MAJOR ARTICLE

Late Treatment with a Protective Antigen–Directed Monoclonal Antibody Improves Hemodynamic Function and Survival in a Lethal Toxin–Infused Rat Model of Anthrax Sepsis

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Background. In animal models, treatment with 5H3, a fully human protective antigen–directed monoclonal antibody (PA-MAb), improved survival when administered close to the time of *Bacillus anthracis* lethal toxin (LeTx) bolus or live bacterial challenge. However, treatment with PA-MAb would be most valuable clinically if it were beneficial even when administered after the onset of shock and lethality due to LeTx.

Methods. We investigated the effects of PA-MAb versus placebo administered in rats ($n = 324$) at the time of or 3, 6, 9, or 12 h after the initiation of a 24-h LeTx infusion.

Results. In rats receiving placebo, mean arterial blood pressure (MBP) and heart rate (HR) were decreased in nonsurvivors, compared with those in survivors, at 6 h and then worsened further, with lethality first evident at 8 h (median, 16 h; range, 8–152 h). At each treatment time, survival rates were greater for PA-MAb than for placebo, although improvement was decreased at later treatment times ($P = .001$, for the effect of time). Compared with placebo, PA-MAb significantly increased MBP during the 12 h after the initiation of treatment, but the increase was greatest for treatment at 3 h; similarly, PA-MAb significantly increased HR at all treatment times.

Conclusion. In this rat model, improvements in outcome due to PA-MAb were significant when it was administered up to 6 h (and approached significance when administered up to 12 h) after initial exposure to LeTx. Clinically, PA-MAb may be beneficial even when administered after the onset of shock and lethality due to LeTx.

Inhalational *Bacillus anthracis* infection is a highly lethal bioterrorism-related health threat today [1–4]. During the recent outbreak of inhalational *B. anthracis*, death in patients was complicated by progressive septic shock, despite treatment with appropriate antibiotics and aggressive hemodynamic support [3, 4]. Production and release of lethal toxin (LeTx) into the intravascular space and subsequent systemic spread were believed to be central to the pathogenesis of the shock and organ injury that occurred during these infections [5, 6]. LeTx consists of 2 components: protective antigen (PA), which is necessary for the receptor-specific uptake of toxin by host cells, and lethal factor, the enzymatic moiety. In several different animal models, administration of purified preparations of LeTx is lethal [7, 8]. Furthermore, strains of *B. anthracis* in which either PA or lethal factor has been inactivated are 1000 times less lethal than fully active strains [9]. Thus, adjunctive therapies designed to inhibit the effects of LeTx may improve outcome during conventional treatment of anthrax sepsis. Because the patients infected with anthrax during the recent outbreak frequently presented with evidence of established shock, these adjunctive therapies must be effective even when initiated during advanced stages of infection.

5H3 is a fully human PA-directed monoclonal antibody (PA-MAb). Administration of PA-MAb before

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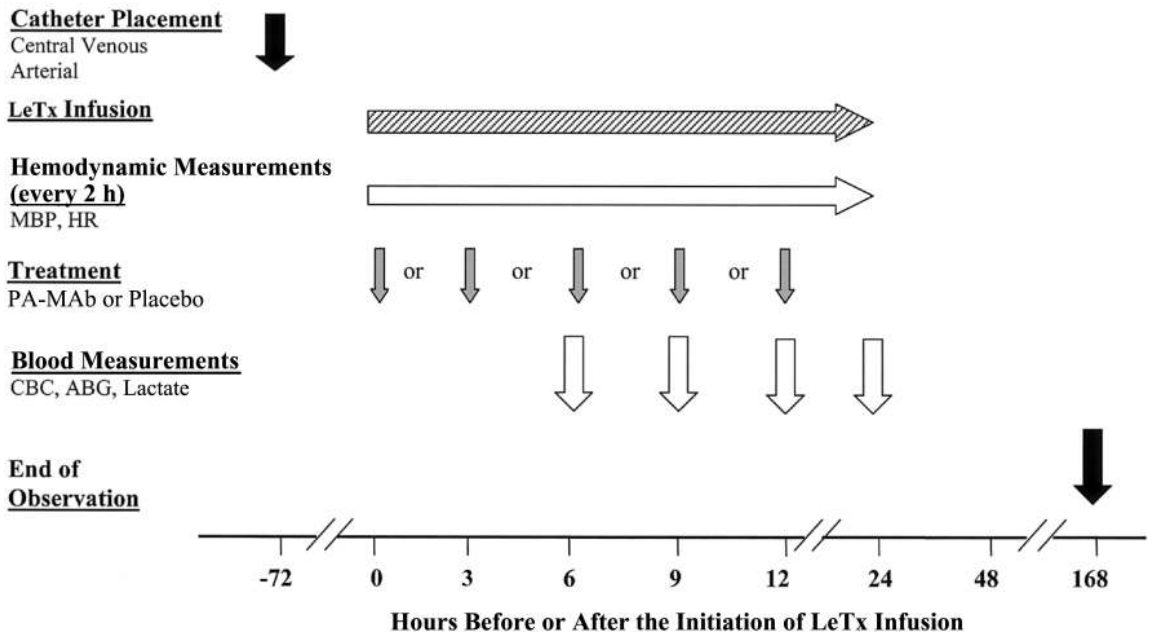

Figure 1. Time course of catheter placement, *Bacillus anthracis* lethal toxin (LeTx) infusion, administration of protective antigen–directed monoclonal antibody (PA-MAb) or placebo (nonspecific antibody), and laboratory measurements. Catheters were placed in rats under isoflurane anesthesia, and the rats were equilibrated for a total of 72 h. They then received 1 dose of ketamine and xylazine (see Materials and Methods) for attachment of catheters to infusion lines and transducers, were awakened, and received no other anesthesia during the remainder of the study. Treatment was administered at the time of or 3, 6, 9, or 12 h after the initiation of LeTx infusion. ABG, arterial blood gas; CBC, complete blood cell count; HR, heart rate; MBP, mean arterial blood pressure.

or within 1 h of inhalation of *B. anthracis* spores improved survival significantly in rabbits and primates [10]. In a conventional Fisher 344 rat model of anthrax sepsis that used an intravenous (iv) bolus of LeTx, administration of PA-MAb before or within 30 min of injection of LeTx also improved survival significantly [11]; however, for later treatment with PA-MAb, similar beneficial effects were not observed in this rat model, likely because rapid death (mean time to death, 1.5 h) after injection of LeTx negated the possibility of observing significant improvements in survival.

To test the effects of later treatment with PA-MAb after challenge with LeTx, we used a conscious Sprague-Dawley rat model in which LeTx was administered via a 24-h infusion rather than a rapid bolus. This model was originally developed to simulate the gradual intravascular release of LeTx that occurs during sepsis due to infection with *B. anthracis* via inhalation or other routes [12]. In this model, levels of LeTx are comparable with those measured in lethal rodent models of live bacterial infection [12]. During the original development of the model, experiments were conducted to establish a dose of LeTx that, when infused over the course of 24 h, would result in 50% mortality, with the initial deaths not occurring until later time points [12]. This 24-h infusion produced a mortality time course (initial death at 9 h) that was very different from the rapid one (1–2 h) produced by a similarly lethal iv bolus of LeTx. Infusion of LeTx caused reductions in blood pressure that were first evident at 6 h and that worsened at later time points as mortality became evident.

In the present study, Sprague-Dawley rats were infused with LeTx according to a regimen that was similar to the one studied previously. Rats were then treated with PA-MAb or placebo at the time of (0 h) or 3, 6, 9, or 12 h after the initiation of LeTx infusion. This model permitted us to study the effects that later treatment with PA-MAb has on survival rates and cardiovascular dysfunction.

MATERIALS AND METHODS

Animal care. The protocol used in the present study was approved by the Animal Care and Use Committee of the Clinical Center of the National Institutes of Health. During the study, every effort was made to minimize the suffering of rats. The research protocol required the veterinarian staff or investigators to kill any rat that became moribund or experienced unexpected pain or distress.

Study design. Sprague-Dawley rats ($n = 324$; weight, 200–250 g), under isoflurane anesthesia, had carotid arterial and internal jugular central venous catheters placed by an animal supplier (Charles River Laboratory). The rats were awakened, equilibrated for 36 h, shipped for laboratory use, and equili-

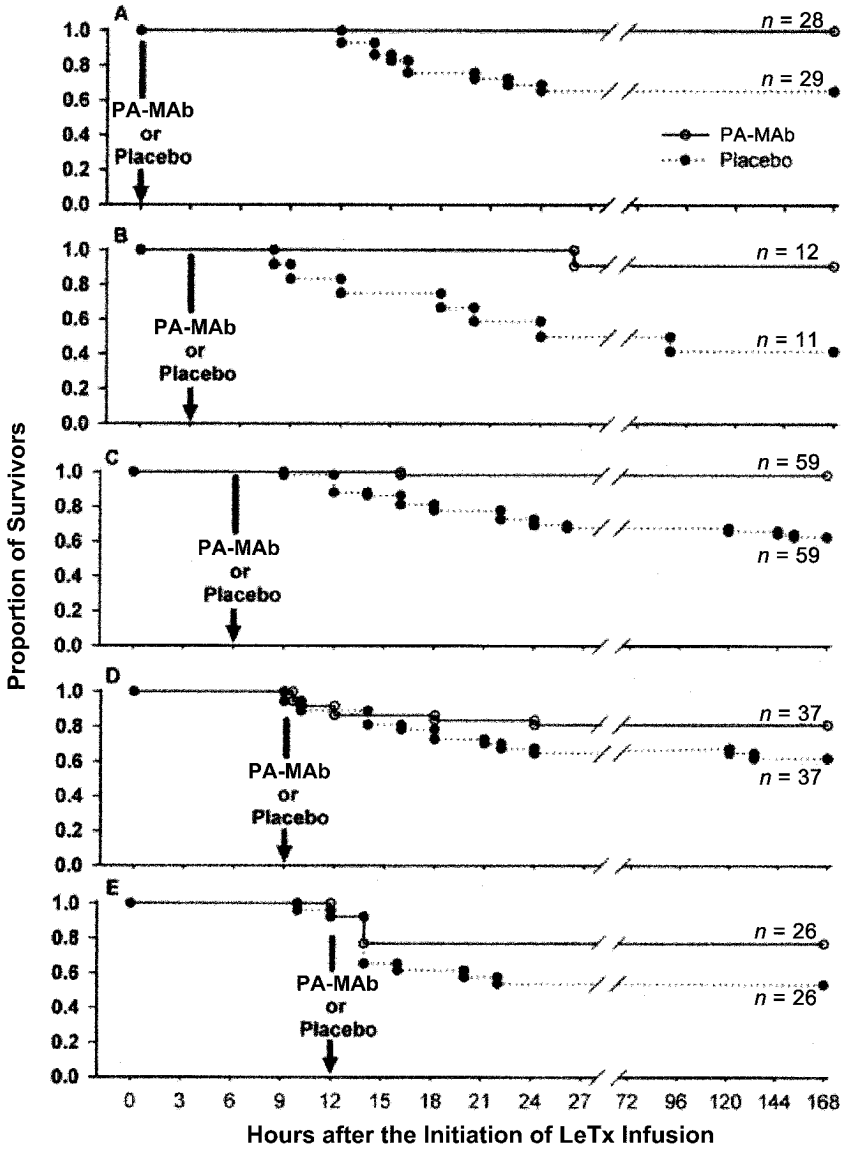

Figure 2. Proportion of rats surviving over time after administration of protective antigen–directed monoclonal antibody (PA-MAb) or placebo (nonspecific antibody) at the time of or 3, 6, 9, or 12 h (*A–E,* respectively) after the initiation of *Bacillus anthracis* lethal toxin (LeTx) infusion. Levels of significance for the effects that PA-MAb had on survival rates for each of these treatment times are shown in figure 3.

brated for an additional 36 h. The rats were then anesthetized by use of a single dose of ketamine (40 mg/kg) and xylazine (10 mg/kg), and the indwelling catheters were connected to transducers and infusion systems (figure 1). Immediately after the catheters were connected, the rats began receiving 24-h LeTx infusions, with total doses of lethal factor and PA of 150 and 300 μg/kg, respectively, administered in 0.5 mL of PBS/hour. The rats awakened during the 1–2-h period after the connection of catheters and received no other anesthesia during the remainder of study. At the time of or 3, 6, 9, or 12 h after the initiation of LeTx infusion, the rats were randomized to be treated iv either with PA-MAb in a single bolus that was 10 times the molar amount of PA administered (hereafter, "10×") or with an equivalent dose of placebo (nonspecific antibody) in 0.5 mL of diluent. At 2-h intervals during the LeTx infusion, mean arterial blood pressure (MBP) and heart rate (HR) were measured in the rats. The rats receiving treatment at 3 h did not have hemodynamic measurements obtained at 12–22 h. In randomly selected rats ($n = 52$) treated with placebo or PA-MAb at 6 h, in addition to hemodynamic measurements, complete blood cell counts and arterial blood gas measurements were obtained immediately before treatment and at 9, 12, and 24 h after the initiation of LeTx infusion. At each sampling, 0.45 mL of blood was removed and then replaced with an equivalent volume of normal saline. The volumes of blood drawn and replaced with saline were similar in all rats. To study

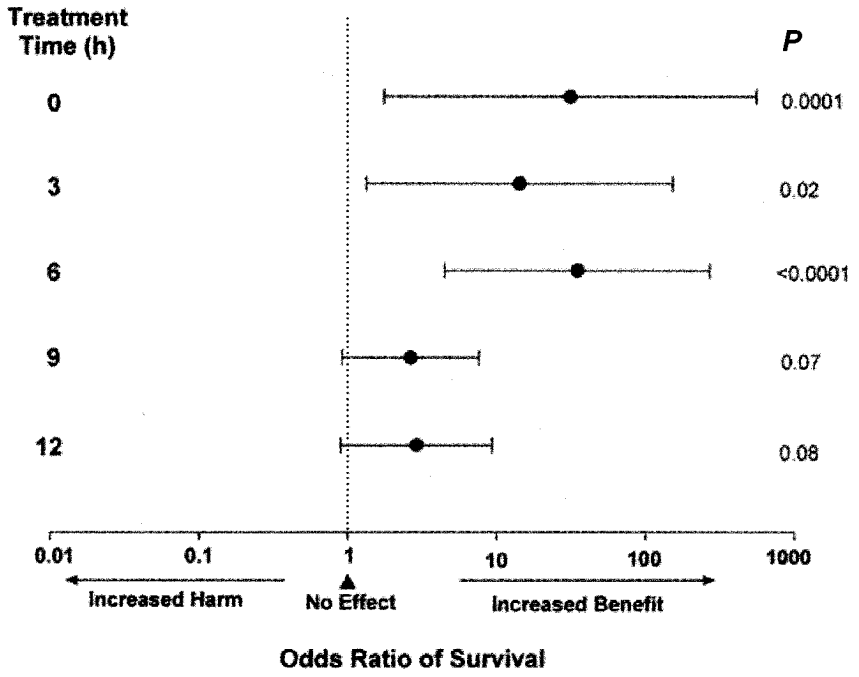

Figure 3. Effects that protective antigen–directed monoclonal antibody (PA-MAb) administered at the time of (0 h) or 3, 6, 9, or 12 h after the initiation of *Bacillus anthracis* lethal toxin (LeTx) infusion had on the odds ratios (ORs) of survival. At each treatment time, the ORs of survival was increased in the rats receiving PA-MAb, although the increases were not as great for treatment at 9 and 12 h ($P < .001$, for the effect that PA-MAb had on treatment time). Bars indicate 95% confidence intervals.

the effect of reducing the dose used for later treatment with PA-MAb, in additional experiments, 6 h after the initiation of LeTx infusion, rats ($n = 115$) were randomized to receive either PA-MAb (in doses of 10×, 1×, 0.5×, 0.1×, or 0.05×) or placebo, each in a similar volume of 0.5 mL. In individual experiments, although the rats receiving each dose were compared directly with control rats receiving placebo, not all rats receiving the doses were compared with the same control rats. All rats were observed for 168 h.

LeTx preparation. The PA and lethal factor used were recombinant proteins prepared from *B. anthracis* under lipopolysaccharide (LPS)–free conditions, as described elsewhere [13, 14]. Rat albumin (25 μg/mL) was used to maintain the stability of PA and lethal factor in LPS-free PBS.

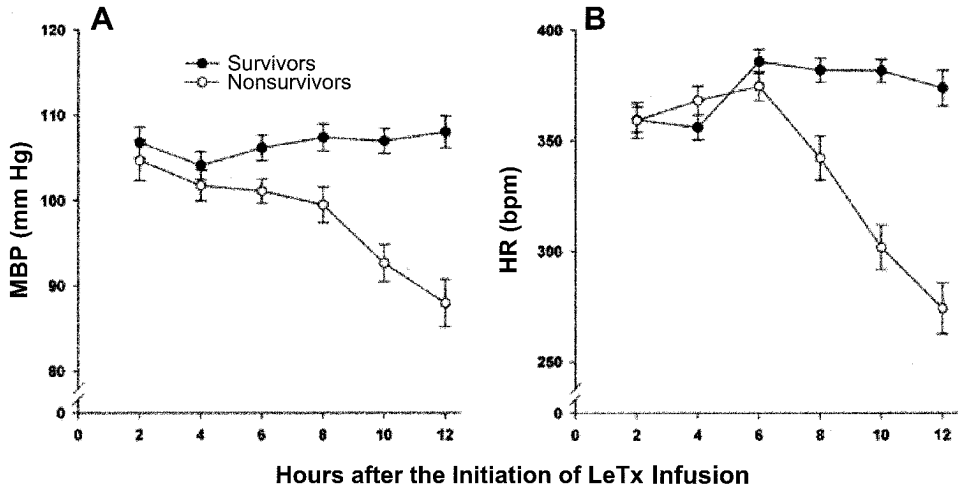

Figure 4. Mean arterial blood pressure (MBP; *A*) and heart rate (HR; *B*) during the first 12 h of *Bacillus anthracis* lethal toxin (LeTx) infusion. Shown are surviving and nonsurviving rats that received placebo only. From 6 to 12 h, both parameters decreased in nonsurvivors, compared with those in survivors ($P < .001$, for both parameters when the values were averaged over the period from 6 to 12 h). Data are mean ± SE. bpm, beats per minute.

Table 1. Arterial pH, base excess, and lactate levels (mean ± SE) in surviving and nonsurviving rats receiving placebo (nonspecific antibody) only at 6, 9, and 12 h after the initiation of *Bacillus anthracis* lethal toxin (LeTx) infusion.

	Hours after the initiation of LeTx infusion		
Parameter, survival group	6	9	12
pH level			
Survivors	7.500 ± 0.005	7.491 ± 0.008	7.469 ± 0.004
Nonsurvivors	7.487 ± 0.005	7.436 ± 0.007	7.369 ± 0.002
Base excess level, mmol/L			
Survivors	0.61 ± 0.48	−0.47 ± 0.95	−1.31 ± 0.41
Nonsurvivors	1.61 ± 0.49	−0.83 ± 0.65	−5.10 ± 2.65
Lactate level, mmol/L			
Survivors	0.91 ± 0.04	1.24 ± 0.28	0.70 ± 0.04
Nonsurvivors	1.02 ± 0.07	0.80 ± 0.14	2.30 ± 1.01

PA-MAb preparation and dose selection. 5H3 (Human Genomic Sciences) was used. The volumes of PA-MAb and placebo administered at each time point were the same. Initial pilot studies indicated that doses of PA-MAb of 1–10× improved survival when administered at the time of the initiation of LeTx infusion. To test the effects of later treatment with PA-MAb, the higher dose from these initial pilot studies was used, to maximize the chances of observing a beneficial effect. In the later experiments, in which treatment was administered at 6 h after the initiation of LeTx infusion, doses of PA-MAb as low as 0.05× were tested.

Laboratory measurements. Catheters protected by coiled tethers (Lomir) were attached to exteriorized arterial and central venous access ports on each rat. For infusion of LeTx, central venous catheters were attached to syringe pumps (PHD 2000; Harvard Apparatus) via 3-way stopcocks. Arterial catheters were connected to pressure transducers (Maxxim Medical), for determination of arterial blood pressure (systolic, diastolic, and mean) and HRs (BioSystem XA software; version 3.0; Buxco) and for collection of blood. After equilibration of the rats, continuous measurements of each hemodynamic parameter were obtained for a 5-min period, and the mean of the measurements for that period was recorded. Arterial blood was collected for blood gas analysis (iSTAT Portable Clinical Analyzer; Abbott), lactate measurements, complete blood cell counts, and white blood cell differentials. Alveolar-to-arterial oxygen gradients and arterial base excesses were calculated by use of standard formulas.

Statistics. A Cox proportional-hazards model or Mantel-Haenszel χ^2 test was used to compare survival rates among rats receiving either PA-MAb at differing times and dose levels or placebo. The effect that PA-MAb had on hemodynamic function was analyzed on the basis of changes from baseline values, which were calculated for individual rats immediately before treatment was initiated. Because pretreatment data could not be obtained for the rats receiving PA-MAb at the time of initiation of LeTx infusion, hemodynamic data are shown but were analyzed separately. The effects that PA-MAb had on other laboratory parameters were analyzed only on the basis of changes after the initiation of treatment, because the quantity of blood required for each sample limited the number of samples that could be obtained from individual rats. Changes were compared by use of 4-way analysis of variance (ANOVA), with the time of measurement and the time, type, and dose of treatment taken into account. Two-way ANOVA was used to compare MBP, HR, and other laboratory measurements between survivors and nonsurvivors across the measurement times. An alternative Tukey test was used where appropriate. Treatment effects were calculated by subtracting the mean changes in the control group from the mean changes in the treatment group. Data are presented as mean ± SE; $P \leq .05$ was considered to be significant.

RESULTS

Comparison of the effects that treatment with PA-MAb at differing times after the initiation of LeTx infusion had on survival rates. Two rats, 1 receiving placebo and 1 receiving PA-MAb, were killed during the study because they became moribund; they were included in the analysis. For the rats receiving placebo, survival rates and median times to death were not significantly different among nonsurvivors, regardless of the treatment time (i.e., when each individual treatment time was compared with the other treatment times). The overall survival rate (mean ± SE) for the rats receiving placebo was 60% ± 2%, and the median time to death for these rats was 16 h (range, 8–152 h) (figure 2). Survival rates for the rats receiving PA-MAb at 0, 3, or 6 h were increased significantly, compared with those in the rats receiving placebo ($P = .001$, $P = .01$, and $P = .001$, respectively) (figures 2 and 3). Treatment with PA-MAb at 9 and 12 h resulted in increased survival rates that approached significance ($P = .07$ and $P = .08$, re-

Table 2. Arterial blood pressure (mean ± SE) over the course of 24 h of measurement in rats receiving either protective antigen–directed monoclonal antibody (PA-MAb) or placebo (nonspecific antibody) at the time of or 3, 6, 9, or 12 h after the initiation of *Bacillus anthracis* lethal toxin (LeTx) infusion.

		Hours after the initiation of LeTx infusion											
Treatment group	Time, h	2	4	6	8	10	12	14	16	18	20	22	24
Placebo (n = 16)	**0**	115 ± 4	104 ± 4	108 ± 3	108 ± 3	107 ± 3	104 ± 11	90 ± 15	105 ± 15	119 ± 8	102 ± 23	109 ± 7	111 ± 5
PA-MAb (n = 17)		106 ± 2	103 ± 2	110 ± 3	112 ± 3	115 ± 3	119 ± 6	119 ± 3	120 ± 6	116 ± 5	122 ± 5	117 ± 4	115 ± 4
Placebo (n = 8)	**3**	92 ± 2	**99 ± 2**	**95 ± 3**	**100 ± 4**	**93 ± 3**	ND	ND	ND	ND	ND	ND	105 ± 7
PA-MAb (n = 7)		96 ± 5	**100 ± 5**	**102 ± 3**	**106 ± 4**	**109 ± 4**	ND	ND	ND	ND	ND	ND	100 ± 4
Placebo (n = 56)	6	105 ± 2	103 ± 2	103 ± 1	**106 ± 3**	**100 ± 2**	**101 ± 3**	**100 ± 3**	**105 ± 3**	**106 ± 3**	107 ± 2	105 ± 2	104 ± 2
PA-MAb (n = 56)		105 ± 2	105 ± 2	104 ± 1	**104 ± 2**	**105 ± 2**	**108 ± 2**	**108 ± 2**	**108 ± 2**	**108 ± 2**	105 ± 2	106 ± 3	106 ± 2
Placebo (n = 33)	9	106 ± 4	106 ± 4	109 ± 3	95 ± 3	**107 ± 3**	**107 ± 4**	**105 ± 4**	**112 ± 3**	**112 ± 4**	**114 ± 5**	119 ± 6	110 ± 3
PA-MAb (n = 36)		106 ± 4	108 ± 3	107 ± 3	99 ± 3	**106 ± 4**	**114 ± 4**	**110 ± 4**	**115 ± 4**	**114 ± 4**	**114 ± 4**	117 ± 4	110 ± 3
Placebo (n = 23)	12	108 ± 4	102 ± 3	103 ± 2	101 ± 3	105 ± 2	98 ± 3	**101 ± 2**	**100 ± 3**	**102 ± 2**	**104 ± 3**	**106 ± 3**	**100 ± 3**
PA-MAb (n = 23)		107 ± 3	104 ± 3	105 ± 2	107 ± 4	104 ± 3	106 ± 4	**111 ± 4**	**115 ± 4**	**112 ± 3**	**115 ± 3**	**116 ± 3**	**110 ± 3**

NOTE. Boldfaced values were used to compare, over similar periods, the effect of PA-MAb administered at different time points (see Materials and Methods and Results). Significance levels for the overall effects of PA-MAb during these periods are shown in figure 5. ND, not done.

Table 3. Heart rate (mean ± SE) over 24 h of measurement in rats receiving either protective antigen–directed monoclonal antibody (PA-MAb) or placebo (nonspecific antibody) at the time of or 3, 6, 9, or 12 h after the initiation of *Bacillus anthracis* lethal toxin (LeTx) infusion.

		Hours after the initiation of LeTx infusion											
Treatment group	Time, h	2	4	6	8	10	12	14	16	18	20	22	24
Placebo (n = 16)	**0**	351 ± 10	351 ± 10	366 ± 16	362 ± 18	328 ± 26	317 ± 80	292 ± 91	303 ± 89	373 ± 68	308 ± 69	390 ± 10	359 ± 19
PA-MAb (n = 17)		362 ± 11	362 ± 11	386 ± 13	397 ± 15	404 ± 19	455 ± 45	362 ± 29	413 ± 29	410 ± 30	420 ± 67	407 ± 37	404 ± 16
Placebo (n = 8)	**3**	363 ± 14	**363 ± 14**	**364 ± 19**	**386 ± 11**	**362 ± 19**	ND	ND	ND	ND	ND	ND	385 ± 11
PA-MAb (n = 7)		353 ± 12	**353 ± 12**	**370 ± 17**	**380 ± 16**	**413 ± 17**	ND	ND	ND	ND	ND	ND	407 ± 15
Placebo (n = 56)	6	370 ± 8	370 ± 8	390 ± 6	**380 ± 9**	**355 ± 9**	**355 ± 12**	**353 ± 13**	**369 ± 15**	**373 ± 11**	371 ± 12	372 ± 10	381 ± 8
PA-MAb (n = 56)		382 ± 7	382 ± 7	386 ± 6	**380 ± 12**	**393 ± 7**	**409 ± 6**	**398 ± 10**	**394 ± 10**	**391 ± 9**	388 ± 13	384 ± 12	392 ± 7
Placebo (n = 33)	9	353 ± 8	353 ± 8	380 ± 9	344 ± 16	**356 ± 8**	**338 ± 13**	**336 ± 12**	**359 ± 16**	**363 ± 20**	**364 ± 17**	360 ± 23	369 ± 14
PA-MAb (n = 36)		373 ± 8	373 ± 8	371 ± 7	348 ± 18	**349 ± 9**	**355 ± 15**	**362 ± 11**	**381 ± 11**	**379 ± 13**	**398 ± 9**	397 ± 14	397 ± 6
Placebo (n = 23)	12	359 ± 10	359 ± 10	385 ± 9	350 ± 14	354 ± 17	330 ± 19	**337 ± 18**	**361 ± 15**	**358 ± 15**	**367 ± 18**	**375 ± 12**	**375 ± 13**
PA-MAb (n = 23)		349 ± 11	349 ± 11	387 ± 9	374 ± 11	382 ± 16	352 ± 15	**386 ± 10**	**381 ± 11**	**384 ± 11**	**379 ± 13**	**406 ± 11**	**368 ± 11**

NOTE. Boldfaced values were used to compare, over similar periods of time, the effect of PA-MAb administered at different time points (see Materials and Methods and Results). Significance levels for the overall effects of PA-MAb during these periods of time are shown in figure 5. ND, not done.

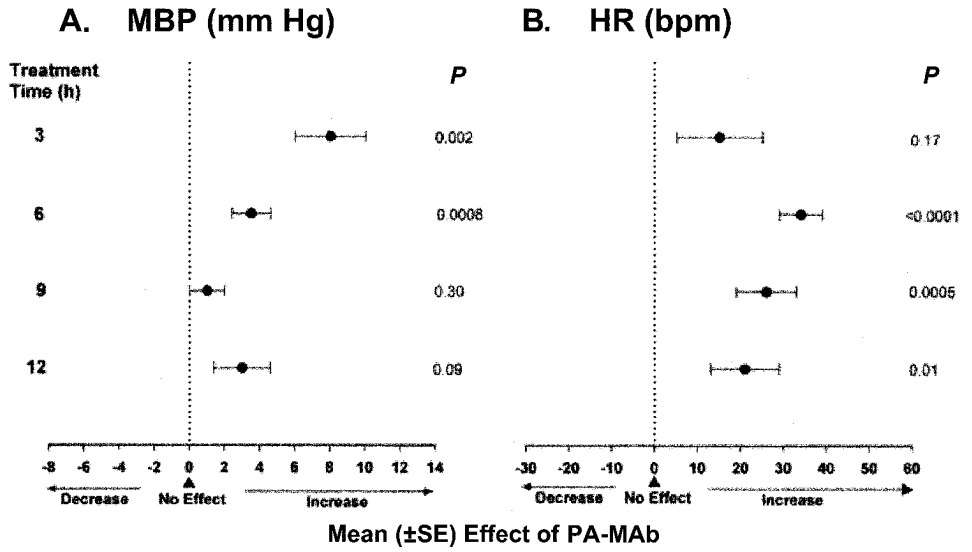

Figure 5. Overall effects that protective antigen–directed monoclonal antibody (PA-MAb) administered 3, 6, 9, or 12 h after the initiation of *Bacillus anthracis* lethal toxin (LeTx) infusion had on changes in mean arterial blood pressure (MBP, *A*) and heart rate (HR, *B*) from baseline when values were averaged over the 12-h periods after treatment. These effects were calculated by subtracting the mean changes in the control group from the mean changes in the treatment group. Measurements for treatment at 3 h were obtained over the subsequent 7-h period. Data are mean ± SE. *P* values for the effects that PA-MAb had on MPB and HR are shown.

spectively) (figures 2 and 3). Overall, treatment time influenced the beneficial effects that treatment with PA-MAb had on survival rates ($P < .001$).

Comparison of the effects that LeTx infusion had on hemodynamic and acid-base parameters in survivors and nonsurvivors receiving placebo. In a previous study, compared with the values observed in control rats receiving an infusion of vehicle only, infusion of LeTx resulted in reductions in MBP and HR that began at 6 h and progressed at later time points [12]. These reductions were much greater in nonsurvivors than in survivors. A vehicle-control challenge was not used in the present study; however, as in the previous study, the nonsurvivors receiving placebo experienced a shock-like state that was accompanied by reductions in MBP and HR (compared with those in the survivors receiving placebo) as early as 6 h after the initiation of LeTx infusion, and the reductions increased during the fol-

Table 4. Arterial pH, HCO_3, base excess, lactate, and alveolar-arterial O_2 gradient ($A\text{-}aO_2$) levels (mean ± SE) in rats receiving either protective antigen–directed monoclonal antibody (PA-MAb) or placebo (nonspecific antibody) 6 h after the initiation of *Bacillus anthracis* lethal toxin (LeTx) infusion.

	Hours after the initiation of LeTx infusion			
Parameter, treatment group	6	9	12	24
pH level				
Placebo	7.496 ± 0.004	7.473 ± 0.005	7.443 ± 0.009	7.462 ± 0.010
PA-MAb	7.492 ± 0.004	7.471 ± 0.005	7.479 ± 0.004	7.478 ± 0.004
HCO_3 level, mmol/L				
Placebo	24.3 ± 0.4	22.6 ± 0.4	21.8 ± 0.7	24.4 ± 0.5
PA-MAb	24.7 ± 0.4	23.7 ± 0.4	22.6 ± 0.4	24.9 ± 0.5
Base excess level, mmol/L				
Placebo	1.06 ± 0.37	−0.92 ± 0.43	−2.28 ± 0.77	0.66 ± 0.57
PA-MAb	1.38 ± 0.40	0.22 ± 0.47	−1.00 ± 0.38	1.47 ± 0.47
Lactate level, mmol/L				
Placebo	0.99 ± 0.04	1.03 ± 0.12	1.12 ± 0.28	0.88 ± 0.09
PA-MAb	1.04 ± 0.07	0.93 ± 0.06	0.84 ± 0.04	0.87 ± 0.08
$A\text{-}aO_2$ level, mm Hg				
Placebo	15.8 ± 1.2	16.6 ± 1.4	17.8 ± 2.2	15.3 ± 1.5
PA-MAb	14.9 ± 1.1	14.7 ± 1.5	18.7 ± 1.0	15.0 ± 1.1

Table 5. White blood cell, neutrophil, lymphocyte, and monocyte counts and hemoglobin concentrations (mean ± SE) in rats receiving either protective antigen–directed monoclonal antibody (PA-MAb) or placebo (nonspecific antibody) 6 h after the initiation of *Bacillus anthracis* lethal toxin (LeTx) infusion.

	Hours after the initiation of LeTx infusion			
Parameter, treatment group	6	9	12	24
White blood cell count, 10^3 cells/μL				
Placebo	10.7 ± 0.5	12.0 ± 0.6	12.1 ± 0.6	15.6 ± 1.1
PA-MAb	11.3 ± 0.6	12.4 ± 0.5	12.8 ± 0.6	12.8 ± 0.6
Neutrophil count, 10^3 cells/μL				
Placebo	7.9 ± 0.3	7.8 ± 0.4	6.9 ± 0.4	5.5 ± 0.8
PA-MAb	8.4 ± 0.4	7.8 ± 0.4	6.6 ± 0.3	4.3 ± 0.3
Lymphocyte count, 10^3 cells/μL				
Placebo	2.4 ± 0.2	3.8 ± 0.3	4.7 ± 0.5	9.2 ± 0.7
PA-MAb	2.4 ± 0.3	4.1 ± 0.3	5.6 ± 0.4	7.8 ± 0.5
Monocyte count, 10^3 cells/μL				
Placebo	0.24 ± 0.04	0.29 ± 0.04	0.36 ± 0.04	0.57 ± 0.08
PA-MAb	0.27 ± 0.04	0.36 ± 0.04	0.35 ± 0.03	0.40 ± 0.04
Hemoglobin concentration, g/dL				
Placebo	13.2 ± 0.4	12.2 ± 0.2	11.8 ± 0.4	11.2 ± 0.4
PA-MAb	13.3 ± 0.3	11.8 ± 0.2	11.3 ± 0.2	11.0 ± 0.2

lowing 6 h as the first deaths occurred ($P < .001$, for both parameters when the values were averaged over the period from 6 to 12 h) (figure 4). These reductions in hemodynamic function in nonsurvivors during the period from 6 to 12 h were also associated with significant decreases in arterial pH and base excess levels and increases in lactate levels, compared with those in survivors ($P < .05$, for all 3 parameters when the values were averaged over the period from 6 to 12 h) (table 1); this finding is consistent with reduced tissue perfusion.

Comparison of the effects that treatment with PA-MAb at differing times after the initiation of LeTx infusion had on MBP and HR. Of the rats that eventually died, few were still alive 2 h after treatment with PA-MAb from which to obtain measurements (0 of those receiving treatment at 0 and 12 h, 1 of those receiving treatment at 3, 1 of those receiving treatment at 6 h, and 4 of those receiving treatment at 9 h). Furthermore, there were no significant differences in the effects that treatment with PA-MAb had on hemodynamic and other parameters between the survivors and nonsurvivors, both for each individual treatment time (when there were survivors and nonsurvivors to compare) and for the data resulting from the combination of treatment times. Therefore, to compare the effects of treatment with PA-MAb with those of administration of placebo, the data for the survivors and nonsurvivors were combined.

For the rats treated at time 0, pretreatment data were not available by which to assess changes from baseline in either MBP or HR. However, during the 12-h period after treatment, both parameters significantly increased in the rats receiving PA-MAb, compared with those in the rats receiving placebo ($P < .001$, for both parameters when the values were averaged over the 12-h period) (tables 2 and 3).

Before treatment at 3, 6, 9, and 12 h, MBP and HR were not significantly different in the rats receiving placebo and in those receiving PA-MAb (tables 2 and 3). Because the time of measurement did not significantly alter the effect that treatment with PA-MAb had on either parameter, the values were averaged over similar 12-h measurement periods beginning immediately after treatment. For treatment at 3 h, measurements from 4 to 10 h were averaged. The effects of treatment with PA-MAb during this shorter period of observation were not significantly different from those observed for treatment with PA-MAb at other time points and were included in the analysis. The average effects of PA-MAb over these periods are shown in figure 5. Compared with the administration of placebo, treatment with PA-MAb at 3, 6, 9, and 12 h were all associated with increases in MBP, but these changes were greatest for treatment with PA-MAb at 3 h, compared with those for later treatment times ($P = .004$, for the influence of treatment time on the effects that PA-MAb had on MBP). Increases in MBP with PA-MAb at 9 and 12 h were not significant. Compared with the administration of placebo, treatment with PA-MAb was associated with significant increases in HR at 6, 9, and 12 h ($P \leq .01$, for all time points) as well as for all treatment times combined ($P < .0001$).

Effects that treatment with PA-MAb at 6 h after the initiation of LeTx infusion had on laboratory measurements. Six hours after the initiation of LeTx infusion and immediately before treatment, arterial blood gas levels and complete blood cell counts did not significantly differ between the rats receiving placebo and those receiving PA-MAb (tables 4 and 5). Com-

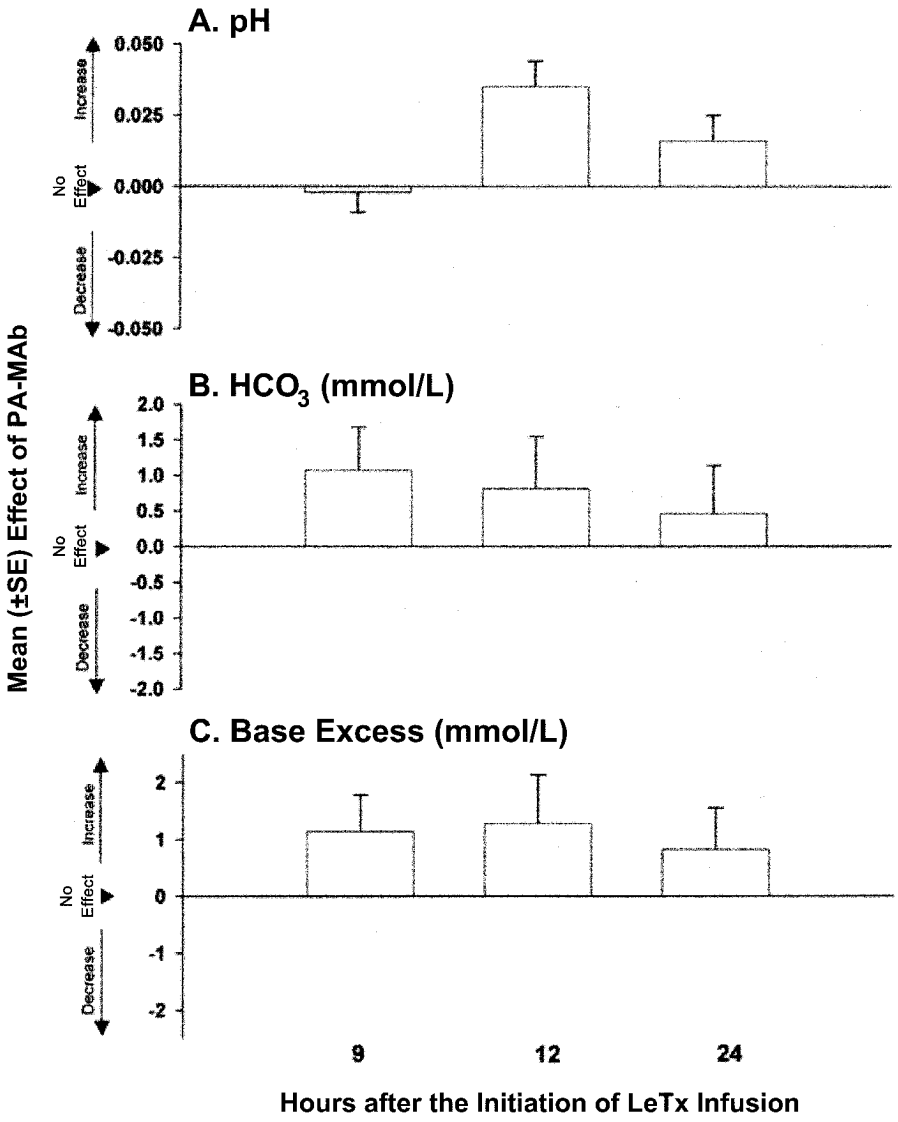

Figure 6. Effects that protective antigen–directed monoclonal antibody (PA-MAb) administered 6 h after the initiation of *Bacillus anthracis* lethal toxin (LeTx) infusion had on arterial pH *(A),* HCO_3 *(B),* and base excess *(C)* levels at 9, 12, and 24 h. These effects were calculated by subtracting the mean changes in the control group from the mean changes in the treatment group. Data are mean ± SE.

pared with the administration of placebo, treatment with PA-MAb increased arterial pH levels at 12 and 24 h but not at 9 h ($P = .01$, for the differing effects of PA-MAb at these time points) and increased arterial bicarbonate and base excess levels at all 3 time points ($P = .05$ and $P = .01$, respectively, for each parameter when the values were averaged across time points) (table 4 and figure 6). Compared with the administration of placebo, treatment with PA-MAb caused reductions in arterial hemoglobin at 9, 12, and 24 h that approached significance ($P = .08$, when the values were averaged across time points) (table 5). Because the volumes of blood drawn and replaced with saline were the same for all rats, blood sampling itself was not the basis for these differences. Compared with the administration of placebo, treatment with PA-MAb at 6 h increased circulating leukocyte and lymphocytes counts at 9 and 12 h but not at 24 h and circulating monocyte counts at 9 h but not at 12 or 24 h ($P = .02$, $P = .05$, and $P = .02$, respectively, for each parameter) (table 5).

Comparison of the effects that treatment with PA-MAb in differing doses at 6 h after the initiation of LeTx infusion had on survival rates. Compared with control rats receiving placebo, decreasing dose levels of PA-MAb (10×, 1×, 0.5×, 0.1×, or 0.05×) administered 6 h after the initiation of LeTx infusion resulted in dose-ordered reductions in the treatment's beneficial effects on survival rates (table 6) ($P = .001$, for the differing effects of PA-MAb across doses).

Table 6. Effects that differing doses of protective antigen–directed monoclonal antibody (PA-MAb) administered 6 h after the initiation of *Bacillus anthracis* lethal toxin infusion had on survival rate in rats.

PA-MAb dose[a]	No. of survivors/total no. of rats studied (%)
10×	23/23 (100)
1×	17/17 (100)
0.5×	21/22 (95)
0.1×	15/18 (83)
0.05×	4/6 (67)
0× (placebo)	35/48 (73)

[a] Doses are the indicated number of times the molar amount of protective antigen that was administered.

DISCUSSION

We had shown previously that abnormalities of cardiovascular but not pulmonary function are associated with the lethal effects of LeTx in this rat model [12]. Consistent with that finding, in the present study, improved survival rates due to treatment with PA-MAb were associated with increases in MBP and HR and improved acid-base status but not with alterations in arterial oxygenation.

Reductions in hemodynamic function in nonsurvivors receiving placebo in the present study were evident as early as 6 h after the initiation of LeTx infusion and worsened during the following 6 h. At the time of the first deaths in rats, MBP had decreased to ⩽90 mm Hg and were almost 20 mm Hg lower than the level in survivors. These reductions were associated with developing arterial acidosis suggestive of worsening tissue hypoperfusion. Thus, in this rat model, it appears that a shock-like state develops at these MBP values. These changes in nonsurvivors are consistent with those noted in a previous study, in which this model was developed [12]. Despite the presence of established hemodynamic instability and developing shock, treatment with PA-MAb as late as 12 h after the initiation of LeTx infusion still resulted in beneficial hemodynamic effects and improved survival rates (which approached significance). However, the beneficial effects that treatment with PA-MAb had on MBP were greatest when treatment was administered at 3 h, compared with the beneficial effects at later treatment times, whereas the beneficial effects that treatment with PA-MAb had on HR appeared to be consistent across all treatment times. During the recent contaminated mail–related outbreak of inhalational *B. anthracis* in humans, several patients presented with evidence of established shock [2–4]. Furthermore, despite treatment with appropriate antibiotics, the shock persisted and progressed in some of the patients. Although there are several possible explanations for this phenomenon, one is that persistent circulating LeTx contributed to ongoing cardiovascular instability despite bacterial killing. Such a condition has been shown to occur in animals with live bacterial infection and could explain the potentially injurious effects of LeTx [15]. However, the findings from the present study suggest that, even in patients in whom anthrax infection has progressed to the point where systemic shock is evident, neutralization of LeTx may still have beneficial effects if achieved in combination with the use of antimicrobial agents that would limit further bacterial growth.

Nonetheless, in this model of *B. anthracis* sepsis, the basis for the protective effects of PA-MAb is not clear, primarily because the pathogenic LeTx-triggered events that cause cardiovascular dysfunction and death are not known. Septic shock related to infection with other bacteria—such as the gram-negative ones with which critically ill patients are frequently infected—is attributed, in large part, to excessive release of inflammatory mediators in the host [16]. Tumor necrosis factor and nitric oxide, 2 such mediators, have been strongly implicated with respect to the endothelial and myocardial injury related to the production of LPS by gram-negative bacteria [12]. The events that occur after LeTx is bound and introduced into the cell appear to be very different from those for LPS, however [17]. Lethal factor is a zinc protease that cleaves and inactivates several members of the mitogen-activated protein kinase kinases family [18, 19]. Although early studies suggested that lethal factor might cause macrophage lysis and the release of cytokines and other injurious host mediators, further data that might support a contributory role for excessive inflammatory response in the pathogenesis of shock due to LeTx have been inconsistent [20–25]. In our previous study, intravascular release of inflammatory cytokines and nitric oxide was markedly increased during 24-h LPS infusions but was not altered during 24-h LeTx infusions [12]. Although it is possible that LeTx may affect local cytokine production, in our previous study, histologic examination of several different organs did not demonstrate any evidence of inflammatory tissue injury due to LeTx [12]. Other recent work has suggested that LeTx may actually inhibit components in the inflammatory response [26].

In the present study, it was also not clear whether the protective cardiovascular effects of PA-MAb were related primarily to alterations in the peripheral vasculature or in the heart itself. It has been proposed that the loss of vascular integrity and the increased permeability and extravasation of fluid contribute to the cardiovascular compromise associated with LeTx [27–29]. Recent work has suggested that anthrax LeTx is capable of inducing endothelial cell apoptosis in both microvascular and large vessel cells through inhibition of the extracellular signal-regulated kinase [30]; it was believed that hemoconcentration reflective of endothelial cell dysfunction and increases in hemoglobin concentration observed in previous animal studies as well as in the present rat model reflected this process [12].

Of note in the present study, although not significant, hemoglobin concentrations were lower in rats treated with PA-MAb, suggesting that one protective effect of treatment may be related to improved vascular integrity.

However, it is also possible that PA-MAb had a primary effect on myocardial function, because HRs were increased consistently across all treatment times—unlike the effects on MBP, which were greatest for earlier treatment. Although, at present, few data describing the specific effects that LeTx has on cardiac cells are available, the effects on endothelial cell apoptosis described above would be important for myocardial function as well [30]. Indirect evidence suggesting a protective mechanism for PA-MAb comes from studies showing that other mitogen-activated protein kinases targeted by LeTx can contribute to cardioprotection [31, 32]. However, this is a complex area, because, in other models, similar pathways have been implicated in cardiac injury [33]. Arguing against a direct protective effect of PA-MAb on cardiac function in the present model was the absence in our previous study of histologic evidence of myocardial injury associated with LeTx [12]. It is also noteworthy that, in previous autopsy studies, myocardial injury appeared to be minimal during live *B. anthracis* infection [12, 34].

The dose of PA-MAb (i.e., the 10× dose) tested at each of the 5 different treatment times was calculated to be a 10-fold molar excess of the serum concentrations of LeTx that were estimated to occur in the present model. However, in experiments in which treatment with PA-MAb was delayed as late as 6 h after the initiation of LeTx infusion, reductions in the dose of PA-MAb to as low as 0.5× were associated with improved survival rates. During clinical administration of PA-MAb, an estimate of circulating LeTx levels may be necessary to determine the most effective dose.

In conclusion, in rats receiving a 24-h LeTx infusion, PA-MAb administered up to 6 h after the initial exposure to LeTx was very protective. Given up to 9 and 12 h afterward, PA-MAb improved hemodynamic function and survival rates. A previous study [12] using this model, as well as the present one, showed that the onset of shock and lethality due to LeTx was already present by these later time points. These findings suggest that, clinically, PA-MAb may reduce morbidity and mortality due to LeTx even if administered in patients who present after the onset of septic shock due to infection with *B. anthracis*.

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