Development and Validation of a Quasi-Quantitative Bioassay for Neutralizing Antibodies Against CP-870,893

Daniel J. Baltrukonis, Deborah Finco-Kent, and Thomas T. Kawabata
Worldwide Safety Sciences, Pfizer Global Research and Development, Groton, Connecticut, USA

Martin Poirier and Lynne LeSauter
Preclinical Services Montreal Inc., Charles River Laboratories, Senneville, Quebec

The human monoclonal antibody CP-870,893 is a CD40 receptor agonist currently being developed for the treatment of cancer. A bioassay to measure neutralizing antibodies (Nab) to CP-870,893 in 5% human serum matrix was developed and validated utilizing the Daudi cell line and flow cytometric detection. Additionally, samples from CP-870,893 treated cynomolgus monkeys were analyzed in the bioassay and compared to results obtained using a competitive receptor-binding (CRB) Nab immunoassay to determine if the CRB assay may be used in place of the bioassay. Treatment of Daudi cells for 2 d with CP-870,893 leads to a concentration-dependent increase in CD54 surface cell expression. The presence of antidrug Nab attenuates CP-870,893 binding to CD40 and the induction of CD54. An anti-idiotypic monoclonal antibody (Mab) and a monkey sera pool were identified as positive controls for neutralization of CP-870,893. During development, it was observed that the assay robustness was altered by culture media and FBS substitutions. For validation the following parameters were established: cutpoint factors in the presence (0.779) and absence (1.282) of 50 ng/ml CP-870,893, linear region of the concentration-response (1–100 ng/ml CP-870,893), intra- and inter-assay precision (CV ≤ 25%), specificity and recovery (± 25%), sensitivity (~500 ng anti-idiotypic Mab per ml serum), technician to technician ruggedness (CV ≤ 25%), and stability (positive control, CD54 labeling, and cell line). A concentration dependent increase in CP-870,893 neutralization was observed in a 3-mo toxicity study in monkeys using both the Bioassay and CRB assay (R² = 0.94) suggesting the CRB Nab assay may be a suitable alternative to a bioassay. Based on the precision, specificity, sensitivity, and robustness, the validated bioassay is suitable for quasi-quantitative analysis of neutralizing anti-CP-870,893 antibodies in human serum.

Keywords assay validation, bioassay, Daudi cell line, neutralizing antibody, CP-870,893

INTRODUCTION

CP-870,893, a human monoclonal IgG2 antibody, is a CD40 receptor agonist currently under development for the treatment of cancer. CD40 is a member of the Tumor Necrosis Factor receptor superfamily, a key regulator of both cellular and humoral immune responses, and expressed on antigen presenting cells such as B-cells, dendritic cells, and monocytes. In vitro and nonclinical models, stimulation of CD40 has been shown to inhibit tumor cell growth and stimulate host antitumor responses (Hirano et al., 1999; Tong and Stone, 2003; Eliopoulos and Young, 2004). Clinically, partial responses and disease stabilization have been demonstrated in Phase I trials with recombinant human CD40 ligand (Vonderheide et al., 2001).

For the development of biological therapeutics, the potential impact of immunogenicity on safety and efficacy must be evaluated. Anti-drug responses could decrease efficacy and/or safety by altering pharmacokinetics and pharmacodynamics or mediating hypersensitivity reactions. Regulatory agencies expect validated immunogenicity assays be used to assess both the incidence and characteristics of antibody formation (U.S. Department of Health and Human Services, 1997a, 1997b, 2005, 2006).

The presence of antibodies that bind to drug in samples is typically assessed by immunoassay. Further characterization of these antibodies as neutralizing may be assessed using either a competitive receptor binding assay or functional cell based bioassay. In order to support Phase I and II Clinical Studies with CP-870,893, a validated bioassay to measure neutralizing anti-CP-870,893 antibodies was needed.

The bioassay developed uses Daudi cells that are treated with CP-870,893 for approximately 2 d in the presence of 5% human serum. Drug-induced increases in CD54 (ICAM-1) cell surface...
expression is detected by flow cytometry using FITC-conjugated anti-CD54 antibodies. The presence of neutralizing antibodies to CP-870,893 results in attenuation of the CP-870,893-induced expression of CD54 on the cell surface.

Described herein is the optimization and validation of the CP-870,893 bioassay. Cell line and receptor identification, flow cytometry setup and acquisition, the minimal required dilution (MRD), and identification of neutralizing positive control antibodies were assessed during optimization. For validation, the cutpoint, linear region of the concentration-response curve, intra- and inter-assay precision, specificity and recovery, sensitivity, ruggedness, and stability were assessed. Finally, plasma samples from a 3-mo toxicity study in cynomolgus monkeys treated with CP-870,893 were analyzed in the bioassay retrospectively and compared to results obtained using a CRB Nab immunoassay to determine if the CRB assay may be used in place of the bioassay.

MATERIALS AND METHODS

Materials

The following materials and reagents were utilized in these experiments: CP-870,893 human IgG2 Mab therapeutic and human IgG2 anti-KLH Mab (Pfizer, Inc), mouse anti-idiotype Mabs (Green Mountain Antibodies: Burlington, VT), FITC-conjugated mouse IgG1 anti-CD54 and FACSFlow (BD Biosciences: San Jose, CA), FITC-conjugated mouse IgG1 isotype control (Caltag: Burlingame, CA), Daudi cell-line and RPMI-1640 media (ATCC: Manassas, V A), sodium azide (Sigma-Aldrich: St. Louis, MO), and normal human serum (Bioreclamation: East Meadow, NY and Rockland: Rockland, ME).

Cell Culture

Daudi cells, a human B-lymphoblastoid line derived from a Burkitt lymphoma, were cultured in 90% RPMI-1640 and 10% heat-inactivated fetal bovine serum (HyClone: Logan, UT and Medicorp, Inc: Montreal, Quebec), propidium iodide and PBS without magnesium and potassium chloride (Invitrogen: Carlsbad, CA and Sigma-Aldrich: St. Louis, MO), sodium azide (Sigma-Aldrich: St. Louis, MO), and normal human serum (Bioreclamation: East Meadow, NY and Rockland: Rockland, ME).

Nab Bioassay

The anti-KLH monoclonal antibody (Mab) was the negative control for CP-870,893 and was used at the highest CP-870,893 concentration used. As a positive control (PC), serum from monkeys immunized with CP-870,893 was used (referred to as PAHA, primate anti-human antibody). Monoclonal antibodies against the Fab fragments (anti-ID) were generated and also used as a PC. Validation was performed with 5% normal human serum (NHS). For nonclinical sample analysis, 10% normal cynomolgus plasma (NCP) was used. All labeling and washes were performed in ice-cold FACS buffer (2% FBS and 0.025% NaN3 in PBS).

Serum samples were combined with CP-870,893 (50 ng/ml) and PC in sterile culture tubes (12 × 75 mm). The tubes were then seeded with Daudi cells (1 × 10^5 cells) to a final volume of 0.5 ml and incubated for approximately 44 hr at 37°C, 5% CO2 with orbital shaking at approximately 60 RPM. CP-870,893 standard curves ranging from 0 to 100 ng/ml were included in all experiments.

After the incubation period, cells were isolated by centrifugation for 5 min at 170 × g at 4°C. Cells were labeled by resuspension in 125 µl of anti-CD54-FITC or anti-Isotype-FITC (1.6 µg/ml) for 30 min on ice, followed by 2 washes with 1.0 ml ice cold FACS buffer. Samples were resuspended in 350 µl of FACS buffer with or without propidium iodide (10 µg/ml), stored on ice, and acquired by flow cytometry within 3 hr.

The acquisition of samples was performed on either a FACSort or FACSCalibur flow cytometer using CellQuest software version 4.0.2 (BD Biosciences). The population corresponding to CD54 positive live Daudi cells was gated and analyzed for geometric mean fluorescence. The fold-change in CD54 expression was calculated by dividing the geometric mean fluorescence of the sample by the average geometric mean fluorescence of the anti-KLH negative control. Data was tabulated using EXCEL (Microsoft); concentration-response curves and back-calculated values were obtained using SOFTmax PRO software versions 4.0 and 4.7.1 (Molecular Devices Corporation).

OPTIMIZATION

Assay Parameters

Cell surface expression of CD80, CD23, CD54, and CD86 was examined in three B-lymphoblast cell lines (HS-Sultan, Raji, and Daudi) by seeding 200,000 cells/ml and treating with 1,000 ng/ml CP-870,893 for 24 hr.

Drug-induced expression of CD54 in the Daudi cell line was evaluated from 1 to 300 ng/ml CP-870,893 in 10% normal human serum after incubation for 1, 2, or 3 d. Samples seeded with 100,000 or 200,000 cells/sample were compared after incubation for 2 d.

Flow Acquisition Parameters

CP-870,893 concentration dependent increases in CD54 expression were examined under various gating conditions: all cells, live/dead cells, and isotype positive/negative. The minimum number of gated events acquired per sample was evaluated from 2,000 to 10,000. Optimal labeling for CD54 was explored by comparing samples labeled with 2 to 60 µl of anti-CD54 FITC.

Minimal Required Dilution

Daudi cells were treated with 0–100 ng/ml CP-870,893 in the presence of 0, 2, 5, 10, 20, and 40% NHS or 10% NCP pool for 2 d.
Positive Control Identification

Five anti-ID monoclonal antibodies and a pooled cynomolgus monkey sera sample (PAHA) positive for CP-870,893 neutralizing antibodies were examined. Daudi cells were treated with 50 ng/ml CP-870,893 plus anti-Id (50 ng/ml) or PAHA (1/100, 1/1000) for 48 hr in the presence of 10% human serum.

VALIDATION

Cutpoints

Fifty-three individual NHS samples tested on three separate days were used to calculate cutpoint factors in the presence of 50 ng/ml CP-870,893. The cutpoint factor was calculated as follows:

\[
\text{Cutpoint factor}_{(\text{Nabs})} = \frac{\text{mean fold-change} - (1.645 \times \text{Std dev})}{\text{mean fold-change}}
\]

This factor was applied to each assay to calculate the cutpoint for each analytical run and samples less than the Cutpoint_{(\text{Nabs})} were considered positive for neutralizing antibodies.

In addition to establishing a cutpoint for Nabs, a cutpoint factor to confirm the presence or absence of drug in the sample was established. Fifty-three individual NHS samples were used to calculate a cutpoint factor in the absence of CP-870,893. The cutpoint factor was calculated as follows:

\[
\text{Cutpoint factor}_{(\text{drug})} = \frac{\text{mean fold-change} + (1.645 \times \text{Std dev})}{\text{mean fold-change}}
\]

Samples less than the Cutpoint_{(\text{drug})} were considered negative for CP-870,893.

Based on the analyses of these data, a negative control pool of serum was prepared by combining equal volumes of selected NHS samples. This serum pool was used as a NHS negative control and sample diluent.

Concentration Response Curves

The concentration response curves for both CP-870,893 and the anti-Cp-870,893 PCs were evaluated. For the CP-870,893 concentration-response curve, 1 to 100 ng/ml CP-870,893 was tested by diluting in serum at the MRD. For neutralizing activity, increasing concentrations/dilutions of the anti-Id and PAHA PCs were tested in the presence of 50 ng/ml CP-870,893.

Precision

Intra- and inter-assay precision were evaluated for the CP-870,893 standard curve (0, 1, 3, 7.5, 15, 25, 50, and 100 ng/ml), PAHA PC (1/800, 1/400, 1/200 dilutions), and anti-ID PC (141 and 242 ng/ml). Intra-assay precision was assessed by comparing 5 replicates per experiment (three independent experiments) and inter-assay precision was examined across 12 independent experiments. For the standard curve and positive control, precision was reported as percent coefficient of variation of the fold-change in CD54 and of the concentration of CP-870,893 neutralized per ml of serum, respectively.

Specificity and Recovery

Ten individual lots of NHS were used to determine assay specificity and recovery. Specificity was determined using drug, drug plus anti-Id, and drug plus anti-KLH Mab. For recovery, the amount of drug neutralized per ml of NHS versus cell culture media was compared.

Sensitivity

Assay sensitivity was defined as the lowest concentration of the anti-ID PC to produce a neutralization response below the cutpoint. The anti-ID PC was tested at concentrations ranging from 1.25 to 100 ng/ml spiked into NHS at the MRD. The relative sensitivity of the assay was determined by adjusting for the MRD (relative sensitivity = anti-ID PC concentration/MRD) and reported as ng anti-ID per ml of serum.

Robustness

Assay robustness in terms of technician-to-technician variability was evaluated by comparing the amount of drug neutralized by the PAHA PC (1/400).

Stability

Serum sample (freeze/thaw, room temperature, and 4°C) and cell line stabilities were assessed by evaluating the amount of drug neutralized by the PAHA PC spiked into 5% NHS. For immunostaining stability, neutralization was assessed using the anti-ID PC.

PRECLINICAL STUDY

Animal Care and Use

Cynomolgus monkeys (Charles River Primates, BRF: Houston, TX) were pair housed in the following environmental conditions: relative humidity of 50 ± 10%, temperature of 21.1 ± 2.8°F, and a 12-hr light/dark cycle. Certified Primate Diet 5K91 (PMI Nutrition International: St. Louis, MO) supplemented with fruit and vegetables was provided once daily and municipal drinking water, purified by reverse osmosis, was provided ad libitum. The animal care and experimental procedures of this study were conducted in compliance with the U.S. Animal Welfare Act and the ILAR Guide (1996).

3-Month Toxicity Study

CP-870,893 was administered to cynomolgus monkeys (5/sex/dose) by bolus intravenous injection every other day for 3 mo at doses of 0.03, 0.1, and 1.0 mg/kg. To assess reversibility, a subset of animals (2/sex/dose) was monitored for an additional 1-mo recovery period. From each animal, blood was collected...
into 2 ml sodium heparin vacutainer tubes prior to randomization and on Study Days 23, 29, 43, 57, 71, and 93.

**Neutralizing CRB Assay**

A subset of samples with CP-870,893 levels below the LLOQ were tested for neutralizing activity in a Nab competitive receptor binding assay and bioassay. For the CRB assay, ELISA 96-well plates were coated with CD40 human IgG fusion protein in carbonate/bicarbonate buffer. Plasma samples from monkeys were added along with CP-870,893. Drug binding to CD40 was detected using a mouse anti-human-IgG-Biotin, streptavidin-HRP, and TMB substrate. Anti-ID ID21.4B 4D10F9 was used as a positive control. Neutralizing antibodies to CP-870,893 would prevent or decrease binding of the drug to CD40 resulting in decrease of optical density (OD) when compared to plasma from naïve monkeys. Plasma samples from 43 naïve monkeys were used to calculate the cutpoint (mean OD – [2 × Std dev]). This cutpoint determination was made prior to the recommendations by Mire-Sluis et al. (2004) and utilized 2 rather than 1.645 standard deviations. Data was reported as a percentage relative to the signal obtained for NCP controls.

**RESULTS**

**Optimization**

**Flow Acquisition Parameters.** Flow cytometric gating for CD54 positive live cells while excluding isotype positive cells resulted in a significant increase in signal (Figures 1 and 2). Under these parameters, an approximate 2- to 9-fold linear increase in CD54 expression was observed between 10 to 100 ng/ml CP-870,893, respectively. No difference in CD54 expression was observed when comparing the total gated events collected or concentration of anti-CD54 FITC reagent used per sample (data not shown).

**Assay Parameters.** Daudi CD54 cell surface expression showed the greatest fold increase of all receptors and cell lines examined after treatment with CP-870,893 for 24 hr (data not shown). Maximum CD54 expression occurred after incubation for 2 d (Figure 3A) using a seeding density of 100,000 cells/sample (data not shown).

**Minimal Required Dilution.** The addition of up to 10% NHS from a single donor did not attenuate the CP-870,893 concentration-response (Figure 3B). For validation studies, 5% NHS was defined as the MRD. Results obtained with a 10% NCP pool were similar to those with the 10% NHS (Figure 3C). Analysis of monkey samples was performed with 10% NCP as the MRD.

**Positive Control Identification.** The anti-idiotypic monoclonal antibody ID21.4B 4D10.F9 demonstrated the greatest neutralization of CP-870,893 after incubation for 2 d with 10% NHS. The PAHA PC at dilutions of 1:1000 and 1:100 also demonstrated strong neutralization of CP-870,893 after incubation for 2 d in the presence of 10% NHS (data not shown).
NEUTRALIZATION BIOASSAY FOR ANTI-CP-870,893 ANTIBODIES

VALIDATION

Cutpoint. For the establishment of cutpoint factors for neutralizing antibodies and the presence of drug, 53 NHS (5%) samples were tested with and without drug. The cutpoint factors were established at 0.779 and 1.282, respectively (Figure 4A and B).

Concentration-Response Curves. Sigmoidal concentration-response curves were observed for CP-870,893, anti-ID PC, and PAHA PC (Figure 5A-C). Standard concentrations for CP-870,893 ranging from 1 to 100 ng/ml were selected based on back-calculated concentrations using a 4-parameter fit curve.

Precision. All standards for the CP-870,893 concentration-response curve met the intra- and inter-assay acceptance criteria (CV ≤ 25%) with the exception at 3 ng/ml (inter-assay, CV = 28%) and on one occasion at 15 ng/ml (intra-assay, CV = 25.8%). Intra and inter-assay precision results obtained with
FIG. 5. Representative concentration-response curves for CP-870,893 (A), anti-ID positive control (B), and PAHA positive control (C). Concentrations of anti-ID and dilutions of PAHA were incubated in the presence 5% normal human serum and 50 ng/ml CP-870,893 for 2 d and the fold-change in CD54 expression was determined. The neutralizing activity of the anti-ID and PAHA positive controls was linear from approximately 1–30 ng/ml of detected CP-870,893.

the anti-ID and PAHA PCs were within the acceptance criteria (CV ≤ 25%) for the concentrations and dilutions tested (Table 1).

**Specificity and Recovery.** To determine the assay specificity, 10 lots of NHS were tested in the presence of drug with and without anti-KLH Mab. For all 10 lots, both conditions resulted in fold-changes above the assay cutpoint, demonstrating good specificity. Incubation of drug in the presence of anti-ID PC resulted in fold-changes below the assay cutpoint for all 10 lots (data not shown).

For recovery, all 10 lots of human serum spiked with 50 ng/ml CP-870,893 gave a recovery within 8% when compared to a similar sample in cell culture media spiked with 50 ng/ml CP-870,893 (data not shown).

**Sensitivity.** The lowest concentration of anti-ID to produce a mean fold-change below the cutpoint was 25 ng/ml. Adjusting for the assay MRD (5%), the relative sensitivity of the assay is approximately 500 ng of anti-ID per ml of serum (Figure 6).

**Robustness.** In independent experiments performed by two analysts, the amount of drug neutralized per ml of serum by the PAHA PC differed by 2.6% (33,817.9 ± 403 vs. 34,721.9 ± 411 ng/ml).

**Stability.** The PAHA PC diluted (1/800 and 1/200) in NHS was stable up to 24 hr at room temperature (CV = 0.8 and 24.8%), 24 hr on wet ice (CV = 1.2 and 0.1%), and after five freeze-thaw cycles at approximately −80°C (CV = 0.2 and 4.3%) when compared to freshly prepared PC (data not shown).

Daudi cell line stability was assessed by comparing the amount of drug neutralized by the PAHA PC at passages 15 and 87. Between these passages a difference of 2.0, 0.2, and 0.1% was observed at PAHA PC dilutions of 1/800, 1/400, and 1/200, respectively. The Daudi cell line is stable up to 87 passages in this bioassay (data not shown).

Immunostaining stability was assessed at 3 and 6 hr post-labeling for CD54 by comparing results obtained with the anti-ID PC. Treatment with 30.25, 121, and 242 ng/ml anti-ID

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<th>Treatment</th>
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<td>% CV range</td>
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<td>CP-870,893 (ng/mL)</td>
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<td>3.3–9.3</td>
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<td>Anti-ID (ng/mL)</td>
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<td>141</td>
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<td>242</td>
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<td>PAHA (1/x)</td>
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obtained with the bioassay (R2 increase in CP-870,893 neutralization by the CRB assay correlated to results percent control reflects an increase in drug neutralization. A dose-dependent analysis of all individual samples analyzed by both the serum dilution, the relative sensitivity of the assay was approximately 500 ng anti-ID per ml of serum.

resulted in a difference of 1.0, 1.5, and 1.7% (3 hr) and 2.9, 3.5, and 3.8% (6 hr), respectively (data not shown). Samples are stable on ice for up to 6 hr post-CD54 staining.

PRECLINICAL STUDY
Using 51 NCP samples, a cutpoint factor for neutralizing antibodies using 50 ng/ml CP-870,893 in 10% plasma was established at 0.882 (data not shown). Retrospective analysis of samples from a 3-mo study in monkey showed a dose-dependent increase in CP-870,893 neutralization (data not shown). A comparison of results of all individual samples analyzed by both the bioassay and CRB assay showed a strong correlation between the two assays (Spearman Rank Order Correlation: R² = 0.94, p < 0.005) (Figure 7).

DISCUSSION AND CONCLUSIONS
Currently there are no formal regulatory guidelines for the design, development, and validation of assays used to detect neutralizing antibodies to biological therapeutics. Recommendations have been published for immunoassays (Mire-Sluis et al., 2004; Geng et al., 2005) and recommendations for cell-based assays are forthcoming. Using the recommendations by Mire-Sluis as a guideline, a cell-based assay was developed and validated for neutralizing antibodies to the therapeutic monoclonal antibody CP-870,893.

The development of the bioassay was performed at Pfizer, Inc. and transferred to Charles River Laboratories for validation. During initial transfer of the bioassay, a significant decrease in the dynamic range of the CP-870,893 concentration-response was observed. This decreased response was attributed to the culture media, FBS, and selection of the MRD. The culture media and FBS were obtained from multiple sources and although chemically identical, the dynamic range of the CP-870,893 concentration-response could not be replicated across sites using these reagents. When the same reagent sources were used, comparable results were obtained between sites (data not shown). While not addressed in the validation, the robustness of this assay is clearly impacted by reagent selection, specifically the source of culture media and FBS.

The dynamic range of the bioassay was also affected by selection of the MRD, which was initially determined from a single individual and defined as 10% NHS. When the MRD was later assessed in multiple individuals, serum interference varied across individuals; thus, the MRD was redefined as 5% NHS. Based on this observation, it is recommended that the selection of the MRD be based on the entire drug curve performed in multiple individuals rather than a single individual or pool.

In the validation of this bioassay, a novel cutpoint for the presence or absence of drug was introduced. During validation of immunogenicity assays, determination of the cutpoint for identifying samples as positive or negative for Nabs is a standard practice. The design of this assay calls for samples to be tested for neutralizing antibodies only if they are positive for anti-drug antibodies and have drug levels below the LLOQ of the Pharmacokinetic (PK) assay. Since there is a difference in sensitivity between the PK and Nab Assay, it is possible that samples with drug levels below the LLOQ may still interfere with the bioassay.

In addition to testing patient samples in the presence of 50 ng/ml CP-870,893, patient samples are tested without drug to determine whether there is drug present that increases CD54 expression. Samples are reported as positive or negative for Nabs. In cases where a sample tests positive for drug, the result would be flagged with a comment indicating the accuracy of the reported result is potentially impacted by the presence of detectable drug in the sample.

Generally, non-cell-based competitive receptor binding and cell-based assays are the two main testing strategies for neutralizing antibodies. While competitive receptor binding assays tend to be more sensitive and easier to develop, functional cell-based
assays have been recommended when the biological therapeutic is an agonist. Results obtained using a CRB assay and the bioassay (modified for use with monkey plasma) were compared from a 3-mo toxicity study in monkeys. A correlation between the percent difference from control (CRB) and amount of drug neutralized per ml of serum (bioassay) was observed ($R^2 = 0.94$). This correlation suggests that the CRB assay can be used in lieu of the cell-based assay. Since bioassays tend to be more difficult to develop and validate than CRB assays (i.e., cell line and signal identification, time and resource requirements, and assay transfer), the added value of a bioassay in comparison to the CRB assay needs to be considered. If clinical samples with neutralizing antibodies become available, this relationship will be explored further using validated procedures.

In conclusion, a bioassay to measure neutralizing antibodies to CP-870,893 in 5% human serum matrix was developed and validated utilizing the Daudi cell line and detection of CD54 expression by flow cytometry. Bioassay precision, specificity, recovery, robustness, and stability were within acceptable limits and the relative sensitivity of the assay was approximately 500 ng/ml of human serum. Recommendations are made for the selection of the MRD based on multiple individual serum samples and the establishment of a cutpoint for the presence of drug. In addition, retrospective analysis of plasma samples from CP-870,893 treated monkeys showed a strong correlation to results obtained using a competitive receptor binding assay for neutralizing antibodies. This correlation suggests a receptor-binding assay may be a suitable alternative to a bioassay, but the relationship still requires further evaluation.

REFERENCES


