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Strategy to confirm the presence of anti-erythropoietin neutralizing antibodies in human serum

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ABSTRACT

Functional cell-based assays are the preferred method to test for the presence of anti-rHuEPO neutralizing antibodies (NAbs). However, due to the unpredictable nature of test serum matrix effects on cell-based assays, confirmatory assays are essential for verifying NAb positive results observed during the course of sample testing. The cell-based assay used for the detection of NAbs described by Wei et al. [1] used 32D-EPOR cells, a murine myeloid cell line transfected with the human EPO receptor (EPOR). The 32D-EPOR cell line responded to either rHuEPO or murine interleukin 3 (mIL-3) with proliferation. NAbs were expected to only inhibit rHuEPO-induced cell proliferation and not mIL-3 induced proliferation. Due to reliance on proliferation, the results from this cell-based assay can be confounded by the presence of non-antibody inhibitory serum factors. This paper describes a strategy for confirming that the inhibition of rHuEPO-induced proliferation in a cell-based assay is only attributable to NAbs. The strategy of antibody depletion uses a resin mixture composed of Protein G Sepharose and Protein L Sepharose (Protein G/L resin) to significantly reduce the concentration of immunoglobulins of IgG, IgM and IgA isotypes from human serum prior to testing in the cell-based assay. If the reduction in immunoglobulins in a serum sample corresponds to a reduction in inhibition of EPO-induced proliferation, it would infer that EPO neutralizing activity is antibody-mediated and not due to non-antibody inhibitory serum factors.

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1. Introduction

Red blood cell production is regulated by the expression of the glycoprotein hormone, erythropoietin (EPO) [2]. Endogenous EPO is produced primarily in the kidney and, upon entering the circulatory system, acts within the spongy tissue of the bone marrow where it binds to and activates the EPO receptors (EPOR) expressed specifically on the surface of erythroid precursors, inducing proliferation and differentiation [3].

The discovery and subsequent cloning of the human EPO gene in the early 1980s led to the production of recombinant human EPO (rHuEPO) [4]. In 1989, the FDA granted rHuEPO approval as a therapeutic protein for the treatment of anemia associated with chronic renal failure. In the United States, a version of rHuEPO, epoetin alfa [5], is manufactured by Amgen, and marketed as EPOGEN® (epoetin alfa) by Amgen and as PROCRIT® (epoetin alfa) by Centocor Ortho Biotech Products. In Japan and China, epoetin alfa is manufactured by Kyowa Hakko Kirin and marketed as ESPO®. For the remaining international markets where the presence of biosimilar products has grown, epoetin alfa is manufactured by several entities including Johnson & Johnson whose own version is marketed under a variety of different names (EPREX®, ERYPRO®, EPOPEN®, EPOXITIN® and GLOBUREN®).

In 2002, EPREX® was found to induce pure red cell aplasia (PRCA) in 13 French patients [6]. PRCA, a condition characterized by a decrease in circulating red blood cells, can be provoked by many factors including, but not limited to, an autoimmune condition, leukemia or a viral infection. The patients diagnosed by Casadevall et al. [6] were unresponsive to increasing doses of EPREX®, and developed a form of PRCA found to be mediated by anti-rHuEPO neutralizing antibodies (NAbs). NAbs not only neutralized the biological activity of rHuEPO, but also cross-reacted with the endogenous protein.

As a consequence, increasing importance has been placed on the sensitivity and specificity of immunogenicity assays designed to detect the presence of NAbs in patients receiving rHuEPO therapy.

Abbreviations: CPM, counts per minute; EPO, erythropoietin; EPOR, erythropoietin receptor; FBS, fetal bovine serum; Ig, immunoglobulin; DA, darbepoetin alfa; mIL-3, murine interleukin-3; M, rHuEPO-induced maximum proliferation control; N, background control; NAb, neutralizing antibody; P, positive control; PNHS, pooled normal human serum; PNRS, pooled normal rat serum; PRCA, pure red cell aplasia; RAC, relative antibody concentration; RH, relative humidity; rHuEPO, recombinant human erythropoietin; RU, response units.

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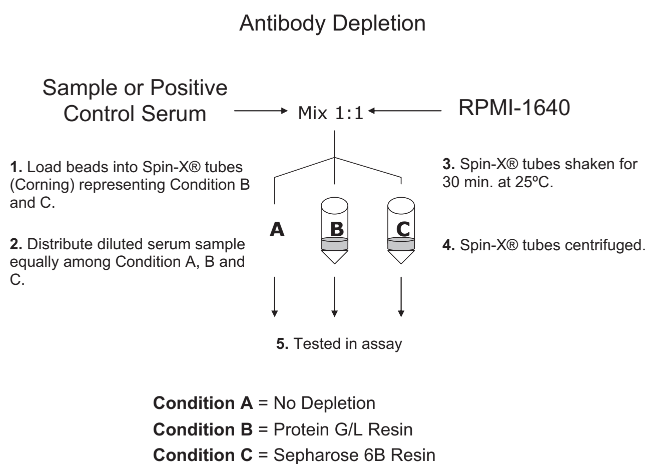

Fig. 1. Antibody depletion.

Testing for the detection of NAbs generally follows a tiered testing strategy [7,8]. Once serum samples are tested in an immunoassay to detect the presence of binding antibodies to rHuEPO [9], reactive or positive serum samples are tested in a functional cell-based assay to assess anti-rHuEPO neutralizing activity and specificity [1].

The procedure described by Wei et al. [1], capable of detecting 500 ng/mL of a positive control neutralizing antibody, used the 32D-EPOR cell line. This cell line responded to the presence of rHuEPO or mIL-3 with proliferation. The rHuEPO-induced proliferation of 32D-EPOR cells was inhibited upon addition of a human serum sample known to contain neutralizing antibodies against EPO; while IL-3-induced proliferation remained unaffected. The authors [1] acknowledged that in certain scenarios this approach could yield false negative results (if cytotoxic factors were present in the sample that inhibited both rHuEPO and mIL-3-induced proliferation) and offered recommendations for developing appropriate confirmatory steps to determine if NAbs were present or not. An accurate determination of NAb presence in an *in vitro* assay is particularly important in cases where a patient is showing clinical sequelae associated with anti-rHuEPO antibodies and the immunoassay result for the sample is positive. The work herein builds on the work conducted by Wei et al. [1] by presenting a method that allows the detection of specific neutralizing antibodies even in the presence of non-antibody inhibitory serum factors, like cytotoxic factors.

This paper describes a confirmatory assay approach to confirm the presence of NAbs in human serum and utilizes the well-known immunoglobulin-binding properties of Protein G [10] and Protein L [11] to deplete most antibodies from human sera including those that have rHuEPO-neutralizing activity. After treatment of NAb-positive samples with a resin mixture of Protein G Sepharose and Protein L Sepharose (Protein G/L resin), the sera are expected to lose their ability to inhibit rHuEPO-induced cell proliferation. However, if significant inhibition is still observed after antibody depletion with Protein G/L resin, without saturation of the binding capacity of the resin, it can be inferred that the rHuEPO neutralizing activity is non-specific and not antibody mediated.

2. Materials

RPMI 1640 serum-free medium and 100 × penicillin/streptomycin/L-glutamine were purchased from GIBCO-BRL (Grand Island, NY). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT). [Methyl-^{3}H] thymidine was purchased from MP Biomedicals (Solon, OH). Protein G Sepharose and Protein L Sepharose were purchased from Pierce Biotechnology (Rockford, IL). Sepharose 6B resin (Unbound beaded agarose) was purchased from Sigma Chemicals (St. Louis, MO). Spin-X® plastic centrifuge tube filters were purchased from Corning (Corning, NY). Pooled normal rat serum (PNRS), pooled normal human serum (PNHS), and individual normal human serum were obtained from Bioreclamation (Hicksville, NY). Recombinant human EPO (EPOGEN®) and the 32D-EPOR cell line were obtained from Amgen Inc. (Thousand Oaks, CA). Murine interleukin-3 was obtained from R&D Systems (Minneapolis, MN). The neutralizing positive control antibody was an affinity-purified rabbit polyclonal antibody to darbepoetin alfa (DA), a novel EPO analog. This antibody was developed at Amgen Inc. (Thousand Oaks, CA) and cross reacts with rHuEPO and neutralizes its biological activity.

Table 1
Statistical Summary of B/C Ratio.

n	50
LS-mean	3.17[a]
Max	14.34
Min	1.49
Lower Bound on	
95%Prediction Limit	1.86[a]
99%Prediction Limit	1.47[a]

[a] Analysis was done using 49 donors.

3. Methods

3.1. Maintenance of cell culture

32D-EPOR cells were maintained in vented cap, canted neck, cell culture flasks (Corning, Corning, NY) at 37 °C, 5% CO_2 and 95% relative humidity (RH). The growth medium consisted of RPMI 1640 supplemented with 15% heat-inactivated FBS, 2 mM L-glutamine, and 1% (v/v) penicillin/streptomycin mixture. The growth medium was supplemented with 10 U/mL of rHuEPO. Cells were subcultured two to three times a week to maintain cell densities between 3×10^4 and 1×10^6 cells/mL. Healthy growing cells were generally 98–100% viable as measured by Trypan Blue exclusion method [12]. Cells were cryo-preserved and used in assays for up to 30 days after thawing.

3.2. Antibody depletion

Protein G/L resin was generated by combining equal volumes of Protein G Sepharose and Protein L Sepharose. Samples and positive control serum (consisting of pooled normal human serum spiked with 500 ng/mL of neutralizing positive control antibody) were each diluted 50% with RPMI 1640 generating a total volume of 320 μL. To remove any unbound material, Protein G/L resin and Sepharose 6B resin were separately washed three times with RPMI 1640 and centrifuged. Spin-X® tubes labeled as Condition B (to contain Protein G/L resin) or Condition C (to contain Sepharose 6B resin) were loaded with an appropriate quantity of washed resin to create a 60 μL pellet by volume (pellet volume optimization data not shown). Following a final centrifugation to remove any remaining liquid and replacement of the Spin-X® recipient tube, a 100 μL aliquot of 50% sample or positive control serum was continuously mixed with the pellets in either Condition B or Condition C for 30 min at 25 °C. The remaining 50% positive control serum or sample (approximately 120 μL) was not filtered through any resin and was labeled as Condition A. After 30 min of mixing, the Spin-X® tubes were centrifuged 5–7 min and the filtrates were collected.

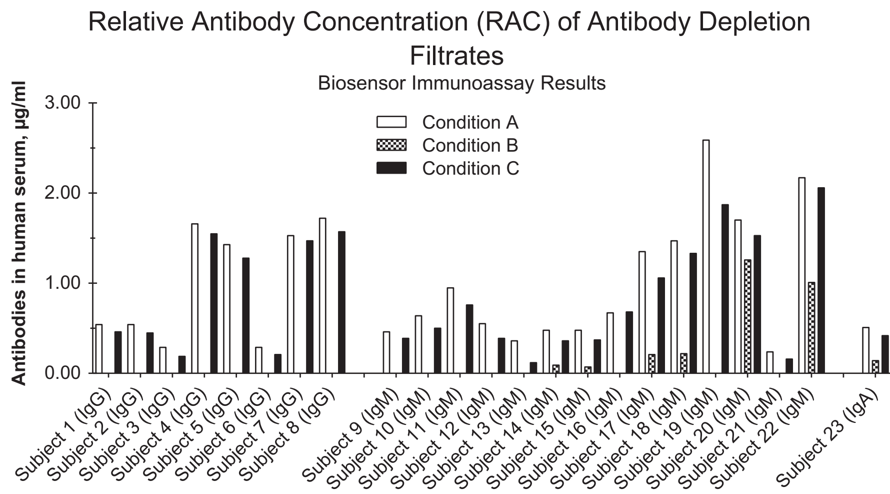

Fig. 2. Protein G/L antibody depletion was performed on a panel of 23 individual human subjects identified as having binding antibodies against rHuEPO. The panel was composed of three distinct isotype groups characterized as predominately IgG (8), IgM (14) or IgA (1). Relative antibody concentration (RAC) of anti-rHuEPO antibodies was extrapolated from a standard curve using the biosensor immunoassay. For graphing purposes, concentrations below the quantitation limit of the biosensor immunoassay were considered zero.

3.3. Biosensor immunoassay: assessment of relative antibody concentration

Relative antibody concentration (RAC) measurements, used to assess the levels of immunoglobulin before and after antibody depletion, were performed with a Biacore® 3000 using a research-grade CM5 biosensor chip. Recombinant human erythropoietin was immobilized to the chip surface using amine-coupling chemistry according to the following method. Flow cells were activated for 7 min with an equal-volume mixture of 0.4 M EDC (3-(N,N-dimethylamine) propyl-N-ethyl-carbodiimide) and 0.1 M NHS (N-hydroxysuccinimide) at a 5 μL/min flow rate. This was followed by an injection of 0.4 mg/mL rHuEPO in 10 mM sodium acetate pH 4.0 (150 μL over 30 min). Residual surface esters were inactivated by passing 1 M ethanolamine hydrochloride pH 8.5 (50 μL for 10 min). The immobilization method was completed with an injection of 50 mM sodium hydroxide (5 μL for 1 min) to condition the surface. Recombinant human erythropoietin was immobilized to a density of 2064.0 response units (RU). PNHS was used as the negative control. Positive controls were prepared by spiking positive control antibody into PNHS to 10 μg/mL or 0.25 μg/mL. A 9-point standard curve was prepared by spiking positive control antibody in PNHS at a concentration of 10 μg/mL and making serial dilutions reaching 0.039 μg/mL. All samples, controls and standards were diluted 50% with RPMI-1640 serum-free medium and filtered through Spin-X® tubes. Following a previously described method of analysis [9], human donor serum (before and after depletion), standards and controls, were prepared and injected into the biosensor flow cell. RAC for each sample was extrapolated from a standard curve using a weighted (1/conc) quadratic regression formula (Microsoft Excel®).

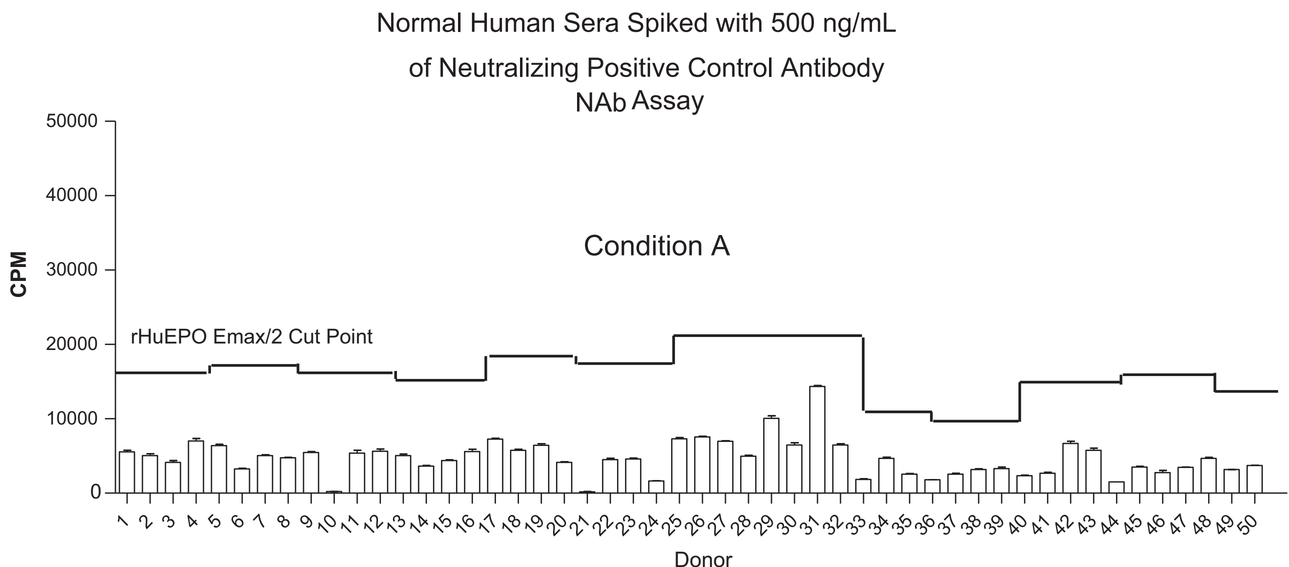

Fig. 3. Serum from 50 normal human donors was spiked with 500 ng/mL of Positive Control Antibody. Each of the 50 donors had a Condition A response below the corresponding rHuEPO Emax/2 cut point, thereby confirming the presence of neutralizing activity. All 50 donors were tested over the span of several assay plates, therefore the Emax/2 cut point changed with each assay plate.

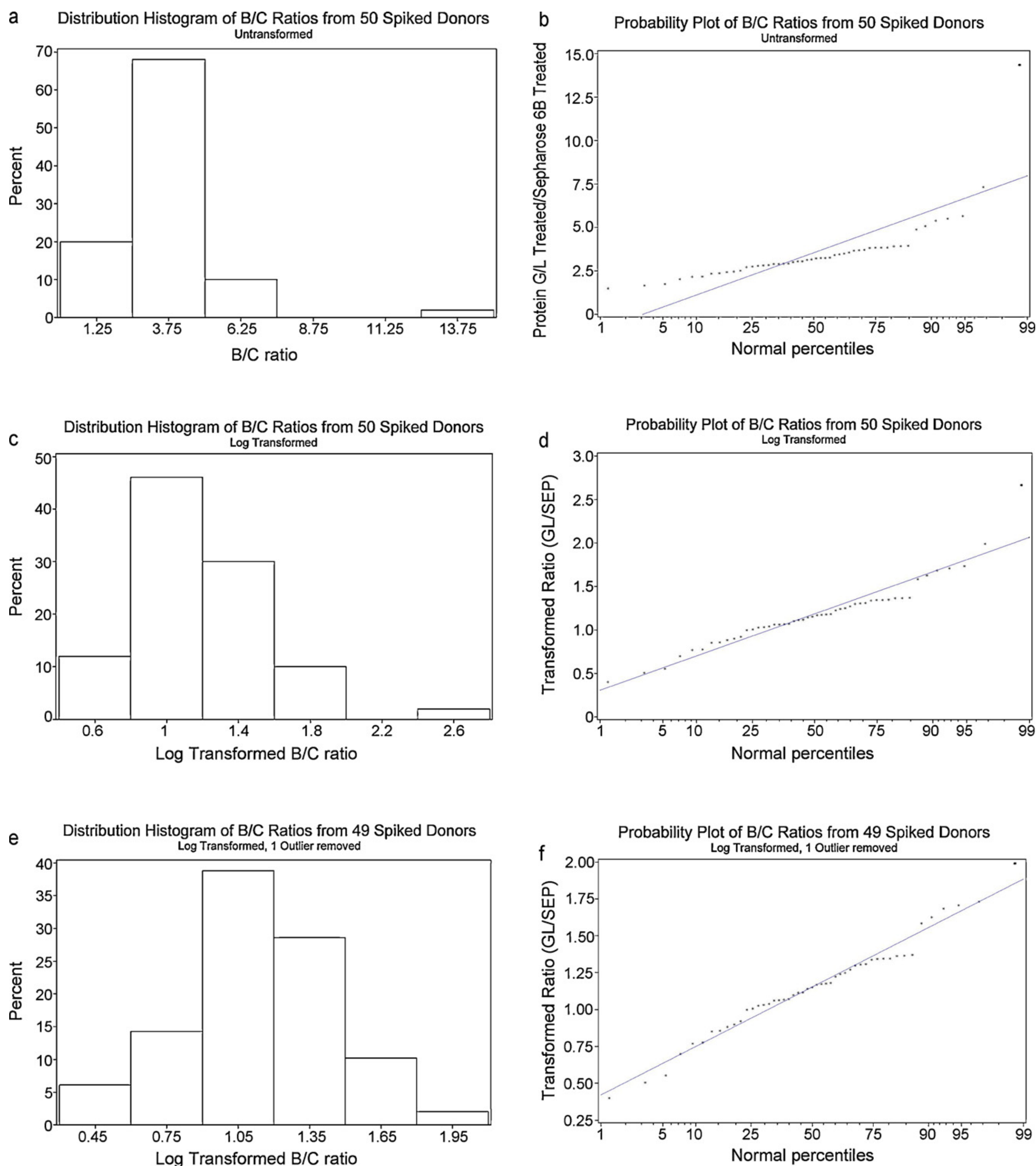

Fig. 4. Using an untransformed scale, the distribution histogram for B/C ratio values from all 50 spiked donors was found to be skewed and not normally distributed (a). The untransformed data on the probability plot was also found not to be in good agreement with the reference line (b). Following a log transformation, the distribution histogram and probability plot (c and d) indicated that the B/C ratio value from one donor (Donor 21) was found to not be drawn from a common normal distribution. The result from Donor 21 was identified as an outlier and excluded from subsequent statistical analysis. The distribution histogram and the probability plot for the remaining transformed B/C ratio values (e and f) appeared normally distributed and the probability plot showed a good agreement with the point pattern and the reference line.

3.4. Biosensor immunoassay: assessment of antibody isotype

Samples containing binding antibodies to rHuEPO were analyzed to determine antibody isotype with a Biacore® 3000 using a research-grade CM5 biosensor chip. Recombinant human erythropoietin was immobilized to the chip surface in the same manner as described above. To determine the isotype of antibodies in the serum samples, TBS-EP buffer (25 mM Tris, 300 mM NaCl, 3 mM EDTA, 0.005% Polysorbate-20 (P20), pH 9.0) was used as a running buffer. The negative control and positive controls (0.25 and 10 μg/mL positive control antibody) were prepared as described above. Samples and controls were diluted 50% with TBS-EP diluent buffer (50 mM Tris, 500 mM NaCl, 5.48 mM EDTA tetrasodium salt, 0.01% P20, 1% carboxymethyl dextran, pH 9.0) and filtered through

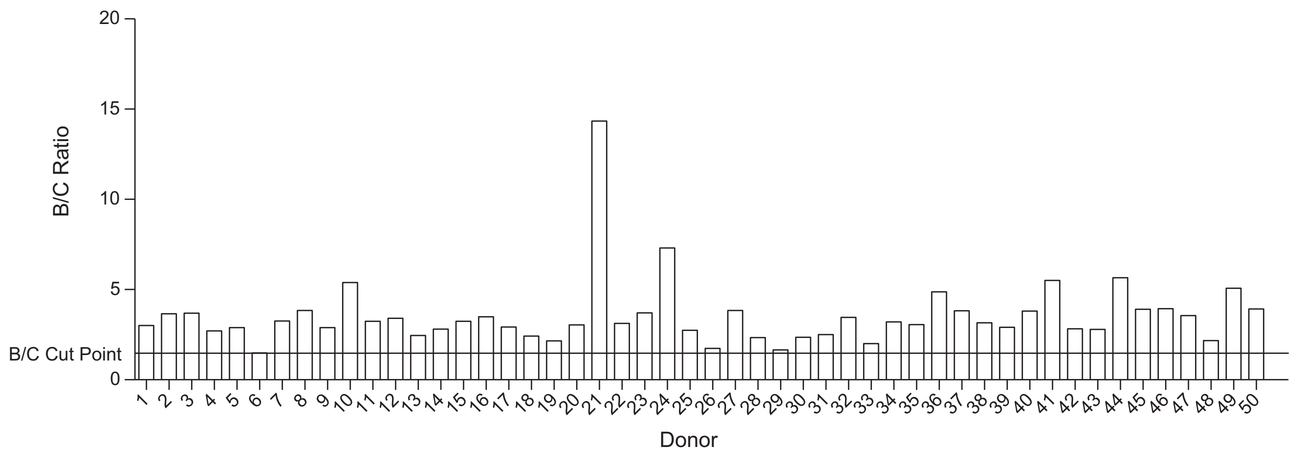

Fig. 5. All 50 normal human donors spiked with 500 ng/mL of Positive Control Antibody were found to have B/C ratios above the statistically derived B/C cut point of 1.47 (99% prediction interval).

Spin-X® tubes. Each sample was passed over the rHuEPO surface at 5 μL/min for 10 min allowing antibodies present in the serum sample to bind to the antigen. Bound antibodies were confirmed to be of a specific isotype by flowing mouse anti-human isotypes (IgG, IgM, or IgA) at 5 μL/min for 4 min. Mouse anti-human IgG, and mouse anti-human IgM were first diluted to a concentration of 0.1 mg/mL in HBS-EP buffer. Mouse anti-human IgA was diluted to 0.2 mg/mL in HBS-EP buffer. The surface was regenerated between cycles by flowing a solution of 50 mM HCl and 5% P20 over the biosensor surface at a rate of 5 μL/min for 1 min. Sample antibodies specific to rHuEPO were confirmed to be of a particular isotype by demonstrating at least 100 RU of binding to the rHuEPO surface and an additional 100 RU of anti-human isotype confirmatory binding.

3.5. Anti-rHuEPO NAb assay: sample and control preparation

The anti-rHuEPO NAb assay (NAb assay) controls included the background control (N) that only comprised cells, and a maximum proliferation control (M) comprised of cells and 1 ng/mL rHuEPO. Control (P) was comprised of cells, 1 ng/mL rHuEPO and 500 ng/mL neutralizing positive control antibody. Except for control (N) which contained no rHuEPO, all the wells contained 5% human serum, 15% rat serum and 1 ng/mL rHuEPO.

Sample and control reaction mixtures were prepared in a deep well microtiter plate. Control (N) was prepared by combining 40 μL PNHS, 240 μL growth medium and 120 μL PNRS. Control (M) was a combination of 40 μL PNHS, 120 μL rHuEPO at 6.67 ng/mL, 120 μL growth medium and 120 μL PNRS. Control (P) was a combination of 80 μL of Condition A generated from the 50% positive control serum, 120 μL rHuEPO at 6.67 ng/mL, 80 μL growth medium and 120 μL PNRS. Reaction mixtures utilizing Condition B and C filtrates for the 50% positive control serum and Conditions A, B and C filtrates for the 50% sample were prepared in the same manner as control (P). The deep well microtiter plate containing the 400 μL reaction mixtures was shaken for 30 min at 25 °C before being used in the cell proliferation assay. To maintain continuity with the original method presented by Wei et al. [1], the plate-specific cut point representing 50% of the rHuEPO-induced response termed rHuEPO Emax/2 was used. This cut point was derived by dividing the sum of the mean counts per minute (CPM) obtained with control (N) and control (M) by 2.

3.6. mIL-3 specificity assay: sample and control preparation

The mIL-3 specificity assay (specificity assay) controls included the background control (N) comprised of cells only, and a maximum proliferation control (E) consisting of cells and 30 pg/ml mIL-3. The preparation of control (N) was identical as described above in the NAb assay. The specificity assay control (E) and samples were prepared in a similar manner as control (M) and samples in the NAb assay, respectively, except that 120 μL of mIL-3 at 200 pg/ml was used instead of rHuEPO at 1 ng/mL. The deep well microtiter plate containing the 400 μL reaction mixtures was shaken for 30 min at 25 °C before being used in the cell proliferation assay. To maintain continuity with the original method presented by Wei et al. [1], the plate-specific cut point representing 50% of the mIL-3 induced response termed mIL-3 Emax/2 was used. This cut point was derived by dividing the sum of the mean CPM obtained with control (N) and control (E) by 2.

3.7. Cell proliferation assay

Following the procedure described by Wei et al. [1], the 32D-EPOR cells were staged by depriving the cells of rHuEPO for 16–24 h prior to the assay. Cell staging was initiated by transferring the cells into a polypropylene tube and centrifuging at 200–300 × *g*. The supernatant was discarded and the cell pellet was resuspended in growth medium and centrifuged. The final cell pellet was resuspended in growth medium lacking rHuEPO and the cell density was adjusted to 5×10^5 cells/ml. Cells were cultured by incubating at 37 °C, 5% CO_2, and 95% RH for 16–24 h. After 16–24 h, the cells were transferred into a polypropylene tube, centrifuged and resuspended in growth medium and counted.

Twenty-thousand staged 32D-EPOR cells in 100 μL volume were incubated with 100 μL of control or sample reaction mixtures in a U-bottom, 96-well assay plate for 44 ± 1 h at 37 °C, 5% CO_2, and 95% RH. Controls and samples were tested in triplicate. At the end of the incubation, 2 μCi [Methyl-^{3}H] thymidine diluted in 50 μL growth medium were added to each experimental well and the plate was incubated for an additional 4 ± 1 h in the incubator. The contents of the plate were harvested onto a filter plate using a plate harvester (Packard Instruments, Downers Grove, IL). The filter plate was air-dried and 25 μL of scintillation fluid was added to each well before counting on a beta particle counter (TopCount, Packard Instruments, Downers Grove, IL).

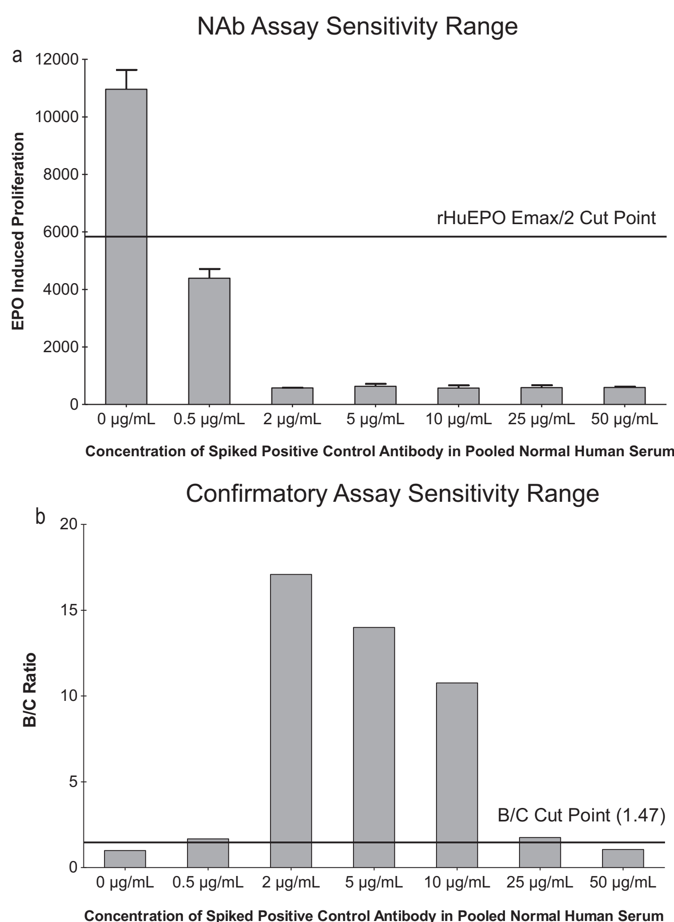

Fig. 6. PNHS was spiked with neutralizing positive control antibody and tested in the NAb assay. Within the antibody concentration range of 0.5–50 μg/mL (a), the assay was able to detect the presence of neutralizing activity (below rHuEPO Emax/2 cut point). The same samples in (a) were also tested in the confirmatory assay and the B/C ratios that were generated indicated a confirmatory assay sensitivity range of 0.5–25 μg/mL (b).

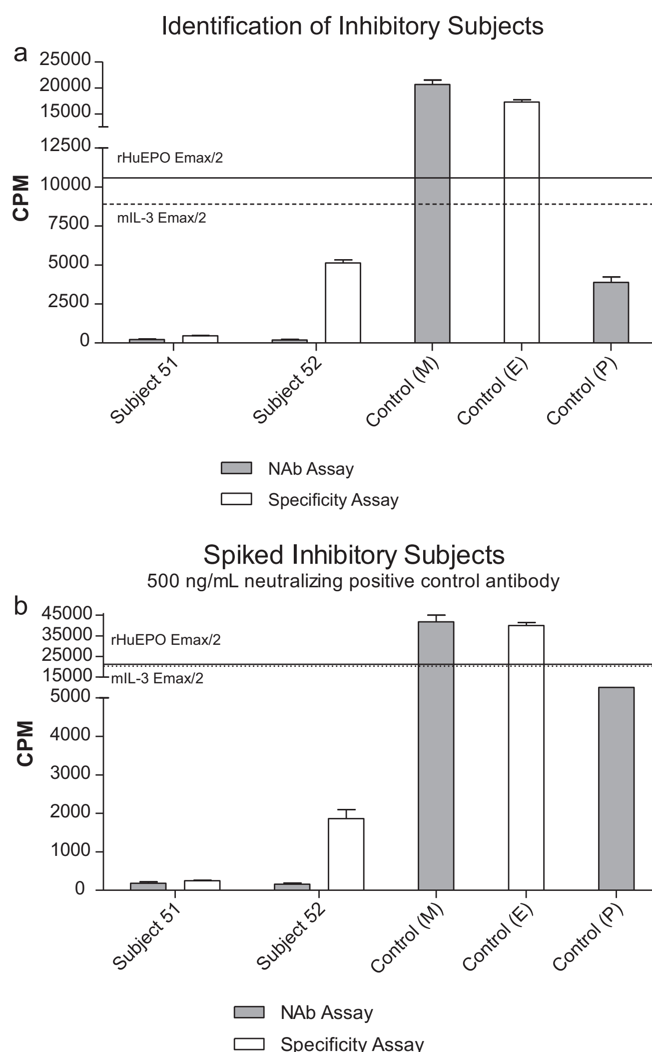

Fig. 7. Control (M) is a maximum proliferation control for the NAb assay and consists of 32D-EPOR cells grown in the presence of 1 ng/mL rHuEPO. Control (E) is a maximum proliferation control for the specificity assay and consists of 32D-EPOR cells grown in the presence of 30 pg/mL of mIL-3. Control (P) is a neutralizing positive control for the NAb assay and consists of 32D-EPOR cells in the presence of 1 ng/mL rHuEPO and 500 ng/mL of neutralizing positive control antibody. rHuEPO Emax/2 is the cut point for the NAb assay; mIL-3 Emax/2 is the cut point for the specificity assay. Sera from two subjects (subjects 51 and 52) found to be inhibitory in the NAb and specificity assays (a) was retested spiked with 500 ng/mL of neutralizing positive control antibody (b). The inhibitory sera from subjects 51 and 52 continued to interfere with the NAb and specificity assays preventing the detection of the spiked neutralizing positive control antibody.

3.8. Statistical analysis

The assay value of interest was defined as the B/C ratio which is equal to the NAb assay CPM for Condition B divided by the NAb assay CPM for Condition C. As the statistical methods used to develop the B/C ratio cut point rely on the assumption that the underlying population under consideration is Gaussian, or the assumption of "asymptotic normality", the evaluation for normality is valuable. If the distribution of the B/C ratio values appeared to be non-normally distributed, the Box–Cox procedure was used to estimate an appropriate transformation to normality.

The estimate of the baseline mean for the population of interest was taken as the LS-mean (least squares mean) of the B/C ratio values. The lower limit on the range of expected values for the population was determined by calculating the lower bound of a one-sided 95% prediction interval for the distribution of the B/C ratio values. The form of the equation utilized was:

$$\text{L95} = \text{LS-mean} - \text{TINV}(0.95, n-1) \times \text{SQRT}\left(\text{variance} \times \left(1 + \frac{1}{n}\right)\right).$$

In the above equation, 'L95' stands for lower 95% prediction limit; "LS-mean" is the LS-mean estimate of the B/C ratio values, "Variance" is the variance estimate of the B/C ratio values and 'n' is the number of the donors. 'TINV' returns the 95th quantile of a Student t-distribution, and 'SQRT' is an abbreviation for square root. If a transformation was utilized, the estimated. LS-mean, and the lower bound were back-transformed prior to reporting. In a similar manner, the lower bound of the 99% prediction interval ("L99") was calculated.

4. Results

4.1. Depletion of rHuEPO binding antibodies from human serum

The ability of Protein G and L to deplete different isotypes of anti-rHuEPO antibodies present in human serum was evaluated using the biosensor immunoassay. Individual sera from 23 subjects that had previously tested positive for anti-rHuEPO antibodies were identified. All 23 subjects had tested positive for binding antibodies that did not neutralize the biological activity of rHuEPO in the NAb assay. In this pool of 23 subjects, 8 subjects contained binding antibodies that were predominantly of the IgG isotype, 14 subjects

Table 2
Control (P) was subjected to antibody depletion by Protein G/L and tested on 2–4 plates every day for 5 days. [Emax/2]/P represents the cut point for the NAb assay and B/C ratio represents the cut point for the confirmatory assay. SD, standard deviation. %CV, %coefficient of variation [(SD/Mean) × 100].

		Intraday repeatability					Interday repeatability
		Assay day 1	Assay day 2	Assay day 3	Assay day 4	Assay day 5	All assay days
[Emax/2]/P	Mean	2.50	2.75	2.32	2.56	2.79	2.58
	SD	0.18	0.14	0.09	0.49	0.13	0.19
	%CV	7.23	5.09	4.07	19.16	4.59	7.46
B/C Ratio	Mean	2.49	2.76	2.58	2.83	2.51	2.64
	SD	0.32	0.23	0.82	0.35	0.55	0.15
	%CV	12.85	8.37	31.96	12.46	21.87	5.83
n (plates)		4	2	2	2	3	13

contained binding antibodies that were predominantly of the IgM isotype and one subject contained binding antibodies that were predominantly of the IgA isotype. The serum for all 23 subjects underwent antibody depletion (Fig. 1). The filtrate products (Conditions A, B and C) from the antibody depletion of each subject were tested in the biosensor immunoassay to assess the remaining RAC (Fig. 2). As expected, for each subject, the Condition A filtrates contained detectable levels of binding antibodies (IgG, IgM or IgA), with concentrations ranging from 0.24 to 2.59 μg/mL. Condition B filtrates did not show any detectable binding antibodies for all 8 subjects that had binding antibodies that were predominantly of the IgG isotype thereby confirming that the resin was highly efficient for depleting IgGs. For the remaining subjects, antibody depletion of IgA and IgM was successful to varying degrees. In the single subject containing the predominate IgA isotype, 70% of the binding antibodies were removed. In the subjects containing the predominate IgM isotype, the effectiveness of binding antibody removal varied from 100% to as low as 20% in one sample. The Sepharose 6B resin used for Condition C was expected not to have specific affinity toward immunoglobulin therefore it served as a negative control (depletion background control) for Condition B. As expected, Condition C filtrates had detectable levels of binding antibodies similar to those found in all Condition A filtrates regardless of isotype. Some non-specific binding was observed in the Condition C filtrates but not to a similar degree as specific binding observed with the Condition B filtrates.

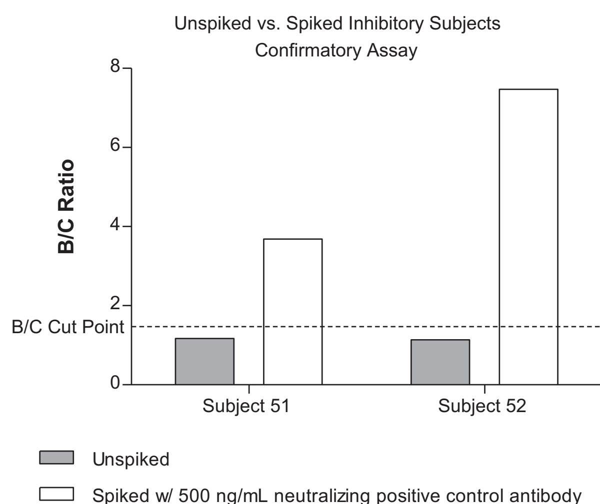

Fig. 8. By applying the statistically derived B/C cut point value of 1.47, the unspiked inhibitory subjects are correctly identified as negative for NAbs while the spiked inhibitory subjects are correctly identified as positive for such antibodies.

4.2. Determination of confirmatory assay cut points

Individual sera from 50 healthy human donors (25 male and 25 female) were spiked with 500 ng/mL of neutralizing positive control antibody (the assay's lower limit of reliable detection). Positive control serum and each spiked individual donor serum underwent antibody depletion. The filtrate products (Conditions A, B and C) from the antibody depletion were tested in the NAb assay.

The rHuEPO Emax/2 cut point used to identify rHuEPO inhibitory activity in serum samples has been described previously [1]; this cut point was applied to Condition A. In all 50 spiked donors tested, the counts per minute (CPM) for Condition A of each spiked donor were below the rHuEPO Emax/2 cut point, correctly indicating the presence of neutralizing activity against rHuEPO (Fig. 3).

For each of the 50 spiked donors, a B/C ratio was generated by dividing the Condition B value by its corresponding Condition C value. This ratio was derived to account for any non-specific binding of the sample to the Sepharose 6B resin. The distribution histogram and probability plot for the untransformed data are provided (Fig. 4a and b). Based on the results of the Box–Cox analysis (results not shown), a log transformation was applied to the B/C ratio values (Fig. 4c and d). On a log transformed scale, the distributions remained skewed due to the B/C ratio value from one spiked donor (Donor 21). The probability plot indicated that Donor 21 yielded an outlier value since its difference from the mean was more than three standard deviations. After removal of this outlier, the distribution histogram of the remaining transformed B/C ratio values appeared normally distributed, and the probability plot showed good agreement with the reference line (Fig. 4e and f). A statistically derived cut point based on the B/C ratio was calculated from the remaining 49 spiked donors. Lower bounds of the one-sided 95% and 99% prediction intervals were calculated. To minimize the number of samples positive for NAbs that would be reported as negative, a conservative B/C cut point corresponding to the 99% prediction limit was chosen (Table 1). Any sample with a Condition A value below the rHuEPO Emax/2 cut point and a B/C ratio value greater than the B/C cut point of 1.47 would be considered confirmed to contain NAbs. All 50 spiked samples were found to have Condition A values below the corresponding rHuEPO Emax/2 cut point (Fig. 3) and B/C ratio values above the B/C cut point of 1.47 (Fig. 5) thereby confirming the presence of NAbs (no false negatives).

4.3. Confirmatory assay range of NAb detection

To determine the assay sensitivity of the confirmatory assay (antibody depletion followed by NAb assay), mock positive samples were prepared by spiking PNHS with 0.5 μg/mL, 2 μg/mL, 5 μg/mL, 10 μg/mL, 25 μg/mL and 50 μg/mL of neutralizing positive control antibody. The lowest concentration represented the assay's sensitivity. These mock samples, along with a sample of

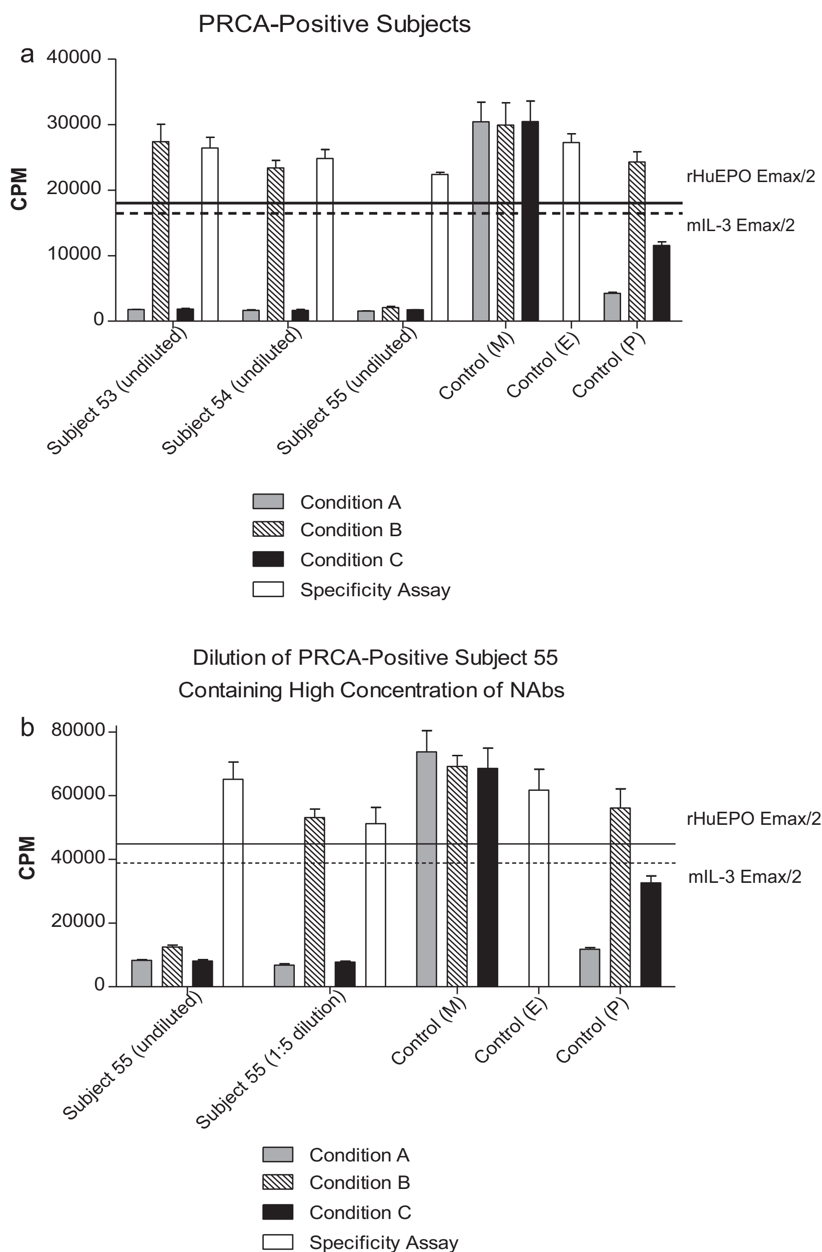

Fig. 9. Following antibody depletion, the sera from three PRCA-positive human subjects (subjects 53, 54 and 55) were tested in the NAb and specificity assay (a). The serum from subject 55 was diluted 1:5 in PNHS, antibody depleted and retested in the NAb and specificity assays (b). rHuEPO Emax/2 and mIL-3 Emax/2 are the cut points for the NAb and specificity assays, respectively. Controls (M), (E) and (P) are assay suitability controls.

PNHS not spiked with neutralizing positive control antibody, were tested in the confirmatory assay. When the rHuEPO Emax/2 cut point was applied to Condition A, inhibition of rHuEPO-induced proliferation was detected at all neutralizing positive control antibody concentrations equal to and greater than 0.5 μg/mL (Fig. 6a). However, when the B/C ratio cut point (1.47) was applied, NAbs were only confirmed within the neutralizing positive control antibody concentration range of 0.5–25 μg/mL (Fig. 6b). The 0.5 μg/mL and 25 μg/mL mock positive samples were barely above the rHuEPO Emax/2 cut point and reveal the lower- and upper-bound confirmatory assay detection limits, respectively. Antibody concentrations below the lower-bound limit and above the upper-bound limit are not detectable due to NAb assay sensitivity and incomplete depletion by the Protein G/L resin, respectively.

4.4. Characterization of repeatability and reproducibility of the confirmatory assay

Assay repeatability was assessed on the NAb assay component and antibody depletion component of the confirmatory assay. Thirteen assay plates, each containing Control (P), were tested in the confirmatory assay over the span of five assay days. The mean, standard deviation and the coefficient of variation (%CV) were calculated for Control (P) in the normalized forms of [Emax/2]/P and the B/C ratio for the NAb assay component and the antibody depletion component, respectively (Table 2). The intraday and interday variation (%CV's) of [Emax/2]/P was less than 20% and 8%, respectively. The intraday and interday variation (%CV's) of the B/C ratio was less than 32% and 6%, respectively.

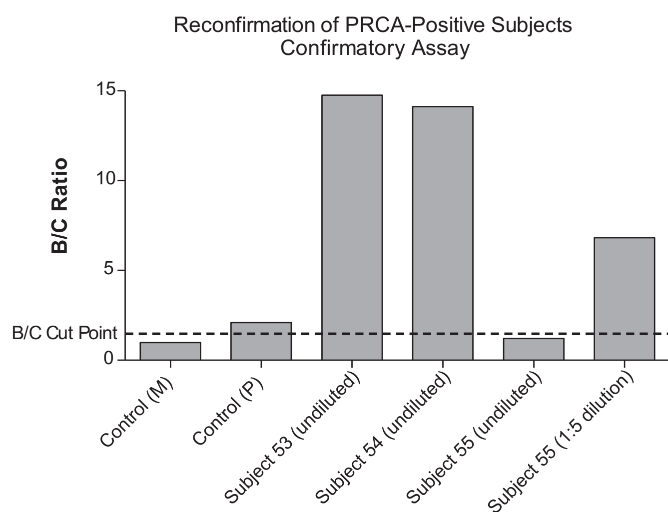

Fig. 10. The results from controls (M) and (P) and subjects 53, 54 and 55 were re-expressed as B/C ratios (confirmatory assay values). Control (P) was detected and all three PRCA-positive human subjects reconfirmed as positive for NAb.

4.5. Detection of NAb in serum containing factors that inhibit cell proliferation

To test the use of the B/C cut point to confirm the presence of NAb, we sought to detect NAbs in the presence of non-antibody inhibitory serum factors. Two rHuEPO-naïve human subjects (subjects 51 and 52) were identified as exhibiting strong inhibition of both rHuEPO and mIL-3-induced cell proliferation of the 32D-EPOR cell line (Fig. 7a). Both subjects were subsequently spiked with 500 ng/mL of neutralizing positive control antibody, followed by testing in the NAb and specificity assays. Using only the Emax/2 cut points for NAb and specificity assays, the sera spiked with NAbs still had signals well below the corresponding Emax/2 cut point for both the NAb and specificity assays (Fig. 7b). If no further testing were to be done, such samples would be erroneously reported negative for NAbs. After applying the confirmatory assay strategy and the B/C cut point criteria, both inhibitory sera spiked with neutralizing positive control antibody were correctly identified as positive for NAbs while the unspiked inhibitory sera were confirmed as negative for such antibodies (Fig. 8).

4.6. Confirming the presence of NAbs in clinically relevant serum samples

To test the use of the B/C cut point to confirm the presence of NAbs in clinically relevant samples, sera from three previously identified PRCA-positive subjects [6] were tested. Biosensor immunoassay RAC results placed the NAb concentrations for subjects 53, 54 and 55 at 7.00 μg/mL, 10.03 μg/mL and 27.56 μg/mL, respectively (data not shown). Serum from these subjects was tested in the confirmatory assay and specificity assay. As expected, subjects 53, 54 and 55 were found to have specific anti-rHuEPO neutralizing capacity but only subjects 53 and 54 confirmed positive for NAb (Fig. 9a). Due to an unusually high RAC (27.56 μg/mL) detected earlier using the biosensor immunoassay, Subject 55 required a 1:5 dilution in PNHS to be successfully antibody depleted (Fig. 9b). Once the B/C ratios were calculated, all 3 PRCA-positive subjects were reconfirmed to contain NAbs (Fig. 10).

5. Discussion

The functional cell-based assay is the most biologically relevant method for assessing the presence of neutralizing antibodies in human serum samples [13]. However, cell-based assays, particularly those that rely on cell proliferation, can be confounded by the presence of non-antibody inhibitory serum factors (e.g., cytotoxic factors) leading to false negatives. Given the importance of adverse-event reporting, an alternative method to detect neutralizing antibodies that is minimally impacted by the presence of non-antibody inhibitory serum factors, would be useful. In December 2009, a draft guidance document released by the FDA recommended that antibody depletion assays should be considered for confirming whether neutralizing activity is truly due to antibodies [15]. We developed a confirmatory assay that uses a resin mixture of Protein G Sepharose and Protein L Sepharose to deplete most immunoglobulins from a human serum sample prior to testing in an assay designed to detect the inhibition of rHuEPO-induced cell proliferation.

In the initial proof of principle experiments using the biosensor immunoassay (Fig. 2), incomplete depletion of IgM and IgA binding antibodies (Condition B) was observed for some of the subjects and could be attributable to the inherent inability of the Protein G Sepharose component in the Protein G/L resin to bind to those particular antibody isotypes. Despite the lack of complete IgM and IgA binding, we concluded that the Protein G/L resin was most suitable for antibody depletion due to its additional capacity to deplete some IgM and IgA compared to Protein G Sepharose alone. The possibility of the occurrence of clinically relevant levels of IgM and IgA rHuEPO NAb is extremely low; however, it was important to determine that if such a scenario was encountered, the Protein G/L antibody depletion step would allow their detection. Since IgG is the most commonly occurring isotype during a secondary immune response, it was important to ensure its complete removal by the Protein G/L resin. Sepharose 6B resin was included as a negative control (depletion background control) for antibody depletion as this resin lacked any capacity to specifically bind immunoglobulins. If an inhibitory factor were to bind to the Sepharose 6B resin and be depleted from a sample, the binding would be defined as non-specific, and would likely sterically interfere with the biological activity of a therapeutic protein such as rHuEPO.

Using sera from 50 normal human donors spiked with 500 ng/mL of neutralizing positive control antibody, we statistically established a B/C cut point for the confirmatory assay. Minimal non-specific binding to the resin was observed however its impact was not significant: all non-specific binding was taken into account by normalizing the signal obtained after treatment with Protein G/L resin (Condition B) by the signal obtained after treatment with Sepharose 6B resin (Condition C). As described previously [1], the rHuEPO Emax/2 criteria remained suitable for detecting the inhibition of rHuEPO-induced cell proliferation by the positive control antibody and PRCA disease state samples. The statistically derived B/C cut point criteria helped determine whether that rHuEPO inhibitory activity was antibody mediated in the presence or absence of non-antibody inhibitory serum factors that can inhibit rHuEPO-induced cell proliferation. In all 50 samples tested, the B/C cutpoint of 1.47 correctly identified all NAb-spiked samples as positive. Despite the complex sample handling involved in the procedure, the assay showed good repeatability and reproducibility (Table 2).The sensitivity for this confirmatory assay was found to be in the range of 0.5 μg/mL to 25 μg/mL for the positive control antibody (Fig 6b). An antibody concentration beyond 25 μg/mL would likely be undetectable due to the inability of Protein G/L resin to completely remove all immunoglobulin content from a test sample. As an example, if, after treatment with Protein G/L, 1% of 50 μg/ml of anti-EPO neutralizing antibodies remains in the test sample, sufficient antibody will remain (0.5 μg/mL) to elicit detectable inhibition of rhuEPO-induced proliferation. In our experience most of the tested serum samples from antibody-mediated PRCA subjects have had RACs that tend to be lower than 25 μg/ml

(data not shown). However, concentrations higher than 25 μg/ml have been detected in some antibody-mediated PRCA subjects, like subject 55 described in this paper. The serum from subject 55 showed strong inhibitory activity that could not be sufficiently depleted and required pre-dilution for NAb detection using this strategy; this was not unexpected and agreed with the biosensor immunoassay RAC results (27.56 μg/mL). Knowledge of RAC results via the biosensor immunoassay prior to testing in this confirmatory assay can provide useful guidance regarding the need and extent of sample dilution.

One considerable advantage of the confirmatory assay is the ability to detect NAbs even in the presence of non-specific inhibitory serum factors, like cytotoxic factors. Serum from two inhibitory human subjects (subjects 51 and 52) was used to test the ability of the confirmatory assay to detect NAbs in the presence of non-antibody inhibitory serum factors. While the reason for the non-specific inhibition of 32D-EPOR proliferation in the presence of rHuEPO or mIL-3 is not clear, these inhibitory subjects represent a likely situation where non-antibody inhibitory serum factors may mask the detection of neutralizing antibodies (Figs. 7a and b, and 8).

We also wanted to demonstrate that this confirmatory assay approach was capable of confirming the presence of NAbs in previously confirmed cases of antibody-mediated PRCA. Serum samples from three subjects previously diagnosed with antibody-mediated PRCA were tested using this new confirmatory assay. The serum sample from one subject (subject 55) was found to contain a high concentration of NAb (27.56 μg/mL) and, therefore, needed to be diluted with PNHS to be effectively antibody depleted. Ultimately, all three antibody-mediated PRCA subjects were reconfirmed as positive for NAb (Fig. 10).

Neutralizing antibodies against therapeutic proteins can present serious safety issues if the therapeutic has an endogenous counterpart. In the case of recombinant human erythropoietin, the emergence of follow-on biologics has the potential of exacerbating rHuEPO-specific immunogenicity. As the market in certain regions for biosimilars has grown, so have the number of companies each developing and marketing rHuEPO produced under different proprietary manufacturing practices and different regulatory landscapes. Despite having similar therapeutic effects, these follow-on biologics may have slightly different biochemical qualities eliciting varying degrees of immunogenicity [14]. This places increasing importance on the accuracy, sensitivity and specificity of immunogenicity assays. The confirmatory assay presented here addresses the impact of non-antibody mediated inhibitory factors on the detection of rHuEPO NAbs where cell proliferation is used as an assay endpoint.

Conflict of interest

All the authors are employees and stockholders in Amgen Inc.

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