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*J Immunol* 2008; 181:1282-1287; doi: 10.4049/jimmunol.181.2.1282

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*The Journal of Immunology* is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
The Potency of Erythropoietin-Mimic Antibodies Correlates Inversely with Affinity

Susan E. Lacy,* Peter J. DeVries,† Nancy Xie,† Emma Fung,* Richard R. Lesniewski,† and Edward B. Reilly1†

Preclinical animal studies have shown that Ab12.6, an agonistic human Ab targeting the erythropoietin receptor (EPOR), exhibits several potential dosing and safety features that make it an attractive clinical candidate for the treatment of anemia. Ab12.6 was derived by yeast display affinity maturation of parental Ab12, a strategy initially intended to improve off-rate and affinity for EPOR, thereby enhancing erythropoietic activity. Analysis of full-length IgGs derived from yeast clones identified sequences within Ab12 CDRH2 that independently influenced both affinity and potency. The Ab12.6 derivative displayed improved in vitro potency and in vivo efficacy, although its binding affinity to the EPOR was lower than that of the parent Ab12. Additional Ab12 derivatives also exhibited an inverse correlation between affinity and potency. These results suggest that for this class of agonistic Abs, faster off-rates may permit continuous receptor stimulation and more efficient erythropoiesis.

Erythropoietin (EPO), a naturally occurring hematopoietic growth factor produced by the kidney, is the primary regulator of erythropoiesis (1). Recombinant human EPO (rHu-EPO) has important clinical uses in patients with anemia associated with renal disease and cancer (2). We have previously shown that a human EPO-mimic Ab, Ab12.6 (also known as ABT007), is a potent stimulator of erythropoiesis in a human EPOR-expressing transgenic mouse model (3). Based on its ability to support potent, more sustained, and less pulsatile elevation of hematocrit, while requiring less frequent dosing compared with standard doses of rHu-EPO, Ab12.6 may provide both potential medical benefits and improved patient convenience. It is unlikely that Ab12.6 would induce Ab-mediated pure red cell aplasia, a condition associated with some forms of rHu-EPO due to the formation of rHu-EPO-induced neutralizing Abs (4). Although the extended serum half-life of the human Ab may enhance its in vivo properties, the potency of Ab12.6 resides primarily in a novel mechanism of receptor activation based on a unique, Ab-imposed, conformational change (3).

Ab12.6 was originally derived from a parental human Ab, Ab12, which was raised to the extracellular domain (ECD) of EPOR using XenoMax technology (5). Ab12 is capable of stimulating the proliferation of EPO-responsive cells both in vitro and in vivo. This activation is influenced by Ab12/EPOR on-off rate kinetics and binding affinity. Since the \( K_d \) of Ab12 for EPOR is 10-fold higher than the 1 nM dissociation constant observed between EPO and the high-affinity EPOR binding site (6), we reasoned that improving the off-rate and, therefore, the overall affinity of Ab12 may enhance its erythropoietic activity. Improvements in Ab affinity often correlate with improvements in potency (7–13), although the correlation between changes in agonistic Ab affinity and biologic potency is less well characterized. Using yeast display affinity maturation to engineer single-chain variable fragment (scFv) Ab12 variants (14), various derivatives were isolated, constructed as full-length IgG2\( \kappa \) Abs, and tested for affinity and potency to EPOR. Sequences within CDRH2 were identified that independently affected both affinity and potency. Ab12.6 emerged from these studies as a potential clinical candidate based on enhanced biological efficacy. Despite its improved potency, however, Ab12.6 had a \( K_d \) value less than that of the parental Ab12, indicating that for this pair of closely related Abs, potency and affinity are inversely correlated.

Materials and Methods

Generation and characterization of Ab12

A soluble form of mature human EPOR ECD representing residues 1–225 was expressed in Escherichia coli, refolded, and purified as described (15). XenoMouse (XenoMouse XG2; Amgen) were immunized with the EPOR ECD, and Ab12 was generated using XenoMax technology as previously described (3, 5, 16). Ab12 and its variants were tested for erythropoietic activity in cell proliferation assays with EPO-responsive cells (17, 18) and by their ability to support the formation of erythroblastic colonies (CFU-E) from human bone marrow containing CD34+ progenitor cells (3). In vivo efficacy was evaluated in an EPOR+ human EPOR+ transgenic mouse model (19, 20). All animal studies were conducted in accordance with the guidelines established by the Abbott Laboratories Institutional Animal Care and Use Committee.

Expression of Ab12 scFv on yeast surface

The yeast cell (Saccharomyces cerevisiae) serves as a living scaffold permitting surface display of diverse populations of scFvs as inducible fusion proteins with the Aga2p mating agglutinin protein (14). Two individual DNAs encoding Ab12 V\(_{\text{H}}\) and V\(_{\text{L}}\) domains were amplified by PCR with oligonucleotide primers. The V\(_{\text{H}}\) domain was amplified using forward primer 5'-AGTACAGCTTGGCTTGGATTGCGAG-3' and reverse primer 5'-TCTCGAGAGGACGGTGACAGGTTCC-3', and the V\(_{\text{L}}\) using forward primer 5'-CCGCTGTCGAGACAGCATGCGGGAAGCAG-3' and reverse primer 5'-CTCGAGCCCGTACGCCGAGGACAGCATGCGGGAAGCAG-3'.
ACCAATCT-3′ and reverse primer 5′-GTCGATTTTGGTACAC TTCAC-3′. One microgram of each of these PCR products was mixed in a trans-PCR reaction with the outermost forward and reverse primers 5106 and 5107, respectively. The resulting scFv DNA encoded from 5′ to 3′ an SfiI restriction site, the V₅ domain, a linker sequence, the V₄ domain, and a NotI restriction site. The linker (designated linker 40) encoded the amino acid sequence GAPEPTLPAEKVS. The cDNA was then ligated into the SfiI and NotI sites of a modified vector derived from pYD1 (Invitrogen). The pYD1 vector was modified to encode substitution of the Gly(Ser)(3′ of the Aga2p sequence) with linker sequence GENKVEYPALMALS, designated linker sequence 41. The Ab12 scFv fusion construct also included vector encoded N-terminal Xpress and C-terminal V5 and poly-His epitope tags to allow normalization of scFv surface copy number. In summary, the Ab12 scFv construct encoded Aga2p–linker 40–Ab12 V₄–linker 41–Ab12 V₅–Xpress epitope–V5 epitope–poly-His epitope. The vector was introduced into S. cerevisiae using a Geitz yeast transformation kit (Tetralink), and transformants were plated on dextrose-containing tryptophan (–) and uracil (–) selective agar plates. Several emergent colonies were expanded into dextrose-containing tryptophan (–) and uracil (–) selective liquid culture. Approximately 1 × 10⁶ yeast cells were transferred to selective media supplemented with galactose to induce scFv expression. After 16–20 h incubation at 30°C, scFv expression was measured by flow cytometric analysis of the FL2 signal generated following incubation with murine anti-V5 mAb (Invitrogen) for 30 min on ice, washing cells three times at 4°C with PBS containing 1 mg/ml BSA, and incubation at 4°C with anti-mouse PE (Jackson ImmunoResearch Laboratories) for an additional 30 min. All samples were analyzed on an Epics XL flow cytometer (Beckman Coulter). Robust scFv expression was noted on 60% of the cells, typical of inducible scFv protein expression on yeast. A single stock clone was selected and expanded for expression studies.

Off-rate analysis of Ab12 scFv on yeast

Off-rate measurements were performed by incubating Ab12 scFv yeast cells with saturating quantities of EPOC ECD followed by addition of a 10,000-fold excess of Ab12 IgG1/κ as a competitor. (Ab12-IgG1 is a derivative of Ab12 that was genetically engineered to encode an IgG1 iso-type recombinant heavy chain sample aliquots were incubated at 37°C, washed, and drawn at defined time points and placed on ice until all samples were ready for incubation with detection reagents. The soluble EPOR remaining bound to Ab12 scFv was measured by addition anti-EPO reactive mAb307 (R&D Systems) followed by anti-mouse PE Ab. All samples were analyzed on an Epics XL flow cytometer and the mean fluorescence of the initial signal in each sample was then plotted against the time allowed for dissociation to generate an off-rate curve.

Generation of Ab12 scFv CDR mutant libraries

All six CDRs of Ab12 scFv (three in the H chain and three in the L chain) were subjected to a mutagenesis scan by randomization of three amino acids per library, theoretically creating 8000 versions of Ab12 per library. All six CDRs of Ab12 scFv (three in the H chain and three in the L chain) were subjected to a mutagenesis scan by randomization of three amino acids per library, theoretically creating 8000 versions of Ab12 per library. The libraries were assessed for quality following the selection and sequencing of yeast plasmid DNA encoding the mutant Ab12 scFv sequences. Plasmid was isolated using a Y-PER kit (Promega). Selection of Ab12 scFv libraries by FACS

Selected scFvs were converted into full-length IgG2/κ Abs by PCR amplification of the mutant Ab12 V₅ or V₄ domains. These products were ligated into an intact IgG2 constant region or κ region present in the vector pBOS (21). pBOS plasmids encoding both H and L chain regions were co-transfected transiently into COS cells, and resulting supernatants from 3-day cell cultures were purified over a protein A-Sepharose column (Amersham Biosciences). Purified Abs were dialyzed into PBS and concentrations were determined using absorbance at 280 nm.

Surface plasmon resonance analysis of Abs derived from yeast display

Real-time binding interactions between Abs captured on a biosensor matrix with goat anti-human IgG and soluble EPO were measured by surface plasmon resonance using the Biacore 3000 system (Biacore Life Sciences) according to the manufacturer’s instructions. Briefly, soluble EPO was diluted to a concentration of 200 nM and loaded through a flow cell containing immobilized Abs at a flow rate of 5 μl/min. The concentrations of EPO for use were 2.5, 5, 10, 25, 40, 50, 80, 100, and 200 nM. Biacore kinetic evaluation software (version 4.0.1) was used to determine the K₅, K₊, and K₉, respectively.

Results

Expression and characterization of Ab12 scFv on the surface of yeast

Ab12 was one of several recombinant Abs identified from the XenMax approach based on its ability to stimulate the proliferation of EPO-responsive cells (3). Affinity measurements of Ab-12 bound to sEPO indicated an on-rate of 1.4 × 10⁷/Ms⁻¹, an off-rate of 1.3 × 10⁻³/s, and an overall K₅ of 9 nM (Table I). The K₅ of Ab12 for EPO is 10-fold higher than the 1 nM dissociation constant observed between EPO and EPOR (6). Since improvements in Ab affinity often correlate with enhanced potency, it was hypothesized that an Ab with higher affinity may result in increased erythropoietic activity. Ab12 was converted into a single-chain format and expressed in S. cerevisiae using the Aga2p tether protein. The Ab12 scFv construct also contained a V5 epitope tag fused in-frame with the scFv. Expression of Ab12 scFv was induced and detected by incubating cells with an anti-V5 Ab followed by an anti-mouse PE secondary Ab. Equilibrium titration data generated

### Table I. Affinity measurement of Ab12 variants

<table>
<thead>
<tr>
<th>Ab</th>
<th>K₅ (1/M · s)</th>
<th>K₊ (1/s)</th>
<th>Kₒ (nM)</th>
<th>Fold Improvement in K₅</th>
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<td>12</td>
<td>1.4 × 10⁵</td>
<td>1.3 × 10⁻³</td>
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<td>12.70</td>
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<td>9 × 10⁻⁵</td>
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<td>9</td>
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with increasing concentrations of EPOR ECD provided an approximate \( K_D \) value of 4 nM for the Ab12 scFv-soluble EPOR interaction (data not shown), consistent with the 9 nM \( K_D \) value for the intact Ab12-sEPOR interaction. An off-rate determination for yeast-displayed Ab12 scFv was performed by measuring remaining prebound sEPOR dissociation over time. The design was such that as EPOR ECD dissociated from Ab12 scFv, it would immediately bind to Ab12 IgG1 competitor mAb (present at a saturating concentration) and would therefore no longer be detected on the surface of yeast. After 20 min of competition at 37°C, no further dissociation appeared to be taking place, consistent with the \( 10^{-3} \) off-rate measured for Ab12 by BIAcore (Table I).

Ab12 scFv CDR mutant libraries and identification of variants with improved binding to EPOR

Forty-four libraries containing randomized mutations within the CDRs were subjected to off-rate analysis (Fig. 1). Eleven of these libraries, including eight CDR H2 libraries, two CDR L2 libraries, and one CDR L3 library (highlighted in gray in Fig. 1), displayed the highest fluorescent shift indicative of EPOR binding. The round 1 and round 2 off-rate FACS outputs for library H2-1–3, displaying the highest fluorescent shift, is shown in Fig. 2. Only two rounds of sorting were necessary to generate a 10,000-fold enrichment from the starting library due to the low diversity contained in each of the libraries and the percentage of cells that were gated in each round. Individual clones from these outputs were recovered following plating on selective media and plasmid DNA isolation. The scFv region of each cloned plasmid was amplified by PCR and sequenced to identify the Ab scFv amino acid mutations resulting in improved off-rates.

Sequence analysis and affinity measurements of Ab12 variants

Overall, clones containing mutations in CDR H2 displayed the highest overall EPOR binding following a 20-min competition with Ab12 IgG1 competitor mAb, and representatives of these were selected for conversion into full-length IgG2\(\alpha\) mAbs to match the original isotype of Ab12. The mAbs were designated Ab12.6, Ab12.17, Ab12.25, Ab12.56, Ab12.61, Ab12.70, and...
Ab12.76. A schematic of the selected clones and the exact locations of their CDR H2 amino acid mutations (in the context of Ab12) are shown in Fig. 3. Essentially two regions in CDR H2 were represented in mutant Ab12 scFvs displaying improved off-rates on the surface of yeast. These regions spanned amino acids YYS and TNY, and in each case two or more of these amino acids were substituted. To determine whether these mutations correlated with improved off-rates in the context of a full-length mutant Ab12, Ab12.6, and an isotype control Ab. The colonies were red in color and identified microscopically. rHu-EPO (EpoGen; Amgen) was used as a positive control. Error bars represent SD calculated from the average of duplicate counts.

**Erythropoietic activity of Ab12.6 vs Ab12**

Preliminary results indicated that Ab12.6, despite having a faster off-rate for the EPOR compared with Ab12, retained significant potency. To further evaluate functional differences between these Abs, their erythropoietic potency was evaluated in an in vitro cell proliferation assay using the F36E EPO-dependent cell line. As shown in Fig. 4A, the maximal proliferative response for Ab12.6 exceeded that for Ab12, indicating that Ab12.6 is a more potent stimulator of F36E cells. The erythropoietic activity of Ab12.6 appeared comparable to the rHu-EPO control, indicating that the YYS mutation in CDRH2, while negatively affecting binding affinity, improved overall agonistic activity. A similar pattern of stimulation was also seen with UT-7, another EPO-responsive cell line (data not shown).

EPO supports the growth and differentiation of erythroid progenitor cells. Ab12 and Ab12.6 were therefore also evaluated for their ability to support the formation of erythroid colonies from human bone marrow containing CD34+ progenitor cells. Consistent with the results observed with EPO-dependent cell lines (Fig. 4A), Fig. 4B indicates that Ab12.6 is more effective at stimulating erythropoiesis, as evidenced by the increased number of erythroid colonies.

**Erythropoietic activity of Ab12 variants**

The eight full-length IgG2/Ab 12 derivatives (Fig. 3) were evaluated to further determine the effect of the two CDRH2 substituted regions on potency. F36E proliferation assay results shown in Fig. 5 indicate that the activities of the Abs could be divided into two main classes dependent on the region in CDRH2 that was mutated. Ab12.56 and Ab12.70, like Ab12.6, displayed maximal proliferative activity at least as comparable to or greater than that for Ab12 (red line graph in Fig. 5A), indicating that overall agonistic activity could be positively impacted by amino acid substitutions of the YYS region. In contrast, proliferation curves from TNY variants 12.17, 12.25, 12.61, and 12.76 shifted primarily to the right of Ab12 (red line graph in Fig. 5B), consistent with reduced potency. Since the YYS and TNY region variants also correlate with opposing off-rate kinetics (Table I and Fig. 3), collectively, these results substantiate the observations made with Ab12 and Ab12.6.
that improvements in agonistic activity are inversely correlated with overall Ab affinity.

A similar pattern of reactivity was also seen following assessment of selected Abs for their ability to support the formation of erythroid colonies from human bone marrow. As shown in Fig. 6, YYS variant Abs 12.6, 12.56, and 12.70 were all more effective, to varying extents, in inducing colony formation than was Ab12. In contrast, the TNY variant, Ab12.17, despite having the greatest binding affinity for EPOR within the existing Ab panel (Table I), was ineffective in stimulating formation of erythroid colonies.

In vivo potency of Ab12.6 vs Ab12

To determine whether the inverse correlation between affinities and erythropoietic activity is also reflected in vivo, Abs 12 and 12.6 were evaluated for the ability to elevate hematocrit in animals. In these experiments the erythropoietic activities of the Abs were compared with that of NESP (Amgen), a long-acting, hyperglycosylated analog of rHu-EPO currently in clinical use for anemia treatment as darbepoetin alfa (22). A transgenic mouse model, generated by rescuing genetic knockout mice lacking the murine EPOR gene with the human EPOR transgene (19, 20), was used since Ab12 and Ab12.6 do not recognize rodent EPOR (3). A single administration of either Ab at concentrations ranging from 0.4 to 1.6 mg/kg resulted in a dose-dependent rise in hematocrit measured on day 21 (Fig. 7). Additional results indicated that these hematocrit increases are sustained beyond 3 wk following dosing with Ab12.6 (3). At every dose tested, Ab12.6 was more effective at elevating hematocrit than was the equivalent dose of Ab12. Additionally, the hematocrit increase observed on day 21 following a single dose of Ab12.6 was at least equivalent to that observed with a clinically relevant dose of NESP (3.75 μg/kg) administered on day 0 and day 14 (23). These results confirm that the in vivo efficacy for Ab12 and Ab12.6 also inversely correlates with affinity.

Discussion

Unlike other murine EPO-mimic Abs that have been described (24, 25), Ab12 is a human EPO agonistic Ab that is capable of stimulating and sustaining erythropoiesis in vivo. We sought to enhance the agonistic activity of Ab12 even further by improving the off-rate and, therefore, the affinity of the Ab to EPO using yeast display affinity maturation. Yeast-displayed Ab12 variants containing CDRH2 amino acid substitutions were isolated at a high frequency and displayed improved off-rate kinetics on the surface of yeast. Several Ab12 variants were subsequently constructed as full-length IgG2κ Abs and were tested for affinity and potency to the EPOR.

Essentially two regions in CDRH2 were interrogated following the generation of full-length IgG: the YYS and TNY regions. Abs containing amino acid changes in the YYS region (Abs 12.6, 12.56, and 12.70) did not demonstrate improved off-rates, contrary to what was observed for the scFv version of these Abs on the surface of yeast. In fact, the affinities of Ab12.6 and Ab12.56 were significantly less than that of parental Ab12, while that of Ab70
was comparable. It is not clear why selected scFv mutants having CDRH2 mutations in the YYS region displayed improved off-rate kinetics on the surface of yeast, yet the corresponding full-length IgG2a versions did not have improved affinity. One possibility is that these scFvs bind and oriented EPOR ECD in such a way as to make it more accessible to the fluorescent-tagged detection reagents, resulting in higher signals mistakenly interpreted as increases in the duration of the off-rate. These findings indicate that the selection criteria used to identify yeast clones with enhanced fluorescent binding may not always yield improved kinetics and ultimately translate into full-length Abs with higher affinity binding.  

When constructed as full-length IgGs, however, Ab12.6 did display enhanced agonistic potency as measured by the ability to support both proliferation of EPO-dependent cell lines and formation of erythroid colonies. Importantly, in vivo results also indicated that Ab12.6 efficacy had been positively impacted by the amino acid substitutions in the YYS region of CDRH2. Other Abs containing substitutions in this same region also displayed increased maximal proliferative activity and potential to support greater erythroid colony formation than did Ab12. Conversely, the TNY Ab12 mutants identified as having slower off-rates of 10–30-fold (Ab12.17, Ab12.61 and Ab12.76) are less efficient in stimulating erythropoiesis, as measured in both in vitro assay formats. Collectively, these data suggest that improvements in agonistic activity for this class of Abs are inversely correlated with overall Ab affinity, as tight binding of Ab to receptor leads to reduced potency. Faster Ab off-rates from the receptor and subsequent re-engagement may permit continuous, more efficient erythropoiesis.  

Although improvements in Ab affinity generally correlate with improved function (7–13), we show herein that, at least for an agonistic EPOR Ab, the reverse is true. However, examples of antagonistic Abs that retain improved potency despite a faster off-rate have been described. Certain CD20-reactive human Abs are antagonistic Abs that retain improved potency despite a faster off-rate have been described. Certain CD20-reactive human Abs are antagonistic Abs that retain improved potency despite a faster off-rate. Certain CD20-reactive human Abs are antagonistic Abs that retain improved potency despite a faster off-rate. Certain CD20-reactive human Abs are antagonistic Abs that retain improved potency despite a faster off-rate. Certain CD20-reactive human Abs are antagonistic Abs that retain improved potency despite a faster off-rate. Certain CD20-reactive human Abs are antagonistic Abs that retain improved potency despite a faster off-rate. 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