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Comparison of Muramyl Dipeptide, Trehalose Dimycolate, and Dimethyl Dioctadecyl Ammonium Bromide as Adjuvants in *Brucella abortus* 45/20 Vaccines†

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The capacity of trehalose dimycolate (TDM), muramyl dipeptide (MDP), and dimethyl dioctadecyl ammonium bromide (DDA)—alone or in combination—to potentiate the immunogenicity of killed *Brucella abortus* 45/20 bacteria was studied in guinea pigs. Bacterins that contained TDM in oil droplet emulsion were as effective in the prevention of brucellosis as those emulsified in Freund complete adjuvant, whereas bacterins that contained a combination of TDM and MDP were most effective. Vaccinal emulsions of bacteria and MDP were ineffective in the prevention of splenic infections. Likewise, DDA was unable to potentiate acquired resistance to *Brucella*. Addition of DDA to 1% oil emulsions of bacteria, TDM, and MDP eliminated protection. Adjuvants without bacteria were not able to nonspecifically protect animals from infection, although TDM was able to significantly reduce the numbers of splenic *Brucella*. A positive correlation ($P < 0.0001$) between splenic infection and splenic weight was found.

Muramyl dipeptide (*N*-acetyl muramyl-L-alanine-D-isoglutamine; MDP) and trehalose dimycolate (TDM; cord factor) are defined microbial components responsible for much of the adjuvant activity of Freund complete adjuvant (3, 4). Adjuvant and biological activities of these two compounds have been described and recently reviewed (1, 6, 10). Both MDP and TDM have been found to enhance cell-mediated immune (CMI) responses when administered in mineral oil emulsions (3, 4, 12).

In a preliminary study, we found both MDP and TDM to increase effectively the immunogenicity of heat-killed *Brucella abortus* 45/20 whole cells when given in oil emulsions (13). This particular strain of *B. abortus* was chosen because of its lack of a smooth lipoplysaccharide responsible for the production of routinely detected agglutinins. Thus, an effective bacterin of this rough strain of *B. abortus* could be used in any age of cattle without interfering with the present bovine brucellosis eradication program and would provide an alternative to live avirulent strain 19 vaccine.

The present study was designed to compare the immunopotentiating capacities of TDM and MDP, either alone or in combination, when emulsified with heat-killed *B. abortus* 45/20 whole cells. We also wanted to compare the adjuvant activities of these two compounds with that of dimethyl dioctadecyl ammonium bromide (DDA). This surface-active lipid has been shown to be effective as an adjuvant for acquired cellular resistance to *Plasmodium berghei* and *Listeria monocytogenes* (2, 11). We felt that combinations of these adjuvants could possibly result in increased immunopotentiation over that observed with any one compound.

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MATERIALS AND METHODS

Animals. Female Hartley guinea pigs (Camm Research Institute, Wayne, N.J.) were used. They weighed 300 to 400 g at the start of the experiment and were maintained on standard guinea pig chow.

Bacteria. *B. abortus* 45/20 was grown and heat killed (60°C for 1 h) at the Veterinary Services Laboratory, U.S. Department of Agriculture, Ames, Iowa. Before preparation of whole cell (WC) vaccines, the cells were washed twice in saline solution, dialyzed against water, and lyophilized. Virulent *B. abortus* 2308 was obtained from the same source and used as the challenge organism.

Adjuvants. TDM (cord factor) was obtained from Rocky Mountain Laboratory, Hamilton, Mont.; synthetic MDP was purchased from Calbiochem, Inc., San Diego, Calif.; DDA was purchased from Eastman Kodak Co., Rochester, N.Y.

Vaccines. Oil droplet emulsions of WC-TDM, WC-MDP, and WC-TDM-MDP were prepared as described previously (12). Final inocula (0.2 ml) contained 300 μg of WC, 150 μg of TDM or MDP (or both) and 1.0% light mineral oil emulsified in 0.2% Tween 80 and normal saline solution. The WC-DDA inocula were prepared with 300 μg of WC and 5 mg of DDA in 0.2 ml of normal saline solution, whereas 5 mg of DDA per dose was mixed with WC-TDM-MDP emulsions to prepare WC-TDM-MDP-DDA vaccines. Suspensions of 300 μg of WC in normal saline solution or

Freund complete adjuvant (FCA) were given to animals in other groups.

Immunity studies. Principal groups of animals were subcutaneously inoculated with respective vaccines. Each animal was intramuscularly challenged, along with unvaccinated control animals, with 2.6×10^4 viable *B. abortus* 2308 at 8 weeks after vaccination. All guinea pigs were killed 6 weeks after challenge, and their spleens were removed. Spleens were then diluted 1:5 (wt/vol) with 1.0% peptone in 0.5% saline and homogenized. Serial dilutions of each spleen were made into pour-plates of Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.). After incubation in 10% CO_2 at 37°C for 6 days, *Brucella* colonies were counted.

To check for possible increased nonspecific resistance to *B. abortus* challenge, additional groups of guinea pigs were given adjuvants in similar vehicles but without killed bacteria. Ten days after injection, they were challenged with 2.2×10^4 virulent *B. abortus* 2308. After an additional 6 weeks, they were sacrificed and their spleens were cultured as described above.

Statistical analysis. Data were expressed as $\log_{10}$ transformation of final *Brucella* colony-forming units (CFU) and splenic weights. Duncan's multiple range test and least squares analysis of variance were performed on transformed data. Fisher's exact test was used to compare infection rates in the various groups. Correlation coefficients were calculated from plots of splenic weight versus splenic bacteria and their $\log_{10}$ transformed values.

RESULTS

Guinea pigs were inoculated with heat-killed whole cells of *B. abortus* 45/20 with or without various adjuvants. Six weeks after challenge with virulent *B. abortus*, all animals were sacrificed. Infection rates, mean splenic *B. abortus* CFU, and mean splenic weights were calculated (Table 1). As compared to the control animals, only WC emulsions with TDM, TDM-MDP, or FCA were able to potentiate acquired resistance ($P < 0.05$). Those preparations without adjuvant and those with MDP or DDA were ineffective immunogens. Addition of DDA to WC-TDM-MDP vaccines eliminated the protective effect.

The 90% protection induced by bacterins prepared with a combination of TDM and MDP was greater ($P < 0.05$) than that provided by those emulsified in FCA. TDM was able to potentiate the protection of WC to a degree equal to ($P > 0.05$) that observed with WC-TDM-MDP and WC-FCA. WC preparations with TDM, TDM-MDP, or FCA were able to reduce splenic bacteria by a significant degree ($P < 0.0001$) as compared to control animals.

Nonspecific resistance to *B. abortus* was not enhanced when the adjuvants without *Brucella* antigens were administered and the numbers of infected animals were compared (Table 2). However, a significant decrease ($P < 0.05$) in mean splenic CFU was found after the administration of TDM but not MDP, DDA, or a combination of TDM-MDP.

Splenic weights also proved to be good indicators of splenic infections. A positive correlation ($P < 0.0001$) between mean splenic weights and mean splenic *Brucella* CFU was found (Fig. 1).

TABLE 1. *Effect of MDP, TDM, and DDA (or combinations thereof) on the immunogenicity of B. abortus* 45/20 WC

Vaccinal group	Infected animals	Avg *Brucella*/spleen	Avg spleen wt
Controls	10/10	7.84×10^5	9.12
WC	6/6	1.03×10^6	9.32
WC-DDA	9/9	9.01×10^5	7.91
WC-MDP	8/10	9.79×10^5	6.06
WC-TDM	3/10	2.40×10^4	2.10
WC-TDM-MDP	1/10	2.00×10^2	1.14
WC-TDM-MDP-DDA	9/9	4.98×10^5	6.08
WC-Freund complete adjuvant	5/8	1.07×10^5	3.08

DISCUSSION

The present study confirms some of the findings of a preliminary trial (13). TDM, commonly known as cord factor, is effective in potentiation of the immunogenicity of heat-killed cells of *B. abortus*, rough strain 45/20. The 70% protection rate of the WC-TDM vaccine was somewhat higher than the 60% previously observed and probably can be attributed to the longer period of time allowed for clearance of the challenge inoculum.

A somewhat surprising finding of the present study is the lack of adjuvanticity of MDP. WC and MDP in a 1% oil emulsion were not effective in preventing infection or in reducing splenic *Brucella*. Perhaps the longer period allowed for clearance was sufficient for the challenge organisms to overwhelm host defenses and multiply. More likely, the 1% oil content may have been insufficient for adequate CMI responses. In our previous study, Freund incomplete adjuvant (50% mineral oil) was used and a significant decrease in mean splenic CFU, but not in ani-

TABLE 2. *Effects of MDP, TDM, and DDA on nonspecific resistance to B. abortus*

Adjuvant group	Infected animals	Avg *Brucella*/spleen
Controls	14/14	9.19×10^6
MDP	5/5	6.59×10^6
TDM	5/5	3.09×10^6
DDA	5/5	6.94×10^6
MDP-TDM	5/5	7.05×10^6

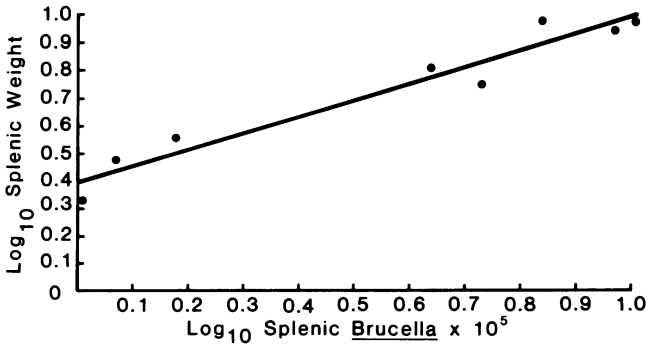

FIG. 1. *Positive correlation between splenic weight and splenic infection with B. abortus.*

mals infected, was found (13). Other investigators have shown the lipophilic character of the vaccinal carrier or MDP molecule to be important in potentiation of CMI (3, 5, 7, 14).

A combination of TDM and MDP adjuvants was effective in potentiation of immunity to brucellosis as only one animal was found to be infected. These compounds have been recently shown to act synergistically in tumor immunotherapy (9). Although WC-TDM-MDP emulsions did not significantly differ ($P > 0.05$) from WC-TMD emulsions in their ability to increase protection, the lower mean splenic weights and splenic *Brucella* suggest that addition of MDP may be of some value. Trials with more animals will be necessary to support or belie this notion.

The absence of an adjuvant effect by DDA was surprising. This surface-active lipid has been shown to be effective in the immunopotentiation of *Listeria* bacterins (11). Because *Listeria* has many properties similar to *Brucella*, we felt that it would be a useful adjuvant. A dosage (5 mg) similar to that found to be optimal in prevention of listeriosis was used (11). Since much of the adjuvant activity of DDA has been attributed to its ability to prevent or delay fusion of phagosomes and lysosomes in macrophages (11), perhaps the amount incorporated into our preparations was excessive and resulted in complete shutdown of antigen processing. However, our studies with 150 μg of DDA in *Brucella* bacterins have not been encouraging (unpublished data).

Immunity induced by WC-TDM-MDP was not observed when DDA was added to the emulsion (Table 1). This may have occurred for the reason discussed above, or it is conceivable that the detergent properties of DDA destroyed the interaction between TDM, MDP, and oil droplets of the emulsion. Interaction of TDM, water-soluble antigens, and oil droplets have been shown to be important in previously described systems (4, 8).

Results of this study show that defined microbial components can replace the immunopotentiating effect of FCA. A combination of TDM and MDP was even more effective in prevention of brucellosis than were bacterins prepared with FCA. Furthermore, this resistance is specific for *Brucella* because the adjuvants themselves were not able to reduce effectively the numbers of infected animals.

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