Passive protection by polyclonal antibodies against Bacillus anthracis infection in guinea pigs.

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Passive Protection by Polyclonal Antibodies against Bacillus anthracis Infection in Guinea Pigs

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The protective effects of polyclonal antiserum produced by injecting guinea pigs with protective antigen (PA), the chemical anthrax vaccine AVA, or Sterne spore vaccine, as well as those of toxin-neutralizing monoclonal antibodies (MAbs) produced against PA, lethal factor, and edema factor, were examined in animals infected with Bacillus anthracis spores. Only the anti-PA polyclonal serum significantly protected the guinea pigs from death, with 67% of infected animals surviving. Although none of the MAbs was protective, one PA MAb caused a significant delay in time to death. Our findings demonstrate that antibodies produced against only PA can provide passive protection against anthrax infection in guinea pigs.

The pathogenesis of anthrax, a disease caused by Bacillus anthracis, is associated with the presence of a plasmid-encoded toxin and two protein exotoxins. The capsule and exotoxins are encoded by plasmids pXO2 (6, 31) and pXO1 (25), respectively. The exotoxins consist of two binary toxins sharing a common binding component and possessing separate effector components, each expressing little or no individual toxicity. Lethal toxin, composed of protective antigen (PA) and lethal factor (LF), kills susceptible animals (1) and is rapidly cytolytic for macrophages in vitro (4). LF has sequences homologous to a zinc metallopeptidase (15, 16). Edema toxin, composed of PA and edema factor (EF), causes edema (3, 29) attributed to the calmodulin-dependent adenylate cyclase activity of EF (17). For activation of either toxin, PA undergoes limited proteolysis after binding to cells, thereby exposing a site on the receptor-bound fragment, PAα3. It is this fragment to which either effector component, LF or EF, can then bind (18). The toxin complex is then taken into cells by receptor-mediated endocytosis (4, 5), and this is followed by translocation of LF or EF to the cytosol.

Protection against anthrax is provided by vaccines that contain PA as the major immunogen (7, 11). The vaccine approved for human use in the United States, AVA, contains aluminum hydroxide adsorbed to filtered culture supernatant fluid from a toxigenic, nonencapsulated strain of B. anthracis. The approved veterinary vaccine consists of spores of the Sterne isolate (containing plasmid pXO1 but not pXO2) of B. anthracis.

To better understand the role that antibodies play in host defense against infection with B. anthracis, we tested polyclonal antiserum produced by injecting guinea pigs with either PA, AVA, or Sterne vaccine for their ability to passively protect against anthrax infection. To specifically examine the relative roles of lethal and edema toxins in pathogenesis, we also tested toxin-neutralizing monoclonal antibodies (MAbs) against PA, LF, and EF. In addition, these experiments were aimed at identifying antigenic regions on relevant proteins essential for induction of immunity to anthrax.

(Portions of this work were presented at the 94th General Meeting of the American Society for Microbiology, Las Vegas, Nev., 23 to 27 May 1994 [22].)

MATERIALS AND METHODS

Polyclonal and monoclonal antiserum. Polyclonal antiserum were produced in Hartley guinea pigs (Charles River Laboratories). Antiserum against PA was prepared by intramuscular (i.m.) injection of PA (20 μg/0.5 ml) emulsified with Ribi adjuvant (Ribi Immunocor Inc., Hamilton, Mont.) at weeks 0, 2, 4, and 12. PA was isolated from Sterne-1(pPAA102) (kindly provided by J. Farchaus, United States Army Medical Research Institute of Infectious Diseases (USAMRIID)), a recombinant strain that does not synthesize capsule, LF, or EF (12). Animals were bled 3 weeks later for antisera. Antiserum against AVA was prepared by injecting guinea pigs i.m. with AVA (0.5 ml; Michigan Biologic Products Institute, Lansing, Mich.) at 0, 4, and 8 weeks. Antiserum was also prepared by injection of spores, prepared at USAMRIID, from the Sterne veterinary vaccine (Burroughs Wellcome Co., Kansas City, Mo.). Spores were prepared by intramuscular injection of PA (20 μg/0.5 ml) emulsified with Ribi adjuvant (monophosphoryl lipid A, trehalose dimycolate, and cell wall skeleton from Mycobacterium phlei; Ribi Immunocor Inc.) at weeks 0, 2, 4, and 12. PA was isolated from Sterne-1(pPAA102) (kindly provided by J. Farchaus, United States Army Medical Research Institute of Infectious Diseases (USAMRIID), a recombinant strain that does not synthesize capsule, LF, or EF (12). Animals were bled 3 weeks later for antisera. Antiserum against AVA was prepared by injecting guinea pigs i.m. with AVA (0.5 ml; Michigan Biologic Products Institute, Lansing, Mich.) at 0, 4, and 8 weeks. Antiserum was also prepared by injection of spores, prepared at USAMRIID, from the Sterne veterinary vaccine (Burroughs Wellcome Co., Kansas City, Mo.). Spores were prepared by intramuscular injection of PA (20 μg/0.5 ml) emulsified with Ribi adjuvant (monophosphoryl lipid A, trehalose dimycolate, and cell wall skeleton from Mycobacterium phlei; Ribi Immunocor Inc.) at weeks 0, 2, 4, and 12. PA was isolated from Sterne-1(pPAA102) (kindly provided by J. Farchaus, United States Army Medical Research Institute of Infectious Diseases (USAMRIID)), a recombinant strain that does not synthesize capsule, LF, or EF (12). Animals were bled 3 weeks later for antisera.

MAbs used in this study, PA II 14B7, PAα3, IG, LF III 10G5, and EF III 9F5, were characterized previously (20, 21, 23, 24). MAb Y-NA05-AH02, produced against Junin virus nucleocapsid (26), served as an unrelated control MAb. All the MAbs were of the IgG1 subclass. IgG was purified from ascitic fluid by using a HiTrap protein G affinity column (Pharmacia Biotech) according to the manufacturer's directions. Purified Ig was diazylated against phosphate-buffered saline (PBS; 10 mM sodium phosphate–0.15 M NaCl, pH 7.3). Quantitation of IgG was performed by radial immunodiffusion analysis with an anti-rabbit anti-mouse or anti-guinea pig IgG and mouse or guinea pig IgG (Sigma Chemical Co.) as standards, respectively.

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as prepared by the Committee on Care and Use of Laboratory Animals, as prepared by the Committee on Care and Use of Laboratory Animals, Commission of Life Sciences-National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

Binding inhibition studies. Inhibition of toxin formation in vitro was performed as described previously. Briefly, inhibition of 125I-PA binding to L6 cells (20) was measured by preincubating the antibody with 125I-PA (100 ng/ml) before adding it to L6 cells and incubating them overnight at 4°C. The radioactivity was counted after the cells were washed and solubilized. To measure inhibition of the binding of 125I-LF (21) or 125I-EF (23) to cell-bound PA, L6 cells were first incubated with PA overnight at 4°C. The cells were then washed and preincubated with antibody samples for 1 h before 125I-LF or 125I-EF was added (50 ng/ml). After 2 h, the cells were washed and solubilized and the cell-associated radioactivity was counted. Counts due to nonspecific binding, determined by incubating the radioactive ligand in the presence of a 250-fold excess of nonradioactive ligand, were subtracted from all sample counts and represented <15% of the total counts bound. All assays were performed in triplicate.

In vitro lethal toxin neutralization assay. In vitro neutralization of the cytolytic activity of lethal toxin, in which antibody samples were preincubated with lethal toxin (400 ng of PA per ml and 40 ng of LF per ml) before being added to...
toxin or edema toxin activity in vitro was also demonstrated in EF III 9F5. The ability of the MAbs to neutralize lethal equivalent effect was observed with tenfold-more PA 2II 14B7 and 0.4 mg of purified MAb IgG or unpurified guinea pig polyclonal-antiserum IgG 6 h obtained from Charles River Laboratories, San Diego, Calif. (23).

Linear regression analysis with the InStat software program (GraphPad Software, San Diego, Calif.) was used to determine the potency of the antibodies in an infection. Figures 2 and 3 show the relative reactivities of the guinea pig antisera against anthrax infection.

### RESULTS AND DISCUSSION

Table 1 summarizes the characteristics of the test antibodies used in this study. With regard to the polyclonal antisera, the cytolytic activity of lethal toxin was neutralized in vitro with 0.3 and 0.4 µg of anti-Sterne vaccine and anti-PA sera per ml, respectively. An equivalent effect was observed with threefold more guinea pig anti-AVA serum. In the in vitro CHO cell assay, edema toxin activity was neutralized with 1 µg of anti-PA serum per ml while an equivalent effect was observed with threefold-more anti-AVA serum or tenfold-more anti-Sterne vaccine serum. The binding inhibition assays demonstrate the ability of the antisera to inhibit toxin formation. Except for anti-AVA, which only blocked the binding of PA to cells, the guinea pig polyclonal antisera blocked the binding of all three components. The antisera produced against PA was slightly more active than the anti-Sterne vaccine serum. With the MAbs, the cytolytic activity of lethal toxin was neutralized in vitro with 0.04 µg of MAb PA 10G3 per ml. An equivalent effect was observed with 20- to 25-fold-more PA 2II 14B7 or LF III 10G3. Passive protection of rats against lethal toxin has been shown to be afforded by MAbs PA 2II 14B7 (20), PA 10G3 (100 µg of IgG) (21), and LF III 10G3 (50 µg of IgG) (23). In the in vitro CHO cell assay, edema toxin activity was neutralized with 1 µg of PA 10G3 per ml. An equivalent effect was observed with tenfold-more PA 2II 14B7 or LF III 10G3. The ability of the MAbs to neutralize lethal toxin or edema toxin activity in vitro was also demonstrated in their ability to inhibit the formation of the toxins. MAb PA 2II 14B7 inhibited the binding of PA to cells (23), while PA 10G3 inhibited the binding of the LF or EF component to PA (24).

The LF and EF MAbs blocked only the binding of their respective components to PA (21, 23).

### Table 1. Characteristics of antibodies used for passive protection against anthrax infection

<table>
<thead>
<tr>
<th>Test antibody</th>
<th>Neutralization of:</th>
<th>Inhibition of binding of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lethal toxin&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Edema toxin&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Anti-PA</td>
<td>0.4</td>
<td>1</td>
</tr>
<tr>
<td>Anti-Sterne</td>
<td>0.3</td>
<td>10</td>
</tr>
<tr>
<td>Anti-AVA</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>PA&lt;sub&gt;10&lt;/sub&gt; 1G3</td>
<td>0.04</td>
<td>1</td>
</tr>
<tr>
<td>LF 10G3</td>
<td>0.05</td>
<td>&gt;100</td>
</tr>
<tr>
<td>EF 10G3</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Y-NA05-AH02</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

<sup>a</sup> Results similar to those of some of these MAbs were reported previously (see the text).
<sup>b</sup> Anti-PA, anti-AVA, and anti-Sterne vaccine (anti-Sterne) polyclonal sera were produced in guinea pigs. Prefixes identify the antigens used to raise MAbs PA 2II 14B7, PA<sub>10</sub> 1G3, LF III 10G3, and EF III 9F5. Y-NA05-AH02 served as a nonrelevant control MAb.
<sup>c</sup> Concentration of IgG required to neutralize the cytolytic activity of lethal toxin in vitro to 50% of the levels of controls.
<sup>d</sup> Concentration of IgG required to inhibit by 80% the elongation of CHO cells in the presence of edema toxin.
<sup>e</sup> Concentration of IgG required to inhibit the binding of 125I-LF to L6 cells by 50%.
<sup>f</sup> Concentration of IgG required to inhibit the binding of 125I-LF to cell-bound PA by 50%.
<sup>g</sup> Concentration of IgG required to inhibit the binding of 125I-EF to cell-bound PA by 50%.

### References

1. Little ET al. INFECT. IMMUN. 2014, 82(2), 412-419.
antiserum prepared against recombinant PA. Figure 2 shows results from two separate experiments in which the data from 23 animals in the two NGP groups for each experiment were combined. Results for each test antiserum were statistically compared to those of the respective NGP control group. As shown in Fig. 2, the guinea pig polyclonal antisera produced against PA and AVA protected 67 and 33%, respectively, of the animals. By Fisher’s exact (two-tailed) test, the difference between the survival rates of the anti-PA group and NGP controls was significant ($P < 0.009$), but that of the anti-AVA group and NGP controls was not. The difference between the survival rates of the anti-PA and anti-AVA groups was not significant. Statistical differences between the delays in time to death, as measured by the log-rank statistic, for the anti-PA and anti-AVA groups compared to NGP controls were significant ($P < 0.001$ and $P < 0.018$, respectively). Differences between the times to death for the anti-PA and anti-AVA groups were also significant ($P < 0.028$). Thus, time-to-death analysis revealed that anti-AVA treatment significantly protected the animals compared to controls and that anti-PA serum was more protective than anti-AVA serum. Antiserum produced against the Sterne vaccine was not protective against death (8% survival), although it did cause a significant delay in time to death compared to controls ($P < 0.011$) (Fig. 2). Differences between the anti-PA and anti-Sterne vaccine groups were significant in terms of survival rates ($P < 0.009$) and time to death ($P < 0.013$). Protection, as measured by survival or a delay in time to death, was not significantly different for the anti-AVA and anti-Sterne vaccine groups.

The relationship between protection and the various measured parameters for each polyclonal antiserum was complex. Correlation analysis showed that the anti-PA titer (Table 2) correlated best with survival rates ($r = 0.9115$) and harmonic mean time to death (HMTTD) ($r = 0.9591$). Also, with a multivariable forward stepwise regression model, the PA ELISA titer was the first variable identified as being associated with increases in both the mean time to death ($P = 0.010$) and survival ($P = 0.031$).

**TABLE 2. ELISA titers of anti-PA, anti-AVA, and anti-Sterne sera against PA, LF, and EF antigens**

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>ELISA titer against $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PA</td>
</tr>
<tr>
<td>Anti-PA</td>
<td>840,760</td>
</tr>
<tr>
<td>Anti-AVA</td>
<td>14,728</td>
</tr>
<tr>
<td>Anti-Sterne vaccine</td>
<td>30,387</td>
</tr>
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</table>

$^a$ ELISA titers are expressed as the reciprocals of dilutions measured at 0.2 OD$_{405}$, derived from Fig. 1 by linear regression analysis.
The toxin neutralization and LF and EF binding inhibition assays were not performed to distinguish between PA-directed and LF- or EF-directed antibodies. The activity of the anti-PA serum was due to the presence of only PA-directed antibodies that inhibited both binding of PA to cells and binding of LF or EF to cell-bound PA (Table 1). LF and EF binding inhibition activity was also present in the anti-Sterne vaccine serum. However, we could not determine whether this activity was due to PA-directed or to LF- or EF-directed antibody, since anti-LF and anti-EF antibodies were present in the anti-Sterne vaccine serum (Table 2). Also, cross-reactivity between anti-LF and anti-EF antibodies cannot be excluded, since LF and EF share regions of homology and compete for binding to PA (18). However, antibodies produced by injecting guinea pigs with Sterne vaccine were not very protective, as demonstrated by the survival rate, which was not significantly higher than that of NGP controls. The activity of the anti-AVA sera was also due mainly to the presence of only PA-directed antibodies (Table 1). Whereas the anti-PA serum antibodies neutralized antigen by interacting with both the cell binding and the LF-EF binding domains, the anti-AVA serum neutralized antigen by interacting with the cell binding domain of PA only. It may be that presenting PA bound to aluminum hydroxide inAVA did not allow induction of antibodies to the LF-EF binding site while administration of PA with Ribi’s adjuvant did. The anti-AVA serum did not inhibit binding of 125I-LF or 125I-EF to cell-bound PA, in contrast to the anti-Sterne vaccine serum, and the ELISA revealed a relatively low anti-LF titer and no detectable anti-EF titer, as was observed by others (30). A comparison of the passive protective efficacy and the antibody titers of the anti-AVA and the anti-Sterne vaccine sera suggests that protection was due to anti-PA antibodies and was unrelated to anti-LF and anti-EF antibodies. This is supported by the observation that the anti-Sterne vaccine serum, which possessed significantly higher titers of antibody to LF and EF while containing a similar anti-PA titer (Table 2 and Fig. 1), gave no additional protection compared to that of the anti-AVA serum (Fig. 2).

Although the MAbs used in this study neutralized lethal toxin or edema toxin activity in vitro (Table 1) or in vivo (20, 21, 24), none of the MAbs protected against death from a lethal spore challenge (Fig. 3). A previous study from this laboratory (32), in which MAbs (except for PA63 1G3) with similar characteristics were used, showed that PA and LF MAbs, but not an EF MAb, protected mice challenged with the attenuated Sterne strain. However, none of the MAbs was protective against a challenge with the fully virulent strain Vollum 1B (pXO1− pXO2+). In this study, guinea pigs treated with PA63 1G3 had a delay in time to death (Fig. 3) that was significant compared to that of the controls (P < 0.0001). The abilities of MAbs PA63 1G3 to inhibit the binding of 125I-LF and 125I-EF to the catalytic binding site on PA and to cause a significant delay in the time to death suggest that this region may be important immunologically.

The role that antibodies play in vaccine-induced resistance to or protection against anthrax infection has not been fully defined. It has been demonstrated that protection against anthrax lethal toxin is antibody mediated, and the use of antitoxin serum as an adjunct with bactericidal antibiotics is recommended for effective treatment of anthrax infection (for a review, see reference 19). Passive administration of antiserum prepared against uncharacterized crude culture filtrates containing PA (2) and against Sterne-type spore vaccines (9) has been reported to provide complete protection of other species, such as rabbits. Our studies in the guinea pig model are the first to report that antisera to recombinant PA, devoid of antibodies to other toxin components, can provide highly significant passive protection against anthrax infection. The level of protection achieved by passive administration of small amounts of anti-PA antibody (67%) was comparable to or better than that achieved after active immunization of guinea pigs with AVA and approaches that seen after active immunization with PA plus Ribi’s adjuvant (10, 11, 13, 30). Studies have shown that protection against anthrax is afforded in the
absence of antibodies to LF and EF and that LF and EF may play only a minor role in protection from anthrax infection (10, 30). Results from the in vitro binding inhibition assays characterized the toxin-neutralizing action of our polyclonal antiserum. We do not know the number of epitopes on PA, or even on LF or EF, that are capable of inducing a protective immune response against infection. We also cannot rule out differences in affinity, avidity, complement binding, or opsonization activity of our test antisera as an explanation for our observations. The ability to protect infected guinea pigs by passive administration of anti-PA antibodies suggests that these antibodies play a major role in resistance to anthrax.

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REFERENCES


