In Vitro and In Vivo Characterization of Anthrax Anti-Protective Antigen and Anti-Lethal Factor Monoclonal Antibodies after Passive Transfer in a Mouse Lethal Toxin Challenge Model To Define Correlates of Immunity[▽]

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Passive transfer of antibody may be useful for preexposure prophylaxis against biological agents used as weapons of terror, such as *Bacillus anthracis*. Studies were performed to evaluate the ability of anthrax antiprotective antigen (anti-PA) and antilethal factor (anti-LF) neutralizing monoclonal antibodies (mAbs) to protect against an anthrax lethal toxin (LeTx) challenge in a mouse model and to identify correlates of immunity to LeTx challenge. Despite having similar affinities for their respective antigens, anti-PA (3F11) and anti-LF (9A11), passive transfer of up to 1.5 mg of anti-PA 3F11 mAb did not provide significant protection when transferred to mice 24 h before LeTx challenge, while passive transfer of as low as 0.375 mg of anti-LF 9A11 did provide significant protection. Serum collected 24 h after passive transfer had LeTx-neutralizing activity when tested using a standard LeTx neutralization assay, but neutralization titers measured using this assay did not correlate with protection against LeTx challenge. However, measurement of LeTx-neutralizing serum responses with an LeTx neutralization assay in vitro employing the addition of LeTx to J774A.1 cells 15 min before the addition of the serum did result in neutralization titers that correlated with protection against LeTx challenge. Our results demonstrate that only the LeTx neutralization titers measured utilizing the addition of LeTx to J774A.1 cells 15 min before the addition of sample correlated with protection in vivo. Thus, this LeTx neutralization assay may be a more biologically relevant neutralization assay to predict the in vivo protective capacity of LeTx-neutralizing antibodies.

Anthrax infection caused by *Bacillus anthracis* may be classified based on the portal of entry into the host (cutaneous, gastrointestinal, or pulmonary), and symptoms may include fever with mild to severe systemic symptoms of malaise and headache (5). In severe forms of anthrax, general toxemia with shock, sepsis, and death may occur. The major virulence factor of *B. anthracis* consists of three proteins, edema factor, protective antigen (PA), and lethal factor (LF) (5, 11, 37). The combination of PA and LF produces lethal toxin (LeTx) that is lethal in several animal models including mice (11, 37). The distribution of *B. anthracis* spores through the mail in the United States in 2001 and the resulting anthrax morbidity and deaths indicate that infectious pathogens may be distributed through the environment for use as bioterrorists (6, 12, 13, 16, 24, 33, 54), and *B. anthracis* is listed as a category A pathogen by the National Institutes of Health (40).

A licensed vaccine that induces protective immunity against anthrax exists, the anthrax vaccine adsorbed (AVA) preparation (BioThrax) (15, 19, 48, 49). New recombinant PA (rPA) anthrax vaccines are also being evaluated (19–21, 28, 36, 53, 57). In guinea pigs and rabbits, protective immunity after im-

munization with AVA correlates with the titer of serum anti-PA antibody induced as well as with the LeTx neutralization titer in a macrophage J774A.1 toxicity assay (47). Protective immunity induced by immunization with rPA correlates with LeTx neutralization titers measured in the macrophage toxicity assay (28, 51). Despite the ability to induce protective immunity with AVA or rPA immunization, widespread immunization against anthrax may not be practical due to the cost required to vaccinate the entire population, and the number of people actively infected after the release of anthrax spores used as a biological weapon may represent only a fraction of the entire population (6, 12, 13, 16, 24, 33, 54). Therefore, the development of effective passive immunotherapies for anthrax is needed, and correlates of protective immunity are needed to ensure that protective levels of immunity are attained after passive immunotherapy.

Passively transferred anti-PA/LF antibodies are able to protect against lethal *B. anthracis* infection (3, 23, 27, 51) and lethal LeTx challenge (23, 29, 30, 34, 56). All antibodies that neutralized LeTx in vivo exhibited LeTx neutralization activity in vitro (30). Recombinant antibodies, scFv or scFv fused to a human constant K domain, specific for PA were able to protect against LeTx in vivo (34, 56). Passive transfer of polyclonal guinea pig anti-PA or anti-AVA antiserum protected 67 and 33%, respectively, of guinea pigs challenged with anthrax spores, while passive transfer of individual anti-PA or anti-LF monoclonal antibody (mAb) did not protect against the spore challenge despite being very potent at neutralizing LeTx in the macrophage toxicity assay (27). Those authors did not deter-

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mine if a combination of the anti-PA and anti-LF mAbs was able to protect against a lethal anthrax spore challenge. A combination of two anti-PA mAbs and one anti-LF mAb protected 100% of mice against challenge with Sterne strain spores, while the combinations of each anti-PA mAb with the single anti-LF mAb provided 0 to 50% protection against a lethal spore challenge (9). Taken together, these reports suggested that (i) polyclonal anti-PA and anti-LF antibodies may be used to provide protective passive immunity against anthrax, (ii) cocktails of anti-PA and anti-LF mAbs may be needed to provide optimal passive immunity, and (iii) the criteria for identifying which mAbs will be therapeutically useful *in vivo* have yet to be fully defined. Additional evidence for the use of mAb cocktails for passive immunotherapy is that individual anti-botulinum neurotoxin type A (BoNT/A) mAbs were not able to protect mice against a lethal challenge with 20 times the 50% lethal dose of BoNT/A, while a mixture of three anti-BoNT/A mAbs protected approximately 50% of mice against 20,000 times the 50% lethal dose of BoNT/A (42). Others also reported that a combination of two human mAbs specific for tetanus toxin provided complete protection against a lethal tetanus toxin challenge in mice, while either antibody alone was not protective (60). The benefit of mAb combinations in the neutralization of virus has also been reported (4, 25, 58).

The present study was performed to determine if a combination of LeTx-neutralizing anti-PA and anti-LF mAbs was superior to individual mAbs in the ability to neutralize LeTx *in vitro* and *in vivo*. More importantly, studies to characterize the LeTx neutralization activity in the serum of mice 24 h after passive transfer were performed to determine if *in vitro* measurements of LeTx-neutralizing antibodies correlated with protection *in vivo*. Our results indicated that measuring LeTx-neutralizing antibody responses in the serum of mice 24 h after passive transfer with a macrophage LeTx neutralization assay performed by adding LeTx to the macrophages 15 min before the addition of the test antibody (26, 30, 34) predicted protection *in vivo*. In contrast, LeTx-neutralizing antibody titers measured with a standard *in vitro* LeTx neutralization assay did not predict protection mediated by passively administered mAbs.

MATERIALS AND METHODS

Hybridoma development and mAb purification. Female (C57BL/6 $\times$ BALB/c) F1 mice were nasally immunized with 4 μ g rPA (List Biological Laboratories, Campbell, CA) and 1 μ g cholera toxin (as a mucosal adjuvant; List Biological Laboratories, Campbell, CA) on days 0, 7, and 14. On days 49 and 57, mice were nasally immunized with 10 μ g PA and 1 μ g cholera toxin. On day 90, mice were immunized intraperitoneally with 50 μ g PA in 100 μ l saline as a final boost before harvesting spleens (on days 95 to 97) to make hybridomas. Female BALB/c mice were similarly immunized with recombinant LF (rLF; List Biological Laboratories, Campbell, CA), and spleens were harvested and used to make hybridomas. Immune spleen cells were fused with mouse myeloma cells using standard procedures routinely used in our laboratory (14, 31, 35, 44, 45, 52, 55). Hybridomas producing anti-rPA or anti-rLF antibodies were screened by enzyme-linked immunosorbent assay (ELISA) using rPA or rLF as the coating antigen, respectively. Controls included ELISA plates coated with irrelevant protein antigens. Once hybridomas were cloned, antibody was purified by protein A/G affinity chromatography (Pierce, Rockford, IL).

ELISA. A fluorescent ELISA was used to measure anti-PA and anti-LF immunoglobulin G (IgG) endpoint titers in the serum of mice after passive transfer. PA or LF was coated onto 384-well plates at a final concentration of 2 μ g/ml in carbonate-bicarbonate buffer, and the assay was performed as previously described (8, 41). The log₂ endpoint titers were used for statistical analysis. Samples

with no detectable anti-PA or anti-LF IgG titers were assigned a value of 1 for statistical analysis.

***In vitro* LeTx neutralization assay.** A macrophage toxicity assay using J774A.1 macrophage cells was used to determine the ability of the anti-rPA and anti-rLF mAbs to neutralize LeTx. The "standard" assay was performed as reported previously by others (7, 28, 36, 39, 50, 59) except that the final concentrations of rPA and rLF in the medium-LeTx-mAb mixtures applied to the J774A.1 cells were 187.5 ng/ml rPA and 187.5 ng/ml rLF. For the "toxin-first" LeTx neutralization assay, LeTx was added to the J774A.1 cells for 15 min before the addition of anti-PA and/or anti-LF mAb (26, 30, 34). For each LeTx neutralization assay, a LeTx standard curve was performed with the rPA/rLF final concentrations starting at 1.5 μ g/ml and progressing by twofold serial dilutions to 5.8 ng/ml rPA/rLF to ensure that the assay was performing reproducibly. The use of rPA and rLF at a final concentration 187.5 ng/ml was always four- to eightfold more toxin than needed to kill 100% of the J774A.1 cells. Others previously used rPA and rLF at a final concentration of 80 ng/ml to produce 100% killing of J774A.1 cells in the LeTx neutralization assay (39). Viability of the cells was determined using CellTiter 96Aqueous (Promega, Madison, WI). Percent neutralization was calculated using the following formula: (sample OD value – LeTx standard OD value)/(cells-only OD value – LeTx standard OD value) $\times$ 100. The optical density (OD) of a medium-only well (i.e., no cells) was subtracted from all values before percent neutralization was calculated. The percent neutralization was plotted versus antibody concentration, and the linear range was used to calculate the concentration of antibody needed to neutralize 50% of LeTx (NC₅₀). Fifty percent neutralization titers (NT₅₀) were similarly calculated for the serum collected from mice. The neutralization concentrations or titers reported represent the final concentration or dilution of antibody when combined with LeTx and the J774A.1 cells. Samples that had no detectable LeTx neutralization activity were assigned a value of 1 for statistical analysis.

***In vivo* LeTx neutralization assay.** To evaluate the ability of anti-rPA and anti-rLF mAbs to protect mice against an LeTx challenge, male BALB/c mice were injected by the intraperitoneal route with the indicated amounts of anti-rPA and/or anti-rLF mAb in 500 μ l sterile phosphate-buffered saline (PBS). Twenty-four hours after passive transfer of mAb, mice were anesthetized with ketamine-xylazine (90/10 mg/kg), and blood was collected. While under anesthesia, mice were injected with 200 μ g rPA plus 200 μ g rLF in 500 μ l of 1 mg/ml bovine serum albumin (BSA) in PBS by the intraperitoneal route. Mice were monitored daily for signs of morbidity. Mice were considered to be moribund when they had lost 15% of their day 0 body weight and were euthanized. Others utilized the intraperitoneal route of toxin administration in mice since a comparison of intravenous (i.v.) and intraperitoneal toxin administrations produced similar survival curves (37).

SPR binding measurements. For surface plasmon resonance (SPR) binding and kinetic measurements, either LF or PA was immobilized on a CM5 sensor chip (BIAcore Inc.). For mAb binding and specificity determinations, about 3,500 response units of LF or PA was immobilized using standard amine coupling as described previously (1, 2). mAbs (500 nM) were injected sequentially over immobilized LF and PA surfaces. For kinetic measurements of Fab binding, about 600 response units of LF or PA was immobilized, and the Fabs were titrated over a concentration series that ranged from 0.0391 to 2.5 μ M. Following each cycle of injection, bound Fab was regenerated using a short injection of gentle elution buffer (Pierce Biotech, Rockford, IL), followed by glycine-HCl (pH 2.0).

Statistics. Due to their normal distribution, the *in vitro* LeTx NC₅₀ values (in μ g/ml) for mAb 3F11, 9A11, and 3F11 plus 9A11 (see Fig. 4) were compared using a two-tailed, two-sample *t* test (assuming equal variance) (39). There were seven to eight replicate measurements per group. The ability of passively transferred antibodies to protect against morbidity after lethal challenge was graded as "1" for no morbidity and "2" for morbidity. These categorical data were used to determine differences in the level of protection between groups following passive transfer and toxin challenge in mice with the use of the Fisher's exact test (two sided) (S-Plus; Insightful Corporation, Seattle, WA), as utilized by others previously (15, 19, 20, 27, 36). Due to the nonparametric distribution of log₂ anti-PA IgG titers, anti-LF IgG titers, and LeTx neutralization titers (standard assay and toxin-first assay) in the serum of mice after passive transfer, these values were compared between the passive transfer groups using an Exact Wilcoxon rank-sum test (S-Plus; Insightful Corporation, Seattle, WA) as reported previously by others (17, 22, 43). Statistical significance was considered to be a *P* value of <0.05 .

FIG. 1. SPR binding of anti-LF mAbs to LF. Anti-LF mAbs 3H3 and 9A11 bound to immobilized LF (A). No binding of anti-PA mAbs 27H11 and 3F11 was observed (A). mAb 3H3 (B) or 9A11 (D) was injected sequentially to saturate the binding sites on immobilized LF (B). This was followed by the injection of either mAb 9A11 (B) or 3H3 (E) over the mAb-saturated LF surface. The overlay of binding of 9A11 (C) or 3H3 (E) with (broken line) and without (solid line) the blocking mAb shows that the two anti-LF mAbs do not bind to overlapping epitopes. Arrows indicate times at which each of the indicated mAbs were injected. Ab, antibody; RU, response units.

RESULTS

Antigen binding characteristics of anti-PA and anti-LF mAbs. In order to select mAbs with higher-avidity binding to PA and LF, anti-PA and anti-LF mAbs were first screened for avidity of binding to immobilized PA or LF in SPR binding assays. Anti-LF mAbs 9A11 and 3H3 bound specifically to immobilized LF (Fig. 1A). In cross-blocking experiments, while mAb 9A11 did not block mAb 3H3 binding to LF, a slightly lower binding response ($\sim 20\%$) of 9A11 was observed when LF was prebound to mAb 3H11 (Fig. 1B and C); thus, mAbs 9A11 and 3H3 bound to distinct sites on LF. The binding avidities of mAbs 3H3 and 9A11 were comparable, 15 and 9 nM, respectively.

Two anti-PA mAbs, 27H11 and 3F11, both bound to PA but with differing kinetics (Fig. 2A). The binding of mAb 27H11 to PA gave fast association and dissociation kinetics, while those of 3F11 were relatively slower, indicating more stable complex formation. This suggested that mAb 3F11 bound to PA with higher avidity. The two anti-PA mAbs also cross-blocked binding to PA (Fig. 2B and C) and therefore demonstrated that these two anti-PA epitopes were spatially close to each other. Based on these initial binding analyses, we selected the higher-avidity anti-LF mAb 9A11 and anti-PA mAb 3F11 for further characterization of binding kinetics and neutralization of LF or PA.

In SPR binding assays, mAbs 3F11 and 9A11 bound specif-

ically to PA and LF proteins, respectively (Fig. 3A and B). No binding of mAb 3F11 to LF or of mAb 9A11 to PA was observed. In order to determine the relative binding K_d (dissociation constant) and rate constants of each of the antibodies, we measured binding kinetics using Fab fragments of 3F11 and 9A11 (Fig. 3C and D). The binding of both Fabs followed a simple two-component interaction model (Langmuir equation) and gave K_d values of 74.8 and 70.1 nM for 3F11 and 9A11, respectively (Table 1). The binding of 3F11 gave relatively slower association and dissociation rates than the 9A11 Fab (Table 2). These binding studies therefore showed that the selected anti-LF (9A11) and anti-PA (3F11) mAbs are specific and bind strongly (K_d in nM) to their respective antigens.

In vitro LeTx neutralization. Anti-PA mAb 3F11, anti-PA mAb 27H11, and anti-LF mAb 9A11 were tested for their ability to neutralize anthrax LeTx using an LeTx neutralization assay employing the murine J774A macrophage cell line. Anti-PA mAb 27H11 did not neutralize LeTx (data not shown). Using a standard assay, serial dilutions of mAbs 3F11, 9A11, and 3F11 plus 9A11 were combined with PA and LF and incubated before being added to J774A cells. The NC_{50} for 3F11 was $0.62 \pm 0.09 \mu\text{g/ml}$, while the NC_{50} for 9A11 was $0.20 \pm 0.03 \mu\text{g/ml}$ of 9A11 ($P = 0.0009$) (Fig. 4A). The combination of 3F11 and 9A11 had an NC_{50} of $0.09 \pm 0.013 \mu\text{g/ml}$, significantly lower than the NC_{50} for 3F11 ($P = 0.0002$) or 9A11 ($P = 0.0107$) (Fig. 4A). Using a toxin-first LeTx neutral-

FIG. 2. SPR binding of anti-PA mAbs to PA. (A) Binding of anti-PA and anti-LF mAbs to immobilized PA. Differences in binding kinetics were observed, with 27H11 (solid line) binding with faster kinetics than 3F11 (broken line) mAb binding. Cross-blocking studies show that 27H11 (B) and 3F11 (C) binding to PA was hindered following saturation of the PA surface with the blocking mAb 3F11 (B) or 27H11 (C), respectively. RU, response units.

ization assay, where LeTx was added to the J774A cells for 15 min before the addition of mAbs, 3F11 and 9A11 neutralized LeTx with NC_{50} values of $4.11 \pm 1.26 \mu\text{g/ml}$ and $0.40 \pm 0.05 \mu\text{g/ml}$, respectively ($P = 0.0107$) (Fig. 4B). With the toxin-first assay, the NC_{50} for the 3F11 and 9A11 ($0.40 \pm 0.165 \mu\text{g/ml}$) was not significantly different from the NC_{50} for 9A11 alone ($P = 0.9966$) (Fig. 4B).

In vivo LeTx neutralization. A passive transfer model was used to evaluate the ability of 3F11, 27H11, and 9A11 to protect mice against a parenteral LeTx challenge. 3F11 (1.5 mg) combined with 27H11 (1.5 mg) was tested to determine if a combination of anti-PA LeTx-neutralizing and LeTx-non-neutralizing mAbs would perform differently from an anti-PA LeTx-neutralizing mAb alone. Combinations of 3F11 and 9A11 were also tested to determine if combining anti-PA and anti-LF mAbs was superior to individual antibodies for passive immunity to LeTx. Twenty-four hours after passive transfer of the antibodies, mice were anesthetized with ketamine-xylazine, a blood sample was collected, and mice were injected with LeTx (200 μg rPA plus 200 μg rLF) by the intraperitoneal route. Mice were monitored daily and euthanized when they became moribund (loss of 15% body weight). All mice that received control mouse IgG (1.5 mg) were moribund and euthanized within 3 days after LeTx challenge (Fig. 5). Passive transfer of 1.5 mg 3F11 resulted in 25% survival (not significant), while 1.5 mg 9A11 provided 100% protection against

moribidity ($P = 0.0079$ versus IgG control) (Fig. 5A). All mice that received 1.5 mg 27H11 or 1.5 mg 27H11 combined with 1.5 mg 3F11 were moribund within 6 days following LeTx challenge (data not shown). 3F11 passively transferred at 0.75 mg provided no protection against moribidity, and all mice were moribund by 6 days after LeTx challenge (Fig. 5B). In contrast, 0.75 mg of 9A11 and 0.75 mg 9A11 combined with 0.75 mg 3F11 provided complete protection, and all animals survived the LeTx challenge ($P = 0.0079$ versus IgG control) (Fig. 5B). Passive transfer of 3F11 at 0.375 mg did not provide protection, while 9A11 at 0.375 mg protected 80% of mice against moribidity ($P = 0.0476$) (Fig. 5C). The combination of 0.375 mg 3F11 and 0.375 mg 9A11 (0.75 mg total IgG transferred) protected 100% of mice ($P = 0.0079$ versus IgG) (Fig. 5C) and was not significantly different than 0.75 or 0.375 mg 9A11 alone.

At the time of LeTx challenge, blood was collected from mice, and serum was tested for the presence of anti-PA IgG, anti-LF IgG, and LeTx-neutralizing antibodies (Table 2). Anti-PA and anti-LF IgG titers were measured in an endpoint ELISA, and data in Table 2 and Fig. 5A to C exclude outliers identified as being mice with an anti-PA IgG or anti-LF IgG endpoint titer more than 1 standard deviation lower than the group geometric mean. Although anti-PA and anti-LF IgG ELISA endpoint titers varied between the various treatment groups based on the amount of antibody transferred, the anti-PA titers were not significantly different between the var-

FIG. 3. Binding analyses of anti-PA and anti-LF mAbs and Fabs. (A) mAb 3F11 bound specifically to rPA (solid line) but not to LF (broken line). (B) mAb 9A11 bound specifically to LF (solid line) but not to PA. Binding of 3F11 Fab to PA and 9A11 Fab to LF is shown in C and D, respectively. Either PA (A and C) or LF (B and D) proteins were immobilized on a CM5 sensor chip as described in Materials and Methods. For Fab binding kinetic studies, 3F11 Fab was injected in concentrations ranging from 0.0391 to 2.5 μ M, while 9A11 was used at 0.0781 to 1.25 μ M. Binding rate constants are given in Table 1. RU, response units.

ious groups that had received 3F11 by passive transfer. Also, the anti-PA titers were not significantly different between mice that received 1.5 mg 3F11 and mice that received 1.5 mg 27H11. However, mice that received 1.5 mg 27H11 plus 1.5 mg 3F11 had anti-PA serum titers that were significantly greater than those in mice that received 1.5 mg 3F11 ($P = 0.0286$) and mice that received 1.5 mg 27H11 ($P = 0.0421$). Anti-LF titers were not significantly different between the various groups that received 9A11 by passive transfer. Since the endpoint ELISA uses twofold serial dilutions, it is likely that the ELISA does not have the needed resolution to determine significant differences in serum ELISA titers between the 3F11 and 9A11 groups, despite up to a fourfold change in antibody transferred between the high (1.5 mg) and the low (0.375 mg) groups.

Serum samples collected at the time of toxin challenge were also tested for their ability to neutralize anthrax LeTx using a standard macrophage LeTx assay (7, 28, 36, 39, 50, 29). Using the standard macrophage LeTx assay, all groups that received anti-PA 3F11 or anti-LF 9A11 antibodies had

detectable LeTx-neutralizing activity in serum at the time of toxin challenge (Table 2). Mice treated with 1.5 mg anti-PA 3F11 or anti-LF 9A11 had LeTx geometric mean NT_{50} titers of 1:100 and $>1:2,048$, respectively ($P = 0.0286$). The neutralization titer in mice receiving 1.5 mg 27H11 ($<1:128$) or 1.5 mg 27H11 plus 1.5 mg 3F11 (1:1,024) was not significantly different than the neutralization titer in mice receiving 1.5 mg 3F11 (1:100) ($P = 0.0689$ and $P = 0.1143$, respectively). However, the neutralization titer in mice receiving 1.5 mg 27H11 plus 1.5 mg 3F11 was significantly greater than the neutralization titer in mice receiving 1.5 mg 27H11 alone ($P = 0.0211$). Mice treated with 0.75 mg 3F11 had an NT_{50} titer of 1:90, while mice treated with 0.75 mg 9A11 had an NT_{50} titer of 1:1,250 ($P = 0.0286$). Mice treated with 0.75 mg 3F11 plus 0.75 mg 9A11 had an NT_{50} titer of 1:1,707, which was significantly greater than the NT_{50} in mice treated with 0.75 mg 3F11 ($P = 0.0286$) and significantly less than the NT_{50} in mice treated with 1.5 mg 9A11 ($P = 0.0286$) but not different from that of mice treated with 0.75 mg 9A11 ($P = 0.4857$). The NT_{50} titer in mice treated with 0.375 mg 3F11 was 1:33, while mice receiving 0.375 mg 9A11 had an NT_{50} titer of 1:634 ($P = 0.0159$). Treatment with 0.375 mg 3F11 plus 0.375 mg 9A11 had an NT_{50} of 1:1,387, which was significantly greater than the NT_{50} in mice treated with 0.375 mg 3F11 ($P = 0.0286$) but not significantly different than the NT_{50} in mice treated with 0.375 mg 9A11 ($P = 0.0635$) or mice treated with 0.75 mg 9A11 ($P = 0.7715$). Thus, LeTx-neutralizing responses were detectable in serum collected 24 h after passive trans-

TABLE 1. Anti-LF and anti-PA Fab binding kinetics^a

Fab/antigen	k_a ($M^{-1} s^{-1}$)	k_d (s^{-1})	K_d (nM)
9A11/LF	1.6×10^4	1.2×10^{-3}	70.1
3F11/PA	4.4×10^3	3.3×10^{-4}	74.8

^a Binding kinetics were derived from global curve-fitting analyses (at least five different Fab concentrations) using the 1:1 Langmuir equation with mass transport limitation as an additional fit parameter. Chi-square values of each fit were below 1.0. Data are representative of two independent experiments.

TABLE 2. IgG ELISA titers (anti-PA and anti-LF) and LeTx neutralization activity of mouse serum collected at the time of LeTx challenge^a

Group	Day 0 body wt (g) $\pm$ SD	Total antibody transferred (mg/kg) $\pm$ SD	Serum anti-PA IgG titer	Serum anti-LF IgG titer	Standard-protocol LeTx NT ₅₀	Toxin-first LeTx NT ₅₀	% Survival
1.5 mg mouse IgG	22.6 $\pm$ 0.8	66.3 $\pm$ 2.5	<1:128	<1:128	<1:128	<1:128	0
1.5 mg 3F11	26.1 $\pm$ 2.0	57.7 $\pm$ 4.7	1:15,464	<1:128	1:100	<1:128	25
1.5 mg 27H11	23.9 $\pm$ 1.9	63.1 $\pm$ 5.4	1:27,544	<1:128	<1:128	<1:128	0
1.5 mg 27H11 + 1.5 mg 3F11	23.8 $\pm$ 0.6	126.4 $\pm$ 3.1	1:82,570	<1:128	1:1,024	<1:128	0
1.5 mg 9A11	22.7 $\pm$ 1.7	66.3 $\pm$ 5.4	<1:128	1:277,732	>1:2,048	1:866	100
0.75 mg 3F11	24.4 $\pm$ 0.6	30.8 $\pm$ 0.8	1:5,161	<1:128	1:90	<1:128	0
0.75 mg 9A11	23.2 $\pm$ 1.8	32.4 $\pm$ 2.5	<1:128	1:82,570	1:1,250	1:347	100
0.75 mg 3F11 + 0.75 mg 9A11 (1.5 mg total)	21.7 $\pm$ 1.7	69.5 $\pm$ 5.8	1:14,596	1:147,123	1:1,707	1:573	100
0.375 mg 3F11	25.1 $\pm$ 1.2	15.0 $\pm$ 0.7	1:10,935	<1:128	1:33	<1:128	0
0.375 mg 9A11	24.1 $\pm$ 1.4	15.6 $\pm$ 0.9	<1:128	1:78,841	1:634	1:154	80
0.375 mg 3F11 + 0.375 mg 9A11 (0.75 mg total)	22.8 $\pm$ 1.0	32.9 $\pm$ 1.4	1:7,732	1:147,123	1:1,387	1:299	100

^a Male BALB/c mice were administered the indicated amount of antibody by intraperitoneal injection (antibodies delivered in 500 μ l sterile PBS). Twenty-four hours after passive transfer of mAb, mice were anesthetized with ketamine-xylazine (90/10 mg/kg), blood was collected and processed, and serum was stored at -20°C until assayed for anti-PA and anti-LF IgG titers by ELISA as well as LeTx-neutralizing antibodies. While under anesthesia, mice were injected intraperitoneally with 200 μ g rPA and 200 μ g rLF in 500 μ l of 1 mg/ml BSA in PBS, mice were monitored daily for signs of morbidity, and mice were euthanized when they had weight loss in excess of 15%. Anti-PA and anti-LF IgG endpoint titers in serum were monitored by ELISA. Animals with anti-PA or anti-LF IgG endpoint titers 1 standard deviation less than the geometric mean titer for their respective group were excluded from analysis.

fer when using a standard LeTx neutralization assay despite a lack of significant protection against LeTx challenge *in vivo*.

Finally, serum collected at the time of toxin challenge was also tested for its ability to neutralize LeTx using the toxin-first macrophage LeTx assay. In contrast to the results obtained when the standard macrophage LeTx assay was performed, none of the serum samples collected 24 h after passive transfer of any dose of anti-PA 3F11 (1.5 mg, 0.75 mg, or 0.375 mg) or 27H11 plus 3F11 had detectable LeTx-neutralizing antibody when tested in the toxin-first LeTx neutralization assay (Table 2). Serum collected from mice that received 1.5 mg anti-LF 9A11 had a geometric mean NT₅₀ titer of 1:866 when tested using the toxin-first neutralization assay, which was significantly greater ($P = 0.0286$) than the NT₅₀ titer (1:347) in the serum of mice that received 0.75 mg 9A11. Mice treated with 0.75 mg anti-PA 3F11 and 0.75 mg anti-LF 9A11 (total antibody dose of 1.5 mg) had an NT₅₀ titer of 1:573, which was not significantly different from that of mice treated with 0.75 mg 9A11 (1:347) ($P = 0.3429$) and not significantly different from that of mice receiving 1.5 mg 9A11 (1:866) ($P = 0.0814$). The NT₅₀ titer of serum collected from mice passively transferred with 0.375 mg 9A11 (1:154) was not significantly different from the NT₅₀ of mice that received 0.75 mg 9A11 (1:347) ($P = 0.1111$). Mice treated with 0.375 mg anti-PA 3F11 and 0.375 mg anti-LF 9A11 (0.75 mg total antibody) had an NT₅₀ of 1:299, which was significantly greater than the NT₅₀ of mice treated with 0.375 mg 9A11 (1:154) ($P = 0.0159$) but not significantly different from the NT₅₀ of mice treated with 0.75 mg 9A11 (1:347) ($P = 0.3836$). Thus, the use of a modified LeTx neutralization assay that adds LeTx to the indicator macrophage cells 15 min before the addition of sample provided a robust LeTx neutralization assay that detected LeTx neutralization responses only in samples collected from mice that were significantly protected against *in vivo* LeTx challenge.

DISCUSSION

In this study, we have shown that anti-PA (3F11) and anti-LF (9A11) mAbs with LeTx-neutralizing activity *in vitro* and similar binding affinities were significantly different in their abilities to provide passive protection against LeTx challenge in mice. Although passive transfer of 1.5 mg anti-PA mAb 3F11 or 1.5 mg 3F11 combined with 1.5 mg anti-PA 27H11 resulted in detectable LeTx-neutralizing activity in the serum of mice at the time of LeTx challenge, as measured with a standard LeTx neutralization assay, there was no significant protection against LeTx-induced morbidity. Mice that received anti-PA mAb (3F11 alone or combined with 27H11) by passive transfer had no detectable LeTx neutralization activity in serum 24 h after passive transfer when a toxin-first LeTx neutralization assay was utilized; the toxin-first assay adds LeTx to the J774A.1 indicator cells 15 min before the addition of antibody and is a more stringent neutralization assay. In contrast to the results obtained with the anti-PA mAbs, the passive transfer of 0.375 mg anti-LF mAb 9A11 provided significant protection against LeTx challenge when transferred to mice 24 h before toxin challenge, and LeTx neutralization activity was detectable in the serum at the time of LeTx challenge as measured by both the standard and toxin-first LeTx neutralization assays. Our results suggest that the presence of LeTx-neutralizing antibody responses as detected by a standard LeTx neutralization assay does not equate with protection against an LeTx challenge and that the use of a toxin-first LeTx neutralization assay provides a more stringent assay for use in predicting protective humoral immunity in the serum of passively immunized hosts.

Passive transfer of anti-PA or anti-LF mAb or polyclonal antibody raised in response to immunization has the potential to protect against anthrax spore challenge (3, 27, 32, 38, 46, 51), and protection correlates with LeTx-neutralizing antibody titers (46, 55). Passive transfer of anti-PA mAb (10, 18, 29, 34, 56) or anti-LF mAb (26, 30, 59) is also able to protect against

FIG. 4. In vitro LeTx neutralization activity of anti-PA mAb 3F11 and anti-LF mAb 9A11 alone and in combination. (A) A standard LeTx neutralization assay was performed by combining 3F11 and/or 9A11 with PA/LF before adding the antibody-PA/LF mixture to the J774A cells (see Materials and Methods for details). Multiple concentrations of each antibody were tested, and percent LeTx neutralization was plotted versus antibody concentration to calculate the concentration of antibody needed to neutralize 50% of the LeTx activity (NC₅₀) (mean $\pm$ standard error). Measurements are based on seven to eight individual neutralization assays. (B) A toxin-first LeTx neutralization assay was performed by adding LeTx to the J774A cells for 15 min before the addition of 3F11 and/or 9A11 (see Materials and Methods for details). Multiple concentrations of each antibody were tested, and percent LeTx neutralization was plotted versus antibody concentration to calculate the concentration of antibody needed to neutralize 50% of the LeTx activity (NC₅₀) (mean $\pm$ standard error). Measurements are based on seven to eight individual neutralization assays.

challenge with LeTx, but correlates of protective immunity after passive transfer have not been evaluated in an LeTx challenge model. The conditions utilized in the performance of the LeTx neutralization assay may be important for the ability of the assay to correlate with protection in vivo. In our LeTx neutralization assay, we utilized a final concentration of rPA and rLF of 187.5 ng/ml. For each LeTx neutralization assay, an LeTx standard curve was performed with the rPA/rLF final concentrations starting at 1.5 μ g/ml and progressing by twofold serial dilutions to 5.8 ng/ml rPA/rLF to ensure that the assay was performing reproducibly. The use of rPA and rLF at a final concentration of 187.5 ng/ml was always four- to eightfold more toxin than what is needed to kill 100% of the J774A.1 cells. Others have used rPA and rLF at a final concentration of

80 ng/ml to produce 100% killing of J774A.1 cells in the LeTx neutralization assay (39). However, the concentrations of PA and LF utilized by others for this assay vary widely, with ranges of 100 to 1,000 ng/ml PA and 0.01 to 10,000 ng/ml LF being used (9, 18, 26, 30, 34, 56, 59).

In our model, we utilized the passive transfer of individual anti-PA and anti-LF mAbs as well as combinations of anti-PA mAbs or anti-PA plus anti-LF mAbs. Of interest was the observation that the combination of anti-PA 3F11 and anti-LF 9A11, both LeTx neutralizing, did not provide additive in vitro LeTx-neutralizing activity in the toxin-first LeTx neutralization assay (Fig. 4B). Since anti-LF 9A11 was so potent in vivo, it was not possible to determine if the combination of anti-PA 3F11 and anti-LF 9A11 provided protection superior to that of

FIG. 5. In vivo protective activity of anti-PA mAb 3F11 and anti-LF mAb 9A11 alone and in combination. Male BALB/c mice were injected by the intraperitoneal route with the indicated amounts of anti-rPA and/or anti-rLF mAb in 500 μ l sterile PBS. Twenty-four hours after passive transfer of mAb, mice were anesthetized with ketamine-xylazine (90/10 mg/kg), and blood was collected. While under anesthesia, mice were injected with 200 μ g rPA plus 200 μ g rLF in 500 μ l of 1 mg/ml BSA in PBS by the intraperitoneal route. Mice were monitored daily for signs of morbidity. Mice were considered to be moribund when they had lost

9A11 alone when using the mouse LeTx challenge model (Fig. 5 and Table 2). The serum LeTx NT₅₀ in mice receiving 0.375 mg 9A11 was 1:154, while the LeTx NT₅₀ in mice receiving 0.375 mg 9A11 plus 0.375 mg 3F11 (total of 0.75 mg antibody) was 1:299, similar to the LeTx NT₅₀ in mice that received 0.75 mg 9A11 alone (1:347) (Table 2). Therefore, it seems likely that the combination of anti-PA 3F11 and anti-LF 9A11 provided no benefit over that provided by simply increasing the dose of anti-LF used alone. Others reported previously that passive transfer of a combination of two anti-PA mAbs and one anti-LF mAb protected 100% of mice against an anthrax spore challenge, while only 50% protection was observed with the best combinations of two of the antibodies and while one combination of two antibodies provided no protection (9). Cocktails of mAbs have also been reported to be superior to individual mAbs for protection against BoNT (42), tetanus toxin (60), and virus neutralization (4, 25, 58). It is likely that many variables influence the ability of antibody combinations to provide additive/synergistic protective activity, including epitope specificity (39), binding affinity of the mAb (34), and antibody isotype.

In our model, anti-PA and/or anti-LF mAb was passively transferred to mice by the intraperitoneal route 24 h before LeTx challenge with 200 μ g rPA plus 200 μ g rLF, also by the intraperitoneal route, as used by others previously (37). This method allowed us to (i) collect serum at the time of LeTx challenge to search for correlates of protective immunity and (ii) utilize a passive transfer method that has potential for clinical application. The transfer of mAb 24 h before LeTx challenge allowed time for the antibody to disseminate throughout the host tissues and also allowed us to determine if passively transferred LeTx-neutralizing antibody would provide protection 24 h after transfer. Passive transfer methods used to evaluate the protective activity of anti-PA or anti-LF mAbs vary considerably. In one mouse model, 20 μ g PA and 4 μ g LF with or without antibody were administered i.v. (59). When rats are used as the host, antibody is often preincubated with PA (40 μ g) and LF (8 μ g) in vitro before the injection of the antibody-LeTx mixture by the i.v. route (26, 29, 30, 56). Other rat models inject the antibody of interest i.v. followed by the injection of LeTx after 5 min (34). Our intraperitoneal challenge model utilized much more PA and LF than is typically used in rat LeTx models. The influence of animal host (rat versus mouse), route of LeTx administration, and amount of PA and LF used for LeTx challenge on protection observed after passive transfer is not clear. We believe that the passive transfer of the antibody of interest 24 h before LeTx challenge provided a more stringent assessment of the ability of the

15% of their day 0 body weight and were humanely euthanized. (A) Survival after passive transfer of 1.5 mg control mouse IgG ($\square$), 1.5 mg 3F11 ($\blacklozenge$) ($P = 0.444$ versus 1.5 mg IgG), or 1.5 mg 9A11 ($\circ$) ($P = 0.0079$ versus 1.5 mg IgG). (B) Survival after passive transfer of 0.75 mg 3F11 ($\blacklozenge$), 9A11 ($\circ$) ($P = 0.0079$ versus 1.5 mg IgG), 3F11 plus 9A11 ($\blacktriangle$) (total, 1.5 mg) ($P = 0.0079$ versus 1.5 mg IgG), or 1.5 mg control mouse IgG ($\square$). (C) Survival after passive transfer of 0.375 mg 3F11 ($\blacklozenge$), 9A11 ($\circ$) ($P = 0.0476$ versus 1.5 mg IgG), 3F11 plus 9A11 ($\blacktriangle$) (total, 0.75 mg) ($P = 0.0079$ versus 1.5 mg IgG), or 1.5 mg control mouse IgG ($\square$).

antibody to protect against LeTx and allowed us a method to better define correlates of immunity.

The affinity (binding of monovalent ligands such as single-chain antibodies or Fab fragments) or avidity (binding of multivalent ligands such as IgG molecules) of anti-PA and anti-LF mAbs may influence the protection observed after passive transfer and challenge with LeTx or anthrax spores. The binding avidity of the intact anti-LF mAb 9A11 used for passive transfer was 9 nM. To better define the binding affinity of each antigen-binding portion of the IgG molecule, Fab fragments were produced for anti-LF 9A11 and anti-PA 3F11, and the K_d values determined by SPR were found to be 70.1 and 74.8 nM, respectively (Table 1). Others reported previously that protection against anthrax toxin by antibody correlates with their binding affinity for antigen (34). Anti-PA single-chain variable fragments (scFvs) and scFvs fused to human constant κ domains with K_d values between 0.25 and 63 nM were tested for their ability to protect rats against an LeTx challenge; scFvs with K_d values of 0.25, 3, and 12 provided measurable protection, while the scFv with a K_d of 63 nM did not (34). Others reported previously that human anti-PA Fabs with K_d values of 0.13 and 0.87 nM provided protection against LeTx in a rat challenge model (56). mAbs that were protective against LeTx challenge in rats had a K_d of 2.62 (26). These results indicate that the binding affinity/avidity of anti-LF or anti-PA mAb is one important variable associated with protection due to the passive transfer of LeTx-neutralizing mAbs. However, other variables, such as epitope specificity, are likely important parameters associated with the neutralization of LeTx in vivo, since Fab fragments produced from our anti-LF 9A11 and anti-PA 3F11 had similar K_d values as determined by SPR (70.1 and 74.8 nM, respectively), and 9A11 provided significant protection in vivo when as little as 0.375 mg/mouse was transferred, while 3F11 did not protect when 1.5 mg/mouse was transferred.

In summary, our results indicate that serum antibody ELISA titers and serum LeTx neutralization titers obtained using a standard macrophage LeTx neutralization assay that preincubated antibody sample with PA and/or LF were not an accurate correlate of protective immunity for LeTx in a mouse model. However, serum LeTx neutralization titers obtained using a toxin-first macrophage LeTx neutralization assay that preincubates LeTx and macrophage target cells for 15 min before the addition of antibody sample provided LeTx neutralization titers that proved to be a more reliable correlate of protective immunity in an LeTx mouse model. This assay may be useful to monitor vaccine-induced and passive immunity to anthrax LeTx.

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