Comparison of Three Anthrax Toxin Neutralization Assays

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Different types of anthrax toxin neutralization assays have been utilized to measure the antibody levels elicited by anthrax vaccines in both nonclinical and clinical studies. In the present study, we sought to determine whether three commonly used toxin neutralization assays—J774A.1 cell-, RAW 264.7 cell-, and CHO cell-based assays—yield comparable estimates of neutralization activities for sera obtained after vaccination with anthrax vaccines composed of recombinant protective antigen (rPA). In order to compare the assays, sera were assayed alongside a common reference serum sample and the neutralization titers were expressed relative to the titer for the reference sample in each assay. Analysis of sera from rabbits immunized with multiple doses of the rPA vaccine showed that for later bleeds, the quantitative agreement between the assays was good; however, for early bleeds, some heterogeneity in relative neutralization estimates was observed. Analysis of serum samples from rabbits, nonhuman primates, and humans immunized with the rPA vaccine showed that the relative neutralization estimates obtained in the different assays agreed to various extents, depending on the species of origin of the sera examined. We identified differences in the magnitudes of the Fc receptor-mediated neutralization associated with the J774A.1 cell- and RAW 264.7 cell-based assays, which may account for some of the species dependence of the assays. The differences in the relative neutralization estimates among the assays were relatively small and were always less than 2.5-fold. However, because toxin neutralization assays will likely be used to establish the efficacies of new anthrax vaccines, our findings should be considered when assay outputs are interpreted.

Inhalation anthrax is one of the most serious of all bioterror threats because of the fatal nature of the disease and the stability and ease of dispersion of Bacillus anthracis spores. Therefore, significant efforts are under way to develop new vaccines for the prevention of anthrax. Many of the new anthrax vaccines being developed specifically target anthrax toxin, which is believed to play a critical role in disease progression and the lethal nature of the disease (1, 4, 14, 18, 26).

Anthrax toxin is a tripartite toxin, composed of a binary combination of three proteins: protective antigen (PA), lethal factor (LF) and edema factor (EF). PA, which by itself lacks toxicity, combines with LF, a zinc-dependent metalloproteinase, to form lethal toxin (LT) and with EF, a calmodulin- and calcium-dependent adenylate cyclase, to form edema toxin (ET). PA binds to cell receptors and mediates the translocation of LF and EF into the cytosol, where LF inactivates mitogen-activated protein kinase kinases, and EF elevates the levels of cellular cyclic AMP (cAMP) (3, 5, 12).

Given the role played by the toxin in the pathology of anthrax, neutralization of the toxin would be expected to prevent or ameliorate anthrax disease. Indeed, numerous animal and in vitro studies have shown that antibodies to toxin components and, in particular, PA confer protection (8, 11, 15, 23, 34). For this reason, many of the new anthrax vaccines under development are based on PA.

Because of the low incidence of anthrax disease in humans, the conduct of human efficacy trials of new anthrax vaccines is not feasible. Challenge studies with humans would be unethical because of the rapid and fatal progression of the disease. Therefore, the approval of new anthrax vaccines by the Food and Drug Administration will be based on the Animal Rule (6). Under this regulation, protection data from relevant animal species may be used to support vaccine efficacy in humans. Studies of the pathology of anthrax and the immunogenicity of vaccines in animals have led to the conclusion that both the rabbit and the nonhuman primate (NHP) could serve as suitable animal models of human anthrax and the response to vaccination (7, 22, 30, 35). Anti-PA antibody levels in immune sera have been shown to correlate with protection in animal models (16, 33) and thus will likely be used to bridge animal protection data to efficacy in humans. Thus, antibody levels will be important in establishing vaccine efficacy, and the assays used to measure these levels are critical to this process.

Two types of serological assays, an anti-PA IgG enzyme-linked immunosorbent assay (ELISA) and the anthrax toxin neutralizing assay (TNA) have been utilized to determine the antibody levels elicited by anthrax vaccines (17, 21, 24, 25). While the anti-PA ELISA measures the total amount of anti-PA IgG in a serum sample, TNA quantifies the anti-PA antibodies that are capable of neutralizing the toxin. Thus, TNA measures the subset of antibodies that are considered functional. Moreover, TNA is considered to be species independent and has been standardized for use with multiple species (10, 13, 20, 36). A species-neutral attribute is important for an assay that is to be used to bridge animal protection data to efficacy in humans.

Three types of TNAs have been developed. Two of the assay formats, the J774A.1 (J774) cell- and the RAW 264.7 (RAW) cell-based TNAs, measure the ability of anti-PA antibodies to neutralize anthrax toxin.
neutralize the cytocidal activity of LT. Both J774A.1 and RAW 264.7 cells are murine macrophage-like cell lines. The third assay format is the CHO cell-based TNA, which measures the ability of anti-PA antibodies to neutralize the ET-induced increase in intracellular cAMP levels. Both the specific toxin and the cell line utilized in the CHO cell-based assay differ from those used in the assays of cytocidal activity. The RAW 264.7 cell-based assay has primarily been used in research studies (see, e.g., references 2 and 19). In addition to their use in research studies, the J774A.1 cell- and CHO cell-based assays have been used to assay serum samples from clinical trials to ascertain the immunogenicities of anthrax vaccines and anti-PA therapeutic monoclonal antibody (MAb) levels, respectively (9, 28).

The general assumption is that in all three TNA, the antibodies elicited by PA-based vaccines recognize the common PA antigen and, therefore, that all three assays should yield the same relative estimate of toxin neutralization activity compared to that of an appropriate reference sample. However, differences in the specific toxin and cell types used in the three assays could result in differences in estimates of neutralizing antibody titers. For example, differences in the specific toxin utilized in the assay (LT versus ET) might affect neutralization estimates if the anti-PA antibodies present in a given serum sample were better able to inhibit the interaction of one of the catalytic components, either LF or EF, with PA than the other. Differences in cell type might also affect estimates of neutralization, since mechanisms of toxin neutralization can differ depending on the cell type. For example, previous studies have shown that cells of myeloid origin, such as J774A.1 and RAW 264.7 cells, express Fc receptors that can contribute to toxin neutralization. Thus, these cells have the potential to display the “classical” toxin neutralization that is seen with other cell types, as well as an Fc receptor-mediated form of toxin neutralization (31, 32). With classical neutralization, antibodies bind to PA and disrupt one or more of the steps involved in toxin action. Fc receptor-mediated neutralization is not well understood but could be due to Fc receptor-bound anti-PA facilitating the endocytosis of immune complexes, leading to their degradation, the sequestration of PA monomers at the cell surface, and/or the enhancement of classical neutralization by increasing the effective concentration of neutralizing antibodies at the cell surface. While Fc receptor-mediated neutralization occurs with both J774A.1 and RAW 264.7 cells, the extent of this type of neutralization differs between cell types (31). Unlike with these macrophage-like cell lines, Fc receptor-mediated neutralization is not expected to occur with CHO cells, an epithelial cell line, since only cells of hematopoietic origin produce Fc receptors.

In this study, we sought to determine whether the three TNA yield comparable estimates of neutralization activity for serum samples. We chose to compare the three TNA formats first by using sera generated in rabbits after the administration of either one, two, or three doses of a recombinant PA (rPA) vaccine in order to determine whether differences in estimates of neutralizing antibody titers might depend on the number of doses of vaccine administered. In addition, we compared the three assay formats using sera from three different species—rabbits, NHPs, and humans—to determine whether the species of origin of the sera might influence neutralization estimates.

We conducted the latter comparison since the TNA will be used to bridge rabbit/NHP protection data to efficacy in humans. Our goal was to determine whether the three assays yield similar estimates of neutralization and thus assess if they can serve equally well to measure the toxin-neutralizing antibody titers in pivotal animal protection studies as well as in human clinical trials.

**MATERIALS AND METHODS**

**Serum samples.** Rabbit anti-rPA serum samples were produced by immunizing rabbits with rPA (10 µg per dose adsorbed to aluminum adjuvant) on days 0, 28, and 56; serum samples were then collected on days 0, 14, 42, and 70. Human and NHP (cynomolgus macaque) sera were provided by NIAID, NIH (Bethesda, MD). Both the human and the NHP sera were from individuals who were immunized with rPA adsorbed to aluminum adjuvant (50 µg rPA per dose for humans; 2.5 or 6.25 µg rPA per dose for NHPs) on days 0 and 28 and from whom serum was collected on day 42. Test samples consisting of rabbit and human serum sample pools were prepared by combining equal volumes of individual rabbit and human test serum samples, respectively. A rabbit anti-rPA reference serum sample (NR-3839) was from the Biodefense and Emerging Infections Research Resources Repository, NIAID, NIH. This rabbit reference serum sample was prepared by pooling sera from 190 rabbits immunized with rPA adsorbed to aluminum adjuvant (50 µg per dose) at 0, 4, and 8 weeks; serum samples were then collected at 6, 8, 10, and 11 weeks. This study complied with all relevant federal guidelines and the Food and Drug Administration’s policies regarding the use of human subjects in research.

**Reagents.** *B. anthracis* recombinant PA (NR-140), recombinant LF (NR-142), and murine macrophage-like 3774A.1 cells (NR-28) were from the Biodefense and Emerging Infections Research Resources Repository, NIAID, NIH. EF was expressed and purified from *Escherichia coli*, as described previously (27). Murine macrophage-like RAW 264.7 cells and epithelioid-like CHO-K1 (CHO) cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA). Rat anti-mouse CD16/CD32 clone 2.4G2 was obtained from BD Pharmingen (Franklin Lakes, NJ). IgG was purified from the rabbit reference serum sample (NR-3839), and F(ab’)2 fragments were prepared from the purified rabbit IgG, as described by Verma et al. (31).

**TNA assay.** J774A.1 and RAW 264.7 cells were cultured in Dulbecco’s modified Eagle media (DMEM) containing 4.5 g/liter D-glucose and 110 mg/liter sodium pyruvate and supplemented with 5% heat-inactivated bovine serum, 2 mM l-glutamine, penicillin (25 U/ml), streptomycin (25 µg/ml), and 10 mM HEPES. The J774A.1 cell-based and RAW 264.7 cell-based assays were performed essentially as described by Li et al. (13) and Hering et al. (10). Briefly, cells cultured in flasks for 72 or 96 h were harvested and seeded in 96-well tissue culture plates at 40,000 cells/well (LT-TNA), followed by 17 to 19 h incubation. Serum samples were prepared in a separate 96-well microtiter plate at 2-fold dilutions (for a total of seven dilutions per sample). Samples were preincubated with a constant concentration of LT (50 ng/ml PA and 40 ng/ml LF for J774A.1 cells or 50 ng/ml PA and 160 ng/ml LF for RAW 264.7 cells), unless otherwise stated, for 30 min prior to addition to the cells. These concentrations of LT, chosen after toxin titration, result in approximately 95% killing of the corresponding cell line in the absence of neutralizing antibodies. The cell-serum-toxin mixture was incubated for 4 h, after which 25 µl/well of 5 mg/ml of a tetrazolium salt, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), was added. After a 2-h incubation, the cells were lysed by using 100 µl/well of acidified isopropanol (90% isopropanol, 0.5% SDS [wt/vol], 38 mM HCl), and the optical density (OD) at 570 nm was determined.

CHO cells were grown in Kighn’s modified F-12 nutrient mixture containing l-glutamine and supplemented with 10% heat-inactivated bovine serum, 2 mM l-glutamine, penicillin (25 U/ml), and streptomycin (25 µg/ml). The CHO cell-based assay was based on the work of Zmuda et al. (36) and was further optimized. In this assay, the cells and serum samples were treated as described for the LT-TNA, except that the plated cells (40,000 cells/well) were incubated for approximately 22 h before addition of the serum-toxin mixture and that ET (50 ng/ml PA and 160 ng/ml LF, unless otherwise stated) was used instead of LT. To prevent cAMP degradation, the serum-toxin mixture contained 750 µM 3-isobutyryl-1-methylxanthine. After the 4-h incubation, the cells were washed three times with medium, and the amount of cAMP was estimated by using a Tropix chemiluminescent cAMP ELISA kit (Applied Biosystems, Foster City, CA), according to the manufacturer’s instructions. The ELISA output was measured as relative luminescence units (RLU) with 1 s integration.
For TNAs performed with Fc receptor-blocking MAb 2.4G2, cells were pre-incubated with 100 μl of 10 mg/ml MAb for 15 min, prior to addition of 100 μl of the serum-toxin mixture, resulting in a final volume of 200 μl/well in the assay. For comparisons of the effects of toxins on the assays (ET-TNA and LT-TNA on J774A.1 cells), J774A.1 cells were plated at 80,000 cells/well, and the concentrations of toxins used were 100 ng/ml for PA and 160 ng/ml for EF and LF. Following treatment with ET, estimation of the amount of cAMP in J774A.1 cells, J774A.1 cells were plated at 80,000 cells/well, and the concentrations of toxins used were 100 ng/ml for PA and 160 ng/ml for EF and LF. Following treatment with ET, estimation of the amount of cAMP in J774A.1 cells was performed as described for the CHO cell assay. All assay incubations were done in an incubator at 37°C with 5% CO2 and 95% relative humidity.

Statistical analyses. The neutralizing activity of the test samples was calculated by curve-fitting analyses performed with GraphPad Prism (version 5) software. Specifically, a four-parameter logistic (4-PL) regression model was used to analyze the OD or the number of RLU versus the reciprocal of the serum dilution. Pairwise analyses of the sample and the reference run on the same plate were performed by use of a parallel-line model in which the slope parameter and the asymptotes of the two curves were constrained to equal values. The inflection point for each curve from this model was reported as the effective dilution at 50% inhibition (ED50) for the corresponding serum sample, and the relative potency (RP) was calculated by dividing the ED50 for the test serum sample by the ED50 for the reference serum sample. An RP value was considered reportable if the test data showed an acceptable goodness of fit to the parallel-line model; the fit was considered acceptable when the squared correlation coefficient (r²) values of the reference and the test serum samples were greater than or equal to 0.95 and 0.90, respectively. RP values were log transformed prior to subsequent statistical analyses.

SAS software was used for all statistical analyses except the comparison of Fc receptor-mediated neutralization, for which GraphPad Prism (version 5) software was used. To assess the effects of the immunization regimen and the species from which the serum samples were obtained on the assays, repeated-measures analysis of variance models were used to analyze the OD or the number of RLU versus the reciprocal of the serum dilution. Specifically, a four-parameter logistic (4-PL) regression model was used to analyze the dose-response data for each of the three assays. Shown are the dose-response data for the rabbit reference serum sample (NR-3839) used for this study and for a serum sample collected from a rabbit 2 weeks after the animal had been immunized with two doses of rPA vaccine given on days 0 and 28. The test and reference sera behaved in similar manners in all three assays, yielding full sigmoid neutralization curves that, for any given assay, had similar slopes and asymptotes. Using the data shown in Fig. 1, we calculated the ED50 for the test serum sample in the J774A.1 cell-, RAW 264.7 cell-, and CHO cell-based assays to be 3,462, 1,740, and 994, respectively, while the ED50 for the reference serum sample in the J774A.1 cell-, RAW 264.7 cell-, and CHO cell-based assays were 2,201, 1,186, and 749, respectively. Differences in the ED50 for a single serum sample in each of the three assays is to be expected for a number of possible reasons, including differences in cell susceptibility to the toxin or differences in assay output parameters (cell viability versus cAMP levels). However, when neutralization is normalized to that for a common reference serum sample and is expressed in terms of RP, similar estimates of RP for any given test serum sample in all three assays would be expected if the three assays yield similar estimates of neutralization.

Effect of immunization regimen on RP estimates. We first examined whether the three different TNAs yield similar estimates of neutralization throughout a multidose immunization regimen. Rabbits were immunized with rPA on days 0, 28, and 56. Sera were obtained 2 weeks after the administration of each dose. We assayed the samples along with the common reference serum sample by each of the three TNAs, calculated the RP values for each sample in each assay, and then determined the RP ratio for that sample for each given assay pair (J774/RAW, J774/CHO, and RAW/CHO cells). If the assays provide identical estimates of neutralization, then we would expect that all ratios would be equal to 1.0.

Sera from rabbits that received only a single immunization (Fig. 2, day 14) exhibited mean RP ratios that ranged from approximately 1.2 to 2.0. For all three assay pairs, the mean RP ratios were significantly different from 1.0 (P < 0.05), since the 95% confidence interval (CIs) did not include 1.0 (Table 1), indicating some differences in the estimates of neutralization between the assays, although the differences was no more than 2-fold. Of note, general heterogeneity between individual serum samples was observed, especially for the ratio for J774 and CHO cells.

When serum samples from the rabbits immunized twice with the rPA vaccine were examined (Fig. 2, day 42), we found that all RP ratios were close to 1.0. The results for certain assay pairwise comparisons were statistically significantly different from 1.0 (P < 0.05), since their 95% CIs did not include 1.0.
in each of the three independent TNAs, and the ED50 value in each assay was determined. RP was calculated as the ratio of the ED50 of test serum collected from rabbits 2 weeks after the first, second, and third immunizations at day 14, day 42, and day 70, respectively. Samples were assayed in each of the three independent TNAs, and the ED50 value in each assay was determined. RP was calculated as the ratio of the ED50 of test serum sample to that of a reference serum sample run on the same plate. Each serum sample was assayed on duplicate plates on three different days. For each serum sample, the geometric mean RPfs from the three independent runs were then used to calculate the RP ratio for each assay pair. Each of the points in the figure represents the RP ratio for an individual serum sample assayed by use of the indicated assay pair. The horizontal lines represent the mean ratio with 95% confidence interval.

Effect of species of origin of sera on RP estimates. The TNA has been considered species neutral and therefore an appropriate assay for use for comparison of the neutralizing antibody levels of different species induced by vaccination (10, 13, 20). This assessment has mainly come from the finding that sera from different species display similar slopes and asymptotes in the J774A.1 cell-based assay (20) and from the belief that PA-specific antibodies should neutralize the action of anthrax toxins in a manner that is independent of the species of origin of the antibody. Such a belief may be justified if the constant region of the antibody plays no role in neutralization. However, recent data suggest that, at least for TNAs that utilize cells that express Fc receptors on their surfaces, such as the cell types used for the J774A.1 and RAW 264.7 cell-based assays, the Fc portion of the antibody may contribute to the total neutralization measured by the assay. In order to further investigate whether any of the three TNAs exhibited a dependence on the species of origin of the sera, we examined sera from rabbits, NHPs, and humans by each of the three TNAs, using a common reference serum sample, and compared the results of the assays. We chose to study sera from these particular species because the rabbit and NHP models will likely be used to generate pivotal protection data for new anthrax vaccines which will be linked to efficacy in humans through a serological bridge.

We obtained sera from humans, rabbits, and NHPs that had been immunized with an rPA vaccine. The sera were matched for the immunization and the bleed schedules. Serum samples were assayed alongside the rabbit anti-rPA reference serum sample, NR-3839, in all three assays. The data for rabbit sera used to investigate the effects of the species source of serum were the same data shown in Fig. 2 with one additional serum sample. The 4-PL curve fits for the NHP and the human sera were similar to those shown in Fig. 1 for rabbit sera and met all set acceptance criteria for curve fitting, indicating that our statistical approach of calculating RP values for sera from various species by use of a rabbit reference serum sample is appropriate. A previous study has also shown that sera from rabbits, NHPs, and humans generate curves with similar slopes and upper and lower asymptotes in the J774A.1 cell-based assay (20).

Figure 3 provides a graphical presentation of the RP ratios for each given pair of assays for individual serum samples from each of the three species. Statistical analyses of these data are summarized in Table 2. As noted above, all three assays yielded RP ratios approximating 1.0 for the rabbit sera examined. In contrast, the NHP sera yielded RP ratios ranging from 0.85 to 1.91, while the human sera yielded RP ratios ranging

| Cells       | 1st dose (day 14) | 2nd dose (day 42) | 3rd dose (day 70) |
|-------------|------------------|------------------|------------------|
| J774/RAW    | 1.63^a (1.34-1.97) | 1.09 (0.99-1.21) | 0.99 (0.90-1.09) |
| J774/CHO    | 2.00^a (1.65-2.42) | 1.29^a (1.17-1.43) | 1.03 (0.93-1.14) |
| RAW/CHO     | 1.23^a (1.01-1.48) | 1.18^b (1.07-1.31) | 1.04 (0.94-1.14) |

^a These data are the geometric mean ratios from 11 rabbits for which valid results were obtained in all three assays at all three time points.
^b The RP ratio is significantly different from 1.0 (P < 0.05), since the 95% CI does not include 1.0.
from 1.06 to 2.41 (Table 2). For each species, the J774/RAW RP ratio was close to 1, suggesting good agreement between the two assays. In contrast, the J774A.1 cell- and RAW 264.7 cell-based assays yielded somewhat higher estimates of neutralization than the CHO-cell-based assay. All pairwise RP ratios reported were constant across the entire range of RP values obtained for the serum samples (data not shown). Of particular note, significant differences between species were noted for most of the pairwise RP ratios, suggesting a species dependence for one or more of the assays (Table 2).

Investigation of differential RP estimates. We sought to further examine why the different TNAs can yield somewhat different estimates of neutralization. Specifically, we investigated whether differences in the specific toxin utilized in the assay or differences in the extent of Fc receptor-dependent neutralization associated with different cell types might contribute to the differences in the neutralization estimates that we observed.

For this investigation, we utilized two serum sample pools, one being a pool of rabbit sera and the other being a pool of human sera. The pools of sera that we utilized for this purpose were composed of equal volumes of the individual serum samples that had previously been examined in the experiment whose results are shown in Fig. 3. Pools of serum samples, instead of individual serum samples, were utilized because of the limited availability of human serum samples. Because of the extremely limited availability of NHP serum samples, we did not use NHP sera for this investigation. In order to ascertain that the serum samples behaved as expected, we examined them in the J774A.1 cell- and the CHO-cell-based assays and compared the results to those seen when the individual serum samples used to make up the pool were examined in the same assays. The J774/CHO RP ratios (geometric means [GMS] of three assay replicates) were 1.34 (95% CI, 1.13 to 1.59) and 2.17 (95% CI, 1.84 to 2.55) for the rabbit serum pool and the human serum pool, respectively. These ratios are in agreement with the mean RP ratios observed for the individual serum samples tested in the same assays, as shown in Fig. 3 and Table 2 (1.27 [95% CI, 1.17 to 1.39] and 2.41 [95% CI, 2.01 to 2.89] for rabbit and human sera, respectively).

We first investigated whether differences in the toxin used (LT versus ET) would contribute to differences in estimates of neutralization. We did this by establishing a modified J774A.1 cell-based assay in which ET instead of LT was utilized and cAMP levels instead of cell viability were measured. The results of the standard J774A.1 cell-based assay (LT-J774), which utilizes LT, were then compared with those of the modified assay that utilizes ET (ET-J774). In this manner, while the toxin types differed for the assays, the cell type was the same. The serum sample pools were assayed in triplicate in each of the two assays. The mean RP ratios for LT-J774/ET-J774 were 1.07 (95% CI, 0.91 to 1.25) for the rabbit serum sample pool and 0.86 (95% CI, 0.74 to 1.01) for the human serum sample pool. Since estimates of neutralization were essentially identical in the two assays (i.e., the RP ratios approximated 1.0 and their confidence intervals included 1.0), the difference in neutralization estimates observed in the LT-J774 cell-based assay and the ET-CHO cell-based assay was most likely not due to the difference in the nature of the toxin used in the assay.

| Cells          | RP ratio on day 42 (95% CI) with sera from: | Between-species comparisons |
|----------------|---------------------------------------------|-----------------------------|
|                | Rabbits (n = 12)                           | NHPs (n = 14)               | Humans (n = 9)               |                          |
| J774/RAW       | 1.08 (0.99–1.18)                           | 0.85* (0.77–0.94)           | 1.06 (0.88–1.27)            | Rabbit vs NHP            | 0.0022                  |
|                |                                             |                             |                             | Rabbit vs human          | 0.8274                  |
|                |                                             |                             |                             | NHP vs human             | 0.0084                  |
| J774/CHO       | 1.27* (1.17–1.39)                           | 1.61* (1.46–1.79)           | 2.41* (2.01–2.89)           | Rabbit vs NHP            | 0.0027                  |
|                |                                             |                             |                             | Rabbit vs human          | <0.0001                 |
|                |                                             |                             |                             | NHP vs human             | <0.0001                 |
| RAW/CHO        | 1.18* (1.08–1.28)                           | 1.91* (1.72–2.11)           | 2.27* (1.89–2.72)           | Rabbit vs NHP            | <0.0001                 |
|                |                                             |                             |                             | Rabbit vs human          | <0.0001                 |
|                |                                             |                             |                             | NHP vs human             | 0.0380                  |

* The RP ratio is significantly different from 1.0 (P < 0.05), since the 95% CI does not include 1.0.
We next investigated whether Fc receptor-dependent neutralization might contribute to the differences in neutralization estimates between different assays and also to differences in the behavior of sera from different species in the different assays. In order to investigate the potential for Fc receptor-mediated neutralization to contribute to differences in the estimates of neutralization between assays, an Fc receptor-blocking antibody (MAb 2.4G2) was utilized. This antibody specifically blocks murine Fc receptors IIb and III. We assessed the magnitude of Fc receptor-dependent neutralization observed in the two TNA formats that could be blocked by this monoclonal antibody using the pooled rabbit and human serum samples described above. We estimated the magnitude of Fc receptor-mediated neutralization blocked by the antibody by comparing the neutralizing titers, expressed as the ED₅₀, obtained in the absence versus the presence of the monoclonal antibody. An ED₅₀ ratio equal to 1.0 would indicate the absence of any Fc receptor-mediated neutralization in the TNA that could be blocked by the monoclonal antibody. The larger that the ED₅₀ ratio is, the greater that the amount of Fc receptor-mediated neutralization is blocked by the monoclonal antibody. The results are shown in Fig. 4 for the J774A.1 cell-based assay (Fig. 4A and B) and for the RAW264.7 cell-based assay (Fig. 4C and D). The GM ratios of the ED₅₀ in the absence of the blocking antibody compared to the ED₅₀ in its presence were 2.8 (GM of three independent assays) for the rabbit serum sample pool and 3.7 (GM of three independent assays) for the human serum sample pool in the J774A.1 cell-based assay. Thus, both the rabbit and human serum sample pools exhibited Fc receptor-mediated neutralization (the ED₅₀ ratios were greater than 1.0 for both), with the human serum sample pool exhibiting significantly more Fc receptor-mediated neutralization than the rabbit serum sample pool (P < 0.002, unpaired t test). For the RAW 264.7 cell-based assay, the GM ratios of the ED₅₀ in the absence of the blocking antibody compared to the ED₅₀ in its presence were 1.4 (GM of three independent assays) for the rabbit serum sample pool and 2.1 (GM of three independent assays) for the human serum sample pool, a statistically significant difference (P = 0.02, unpaired t test). In contrast, since epithelial cells do not express Fc receptors, no Fc receptor-mediated neutralization would be expected in the CHO cell-based TNA. Because the Fc receptor-blocking antibody MAb 2.4G2 is specific for mouse Fc receptors, we confirmed the lack of Fc receptor-
mediated neutralization in the CHO cell-based assay by directly comparing the 50% effective concentration (EC50) values of a purified IgG preparation and those of purified F(ab\textquotesingle)2 fragments isolated from the same IgG preparation. The F(ab\textquotesingle)2 fragments retain the bivalent binding of IgG but lack the Fc portion of the antibody. In contrast, we found that the EC50 estimates for the two preparations were similar. The EC50 values were 31 and 33 for the IgG and F(ab\textquotesingle)2 fragments, respectively (GMs of two independent assays).

**DISCUSSION**

On the basis of the current interest and performance of the TNA in clinical and nonclinical studies, the assay, in either the J774A.1 cell-, RAW 264.7 cell-, or CHO cell-based format, will continue to be utilized in immunoogeneity studies of anthrax vaccines as well as in the evaluation of immunotherapies for anthrax. It is therefore important to determine how the three assays compare in their capacities to estimate the neutralization abilities of various antibodies. Understanding the performance of each assay is critical to choosing the most appropriate assay format and for interpretation of the data.

In our study, the three TNAs yielded various results for the estimates of neutralization for sera (relative to the estimate for the reference serum sample) throughout the course of immunization of rabbits with three doses of rPA. The level of agreement between the assays was good for sera collected after the administration of either two or three doses of the rPA vaccine. After a single immunization, estimates of neutralization differed between the assays by as much as 2-fold. In addition, some heterogeneity in the RP ratios for individual serum samples was observed, especially for the J774/CHO RP ratios. Antibodies induced early after the initial immunization would be expected to be of lower affinity than antibodies present later in the immunization regimen, which may affect estimates of neutralization. For example, Fc receptor-mediated neutralization might be more pronounced for lower-affinity antibodies if an Fc receptor tends to hold the antibody at the cell surface, thereby stabilizing the antibody-PA complex. IgM would also be expected to be more prominent early in the immunization regimen than later, when IgG would be predominant. Such isotype switching might be expected to affect the relative contribution of Fc receptor-mediated neutralization in the J774A.1 cell- and/or RAW 264.7 cell-based assays since Fc receptors are isotype specific. For these reasons, differences in the kinetics of the antibody maturation process in individual animals may contribute to the heterogeneity observed for the RP ratios of sera obtained after a single immunization.

While we obtained comparable estimates of neutralization in the three TNAs when rabbit sera were analyzed, the estimates of neutralization agreed less well when human and NHP sera were analyzed. Moreover, our observation that the J774/CHO as well as the RAW/CHO RP ratios were significantly different for sera from different species suggest that one or more of the TNAs exhibit some species dependence. A possible source for species dependence might be the Fc receptor-dependent neutralization that is known to exist with macrophage-like cells. In support of this explanation, we found that a human serum sample pool exhibited significantly more neutralization that could be blocked by an Fc receptor-blocking monoclonal antibody compared to that of a rabbit serum sample pool in both the J774A.1 and the RAW 264.7 cell assays. We should note that the Fc receptor-blocking antibody used in these studies, MAb 2.4G2, blocks Fc receptor-mediated neutralization, which is specifically dependent on Fcy receptors IIb and III (29), and does not necessarily block all Fc receptor-dependent neutralization that may be present in the assays. Nonetheless, the results observed illustrate the possibility that Fc receptor-mediated neutralization could influence estimates of neutralization and may impart some species specificity to certain of the TNAs.

The difference in the contribution of Fc receptor-mediated neutralization observed for the two serum sample pools might be explained by the fact that the Fc regions of human and rabbit antibodies are not identical. This difference in structure could translate into differential binding of the antibodies to the murine Fc receptors present on the macrophage-like cells. These results should be considered when direct comparisons of the neutralization levels of different species are made by TNA, such as will be done when the Animal Rule is applied to new PA-based vaccines.

At this time, the biological relevance of Fc receptor-mediated neutralization is not known. Additional studies will be needed to assess the extent to which Fc receptor-mediated neutralization might play a role in protection against *B. anthracis* infection *in vivo*. Such information would be useful when choosing the most appropriate assay format for a given purpose.

Our finding that the three TNAs yielded similar estimates of neutralization when rabbit sera were analyzed, especially after two or three immunizations, but not when either human or NHP sera were analyzed may stem from the fact that the common reference serum sample that we used for each of the three assays was derived from rabbits. This reference serum sample was a polyclonal antiserum sample pooled from rabbits that had received either two or three immunizations of the rPA vaccine. Therefore, the reference serum sample would be expected to behave in a manner most similar to the manners of the rabbit test serum samples in our study that were obtained after either two or three immunizations. The behavior of the reference serum sample is critical and likely dictates whether estimates of neutralization, based on RP values, from the three TNAs will be similar or different. In this regard, our findings serve to emphasize the basic tenet that a good reference sample is one that behaves in a manner similar to the manners of the test samples and further illustrate the importance of the choice of reference sample for use in these types of assays. Our results demonstrate that when the suitability of a reference sample is determined, not only should assay output parameters such as slope, upper asymptote, and lower asymptote be considered—which, for a given assay, were similar between the reference sample and all test serum samples used in this study—but more subtle features of the reference serum sample, such as isotype composition and the species of origin, may also be important considerations, since these characteristics might contribute to the behavior of the reference sample, at least in certain TNA formats. Use of a reference serum sample offers many advantages, such as minimizing assay-to-assay variability and better allowing interlaboratory comparisons. While we believe that these advantages outweigh the potential disad-
vantages, the findings presented here should be considered when an optimal reference serum sample for use in TNA is chosen.

The results from this study demonstrate that the Fc receptor-mediated component of neutralization observed in the J774.A1 cell- and RAW 264.7 cell-based assays is associated with some level of species dependence. For many uses of the assays, this may not be a concern. However, for other uses, such as when TNA titers for animals need to be directly compared to those for humans, this finding will be more pertinent. Ultimately, the choice of TNA format that is best for a particular purpose must be regarded in the context of the specific questions that the assay will be used to address. A number of considerations may come into play, some of which involve the biological characteristics of the assay and others of which may be more practical in nature, such as cost and amenability to high-throughput operation.

In conclusion, we have demonstrated that, in general, the level of agreement between the three TNA formats for many purposes is reasonable, but not perfect, at least for the serum samples that were used for the study. The greatest difference in RP estimates observed between the assays was approximately 2.5-fold in magnitude, with some species specificity being noted. Given the differences between the assays in regards to cell type, the specific toxin used, and assay output, as well as the challenges in choosing a common reference serum sample that is suitable for use with all test samples, these results provide reassurance that, regardless of the TNA format used, estimates of neutralization are not dramatically assay dependent. Nonetheless, the differences in estimates of neutralization that were observed in this study and the geneses of these differences should be considered when the TNA output is interpreted.

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