Bactericidal Activity of Blood of Rabbits Vaccinated With Homologous Antigens of Campylobacter fetus (Vibrio fetus)  

MARYON BORDER, L. L. MYERS, AND B. D. FIREHAMMER  
Veterinary Research Laboratory, Montana State University, Bozeman, Montana 59715  

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Rabbits were vaccinated with the following Campylobacter fetus var. venerealis (Vibrio fetus) antigens: whole-cell (WC), autoclaved (A), boiled (B), and purified postgrowth broth (PGB). Bactericidal activity of freshly drawn heparinized blood against the organism was determined after each vaccination. In all cases bactericidal activity of the blood of vaccinated rabbits was higher than for nonvaccinated rabbits. The in vitro bactericidal activity of the blood was determined in two separate experiments. In experiment I the bactericidal activity of the blood of rabbits vaccinated with PGB antigen was the same as that of rabbits vaccinated with WC antigen and higher than that of rabbits vaccinated with A antigen after the third vaccination. In experiment II the bactericidal activity of blood of rabbits vaccinated with PGB antigen was the same as that of those vaccinated with WC antigen after the second and third vaccinations and higher than for rabbits vaccinated with A antigen after the third vaccination. Blood of rabbits vaccinated with A antigen was less bactericidal than blood of rabbits vaccinated with B antigen after the third vaccination, indicating the presence of a surface antigen destroyed by autoclaving but not by boiling. The in vivo and in vitro whole blood bactericidal tests are more sensitive for measuring the response of rabbits vaccinated with WC, B, A, or PGB antigens than is the plate agglutination test.

Campylobacter fetus var. venerealis is strictly adapted to the bovine genital tract and causes enzootic venereal sterility or abortion in cows. Bacterins have been used effectively for the control of bovine vibriosis under range conditions (6). Agglutination, a relatively insensitive test for the presence of antibody (15), is sometimes used for evaluating the potency of C. fetus vaccines. In general, the serum bactericidal assay is more sensitive for measuring antibody than agglutination or passive hemagglutination techniques (1, 8). Since bacteriolysis and opsonization are mechanisms of host resistance to bacterial invasion (5, 14), a whole-blood bactericidal test (5, 10, 19) may be a more accurate measure of the protective capacity of a vaccine than is the agglutination test.

We did some preliminary in vivo studies of bacterial clearance in sheep and rabbits vaccinated with killed whole cells and challenged with homologous and heterologous viable C. fetus var. intestinalis (B. D. Firehammer, R. L. Berg, M. Border, and L. L. Myers, unpublished data). After intravenous challenge, there was marked clearance of homologous C. fetus from the blood of sheep within 10 min and slight or no apparent clearance of heterologous C. fetus at 1.5 to 6 h. Similar results were obtained with rabbits.

In this work, we hoped to gain information concerning the relative ability of various C. fetus antigens, when injected into rabbits, to increase the bactericidal activity of freshly drawn whole blood. We were especially interested in the ability of postgrowth broth (PGB) antigen to stimulate the production of bactericidal or opsonizing antibody. PGB antigen is a glycoprotein (12) released into the broth culture medium during growth of C. fetus. When used in a vaccine, PGB antigen can elicit protection in pregnant ewes against challenge with live homologous C. fetus (13).

MATERIALS AND METHODS

Media. The basal medium contained 2.8% brucella broth, 0.5% yeast extract, 0.2% sodium succinate, and 0.1% magnesium chloride (MgCl2, 6H2O). Semisolid agar was made by adding 0.15% agar to the basal medium; assay medium was made by adding 0.002% ferrous sulfate (FeSO4·7H2O) 0.002 or 0.004% alkaline hemin, and 1.5% agar. The broth cultures were
grown in flasks on a reciprocating shaker at 37 C (7). For the determination of the number of viable organisms, 8 ml of melted assay medium was poured into a screw-capped tube (200 by 25 mm) containing 1 ml of the material to be assayed and mixed well by gentle swirling, and the tubes were placed in a horizontal position to allow the agar to set (M. M. Border, B. D. Firehammer, and L. L. Myers, submitted for publication). The tubes were incubated in the same position, with their bases at 37 C for 5 days with 5% carbon dioxide (National CO2 incubator, National Appliance Company, Inc., Rochester, N.Y.). The cultures on semisolid agar were incubated two days at 37 C in 10% carbon dioxide.

**Cultures and antigens.** *C. fetus* var. *venerealis* (13831, A-sub-1) was isolated from a bovine fetus (3). Defibrinated bovine blood was added to the growth from semisolid agar and stored at ~30 C until used. Except for slight modifications, autoclaved (A), boiled (B), and whole-cell (WC) antigens were prepared by methods described earlier (2, 13). Growth from a 24-h broth culture was centrifuged at 4,080 × g for 20 min and the cells were resuspended in 0.85% saline solution. This suspension was divided into two portions; one was boiled for 2 h (B antigen) and the other was autoclaved for 2 h (A antigen) at 121 C. For the WC antigen, a 24-h broth culture was formalized (0.3% formalin). These were all centrifuged, and the resulting pellets were resuspended in physiological saline to one-half the volume present in the growth medium.

Antigen used in the PGB vaccine was isolated from the growth medium by ammonium sulfate precipitation, followed by preparative polyacrylamide gel electrophoresis, as previously described (12). After electrophoresis, the location of the PGB antigen in the acrylamide gel sheet was determined by using the Rf value (distance antigen migrated through small-pore gel/distancesmall molecules at salt front migrated) of 0.30. The acrylamide gel containing the PGB antigen was forced through an 80-mesh screen into a small amount of distilled water to give a homogeneous suspension of gel particles. Formalin (0.3%) was added to the acrylamide gel particles plus PGB antigen before vaccination of rabbits. Quantitation of PGB antigen present in solution before electrophoresis was done by elution of stain (Ponceau S) after paper electrophoresis as described earlier (12).

**Vaccination of rabbits.** Three, 4 and 5 rabbits were vaccinated twice subcutaneously with B, PGB, or WC antigen, respectively, 7 months before the beginning of experiment I. In experiment I these rabbits were revaccinated subcutaneously with the same antigens as before except that the rabbits given B antigen earlier were vaccinated in experiment I with A antigen. Four rabbits were not vaccinated. Rabbits received 3 mg of PGB antigen in acrylamide gel per vaccination. In experiment I rabbits received one subcutaneous vaccination with 2 ml of vaccine containing one part aluminum hydroxide (2% aluminum hydroxide) gel and one part antigen.

For experiment II, 26 10-week-old rabbits were placed in five groups. Four groups of five rabbits each were vaccinated with either A, B, PGB, or WC antigen. The six rabbits in the control group were not vaccinated. Rabbits were given three subcutaneous vaccinations (1 ml of antigen per vaccination) without adjuvant, 4 and 6 weeks apart.

**Agglutination.** The WC and A antigens were suspended in saline to an optical density of 0.8 at a wavelength of 740 nm in a spectrophotometer (Spectronic 20, Bausch and Lomb, Inc., Rochester, N.Y.). Slide agglutination tests were conducted by using 0.03 ml of antigen added to 0.03 ml of twofold dilutions of serum.

**Bactericidal activity.** In vitro bactericidal activity of freshly drawn blood was determined for rabbits in experiments I and II. The in vitro bacterial clearance rate was also determined for the rabbits in experiment II. Blood was drawn with a disposable 5-ml syringe and a 20- or 22-gauge disposable needle from the median artery of the ear. One portion was added to a tube containing heparin (10 U/ml of blood); the other portion was allowed to clot and the serum was removed for the agglutination test. For the in vitro bactericidal test, 2 ml of heparinized blood was transferred to the standardized bacterial suspension and incubated together at 37 C with gentle shaking. The extent of bacterial inactivation was assessed by determining the number of viable organisms in the incubation mixture at zero time and time intervals during the incubation period. Determinations of viable *C. fetus* were done in duplicate.

In experiment I, 2 ml of heparinized blood was immediately added to 0.1 ml of broth dilution of the bacteriological culture, containing approximately 6 × 10⁷ viable *C. fetus*. Samples of 0.1 ml were removed at 10 and 30 min. Each sample was diluted in broth and assayed for number of viable organisms.

In experiment II, 2 ml of heparinized blood was immediately added to 0.1 ml of broth dilution of the bacteriological culture, containing 2 × 10⁸ to 4 × 10⁹ bacteria. Assay samples were removed and diluted at time intervals up to 30 min.

In the in vivo clearance test (done 7.5 weeks after the third vaccination), 1 ml of broth containing approximately 1.1 × 10⁹ viable organisms was injected into the marginal ear vein of each rabbit. Blood samples were taken from the ear artery at 5, 10, 20, 30, 45, and 60 min after challenge. Samples taken at 5 and 10 min were diluted in broth to facilitate counting of colonies. In the other blood samples, colony-forming units containing in 0.1 ml of undiluted blood were counted.

**Statistical methods.** Analysis of variance (18) of the percentage of bacteria surviving in the bacteriological tests were conducted. The Newman-Keul (18) test was used to compare the means for blood bactericidal activity of rabbits vaccinated with the various antigens. Colony counts for the in vivo clearance test were transformed to log₁₀ values before analysis because the data were not normally distributed.

**RESULTS**

**Experiment I.** Agglutination titers were generally low or absent 4.5 months after the second vaccination (Table 1). Thirteen days after the
third vaccination, an anamnestic response was detected by the agglutination test using WC antigen against antisera from rabbits given WC or PGB vaccine. No anamnestic response was apparent in rabbits vaccinated twice previously.

Table 1. Reciprocal of serum agglutination titers (experiment I)

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>4.5 months after 2nd vaccination</th>
<th>13 days after 3rd vaccination</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WC</td>
<td>A</td>
</tr>
<tr>
<td>Whole cell</td>
<td>10-40</td>
<td>0</td>
</tr>
<tr>
<td>Postgrowth</td>
<td>10-160</td>
<td>0</td>
</tr>
<tr>
<td>broth</td>
<td>0</td>
<td>0-20</td>
</tr>
<tr>
<td>Boiled</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Controls</td>
<td>0</td>
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</tbody>
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*a WC, Whole-cell antigen; A, autoclaved antigen.

The bactericidal activities of blood from rabbits given WC or PGB vaccine were similar (Fig. 1A). The bactericidal activities of blood from control rabbits and those vaccinated with A antigen were similar. The bactericidal activity of blood from rabbits given WC or PGB vaccine was significantly higher ($P < 0.001$) than for control rabbits or rabbits given A vaccine.

**Experiment II.** WC and A antigens were agglutinated with antisera from rabbits given WC or B vaccine (Table 2). Autoclaved antigen was agglutinated with antisera from rabbits given A vaccine, whereas WC antigen was not agglutinated by this antiserum. Neither WC nor A antigens were agglutinated with antisera from rabbits given PGB vaccine except at low titer, after the third vaccination. This agglutination

![Fig. 1. Bactericidal activity of blood. (A) Experiment I, 2 weeks after third vaccination. (B) Experiment II, 2 weeks after first vaccination. (C) Experiment II, 4 weeks after first vaccination. (D) Experiment II, 2 weeks after second vaccination. (E) Experiment II, 5 weeks after second vaccination. (F) Experiment II, 9 days after third vaccination.](http://iai.asm.org/)

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titer was given by the antisera from only one rabbit. The serum agglutination titer at the time the in vivo clearance test was conducted were all low or absent, except for the rabbits given WC vaccine.

In vitro bactericidal activity of the blood of vaccinated rabbits was significantly higher (P < 0.05) than for control rabbits (Fig. 1B–E). Bactericidal activity of the blood of rabbits given PGB vaccine was not significantly different from rabbits given the other vaccines 2 weeks after the first vaccination (Fig. 1B). The bactericidal activity of the blood at 30 min was significantly lower (P < 0.001) for rabbits given PGB vaccine than for rabbits given other vaccines 4 weeks after the first vaccination (Fig. 1C). After the second vaccination (Fig. 1D and E), the bactericidal activity of the blood of rabbits given PGB vaccine was similar to that of rabbits given the other vaccines. After the third vaccination, the bactericidal activity of blood from rabbits given PGB vaccine was similar to rabbits given WC and B vaccine, but significantly higher (P < 0.001) than for those given A vaccine. Bactericidal activity of blood from rabbits given A vaccine was significantly lower (P < 0.001) than that for rabbits given the other vaccines after the third vaccination (Fig. 1F). Bactericidal activity of the blood of rabbits given B vaccine was similar to those given WC vaccine (Fig. 1B–F).

Rabbits given WC or B vaccine cleared C. fetus from their blood significantly faster (P < 0.01) than the controls at 5, 10, 20, and 30 min in the in vivo clearance test (Table 3). Rabbits given A vaccine cleared C. fetus from their blood significantly faster (P < 0.01) than the controls at 5, 10, and 20 min. Clearance rate in rabbits given WC vaccine was significantly faster (P < 0.01) at 10, 20, 30, and 45 min than for any of the other rabbits. The clearance rate in rabbits given PGB vaccine was significantly faster (P < 0.01) than for control rabbits at 5 min but not significantly different from the controls at 10, 20, and 30 min.

**DISCUSSION**

Many gram-negative bacteria are susceptible to the bactericidal action of complement and specific antibody (17). Bactericidal test systems commonly use antisera, complement, and antigen (9, 16, 20). We were unable to demonstrate any bactericidal effect of complement and specific antibody against C. fetus in a system of this type. In a preliminary experiment, we
observed a bactericidal effect of specific antiserum when it was used to opsonize *C. fetus* before incubating with fresh heparinized non-immune whole blood. We concluded that *C. fetus* organisms are not lysed by the action of complement and antiserum alone, but their susceptibility to phagocytosis is increased by incubation with antiserum. Newman et al. (14) observed that under conditions which permit growth of *Haemophilus influenzae*, serum may lack effective bactericidal activity but efficiently promote phagocytic killing of the organism. This mechanism may have been operative in a passive immunization experiment in which immune sera from heifers hyperimmunized with WC antigen of *C. fetus var. venerealis* prevented infection in nonvaccinated heifers challenged intravaginally with viable homologous *C. fetus* (R. L. Berg, B. D. Firehammer, M. Border, and L. L. Myers, unpublished data).

In experiment I, WC agglutination titers of antisera from rabbits vaccinated with PGB vaccine were high, indicating the presence of PGB antigen on the surface of *C. fetus*. In experiment II, WC agglutination titers of PGB antiserum were low or absent. Aluminum hydroxide apparently enhances the antigenicity of PGB antigen since aluminum hydroxide adjuvant was used in experiment I but not in experiment II. The particulate antigens, A, B, and WC, did not need the adjuvant effect of aluminum hydroxide to stimulate the production of agglutinating antibodies.

In experiment I there was a positive correlation between agglutination titers and bactericidal activity. In experiment II sera from rabbits vaccinated with WC vaccine had the highest agglutination titers and those vaccinated with PGB antigen had very low or no agglutination titers. However, the bactericidal activities of blood from rabbits vaccinated with WC and PGB antigens were comparable. PGB antigen is apparently able to stimulate production of an antibody capable of enhancing phagocytosis and intracellular killing even though antibody against PGB antigen is present in amounts too low to detect by agglutination. Soluble conjugated protein antigens (such as PGB antigen) are more apt to stimulate production of immunoglobulin (Ig) G than of IgM antibody (15). Bjornson and Michael (4) have reported that IgG is 10,000 times more effective as an opsonin than IgM. Since PGB antigen is probably stimulating the production of IgG, its activity to promote the killing of *C. fetus* may be due to the opsonic properties of IgG antibody against PGB antigen on the cell surface.

The *C. fetus* strain used in this work has K antigen(s) as indicated by the absence of agglutination of live or formalized cells with antiserum produced against O (somatic) antigen of *C. fetus* 13831. This O inagglutinability was removed by boiling live cells for 15 min. WC antiserum absorbed with autoclaved *C. fetus* 13831 cells agglutinated homologous cells boiled 105 min but not cells boiled 120 min. This heat stability of the K antigen is similar to the heat stability of *C. fetus* 13831 PGB antigen. The PGB antigen is destroyed by boiling or autoclaving 2 h (2) but not by boiling 90 min, as tested in gel double-diffusion analysis using rabbit homologous antiserum against PGB antigen. The finding that the heat stabilities of PGB antigen and K antigen are similar, and the finding that sera from rabbits vaccinated with PGB antigen agglutinated formalized whole cells (Table 1), indicate that PGB and K antigen may be the same. In experiment II, blood from rabbits vaccinated with B antigen produced an in vitro bactericidal effect similar to blood from rabbits vaccinated with WC vaccine. In experiments I and II, after the third vaccination, the bactericidal effect of blood of rabbits given A vaccine was significantly lower than for rabbits given B or WC vaccines. It appears that K antigen of *C. fetus* 13831 is capable of eliciting an increased bactericidal activity of whole blood even after the antigen is boiled 2 h. The heat stability of K antigen was confirmed by the finding that homologous K antiserum agglutinates *C. fetus* 13831 after boiling the antigen 105 min. The heat stability of the K antigen may explain the greater bactericidal activity of blood from rabbits vaccinated with B antigen than blood of rabbits vaccinated with A antigen.

The in vitro test results indicated that antibody against PGB antigen is as active in enhancing phagocytosis as antibody against WC antigen. In the in vivo test, the rabbits vaccinated with PGB vaccine did not clear *C. fetus* as rapidly as those vaccinated with WC vaccine, probably due to lack of an adjuvant in the PGB vaccine and the time delay of 7.5 weeks after the third vaccination. Perhaps PGB antigen (released into the broth during growth) should be given with WC vaccine to increase its protective capacity.

**ACKNOWLEDGMENTS**

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LITERATURE CITED