12A or 5D11A was added to determine whether specific binding could occur. In the case of 1G12A, only the fragment consisting of N-terminal 20 amino acids of rhEPO gave a clear positive signal (Fig. 2A). As an alternative method, we tested whether any of the Lys-C fragments were able to act as competitors against intact rhEPO for binding to the anti-rhEPO mAbs. As shown in Fig. 3A, only the Lys-C fragment consisting of first 20 amino acids of rhEPO was able to compete with rhEPO for binding to 1G12A. Therefore, we conclude that 1G12A recognizes residues within first 20 amino acids of

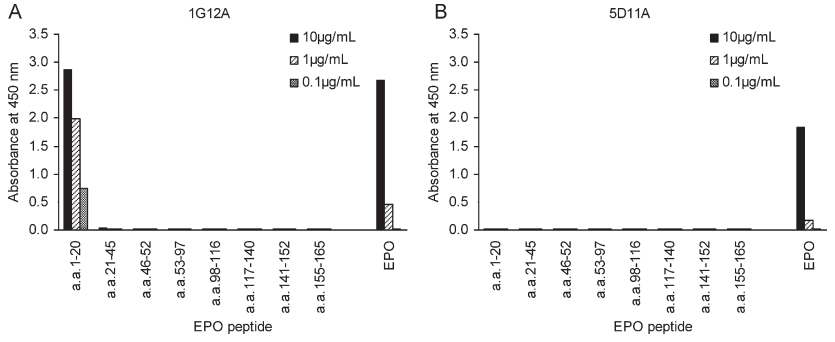

Figure 2. Epitope mapping of 1G12A and 5D11A by peptide immobilization ELISA. Lys-C-digested and intact rhEPOs were immobilized on plates at three different concentrations (10, 1, and 0.1 μg/mL) and then incubated with 1 μg/mL 1G12A (A) or 5D11A (B).

rhEPO. On the other hand, 5D11A did not react with any of the Lys-C fragments (Fig. 2B). Consistent with this, none of the Lys-C fragments inhibited rhEPO from binding to 5D11A (Fig. 3B). These results indicate that 5D11A does not react with any Lys-C fragments. Because the Lys-C fragments used here were still glycosylated at the same positions as the intact rhEPO molecule, these results suggest also that 5D11A does not recognize the carbohydrate portion of the antigen. Furthermore, SDS-denatured rhEPO was shown to react with 1G12A and not with 5D11A in the Western blotting analysis (data not shown). Taken together, we conclude that 5D11A recognizes a conformation-dependent epitope.

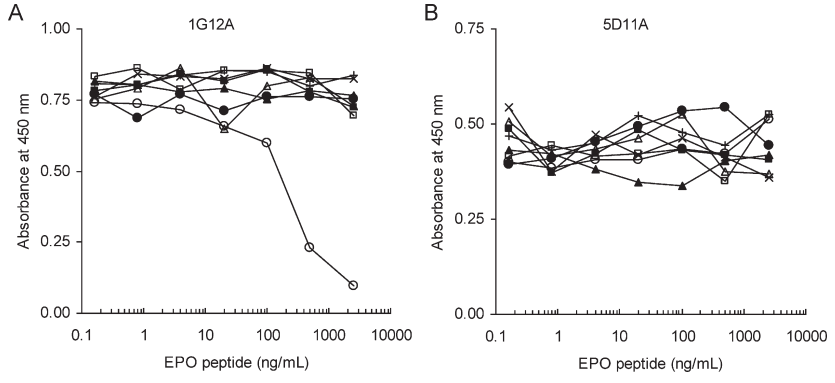

Figure 3. Inhibition of rhEPO binding by Lys-C-digested rhEPO fragments. Microtiter plate well previously coated with 100 ng of rhEPO was incubated with various concentrations of Lys-C fragments of rhEPO and 10 μg/mL 1G12A (A) or 5D11A (B). Lys-C fragments of rhEPO used: aa. 1–20 (○), aa. 21–45 (●), aa. 46–52 (Δ), aa. 53–97 (▲), aa. 98–116 (□), aa. 117–140 (■), aa. 141–152 (×), aa.155–165 (+). The final concentration of each peptide is indicated on the *x*-axis.

EPO-Neutralizing Activities of mAbs

To determine whether the five mAbs isolated in this study could neutralize the biological activity of rhEPO, their abilities to block rhEPO-dependent proliferation of UT-7/EPO cells were evaluated. As shown in Fig. 4, all the mAbs inhibited the rhEPO-dependent growth of UT-7/EPO cells in a concentration-dependent manner. However, the extent of inhibition differed markedly among the mAbs. 5D11A showed the strongest neutralizing activity, 0.02 μg of antibody per mL being required for 50% inhibition of rhEPO-dependent cell proliferation. For the other mAbs, 0.5 to 5 μg of antibody per mL was necessary to achieve the same level of inhibition. Therefore, we conclude that 5D11A exhibits the strongest rhEPO-neutralizing activity, approximately 25-to 250-fold higher than that of the other mAbs.

Development of an ELISA using the 5D11A mAb

As shown above, 5D11A has the highest binding affinity and highest neutralizing activity against bioactive rhEPO (Table 2, Fig. 4). In order to evaluate the ability of 5D11A as the tool for immunochemical detection of rhEPO, we developed an ELISA for rhEPO using 5D11A as a capture antibody. The rhEPO captured by immobilized 5D11A was detected biotin-labeled anti-rhEPO pAb. The rhEPO dose-response curve was linear over the range of 2.5–160 mIU/mL (Fig. 5).

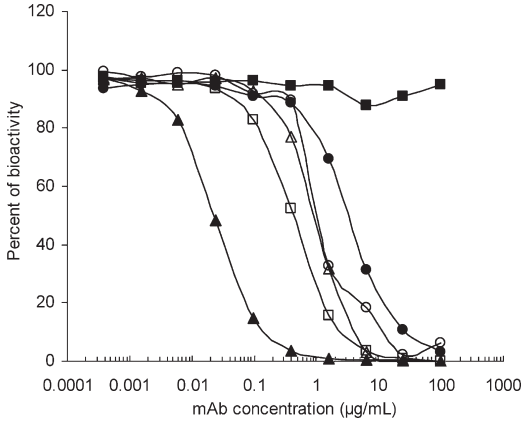

Figure 4. Neutralization of rhEPO bioactivity. A total of 100 μL RPMI1640 medium containing 4% fetal calf serum, 10 mM glutamine and 300 pg/mL rhEPO was incubated with 50 μL of the indicated concentrations of each anti-rhEPO mAb (1G12A (○), 2A2B (●), 4G3C (Δ), 5D11A (▲), or 4H10C (□)), and rhEPO-induced cell proliferation was monitored. AE7A5 (■) is an anti-rhEPO mAb that does not neutralize rhEPO bioactivity (purchased from R&D Systems).

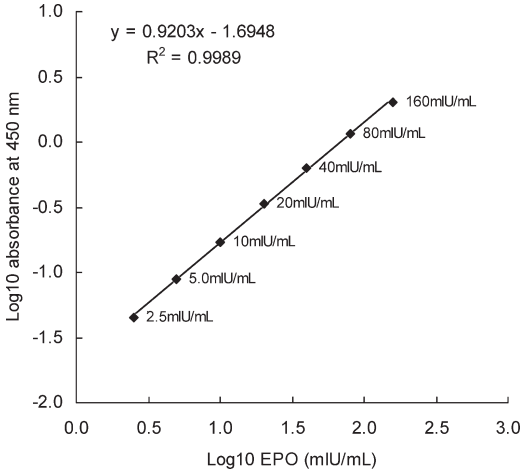

Figure 5. The rhEPO dose-response curve. The rhEPO dose-response curve was created by plotting the Log of absorbance for each standard on the *y*-axis against the Log of the rhEPO concentrations (2.5–160 mIU/mL) on the *x*-axis. The best fit line was determined by the regression analysis.

Intra- and inter-assay precision and accuracy were assessed by analysis of quality control samples prepared at the lowest limit of the standard curve (2.5 mIU/mL) and three additional concentrations spanning the standard curve (5, 20, and 80 mIU/mL). Intra-assay precision and accuracy were evaluated for each concentration by six replicates of the sample in one assay. Inter-assay precision and accuracy were determined for all values obtained along five independent assays. All the estimated parameters were satisfactory to evaluate quality control samples (Table 3). The quantitation limit was determined to be 2.5 mIU/mL, because this concentration was minimum so that the analyte could be quantified with acceptable precision and accuracy. The detection limit defined as the minimal concentration of rhEPO that produced a signal with an intensity equal to the blank signal plus twice standard deviation, was measured to be 0.2 mIU/mL. To test the specificity, rhEPO was artificially mixed at final concentrations of 67 to 680 IU/mL with the crude protein extract (45 μg protein/mL) from an EPO-deficient cell line of the chinese hamster ovary and the resulting mixture was examined by the ELISA. As shown in Table 4, the recovery ranged from 100.5 to 108.0%. These results indicate that 5D11A is suitable for the tool of immunochemical detection of rhEPO.

In order to evaluate the relationship between the measurement results by ELISA and biological activity of rhEPO, heat-denatured and light-exposed rhEPO samples were tested by both ELISA and cell-based potency assay. The obtained results showed a good correlation between the two assays

Table 3. Intra- and inter-assay validation

	Intra-assay					Inter-assay				
EPO concentration (mIU/mL)	n	Mean (mIU/mL)	S.D. (mIU/mL)	Precision C.V. (%)	Accuracy Recovery (%)	n	Mean (mIU/mL)	S.D. (mIU/mL)	Precision C.V. (%)	Accuracy Recovery (%)
2.5	6	2.01	0.16	7.9	80.2	30	2.2	0.23	10.2	88.8
	6	2.41	0.08	3.3	96.3					
	6	2.21	0.31	13.9	88.2					
	6	2.28	0.11	5.0	91.3					
	6	2.20	0.24	11.0	88.0					
5	6	4.4	0.34	7.6	88.7	30	4.6	0.34	7.3	92.3
	6	4.8	0.14	2.9	96.7					
	6	4.5	0.43	9.6	91.0					
	6	4.7	0.16	3.3	93.3					
	6	4.6	0.45	9.8	91.6					
20	6	19.7	1.10	5.6	98.5	30	19.3	1.21	6.3	96.4
	6	19.8	0.76	3.9	99.2					
	6	18.9	1.96	10.4	94.6					
	6	18.6	0.63	3.4	93.2					
	6	19.4	1.13	5.8	96.8					
80	6	76.7	3.56	4.6	95.9	30	72.9	4.90	6.7	91.1
	6	75.8	3.38	4.5	94.8					
	6	71.2	5.53	7.8	88.9					
	6	67.8	2.44	3.6	84.7					
	6	72.8	3.99	5.5	91.0					

Table 4. Recovery of rhEPO in crude protein extract

Expected concentration (IU/mL)	n	Observed concentration			Recovery (%)
		Mean (IU/mL)	S.D. (IU/mL)	C.V. (%)	
680	6	682.8	23.40	3.4	100.5
592	6	616.6	31.50	5.1	104.2
474	6	497.2	24.20	4.9	105.0
337	6	363.6	18.83	5.2	108.0
213	6	226.5	13.73	6.1	106.1
123	6	131.3	9.35	7.1	106.6
67	6	70.7	3.83	5.4	105.9

(Table 5). This indicates that the rhEPO level estimated with the ELISA reflects its bioactiviy.

DISCUSSION

In this report, we described isolation and characterization of five new mAbs against rhEPO. Epitope exclusion ELISA analysis indicated that obtained mAbs can be classified into two groups.

The group 1 mAbs (1G12A, 2A2B, and 4G3C) were shown to recognize residues within the N-terminal 20 amino acids of rhEPO. So far several antibodies against N-terminal region of EPO have been established.[13–16,18–20] All of these antibodies with only one exception have been reported to lack

Table 5. Correlation between ELISA and cell-based potency assay

Denaturing condition	ELISA				Cell-based bioassay			
	n	Mean (%)[a]	S.D. (%)	C.V. (%)	n	Mean (%)[a]	S.D. (%)	C.V. (%)
None	3	100.0	—	—	3	100.0	—	—
50°C 1 week[b]	3	73.0	2.50	3.4	3	72.5	3.04	4.2
50°C 2 weeks[b]	3	52.3	2.19	4.2	3	50.6	4.86	9.6
Light exposure[c]	3	73.7‘	5.02	6.8	3	57.4	3.82	6.6

[a]Residual activity relative to the non-treated sample.

[b]rhEPO was heated for 1 or 2 weeks at 50°C.

[c]rhEPO was exposed to light (overall illumination of 1.2 million lx hours and an integrated near ultraviolet energy of 200 watt hours/square meter).

the EPO-neutralizing activity. This fact suggested that the N-terminal region of EPO does not play an important role in its receptor recognition. On the other hand, single amino-acid substitutions in the N-terminal region of EPO have been shown to affect its function, indicating that this region is functionally important.[25–27] It is noteworthy that all the group 1 mAbs obtained in this study had the EPO-neutralizing activity (Fig. 4). We believe that these mAbs will help in future to understand precise structure-function relationship of the N-terminal region of EPO.

Our data suggest that group 2 mAbs (5D11A and 4H10C) do not recognize a linear epitope but a conformation-dependent epitope of rhEPO. One of them 5D11A showed the highest binding affinity and neutralizing activity against rhEPO. These results let us to evaluate 5D11A as the tool for immunochemical detection of biologically active rhEPO. For this purpose, we established an ELISA using 5D11A as the capture and the biotinylated anti-rhEPO pAb as the detector.

Validity of this ELISA was assessed in several ways. (1) Study on the intra- and inter-assay validation showed that precision and accuracy of this assay were acceptable to measure the concentration of rhEPO (Table 3). (2) The detection limit (0.2 mIU/mL) and quantitation limit (2.5 mIU/mL) of this ELISA were almost comparable to those previously described for other rhEPO ELISAs.[9–12] (3) This ELISA was capable of detecting specifically rhEPO in the samples containing complex matrices such as crude protein extract from an EPO-deficient cell line (Table 4). (4) The measurement results from this ELISA had a good correlation with the bioactive EPO levels measured by the cell-based potency assay (Table 5). Taken together, we conclude that the ELISA developed in this study is a useful tool for efficient and accurate determination of the bioactive rhEPO concentration with full reliability and reproducibility.

ABBREVIATIONS

ELISA, enzyme-linked immunosorbent assay; EPO, erythropoietin; FCA, Freund's complete adjuvant; FIA, Freund's incomplete adjuvant; HRP, Horseradish Peroxidase; Lys-C, endolysine C; mAb, monoclonal antibody; pAb, polyclonal antibody; PBS, phosphate-buffered saline; rhEPO, recombinant human EPO; RT, room temperature; RU, resonance unit(s); SPR, surface plasmon resonance; TMB, 3, 3′, 5, 5′-tetramethylbenzidine.

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