Interferon Protects Mice Against Inhalation Anthrax

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Interferons (IFNs) play a role in innate immunity during many viral, bacterial, and protozoal infections. With the increasing threat of bioterrorist attacks with *Bacillus anthracis*, its high lethality, and the limited effectiveness of antibiotics, alternative treatments are being studied. Antibodies to protective antigen (PA) are promising, as is IFN. During many bacterial infections, production of and protection by IFNs has been reported, including *B. anthracis* *in vitro*. *In vivo*, we find that (1) the type I IFN inducer, Poly-ICLC, strongly and rapidly protects mice; (2) the protection is IFN-mediated since recombinant murine IFN- β can protect, and protection by Poly-ICLC is abrogated in IFN type I receptor knockout mice. The greatest protection by Poly-ICLC was conferred by intranasal treatment. A delay in death was observed with the intramuscular route alone, but was not significant. Together, the results suggest the IFN defense could protect mice, up to 60%, against lethal inhalational anthrax, and thus have important medical implications for therapy of human anthrax.

Introduction

ANTHRAX IS A SERIOUS and often lethal disease that has been a prime strategy in bioterrorism (Dewan and others 2002), and has become a matter of increased concern in the years following the 2001 anthrax attacks in the United States. When *Bacillus anthracis* spores are inhaled, they reach the alveolar spaces of the lung, where they are taken up by phagocytes, such as mediastinal macrophages, and are transported to the lymph nodes where they germinate and release two toxins (edema and lethal toxins, which bind to a receptor via protective antigen (PA) for activation), culminating in systemic disease. The established treatment of exposed or infected individuals is currently limited to antibiotics, which are effective only when administered soon after exposure (Heine and others 2007). For prevention, a cell-free vaccine (BioThrax) against anthrax is available. Improved therapies are urgently needed for the protection of our armed forces and our communities. Experimental treatment with passive antibodies to PA is being tested both *in vitro* and *in vivo* (Peterson and others 2006).

A potential adjunct therapy is the interferon (IFN) component of innate immunity, which is known to be protective experimentally during some bacterial infections (Giron and others 1972; Baron and others 1985; Bogdan, 2000). Two types of IFN proteins are produced in response to many foreign stimuli including bacterial nucleic acids and proteins (Bekisz

and others 2004; Pang and others 2005; Pestka 2007). The IFNs act through cell receptors to induce a number of IFN response genes. The IFN actions are complex and include antiviral, antitumor, and immunomodulatory activities. The antibacterial actions of IFN are often mediated by immunomodulatory activation of macrophages by type II IFN- γ , and also by the type I IFN- α and - β (Belardelli and others 2002). Thus, the activation of macrophages by IFNs may be able to control initial infection of *B. anthracis* and limit the spread of the vegetative bacilli. *In vitro* studies with IFN have demonstrated the protection of macrophages against anthrax spores. In this report, we studied the *in vivo* role of IFNs against inhalational anthrax infection and evaluated the hypothesis that a pre-exposure dose of IFN could protect mice against lethal infection.

Materials and Methods

Intranasal infection

Bacillus anthracis Ames strain spores were prepared and purified by methods established by Peterson et al. (2006). Groups of 5–10, 6–8-week-old Swiss Webster mice (Taconic, Germantown, NY) were challenged with 3.5×10^4 cfu/40 μ L (3 LD₅₀) *B. anthracis* Ames strain spores by pipetting the spore suspension into the nasal passage of the animals

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while under anesthetic (Comer and others 2006; Peterson and others 2006).

IFNAR KO mice

We maintained a breeding colony of interferon type I receptor knockout (IFNAR KO) mice (Muller and others 1994), initially provided by Dr. Virgin, Washington University in St. Louis, that were treated and challenged using the same methods.

Interferon and inducer

Murine recombinant IFN- α , - β , and - γ (PBL Laboratories, Piscataway, NJ) and Poly-ICLC (Oncovir Corporation) were diluted to treatment concentration with 2% EMEM and were administered either 24 h prior to challenge, or at the time of challenge with *B. anthracis* challenge. Various concentrations of IFN or Poly-ICLC were tested in the studies. The route of administration differed among intramuscular, intraperitoneal, and intranasal, and in some studies, multiple routes were administered to observe inhibitory or synergistic effects. When treated intranasally, mice were anesthetized and treatment administered using the same method as the *B. anthracis* intranasal challenge. While each figure is a representative (individual) study, and all studies were repeated to confirm repetitive results, except those with murine IFN due to limited availability. All studies were performed in an animal Biosafety Level 3 laboratory under an approved Institutional Animal Care and Use Committee protocol.

Results

Type I IFN inducer Poly-ICLC protects mice

Poly-ICLC is a synthetic double-stranded RNA complex consisting of polyriboinosine-polyribocytidylic acid stabilized with poly-L-lysine and carboxymethylcellulose. It is a strong inducer of both type I IFN- α and - β (Guerrero-Plata and others 2005; Zhu and others 2007). Swiss Webster mice (5 per group) were given an intranasal and intramuscular dose of Poly-ICLC (25 μ g each route), 24 h prior to intranasal challenge with 3 LD₅₀ (35,000) Ames strain spores. Mice were observed for two weeks for mortality. Figure 1 illustrates a representative survival graph showing protection of 50% of the challenged mice. Our findings are consistent with early onset of protection because when Poly-ICLC was given at the time of challenge, 5 out of 10 Swiss Webster mice survived for three weeks, whereas, mice which were not treated died within 48 h (data not shown).

Is IFN the protective substance?

To determine whether the Poly-ICLC protection was due to IFN, mice were treated with recombinant murine IFNs. Swiss Webster mice (5 per group) were given rMuIFN β either intranasally or intraperitoneally at 150,000, 50,000, or 15,000 U, 24 h prior to intranasal challenge with 3 LD₅₀ (35,000) Ames strain spores. Mice were observed for a period of two weeks for mortality. The intranasal dose was not protective (data not shown), but the intraperitoneal dose showed significant protection (Fig. 2).

FIG. 1. Poly-ICLC protects mice infected with anthrax spores. Protection of mice by Poly-ICLC (25 μ g) given both intramuscularly and intranasally 24 h prior to lethal inhalational anthrax exposure. *Indicates significance from control by Fisher Exact test ($p < 0.05$).

Swiss Webster mice (10 per group) were also given either an intranasal and intraperitoneal dose of murine recombinant IFN- γ (50,000, 5,000, or 500 U), 24 h prior to intranasal challenge with 5 LD₅₀ (58,000) Ames strain spores. Over a period of two weeks, mice were observed for mortality, and all mice treated with IFN- γ showed no protection. (data not shown). The challenge dose is regularly fine tuned, based on the results of the previous experiment to give a constant LD₅₀. Similarly, Swiss Webster mice (5 per group) were given 1×10^5 U of rMuIFN α either intranasally or intraperitoneally, at either 24 h prior to challenge, or at the time of challenge. Intranasal challenge dose was 3 LD₅₀ (35,000) Ames strain spores. Mice were observed for one week for mortality and during this time all mice treated with rMuIFN α also showed no difference from the control mice (data not

FIG. 2. Type I IFN rMuIFN β delays death in mice infected with anthrax spores. Delay in death of Swiss Webster mice when rMuIFN β is given intraperitoneally 24 h prior to lethal inhalational anthrax exposure. *Indicates significance from control by Fisher Exact test ($p < 0.05$).

FIG. 3. Inhalation anthrax is more severe in type I receptor knockout (KO) mice. Type I interferon receptor KO mice have a more severe infection and are not protected by Poly-ICLC compared to wild-type 129S mice. *Indicates significance from the control by Fisher Exact Test ($p < 0.05$).

shown). Together these findings indicate that the Poly-ICLC protection may be mediated at least in part by IFN- β .

Interferon type I receptor KO mice

To further determine whether the protection by Poly-ICLC was due to IFN, IFNAR KO mice were used. Mice lacking the receptor for type I IFN, along with wild-type controls (129S) (6 control mice and 8 KO mice per group), were given Poly-ICLC, 25 μ g intranasally plus 25 μ g intramuscularly, 24 h prior to intranasal challenge with 3 LD₅₀ (59,850) Ames strain spores. Mice were observed for two weeks for mortality. As shown in Figure 3, KO mice succumbed to infection faster than wild-type controls, suggesting a faster disease progression. In addition, IFNAR KO mice that were treated with Poly-ICLC were not significantly protected, unlike the treated wild-type mice. This confirms that type I IFN contributes to the observed survival during anthrax infections.

Intranasal administration of Poly-ICLC provides the strongest protection

Swiss Webster mice (5 per group) were given 25 μ g of Poly-ICLC by various routes, 24 h prior to intranasal challenge with 3 LD₅₀ (35,000) Ames strain spores. Mice were observed two weeks for mortality. Mice given Poly-ICLC intranasally were strongly protected at 80% for two weeks. The group dosed by the intramuscular route showed 60% protection for 12 days. Mice given the intraperitoneal dose were not protected (Fig. 4). While further studies are underway to explain these differences, we believe that activation of the alveolar macrophages or dendritic cells should

FIG. 4. Intranasal administration of Poly-ICLC protects mice infected with anthrax spores better than intraperitoneal and intramuscular routes. Intranasal Poly-ICLC (25 μ g) given 24 h prior to lethal inhalational anthrax exposure offers better protection than intramuscular and intraperitoneal administration. *Indicates significance from control by Fisher Exact test ($p < 0.05$).

provide the best explanation because these cells are directly involved with spore phagocytosis in the lungs. The intraperitoneal administration of Poly-ICLC was added to induce IFN in the peritoneum and in circulation (Guerrero-Plata and others, 2005). Interestingly, we also noted that dosing the animals with an intraperitoneal dose of Poly-ICLC inhibited the protective effect of the intranasal dose, perhaps by causing migration of systemic macrophages to the peritoneal cavity (Fig. 5).

FIG. 5. Intraperitoneal administration of Poly-ICLC eliminates the protective effects of intranasal administration of Poly-ICLC. Intraperitoneal administration of Poly-ICLC (25 μ g) given 24 h prior to lethal inhalational anthrax offers no protective effects against inhalational anthrax and eliminates the protective effects of the intranasal dose (25 μ g) when combined. *Indicates significance from control by Fisher Exact test ($p < 0.05$).

Discussion

The 2001 bioterrorist release of anthrax spores proved that the threat is serious and often leads to a lethal infection (Dewan and others 2002). The current treatment of exposed or infected individuals is antibiotic administration, mainly an extended course of ciprofloxacin or levofloxacin. The effectiveness of antibiotics is dependent on time of initiation of treatment (Heine and others 2007). Current therapy of human patients with inhalational anthrax using antibiotics and supportive care results in 60% survival, but only when treatment is begun during the initial phase of illness (Jernigan and others 2001). Although the USAMRIID's handbook recommends 60 days of antibiotics, some of the survivors of the most recent outbreak were treated for shorter times. Experimental treatment with passive antibodies to PA is being developed and tested (Peterson and others 2006). For these reasons, and for the protection of our communities, improved therapies are needed. A new potential therapy for anthrax is IFN-based stimulation of innate immunity, which is also protective experimentally against other bacterial infections (Giron and others 1972; Baron and others 1985; Bogdan 2000). Interferon protects cultured cells against challenge with *B. anthracis*, but there are only preliminary reports of protection of animals against inhalation anthrax (Gold and others 2004; Gold and others 2007; Mancuso and others 2007). Our study has demonstrated that IFN protects mice against inhalation anthrax. We found that (1) the type I IFN inducer, Poly-ICLC, can elicit strong protection in mice; protection by IFN begins rapidly—within hours; (2) the protection is probably due to the induced IFN since recombinant murine IFN- β can protect, and protection by Poly-ICLC is abrogated in IFNAN KO mice; and (3) the greatest protection by Poly-ICLC was conferred by the intranasal route of administration, although the intramuscular route was also protective. This protective action by IFN may have important medical implications for therapy of human anthrax, which requires early treatment to reduce the high fatality rate. In future studies, we anticipate that higher doses of the IFNs, which would mimic the very high concentrations induced by Poly-ICLC (10,000–50,000 U, persisting for 24–48 h), will result in stronger protection. Type II IFN- γ has a different range of immune functions than type I IFN, and has a lower specific activity, which may help explain why it did not protect mice from infection (Baron and others 2007).

Combination therapy of monkeys with antibiotics and vaccination improved survival (Altboum and others 2002; Vietri and others 2006). The very recent report that IFN- γ can enhance lethality in mice (Gold and others 2007) infected with the Sterne strain indicates that innate immune protection by IFNs may be complex and controlled by variables such as strains of *B. anthracis* that lack important virulence factors, time of treatment, and types of IFN. Study of these variables will help optimize treatment regimens. Further improvement of survival may come from combining standard therapy with antibiotics, with antibodies to the toxins, and with the experimentally effective IFNs. Such studies are in progress in animal models.

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