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TNF Receptor 1, IL-1 Receptor, and iNOS Genetic Knockout Mice Are Not Protected from Anthrax Infection

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Anthrax produces at least two toxins that cause an intense systemic inflammatory response, edema, shock, and eventually death. The relative contributions of various elements of the immune response to mortality and course of disease progression are poorly understood. We hypothesized that knockout mice missing components of the immune system will have an altered response to infection. Parent strain mice and knockouts were challenged with LD95 of anthrax spores (5×10^6) administered subcutaneously. Our results show that all genetic knockouts succumbed to anthrax infection at the same frequency as the parent. TNF antibody delayed death but TNF receptor 1 knockout had no effect. IL-1 receptor or iNOS knockouts died sooner. Anthrax was more abundant in the injection site of TNF-α and iNOS knockouts compared to parent suggesting that attenuated cellular response increases rate of disease progression. With the exception of edema and necrosis at the injection site pathological changes in internal organs were not observed.

Key Words: **anthrax; *Bacillus anthracis;* TNF; TNF-alpha; IL-1; iNOS; nitric oxide; mice; murine; knockout; mortality; death.**

Anthrax infection occurs when spores are introduced into the body via the lungs, skin or gastrointestinal tract (1). Spores germinate after they are engulfed by macrophages (2) and begin to produce toxins that initiate a cascade, leading to a systemic immune response, shock and death (1, 3). Anthrax growing in the body produces three soluble toxins: lethal factor (LF), edema factor (EF) and protective antigen (PA), believed to be principally responsible for invoking the host response. PA functions as a molecular transporter facilitating the entry of LF and EF into cells (reference). While many cells internalize LF, cytotoxic effects are only produced in macrophages (4). Once internalized into macrophages, LF exerts its biological action as a protease that leads to specific inactivation of MAPKK (5, 6), that, in turn, leads to up-regulation of two pro-inflammatory cytokines, TNF and IL-1β (7). The relative importance of macrophages and IL-1β has been demonstrated by studies that have shown that removal of macrophages by administration of silica particles or antagonism of IL-1β (8) protects mice against lethal challenge with combined PA and LF injection. Other studies have also shown that administration of anti-oxidants can delay death in mice from lethal toxin challenge, suggesting that oxidative stress is an important contributor to anthrax pathology (9).

While several elegant studies have been performed with pure toxins, relatively little is known about the immune response following infection with anthrax spores. We hypothesized that since TNF and IL-1β are up-regulated during intoxication with lethal toxin (e.g., lethal factor plus protective antigen), receptor knockouts will be protected or death will be delayed following a lethal challenge with anthrax spores. Because of the requirement for internalization and processing of spores by macrophages at the initial stages of infection and because of the importance of oxidative stress in contributing to anthrax pathology, we reasoned that knockouts missing the free radical generating enzyme iNOS will also be protected. It has been shown that anthrax lethal factor inhibited the release of NO from murine macrophage cell lines, suggesting that this inhibition may be a mechanism of survival during the initial stage of infection (10). Finally we also examined the effects of a TNF neutralizing antibody during a challenge with viable anthrax spores.

The strain of anthrax selected for this study, Sterne, lacks the pX02 plasmid coding for capsule proteins that inhibit phagocytosis of vegetative anthrax (11) but ex-

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presses the pX01 plasmid coding for toxins (12). Thus the biological events that occur during infection with Sterne are similar to those produced during infection with other strains.

MATERIALS AND METHODS

All mice were purchased from Jackson Laboratories (Bar Harbor, ME). Male mice, 7.5–9 weeks old were used in all experiments. The parent strain used was C57BL/6J. Knockouts were derived from this strain of mouse. TNF (B6.129-Tnfrsf1α^{tm1Mak}) and IL-1 (B6.129S7-Il-1r1^{tm1Imx}) are receptor knockouts missing TNF receptor 1 (TNFR1 KO) and Il-1 receptor 1 (IL-1R1 KO), respectively. Thus these mice are expected to produce both TNF and IL-1β but lack signals that are associated with their respective receptors. The inducible nitric oxide synthase (iNOS) knockout (B6.129P2-Nos2^{tm1Lau}) does not express the associated enzyme.

B. anthracis, Sterne strain, was obtained from a commercial veterinary vaccine (Colorado Serum, Denver, CO) and was used for the production of spores. All spores used in the study were propagated in a single batch derived from the vaccine. Sterne strain *B. anthracis* is not pathogenic in humans since it lacks the pX02 plasmid which codes for proteins facilitating production of a capsule which prevents phagocytosis of anthrax. Sterne strain produces anthrax associated toxins and thus causes symptoms similar to those associated with infection by other strains of anthrax. Anthrax from the vaccine was propagated on sporulating agar (AKAgar No. 2, Becton–Dickinson, France) for 4 days at 37°C in a humid incubator at $<0.5\%$ CO_2 and conversion to spores induced by removing growing cultures to room temperature for 24 h. The spores were collected in ice-cold distilled, deionized H_2O (ddH_2O) and filtered through a Whatman No. 2 filter and then heat shocked for 30 min at 65°C to eliminate remaining vegetative anthrax. Spores were washed and centrifuged three times in ice-cold ddH_2O. Microscopic examination of the resulting preparation demonstrated that no vegetative form was present. Spores were stored in 2-ml aliquots in ddH_2O with 1% phenol at 4°C at a concentration of 10^9 spores/ml. Phenol was added to prevent colonization by other bacteria. Prior to inoculation, spores were washed three times with ice-cold ddH_2O before injection to remove phenol. The concentration of spores in the inoculum was determined on each dosing day by determining the number of colony forming units (CFU). CFU's were determined in triplicate by serial dilutions in ddH_2O. Spore inoculum concentration was made using trypticase soy agar (Remel) for 16 h at 37°C at $<0.5\%$ CO_2. Intraday variation using this method is $<5\%$. The same batch of spores was used in all experiments.

Mice used in the experiment were maintained under conditions in accord with AALAC standards. Mice were anaesthetized by placing them in a rodent anesthesia machine (IMPAC[6], VetEquip) equipped with an Isoflurane vaporizer (100% O_2 at 1 liter/min and 4–5 vaporizer setting) for 2 min. Mice were then injected with 200 μL of spore suspension in ddH_2O, subcutaneously between the scapulae. We have previously determined (data not shown) that a dose of 5×10^6 spores per mouse will cause death in 95% of the mouse population. This target dose was administered to all mice in this study. Some mice received a TNF neutralizing antibody. This antibody was produced by a hybridoma cell line expressing V1q, a rat IgD monoclonal antibody that blocks mouse TNF bioactivity (13) was obtained from P. Krammer, German Cancer Research Center. DNA encoding the heavy and light chain variable regions of V1q was cloned using conventional molecular techniques into plasmid vectors that encoded murine IgG2a and kappa constant regions, respectively, and was expressed and purified as intact IgG (TNF neutralizing Ab). Antibody-treated mice received 0.5 mg in 100 μL of PBS containing 0.1% Tween 80 by intraperitoneal injection on the day of inoculation with spores and 1 and 2 days after. The group to which the treatment was compared received the vehicle on these days. Mice were observed for 14 days and then euthanized using CO_2. The mice were observed for formation of edema at the site of injection or for increased lethargy. Mice that were unable to right themselves were considered moribund and were euthanized. Studies were also conducted to determine histological changes that occurred during the course of infection. For this purpose mice were euthanized 6, 24, 48, and 72 h postinfection. Samples of internal organs including lung, liver, spleen, lymph nodes and skin at injection site, were collected, fixed in 10% neutral buffered formalin and processed into slides for microscopic examination. Lungs were perfused with formalin just prior to removal from the thoracic cavity. Slides were subsequently stained with hematoxylin and eosin and evaluated for pathologic changes. A small number of mice also received a sham injection of 200 μl ddH_2O and also were evaluated. Because of limitations in availability, parent, TNFR1 KO and iNOS KO's only were evaluated over time.

Mortality data were analyzed using survival analysis where $P < 0.1$ was deemed to be a significant difference.

RESULTS AND DISCUSSION

Microscopic examination of tissues from the anthrax infected parent strain mice revealed that significant inflammation was limited to the skin and subcutis at the site of injection. Lymphoid necrosis of the spleen and thymus was also observed in several animals. Other tissues were unremarkable. In all groups, bacteria were only observed at injection sites suggesting that death of animals was to due to the systemic effects of the toxin rather than bacteremia.

Histologically, the lesion in the skin was characterized by marked edema and variable numbers of neutrophils with fewer mononuclear cells. Necrosis and hemorrhage occurred in the more severe lesions. Bacilli were often present in high numbers. At the 6-h time point, inflammation and edema of the skin and subcutis was evident, but the lesions were less severe at 6 h than at 24, 48, and 54 h.

In general, histologic findings in TNFR1 KO and iNOS KO mice were similar to those observed in the parent strain. One difference was the difference in relative abundance of bacteria at the injection site. While only 20% of parent strain animals had significant numbers of bacteria evident at the 6-h time point, 80% of iNOS KO and 65% of TNFR1 KO mice showed prominent bacterial proliferation by 6 h. However, by 24 h high numbers of bacteria were seen in most animals from each group. This finding is consistent with a hypothesis that iNOS KO are impaired with respect to phagocytic clearance of bacteria during early infection.

These results are quite different than those observed in the human cases of pulmonary anthrax infection that have been recently documented (14). On autopsy, infected humans show evidence of vegetative forms throughout the body and in the blood. Since mice at the later time points were lethargic and in some cases moribund it seems unlikely that the lack of overt evidence of bacilli is due to premature examination. It is possible that anthrax is not disseminated in the same manner in mice as in humans or rabbits (15). Since

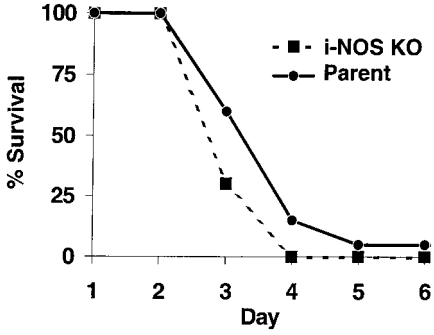

FIG. 1. i-NOS KO survival compared to parent. Groups of 20 mice were challenged with anthrax spores on day 0. i-NOS KO's die earlier than parent, $P < 0.01$.

death nonetheless occurs, toxins could disseminate from the injection site to distant organs altering performance and causing shock, and ultimately, death.

Figure 1 shows that iNOS KO mice die more rapidly than the parent. Table 1 shows that edema occurs earlier in iNOS KO mice. Our original hypothesis was that free radical stress has a deleterious effect and that iNOS KO's would survive longer, since oxidative stress should be reduced (9). Instead, the data suggest that loss of free radical generating capacity results in a more rapid course leading to death. Our observations are consistent with a hypothesis that bacteria numbers increase more rapidly in the iNOS KO's because clearance rate is reduced. These observations are in agreement with a previous finding that shows that survival following Sterne strain anthrax challenge is highly dependent on the early cellular response (16) and that iNOS KO's are less able to repair dermal injuries (17) and are more susceptible to bacterial infections (18).

Figure 2 shows that there is little effect attributable to loss of the TNF R1. However the figure also shows that administration of TNF antibody increases survival. The antibody also has a positive effect on the health and condition of the mice. On day 2 postinfection, 9/10 parent strain mice are lethargic, whereas 5/10 mice receiving the antibody showed a high level of alertness, i.e., were difficult to catch. Our finding is

TABLE 1

Incidence of Observable Edema in the Left Forelimb 1 Day after Subcutaneous Administration of Anthrax Spores

Model	Edema day 1
Parent	48/60 (80%)
iNOS KO	40/40 (100%)
TNFR1 KO	38/40 (95%)
TNF Ab	8/10 (80%)
IL-1R1 KO	9/10 (90%)

Note. By day 2, parent 97% (58/60) show edema and 100% of other types show edema.

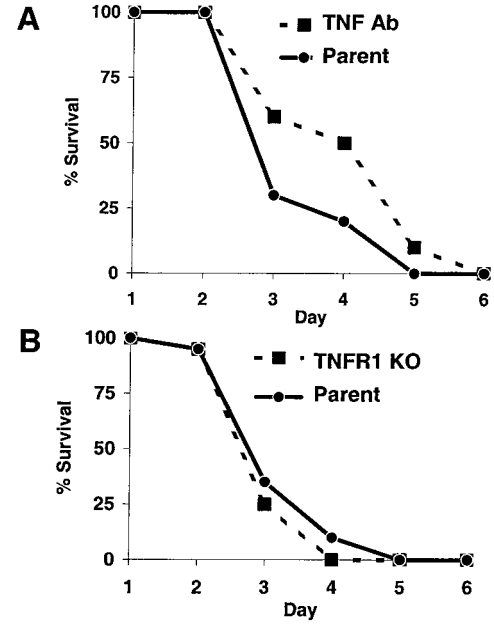

FIG. 2. Effect of TNF-α on survival during anthrax infection in mice. (A) Parent mice dosed with TNF-α antibody, $n = 10$ per group, intraperitoneal injection, 0.5 mg on days 0, 1, and 2, $P = 0.07$. (B) TNFR1 KO, $n = 20$ per group, $P > 0.1$.

somewhat different than that shown of Hanna who showed that administration of a TNF antibody had little effect on mortality of mice given injection of lethal toxin (8). Our finding suggests that other factors besides lethal toxin are important contributors to anthrax pathology. The dose of antibody required to completely inactivate TNF produced in response to anthrax infection has not yet been determined and thus it is possible that increased doses may confer even greater protection.

Figure 3 shows that IL-1R1 KO are not protected against spore challenge but die even more rapidly. This is in contrast to the finding by Hanna (8) which showed that administration of an IL-1β antagonist prevented death from occurring in mice challenged with lethal toxin.

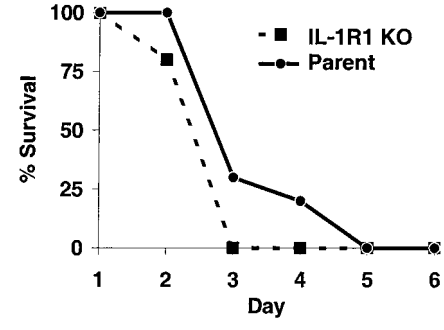

FIG. 3. Il-1R1 KO survival compared to parent. Groups of 10 KO and 20 parent. Survival is significantly reduced in KO's, $P < 0.05$.

Our results show that a subcutaneous challenge with anthrax spores causes death in mice. The genetic knockouts that we have examined do not confer protection. However, administration of a TNF antibody does increase both survival and improves the health of mice during infection. This suggests that this approach has utility as an adjuvant treatment in cases where antibiotics are started late. Administration of TNF antibody may thus be used to allow time for anthrax to be cleared by antibiotics and for toxins to be removed from the circulation thereby preventing death. This hypothesis is currently being tested in our lab.

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