Reduction of Immunogenicity of Anthrax Vaccines Subjected to Thermal Stress, as Measured by a Toxin Neutralization Assay[▽]

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We report that a toxin neutralization assay (TNA) can detect a decrease in the immunogenicity of anthrax vaccines as a consequence of brief exposure to elevated temperature. This attribute of TNA may help in adopting immunogenicity as a replacement of the current potency test, which involves protection from lethal challenge.

All anthrax vaccine that enters the Strategic National Stockpile must maintain its potency through protracted storage periods. Thus, robust, practical, and meaningful potency tests are essential to evaluate vaccine manufacturing consistency and stability. The stability of protective antigen (PA), a major antigen included in anthrax vaccines, is critical to establish the suitability of a formulation for long-term storage. PA is denatured at temperatures as low as 40°C (4, 7, 9), which can jeopardize vaccine potency if the product is heated at any time prior to its administration, even during manufacturing. The current potency assay for anthrax vaccines is an active protection test that consumes many animals and requires security and biosafety measures because of the use of virulent *Bacillus anthracis*. To circumvent these drawbacks, immunogenicity assays have been developed in which lethal challenge is replaced with the measurement of differential antibody induction using *in vitro* tests.

We assessed whether a toxin neutralization assay (TNA) can detect changes in antibody response as a consequence of the exposure of an experimental recombinant PA vaccine (rPAV) and BioThrax, a commercial vaccine, to high temperatures for brief periods. The number of possible time/temperature combinations to which a vaccine can be exposed before use is very high. Therefore, we selected a few combinations to model the possibility of using murine immunogenicity to detect anthrax vaccine exposure to nonideal storage conditions.

We used a published method (3), slightly modified, to measure neutralizing activity in mouse sera. The reference serum and samples were prediluted with Dulbecco's modified Eagle medium (DMEM) and serially diluted (1:2) in a 96-well microtiter plate. Predilution was made to achieve full neutralization curves, i.e., to obtain upper and lower asymptotes. Lethal toxin (LT; 100 ng/ml of PA plus 80 ng/ml of lethal factor in DMEM) was added, and mixtures were incubated (37°C and 5% CO_2) for 30 min. One well in each column contained only the sample at the lowest dilution tested (sample control [SC]). One column contained normal mouse serum diluted 1:25 in DMEM. Toxin activity was confirmed by the addition of LT to four wells (LT control), while four wells were used to verify cell viability (reagent control). Toxin-serum mixtures were added to J774A.1 cells seeded in a second 96-well plate (40% to 60% confluence) and incubated (37°C and 5% CO_2) for 4 h. Cell viability was estimated with a vital dye, MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] (2). The absorbance per well (determined as the change in optical density [ΔOD] at 570 and 690 nm) was transformed to the percentage of neutralization ($N\%$) as follows: $N\% = 100(OD - OD_{min}/OD_{max} - OD_{min})$, where OD_{max} and OD_{min} represent the maximum and minimum ΔOD, respectively, in each titration curve. For incomplete curves (no upper asymptote), SC's ΔOD was used as OD_{max}. Four-parameter logistic regression was performed, constraining the upper and lower asymptotes to 100% and 0% neutralization, respectively.

TNA validity was assessed using the following criteria: the average ΔOD for LT control wells should be <0.4, and the average ΔOD for reagent control wells should be >0.8. The reference curve should have an OD_{max} of >0.8 and an OD_{min} of <0.4, and the value of OD_{max} minus OD_{min} should be >0.8. The coefficient of determination (r^2) for the reference should be ≥0.99. The percent coefficient of variation (%CV) of the two ΔOD values at each dilution of the reference curve must be less than 25%.

Samples with an OD_{max} of ≤0.4 were considered to have 0% neutralization. Nonmonotonic response at the upper plateau was eliminated by imputing 100% neutralization to dilutions lower than those corresponding to an OD_{max} that gave a lower ΔOD reading. Parallelism was assessed by the r^2 (6) and the dilution effect (DE) method (5). The neutralizing activity (U/ml) for samples that met either parallelism criterion was calculated by multiplying the neutralization factor ($NF_{50} = ED_{50 \text{ sample}}/ED_{50 \text{ reference}}$, where ED_{50} represents the reciprocal of the dilution corresponding to 50% neutralization read from each fitted curve [sample and reference serum]) by the activity of the reference serum (MR02-2; 713 U/ml), which is an anti-PA, freeze-dried, immunoglobulin-enriched preparation obtained by immunizing mice multiple times with an experimental rPAV (1).

Initially, we measured the toxin-neutralizing activity in serum samples collected 28 days postimmunization from two groups of mice immunized with a single dose of either Bio-Thrax (0.2 ml intraperitoneally [i.p.]) or rPAV (formulated at

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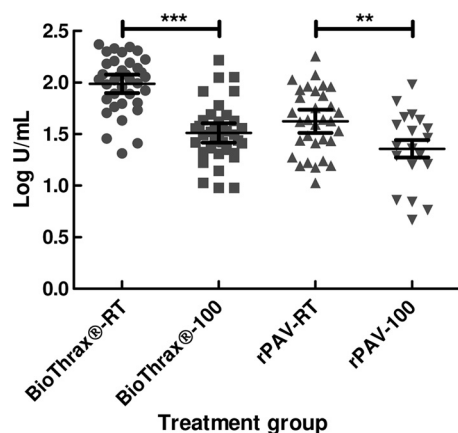
FIG. 1. Serum PA-neutralizing activity in mice (40 animals per group) immunized with anthrax vaccines subjected to high temperature. Vaccines were heated at 100°C for 2 min. RT, control group (BioThrax stored at 2 to 8°C or rPAV freshly formulated at 25°C); 100, exposure to 100°C. Log_{10} neutralizing activity (U/ml) is shown on the y axis. Pairs of mean log_{10} neutralizing activities by vaccine type were compared by Student's t test. $**$, $P < 0.01$; $***$, $P < 0.001$. The central horizontal line in each group represents the mean of the log_{10} neutralizing activities (U/ml), and the two additional horizontal lines represent the limits of the 95% confidence interval (CI) for each estimate. Activities in U/ml (95% CI): BioThrax-RT, 97.9 (78.6 to 115.03); BioThrax-100, 32.4 (26.1 to 40.3); rPAV-RT, 44.4 (34.4 to 57.1); rPAV-100, 21.8 (14.7 to 32.4).

25°C; 3 μg i.p.) and from two groups immunized with each vaccine heated at 100°C for 2 min. We found significant decreases (rPAV, $P = 0.004$, and BioThrax, $P < 0.0001$ [Student's t test]) in immunogenicity in the groups immunized with the heated vaccines (Fig. 1). Interestingly, BioThrax incubated at 100°C for 2 min (Fig. 1, Biothrax-100) elicited neutralizing titers that were similar to the neutralizing titers elicited by unheated rPAV (Fig. 1, rPAV-RT). This effect can be attributed to a larger amount of PA in BioThrax (its exact content is unknown) or to the presence of other antigens in this vaccine that may induce a neutralizing response.

To study the effects of exposure to milder temperatures, we performed a supplementary experiment using 45°C and 70°C, keeping 100°C as a positive control. We found an inversely proportional decrease in immunogenicity with each temperature relative to that elicited by untreated rPAV (Fig. 2A) (45°C, $P = 0.049$; 70°C, $P = 0.011$; and 100°C, $P < 0.001$ [Dunnett's test]). While Reuveny et al. (8) showed that exposure of rPAV to 40°C during 6 days caused a 3-fold decrease of its immunogenicity in guinea pigs, as measured by TNA, we could detect exposure to an analogous temperature for a period as brief as 2 min. Remarkably, relatively elevated neutralizing immunogenicity remained even after exposure of the vaccines to the highest temperature tested (100°C).

The responses induced by heated vaccines generated titration curves that were nonparallel to the reference curve more often than those corresponding to untreated vaccine (Table 1). This was most prominent at the highest temperatures. When the r^2 method was used to evaluate parallelism, significant decreases in immunogenicity were detected only at 70°C and 100°C (Fig. 2B) (45°C, $P = 0.456$; 70°C, $P = 0.029$; and 100°C, $P = 0.019$ [Dunnett's test]). The nonparallelism of antibody
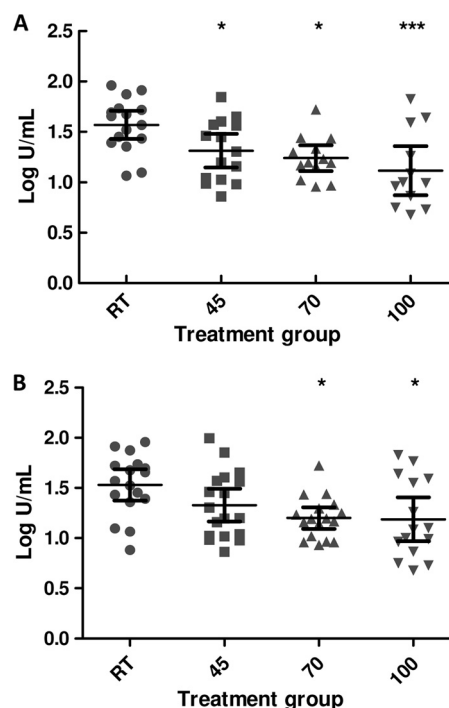

FIG. 2. Serum PA-neutralizing activities in mice immunized with rPAV subjected to different temperatures (20 animals per group). Vaccines were heated at the indicated temperatures (°C, on the x axis) for 2 min. RT, control group (freshly formulated rPAV at 25°C). Log_{10} neutralizing activity (U/ml) is shown on the y axis. (A) Serum activities, excluding nonparallel curves, according to the DE method. (B) Serum activities, excluding nonparallel curves, according to the r^2 method. Mean log_{10} neutralizing activities were compared by Dunnett's multiple comparison test, using the RT group as the control group. $*$, $P < 0.05$; $***$, $P < 0.001$. Activities in U/ml (95% CI): (A) RT, 37.1 (26.9 to 51.3); 45, 20.6 (14.0 to 30.2); 70, 17.4 (12.9 to 23.3); and 100, 13.0 (7.4 to 22.9); (B) RT, 29.8 (19.3 to 46.0); 45, 21.3 (14.6 to 31.1); 70, 14.8 (11.3 to 19.5); and 100, 13.9 (8.3 to 23.2).

titration curves may be additional, albeit indirect evidence of structural alterations of PA.

The data presented herein, along with results obtained by other authors (4, 8, 10), support the concept that TNA can quantify differences among anthrax vaccine batches that have been altered by exposure to high temperature during storage. However, these experiments represent only a first step toward

TABLE 1. Samples excluded from analysis, by treatment group

Treatment group[a]	No. of samples excluded for indicated reason		
	Nonresponder[b]	TNA nonparallel curves[c]	
		DE	r^2
RT	1	3	1
45	1	4	1
70	1	5	1
100	3	5	1

[a] RT, room temperature (25°C); 45, 70, and 100, high temperatures (°C) (2-min exposure).
[b] Serum samples with an OD_{max} of <0.4.
[c] Procedure for curve rejection was as described in references 5 (DE, dilution effect) and 6 (r^2, coefficient of determination).

the development of an alternative potency test for anthrax vaccines, based on their immunogenicity for the mouse. Further refinements of the procedure will include assigning unitage to a standard reference serum, the production of a reference vaccine, and the establishment of acceptance criteria based on the behavior of vaccine lots that have been shown to be efficacious in the target population.

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