Effect of nasal immunization with protective antigen of Bacillus anthracis on protective immune response against anthrax toxin

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Abstract

Anthrax toxin consists of three proteins: protective antigen (PA), lethal factor (LF) and edema factor (EF). PA in combination with LF (lethal toxin) is lethal to mammalian cells and is the major component of human anthrax vaccine. Immunization with PA elicits the production of neutralizing antibodies that form a major component of the protective immunity against anthrax. Recent reports have shown that neutralizing antibody titres can serve as a reliable surrogate marker for protection against anthrax. In the present study, the use of non-invasive routes such as bare skin and nose for immunization with PA on its protective immune response was investigated. Mice were inoculated intranasally (i.n.), subcutaneously (s.c.) or through the skin on days 0, 15 and 28 with purified PA. Intranasal and subcutaneous immunization with PA resulted in high IgG ELISA titers. The predominant subclass in each group was IgG1. High titres of IgA were observed only in i.n. immunized mice. In a cytotoxicity assay these sera protected J774A.1 cells from lethal toxin challenge. The results suggest that non-invasive nasal immunization may be useful in improving vaccination strategies against anthrax. © 2002 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Anthrax is a bacterial disease caused by Bacillus anthracis. The disease is normally confined to herbivores such as sheep, goat and cattle, but can also infect humans [1]. The major virulence factor associated with B. anthracis is a tripartite protein exotoxin called anthrax toxin. The three components of anthrax toxin are protective antigen (PA), lethal factor (LF), and edema factor (EF). Individually, all the three components are non-toxic to mammalian cells. However, in binary combination, PA plus LF (lethal toxin) causes lysis of mouse macrophages [2] and PA plus EF (edema toxin) raises the intracellular cAMP levels causing cellular edema [3].

PA is the most immunogenic component of anthrax toxin and is a necessary component of anthrax vaccine [4,5]. We have previously shown that a non-toxic mutant PA protein completely protected guinea pigs from B. anthracis spore challenge [6]. The ability of this organism to form resistant spores and infect via the aerosol route has led it to be a potential agent of bioterrorism. There has been a continuous demand for a vaccine against anthrax in various countries [7]. The currently available human vaccines are far from ideal; they are expensive to produce, require repeated doses and may invoke transient side effects in some individuals. The current US human anthrax vaccine, anthrax vaccine adsorbed (AVA), consists of aluminium hydroxide-adsorbed supernatant material from fermentor cultures of a toxigenic non-encapsulated strain of B. anthracis, V770-NPI-R. Currently, the human vaccination strategy against anthrax utilizes a course of three subcutaneous injections 2 weeks apart (0–2–4), followed by three injections 6 months apart (6–12–18) and annual booster doses as long as the individual remains at risk [8]. These vaccines cause local pain, edema, erythema and require several boosters [9]. Recent reports have also shown that there has been a shortage in the supply of anthrax vaccine [7]. Thus, there is an urgent need to improve the efficacy of the anthrax vaccine to overcome these limitations.

In recent years, it has been shown that non-invasive routes for vaccine delivery such as the bare skin or the nose improve the efficacy of vaccination against various diseases due to the presence of a large amount of associated lymphoid tissue and antigen presenting cells [10–12]. However, no such studies have been carried out in the case of anthrax. The present study was thus, undertaken to investigate the protective efficacy of intranasal and skin immunization against anthrax toxin.
2. Materials and methods

2.1. Purification of PA

PA was purified from the culture supernatant of *B. anthracis* transformed with plasmid pYS5 as described earlier [13].

2.2. Immunizations and sample collection

Groups of six mice (female Swiss albino) each weighing 18–20 g received three doses of PA at intervals of 2 weeks intranasally (i.n.), subcutaneously (s.c.) and through the skin. PA (10 μg) was administered in a drug carrier consisting of 140 μl of 12.4 mg soya phosphatidyl choline, 1.7 mg sodium cholate and 11.3 mg ethanol in 10 mM phosphate buffer pH 7.4 that was prepared using a previously described protocol [14]. Control groups were immunized either with PA (without the carrier) or with carrier alone (without PA). Mice were immunized on day 0 and boosted two more times on a 2-week schedule. One week after final immunization, mice were sacrificed and serum was separated as described earlier [15]. Sera were stored at −20 °C until used.

2.3. Measurement of antigen-specific immunoglobulins

Sera were examined for the levels of PA specific immunoglobulins by enzyme linked immuno-sorbent assay (ELISA). PA (1 μg per well in 100 μl) was coated on 96 well ELISA plates for 12 h at 4 °C. The wells were then washed twice with wash buffer (PBS + 0.05% Tween 20) and blocked with 2% fat-free milk for 2 h. After washing twice with wash buffer the plates were incubated with different dilutions of sera obtained from mice of control groups or from mice immunized with PA i.n., s.c. and through skin for 12 h at 4 °C. The wells were then washed twice with wash buffer and incubated with horseradish peroxidase conjugated goat anti-mouse secondary antibody (isotype specific as well as IgG whole). Unbound antibodies were removed by washing four times with wash buffer and the bound antibodies were detected using o-phenylene diamine as the substrate along with H2O2. The reaction was stopped by adding 50 μl of 2N H2SO4 and absorbance was read at 490 nm in an ELISA reader (Molecular Devices, CA). The end point dilution was defined as the highest dilution exhibiting absorbance higher than two standard deviations above the negative control (pre-immune sera). Antibody titres were expressed as the reciprocal of end point dilution.

2.4. Competitive inhibition or neutralization assay

In order to test the protective efficacy of the antibodies raised against PA, various dilutions of sera obtained from different routes of immunization were added in combination with PA and LF to J774A.1 macrophage cells and incubated for 3 h at 37 °C. At the end of the experiment, cell viability was measured by MTT assay as described earlier [16].

3. Results and discussion

PA is the main component in the existing vaccine to protect against *B. anthracis*. Although, the currently available animal and human vaccines are effective, they have limitations. Immunization with the human vaccine can induce local pain, edema, and erythema. Vaccines delivered by a non-invasive route such as bare skin and nose offer considerable advantages over injectable vaccines. This is due to the presence of large amounts of associated lymphoid tissue and antigen presenting cells which ensures efficient presentation of antigen to the immunocompetent cells [17,12]. The primary aim of this study was to determine whether a non-invasive route of delivery of anthrax vaccine could be used as an alternative to the parentally administered vaccines. The antigen-specific serum IgG titers obtained from mice immunized with PA i.n. and s.c were 7800 and 5700, respectively (Fig. 1). Very low antibody titer was observed...
in sera of mice immunized with PA through skin. No detectable antibody titer was observed in sera of mice when immunized with the drug carrier alone through the skin or as free protein (without carrier) administered either through the nose (Fig. 1) or s.c. (data not shown). The predominant immunoglobulin subclass was IgG1 in both s.c. as well as i.n. immunized mice, whereas the titres of IgG2a were substantially low. High titres of IgA were seen only in cases of mice immunized i.n., which is indicative of a mucosal immune response (Fig. 2). The results suggest that both intranasal and subcutaneous route of immunization induce antibody generation against PA.

A correlation between protection and neutralization titres of PA was recently observed in guinea pigs [18] and other animal models [19,20], suggesting that this phenomenon is not species specific. It has been shown that passive transfer of PA immunized guinea pig sera into guinea pigs and SCID mice leads to complete protection [21]. In a recent report it has been shown that passive transfer of lymphocytes and sera from mice immunized with recombinant PA provides protection against B. anthracis infection [22]. This ability of anti-PA antibodies to neutralize the cytotoxic effect of lethal toxin complex suggests that neutralizing antibody titres can serve as a reliable surrogate marker in evaluating novel vaccines in preclinical studies [18].

In the neutralization or competitive inhibition assay, where only 27% of the cells incubated with PA plus LF survived, 99% of the cells were protected from lethal toxin challenge even in combination with antibody dilution of 1:1000 obtained from s.c. as well as i.n. immunized mice (Fig. 3). Serum obtained from mice immunized with PA through skin protected more than 50% of the cells at a dilution of 1:100 (Fig. 3). The data presented here show that immunization with PA i.n. raises considerable protective.

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**Fig. 2. Determination of antigen-specific immunoglobulins.** PA (1 μg per well in 10 μl) was coated on ELISA plates for 12 h at 4°C. The wells were then washed twice with wash buffer (PBS + 0.05% Tween 20), blocked with 2% fat-free milk for 2 h, washed twice with wash buffer and incubated with different serum dilutions obtained from control mice (Con. 1, PA alone without carrier; and Con. 2, carrier alone without PA) or mice immunized with PA (10 μg) i.n., s.c. and through skin or without protein for 12 h at 4°C. The wells were then washed twice with wash buffer and incubated with horseradish peroxydase conjugated anti-mouse secondary antibody (isotype specific) raised in goat at a dilution of 1:1000 at 37°C for 3 h. After washing four times with wash buffer, 100 μl of o-phenylenediamine was added along with 1 μl H₂O₂ per well. The reaction was stopped by adding 50 μl of 2N H₂SO₄ per well and absorbance was read at 490 nm in an ELISA reader (Molecular Devices, CA). Antibody titres were determined in serum pools derived from six mice. Each value represents mean titre ± S.E.M. of three different experiments done on triplicate.

**Fig. 3. Competitive inhibition of lethal toxin activity:** J774A.1 cells were incubated with PA (0.1 μg/ml), LF (1 μg/ml) in combination with varying dilutions of pooled sera obtained from control mice (Con. 1, PA alone without carrier; and Con. 2, carrier alone without PA) or mice immunized with PA i.n., s.c. and through skin or without PA (no protein) for 3 h at 37°C. At the end of 3 h, cell viability was determined by MTT assay. Each value represents mean titre ± S.E.M. of three different experiments done on triplicate.
antibody response and can be a substitute for subcutaneous injection. Being a non-invasive method, intranasal immunization will be cost effective with negligible side effects and will be very suitable for mass immunization programs.

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References