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Immune Response to Porin in Cattle Immunized with Whole Cell, Outer Membrane, and Outer Membrane Protein Antigens of *Brucella abortus* Combined with Trehalose Dimycolate and Muramyl Dipeptide Adjuvants

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The immune response of cattle to nonliving vaccines derived from *Brucella abortus* rough strain 45/20 was studied. Vaccines contained trehalose dimycolate and a derivative of muramyl dipeptide, *N*-acetylmuramyl-L-α-aminobutyryl-D-isoglutamine. A factorial experiment was designed to test the effects of type of antigen, quantity of antigen, and quantity of mineral oil on the immune response to porin. Muramyl dipeptide was kept constant at 5 mg per dose, and 1 part of trehalose dimycolate was incorporated for two parts of dry matter. Over a 10-week period, blastogenesis responses to porin were largest in cattle immunized with outer membranes; the highest antibody titers to the porin-lipopolysaccharide complex were achieved by immunization with detergent-extracted outer membrane proteins. There was no advantage in the use of 25, rather than 5, mg of any of the antigens, but antibody responses were improved by increasing the quantity of oil from 0.6 to 1.8 ml per dose. In other animals, blastogenesis and antibody responses were sustained at high levels longer than 3 months after two vaccinations with outer membrane proteins. Intradermal injection of porin evoked inflammatory reactions histologically consistent with delayed-type hypersensitivity. Cross-reactions in cases of delayed-type hypersensitivity occurred with porin derived from a smooth strain of *B. abortus* but were less extensive than in the blastogenesis test. The magnitude of the delayed-type hypersensitivity and blastogenesis responses induced by vaccination exceeded those observed after natural or experimental infections. No ill effects were observed after vaccination. These findings provide a basis for the use of trehalose dimycolate and muramyl dipeptide adjuvants in evaluating nonviable vaccines for bovine brucellosis.

Development of an effective nonviable vaccine for *Brucella abortus* would constitute a major advance toward the goal of eradicating bovine brucellosis from the United States. It is well established that *B. abortus* is a facultative intracellular parasite for which a cell-mediated immune response associated with delayed-type hypersensitivity (DTH) is crucial for protective immunity (8, 14). Such a response can be induced effectively by nonviable vaccines in which trehalose dimycolate (TDM) and muramyl dipeptide (MDP) have been incorporated as adjuvants (for reviews, see references 1 and 7). Woodard et al. (25) have demonstrated that guinea pigs immunized with killed cells of *B. abortus* rough strain 45/20 combined with both TDM and MDP adjuvants developed a relatively high level of resistance to infection with virulent *B. abortus* 2308. The use of these adjuvants would be feasible in a commercial vaccine for cattle because the absence of mycobacteria and the low content of mineral oil minimize local reactions and prevent development of tuberculin hypersensitivity.

We have isolated and characterized the major outer membrane proteins (OMP) of *B. abortus* (21). One of these (group 2) has been identified as porin by functional assays (J. T. Douglas, E. Y. Rosenberg, H. Nikaido, D. R. Verstreate, and A. J. Winter, submitted for publication). *Brucella* porins elicit blastogenic transformation of peripheral blood lymphocytes (C. L. Baldwin, D. F. Antczak, and A. J. Winter, *Third Interna-*

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tional Symposium on Brucellosis, in press) and DTH (A. J. Winter and W. L. Castleman, unpublished data) in experimentally infected heifers and are therefore viewed as having potential usefulness in a subcellular vaccine. However, a number of other protein species (10) as well as lipopolysaccharide (LPS) are exposed on the surface of the *Brucella* cell, and the composition of the most potent vaccine remains to be established. We wished initially to compare a vaccine composed of killed whole cells (WC) (25) with those prepared from outer membranes (OM) or from detergent-extracted OMP of the same strain.

To permit a critical evaluation of protective immunity conferred upon cattle by these vaccines, information was required on the magnitude and duration of the immune responses to different dosages of the antigens in TDM and MDP adjuvants. Besides this, there was no published information on the tolerance of cattle to vaccines containing MDP or TDM in excess of 1.5 mg (24). The present study, therefore, was undertaken to measure immune responses in adult cattle immunized with various quantities of killed WC, OM, or OMP in an adjuvant containing commensurate quantities of TDM and MDP. Immune responses were measured to porin proteins to provide a comparison among the groups, in the belief that the immune response to this antigen was likely to be instrumental in protective immunity.

MATERIALS AND METHODS

Antigens for vaccines. *B. abortus* rough strain 45/20 cells were grown to log phase in a fermentor. The washed cells were inactivated in the frozen state by ^{60}Co radiation. A portion was lyophilized after exhaustive dialysis against deionized water and served as the WC antigen.

WC were disrupted by passage through a high-pressure cell (Sorvall Ribi cell fractionator, model RF-1) as described previously (21). One portion of crude membranes was subjected to density gradient centrifugation to separate OM (21). These were lyophilized after exhaustive dialysis in deionized water.

The remaining crude membranes were extracted sequentially with sodium *N*-lauroylsarcosinate (Pfaltz & Bauer, Stamford, Conn.) and Zwittergent 3-14 (Calbiochem-Behring, La Jolla, Calif.) to yield the major OMP (21). The Zwittergent concentration was reduced below the critical micellar concentration (to 0.01%) by anion-exchange chromatography to permit removal of remaining detergent by dialysis in deionized water. Detergent-free proteins were lyophilized. All lyophilized antigens were held in a desiccator at room temperature.

Antigens for immunological assays. Porins (group 2 proteins) were purified from Zwittergent extracts of *B. abortus* 45/20 and 2308 by sequential anion-exchange and gel filtration chromatography (21). The homogeneity of the preparations was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (21). The Zwittergent concentration was maintained at 0.01% in antigen solutions used in the enzyme-linked immunosorbent assay (ELISA).

For lymphocyte blastogenesis and dermal sensitivity assays, porins in 0.2% Zwittergent were subjected to three cycles of sonication (60 W for 1 min at 4°C) followed by precipitation with 7.2% trichloroacetic acid. Antigens to be used for skin tests were solubilized in phosphate-buffered saline (pH 7.2) containing 0.01% Zwittergent and were dialyzed against this buffer for 48 h before use.

Antigens to be used for lymphocyte blastogenesis tests were dialyzed against deionized water and were held frozen at −70°C in portions sufficient for the tests of a single day.

Adjuvants. TDM was extracted from *Mycobacterium avium* by the method of Azuma et al. (3). The ratio of TDM to other dry matter was held at 1:2 because association of antigen with oil droplets is much reduced at lower ratios.

A derivative of MDP, *N*-acetylmuramyl-L-α-aminobutyryl-D-isoglutamine, was provided as a gift by G. H. Jones, Syntex Research, Palo Alto, Calif. The adjuvant properties of this product had been found to be superior to those of MDP (G. H. Jones, personal communication, 1982). Based on extrapolation of data from laboratory animals, 5 mg was considered sufficient for immunostimulation of cattle yet well below an immunosuppressive dose (7) (G. H. Jones, personal communication, 1982).

Preparation and administration of vaccines. We followed in essentials the method of Granger et al. (6) TDM solubilized in chloroform methanol (95:5, vol/vol) and MDP solubilized in methanol were added to scrupulously dried antigen in a glass homogenizing vessel. After the solvents had been evaporated with a stream of nitrogen, mineral oil (Drakeol 6VR; Penreco, Lyndhurst, N.J.) was added and ground for at least 4 min with a Teflon pestle attached to a mechanical mixer. A solution of phosphate-buffered saline containing 0.4% Tween 80 was then added, and the oil was emulsified by further grinding. Preparations were examined microscopically with phase contrast under oil immersion and, if satisfactory, were used within 2 h. Oil droplets were translucent. Droplets containing TDM and antigen had a speckled and granular appearance. Vaccines were administered intramuscularly in the neck or hind legs with one-half of the emulsion on each side.

Experimental animal groups. A pilot study was performed on four 15- to 16-month-old Holstein-Friesian heifers (heifers 43, 44, 45, and 53) which had not been vaccinated with *B. abortus* 19. These animals were immunized three times over the course of 8 months. Blood samples were taken at 2- to 5-week intervals throughout this period. Skin tests were performed after blood had been drawn at 2 and 6 weeks after the third immunization.

The principal experiment was conducted on 38 Holstein-Friesian cows, ranging in age from 2 to 3 years, housed at the Teaching and Research Center of Cornell University, Ithaca, N.Y. All of these animals had been vaccinated as calves with strain 19. Some of the animals were pregnant at the beginning of the experiment, of which the most advanced were in the fourth month of gestation. Milk weights were recorded on all animals before vaccination and throughout the

course of the experiment. Management practices precluded skin tests on these animals.

The 38 cows were divided randomly into nine groups of 4 principals (Table 1) and one group of 2 controls. The experiment was designed to test the response to three antigens at two dosage levels, 5 and 25 mg. The concentration of MDP was kept constant, but that of TDM was regulated to compose 50% of the dry weight of antigen plus MDP (Table 1). Large and small doses of antigen were tested with the concentration of oil kept constant at approximately 18% (vol/vol) and 1 ml of oil per 25 mg of dry weight constituents (groups 1 and 3, 4 and 6, 7 and 9). A further comparison was made in which the small antigen dose was administered with the same quantity of oil that was used with the high dose (groups 2, 5, and 8).

Blood samples were taken before immunization and at 2, 6, and 10 weeks thereafter. Six principals and one control were sampled on a given test day. Vaccination schedules were staggered over a period of 2 weeks to maintain the desired postimmunization to test intervals for each animal.

Blood sampling. Blood samples were collected from the tail vein into heparinized or untreated vacutainer tubes. Serum was separated from clotted blood by centrifugation and was stored at −20°C.

Lymphocyte blastogenesis. A titration assay was employed (C. L. Baldwin, D. F. Antczak, and A. J. Winter, submitted for publication). Washed mononuclear cells Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, N.J.) gradients were suspended in RPMI 1640 medium (GIBCO Laboratories, Grand Island, N.Y), pH 7.2, supplemented with beef lung heparin (1 U/ml), fetal calf serum (10%), gentamicin (0.01 mg/ml), and 2-mercaptoethanol (2.5×10^{-5}) and were adjusted to concentrations of 4×10^6, 2×10^6, 1×10^6, and 5×10^6 viable cells per ml. Quantities of 0.1 ml of each cell suspension were transferred into round-bottom microtiter plates followed by an equal volume of antigen diluted in the same medium. Each antigen was added at 1 μg per well, which was determined by prior titration to be the optimal concentration for inducing blastogenesis. Control cultures of each of the cell concentrations received 0.1 ml of complete medium without antigen. All cultures were performed in triplicate. Cultures were pulsed with 0.5 μCi of [^{3}H]thymidine (New England Nuclear Corp., Boston, Mass.) 4 h before the end of the 96-h incubation. Means and standard deviations of the counts for each of the replicate cultures were determined. The difference in counts per minute (Δcpm) was calculated by subtracting the mean response of the control cultures from the mean response of the corresponding antigen-stimulated cultures. The largest Δcpm achieved among the four cell concentrations was assigned as the result of an individual test. Prior studies (C. L. Baldwin, Ph.D. dissertation, Cornell University, Ithaca, N.Y., 1983; Baldwin et al., submitted for publication) have shown that the titration assay is better than single-point assays for evaluating lymphocyte proliferation and that maximum Δcpm provides the truest indication of the magnitude of the response.

ELISA. Kinetics-based ELISA (4) were conducted on computer-assisted Gilford PR-50s (Gilford Instruments, Inc., Oberlin, Ohio). Optimal concentrations, volumes, and incubation intervals of antigen, test serum, and conjugate were predetermined. Cuvettes were loaded with 100 μl of antigen diluted to a final concentration of 5 μg/ml in 0.01 M carbonate buffer (pH 9.6), dried for 24 h at 37°C, and stored sealed at room temperature. Before use, cuvettes were rinsed with phosphate-buffered saline (pH 7.4)–0.5% Tween 20 to remove excess unbound antigen. A 1:100 working dilution of each test serum was then placed in duplicate cuvettes and incubated at room temperature for 45 min. The same five positive and negative control sera were included in each run. After washing, 100 μl of horseradish peroxidase-labeled rabbit anti-bovine immunoglobulin G (heavy and light chains) (Cappell Laboratories, West Chester, Pa.) was added to each well and incubated for 30 min. The amount of bound conjugate was determined by establishing the rate of colored product development upon the addition of 400 μl of 2,2′-azino-di-(3-ethylbenzthiazolinesulfonic acid) substrate solution. The slope of the reaction rate was established by computer-timed absorbance readings at 5, 9, and 13 min after the addition of substrate. Based on previous studies, the slope was proportional to bound antibody and was measured in the linear response phase of enzyme-substrate interaction. Data were normalized and were expressed as percentages of increase in slope values over time ([slope of postvaccination sample − slope of prevaccination sample]/

TABLE 1. Composition of vaccines used in the principal experiment

Group[a]	Vaccine composition					
	Antigen		TDM (mg)	MDP (mg)	Oil (ml)	Tween-saline solution (ml)
	Type[b]	Amt (mg)				
1	OMP	25	15	5	1.8	8.2
2	OMP	5	5	5	1.8	8.2
3	OMP	5	5	5	0.6	3.4
4	OM	25	15	5	1.8	8.2
5	OM	5	5	5	1.8	8.2
6	OM	5	5	5	0.6	3.4
7	WC	25	15	5	1.8	8.2
8	WC	5	5	5	1.8	8.2
9	WC	5	5	5	0.6	3.4

[a] Each group consisted of four cows.
[b] All antigens were derived from *B. abortus* 45/20.

[slope of prevaccination sample]). Because we were principally interested in experimental group comparisons, the data were not routinely converted to titers. However, approximate titers for the positive controls were determined by limiting twofold dilutions of positive control serum in negative control serum. A nomograph established by regression analysis provided an estimate of titer as a function of percent increase in slope. Thus, percent increases in slope values of 100, 200, 300, and 400 represented approximate titers of 16, 246, 3.9×10^3, and 6.0×10^4, respectively (Fig. 1).

Skin tests. Quantities of 150 μl were injected into shaved skin on the side of the neck by using tuberculin syringes with 26-gauge needles. Double skin thickness was measured with Hauptner calipers (Jorgensen Laboratories, Loveland, Colo.) before injection and at 6, 24, and 48 h thereafter. An increase in double skin thickness of ≥2 mm at 48 h was considered a positive response (P. Nicoletti, personal communication, 1983). Punch biopsies were taken of selected lesions after the last reading and were fixed in 10% neutral Formalin. Tissue sections were stained with hematoxylin and eosin.

Chemical analyses. Protein was determined by the Peterson modification (15) of the Lowry method with bovine serum albumin as the standard. The assay for 2-keto-3-deoxy sugars described by Weissbach and Hurwitz (23) was performed with the modification of Osborn (12). 2-Keto-3-deoxyoctonate (Sigma Chemical Co., St. Louis, Mo.) was used as the standard, and the results of the assay were taken as a measure of 2-keto-3-deoxyoctonate in the sample after correction for 2-deoxyaldoses (22).

Statistical methods. Data from the principal experiment were analyzed as repeated measures (17). Usual statistical methods which assume independence between responses could not be applied because of the repeated measurements on the same cows. Summary statistics representing the mean, linear, and quadratic trends of each group of cows were calculated and analyzed to determine differences among the treatments.

RESULTS

Antigen treatment. Porins of strains 45/20 and 2308 had ratios of 2-keto-3-deoxyoctonate to protein of 1.5 and 3.4 μg/mg, respectively. After three cycles of sonication and trichloroacetic acid precipitation, ratios were <0.45 μg of 2-keto-3-deoxyoctonate per mg of protein. *Brucella* porins are believed to exist in their native state as trimers and to be converted into monomers by boiling in sodium dodecyl sulfate (21). After sonication and trichloroacetic acid precipitation, porins migrated as monomers in sodium dodecyl sulfate-polyacrylamide gel electrophoresis without a requirement for heating and failed to produce reactions in ELISA or immunoprecipitates with antisera specific for native antigen. However, such denatured antigens have generally been found more effective than native antigens in eliciting lymphocyte blastogenesis (Baldwin, Ph.D dissertation) and so were used routinely for this purpose. Sonicated antigens were also used in dermal sensitivity tests to prevent the primary toxicity caused by *Brucella* OMP containing LPS (A. J. Winter and W. L. Castleman, unpublished data).

Untreated antigen had to be used in ELISA. Although rabbits immunized with the native preparation of strain 45/20 porin in TDM and MDP adjuvant failed to produce detectable precipitins to LPS (J. M. Santos and A. J. Winter, unpublished data), it is possible that some of the antibodies produced by cattle and detected in ELISA were specific for the rough core LPS

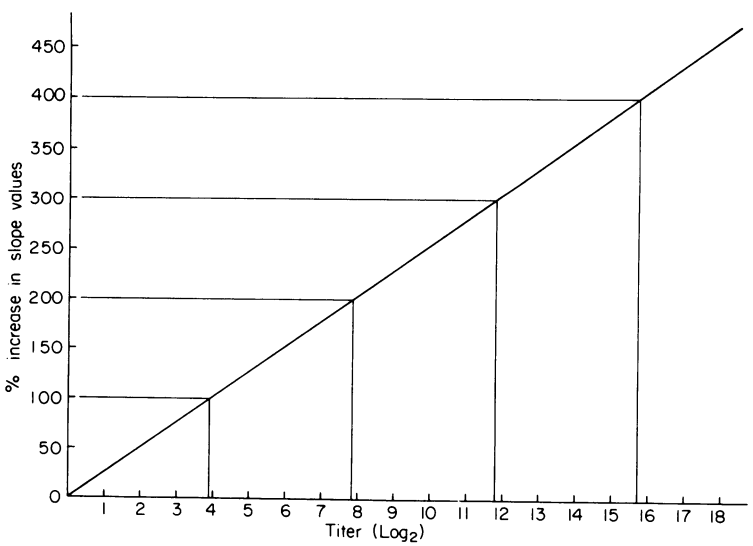

FIG. 1. Estimates of antibody titer as a function of percent increase in ELISA slope values.

determinants. We did not distinguish these specificities, so antibodies were considered specific for the porin-LPS complex.

Pilot study. On day 0, heifers 43 and 44 were immunized with 25 mg of OMP in an adjuvant containing 12.5 mg of TDM, 1.5 ml of oil, and 8.5 ml of Tween-saline. Heifers 45 and 53 received 5 mg of antigen and 2.5 mg of TDM in the same volumes of oil and Tween-saline. On day 38, all heifers received 5 mg of OMP, 5 mg of TDM, and 5 mg of MDP in 1.5 ml of oil and 8.5 ml of Tween-saline. The vaccine given on day 234 was the same, except that the volumes of oil and Tween-saline were reduced to 0.6 and 3.4 ml, respectively. The larger dose received initially by heifers 43 and 44 had no apparent effect on the immune responses measured (Fig. 2, Table 2). No ill effects were observed as a consequence of vaccination throughout the 9-month course of this experiment.

Blastogenesis responses rose modestly after the first immunization and very markedly after the second. Maximum Δcpm above 50,000 were maintained until after day 150 and then gradually diminished (Fig. 2). At the time of the third immunization on day 234, the maximum Δcpm in the four heifers ranged from 400 to 2,800. After the third immunization, there was an increase in the blastogenesis response in all heifers, with maximum Δcpm ranging from 17,700 to 40,400. These responses were, however, much smaller

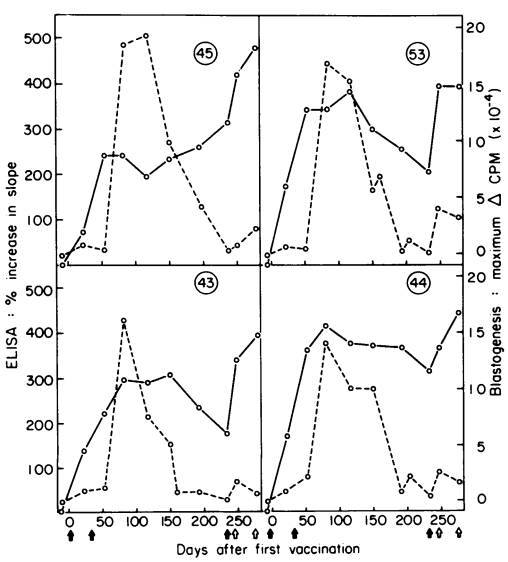

FIG. 2. Pilot study: response of four heifers in ELISA (solid lines) and blastogenesis (broken lines). ELISA data are expressed as percent increase in slope in comparison with the prevaccination value. Blastogenesis responses are expressed as maximum Δcpm × 10^{-4}. Times of vaccination (black arrows) and skin tests (white arrows) are indicated.

that those that had occurred previously (Fig. 2). The relative responsiveness to homologous (strain 45/20) and heterologous (strain 2308) porins was compared by calculating ratios of response (45/20 to 2308) at each test date. The magnitude of responses was similar but favored the homologous antigen in three of four animals, as indicated by means ± standard errors of 1.36 ± 1.20 (heifer 43), 1.50 ± 1.17 (heifer 44), 2.07 ± 2.96 (heifer 45), and 0.90 ± 0.22 (heifer 53).

Levels of antibodies to the porin-LPS complex rose sharply after the first two immunizations, and in two of the heifers (43 and 53) they declined gradually after day 150 (Fig. 2). The third immunization produced an increased antibody response in all heifers, equal to or exceeding a 400% increase in slope values (Fig. 2), which was equivalent to titers of ≥60,000 (Fig. 1).

Intradermal injection of 100-μg quantities of porin produced marked skin reactions in all of the immunized heifers but none in control animals (Table 2). Dose-response titrations with strain 45/20 porin in heifers 44 and 53 demonstrated a significant correlation of the change in skin thickness to $\log_{10}$ dosage (heifer 44, $r = 0.9954$, $P < 0.01$; heifer 53, $r = 0.9832$, $P < 0.05$). In heifer 44, as little as 0.8 μg of protein produced a positive reaction (Table 2). Responses to the homologous porin (45/20) were in all instances greater.

Histological examination of skin from vaccinated heifers biopsied at 48 h postinjection revealed dermal lesions consistent with a DTH response (Fig. 3). There were moderately dense perivascular aggregates of inflammatory cells in the superficial and deep dermis with intervening areas of edema. The inflammatory cells were composed of aggregates of macrophages with associated lymphocytes, as well as neutrophils and eosinophils. Vasculitis characterized by collections of neutrophils, lymphocytes, and monocytes in all layers of arterial and venous walls was occasionally present. The presence of vasculitis and neutrophils, together with the presence of high-antibody titers and the fact that positive skin reactions (>2 mm) had developed by 6 h, suggested that there may have been an arthus-type component to the reaction in addition to DTH. Dermal lesions in nonvaccinated controls were restricted to the superficial dermis and consisted of minimal perivascular collections of macrophages and an occasional neutrophil or eosinophil.

Principal study. Vaccination produced neither anorexia nor even a transient decrease in milk production in any of the cows. All animals remained clinically normal throughout the course of the experiment. All treatments produced clear-cut responses to antigens of strain

TABLE 2. Skin test reactions to porins in heifers after vaccination with *B. abortus* 45/20 OMP in adjuvants containing TDM and MDP

Animal no.	Strain finishing porin	Dosage (μg)	Differences in double skin thickness (mm) at 48 h[a]	
			2 weeks post-infection[b]	6 weeks post-infection[c]
43	45/20	100	19.9	>23.8[d]
	2308	100	13.2	22.0
44	45/20	100	13.4	13.3[e]
		20		9.7
		4		5.5
		0.8		3.0
	2308	100	9.0	10.5
45	45/20	100	15.9	>21.6[d]
	2308	100	11.2	15.0
53	45/20	100	10.3	8.6[e]
		20		4.9
		4		3.2
		0.8		1.2
	2308	100	6.2	7.8

[a] Measurements at 48 h in two control animals injected with 100 μg of each porin were <0.5 mm.
[b] Refers to time after final immunization on day 234 and corresponds to day 248 (Fig. 2).
[c] Refers to time after final immunization on day 234 and corresponds to day 276 (Fig. 2).
[d] Lesions were larger than calipers fully extended.
[e] In antigen titrations, all concentrations of antigen were injected in 0.15 ml of the same diluent.

45/20 in both blastogenesis tests and the ELISA (Fig. 4). Thus, square roots of maximum Δcpm ± standard error of prevaccination samples from the 36 principals were 42.71 ± 3.94. Values for the two controls throughout the experiment were 37.18 ± 10.52 and 22.04 ± 4.65. In the ELISA, slope values of the first serum samples from the two controls did not differ by more than 22% from those obtained at any subsequent time. The magnitude of response in the blastogenesis test to porins of strains 45/20 and 2308 was similar and did not vary over time or with the antigen preparation used. Means ± standard errors of ratios of responses (45/20 to 2308) from the nine treatment groups were 1.21 ± 0.14, 1.26 ± 0.26, and 1.25 ± 0.10 at 2, 6, and 10 weeks postvaccination. When data from all 36 principals were analyzed together, there was a positive correlation between the stage of gestation and the blastogenesis response at 2 weeks (r = 0.341, $P < 0.05$), but not at 6 or 10 weeks, postvaccination. No significant correlations between gestation stage and antibody response occurred in any time period.

Averaged across the three time periods, blastogenesis responses to strain 45/20 porin were significantly higher (P = 0.045) in cattle immunized with OM (groups 4, 5, and 6) than in those immunized with OMP (groups 1, 2, and 3) or WC (groups 7, 8, and 9) (Fig. 4). The slope of the blastogenesis response to OM over time also differed significantly (P = 0.025) from those to the other antigens. Taken together, responses to OM showed a gradual decline, whereas responses to the other antigens increased between 2 and 6 weeks and then declined (Fig. 5A). Thus, at 10 weeks the response to OMP was greater than the response to the other antigens (Fig. 5A) and significantly different (P = 0.027) from the response to WC. With none of the antigens could significant differences in response be ascribed to antigen dosage or quantity of oil, either at individual time periods or over the whole duration of the experiment.

Cattle immunized with OMP (groups 1, 2, and 3) produced significantly higher antibody responses over the course of the experiment than did those receiving OM (groups 4, 5, and 6) (P = 0.044) or WC (groups 7, 8, and 9) (P = 0.002). Considered over time, the response of animals receiving 25 mg of antigen (groups 1, 4, and 7) was no better than that of those receiving 5 mg (groups 2, 5, and 8) (P = 0.356), but groups receiving large amounts of oil (groups 1, 2, 4, 5, 7, and 8) achieved significantly higher responses (P = 0.019) than did those receiving little oil (groups 3, 6, and 9). Furthermore, the slope of increase in antibody production over time was significantly steeper (P = 0.061) in animals immunized with antigens with large amounts of oil (Fig. 5B). Slopes of increase in antibody response over time differed significantly (P = 0.028) between animals receiving OM and OMP, whereas no significant difference (P = 0.833) occurred between groups immunized with OMP and WC. This occurred because responses of the OMP and WC groups increased over time, whereas those of the OM groups had decreased by 10 wk (Fig. 5C).

DISCUSSION

The nature of protective immunity in bovine brucellosis is not yet understood, but based upon evidence from the murine system it is likely that both cell-mediated (14) and humoral (9, 13, 19) immune responses contribute to protection. This study presents evidence that vaccines in TDM and MDP adjuvants could induce the required immune responses without untoward side effects, even with doses that appeared greater than required for this purpose. The magnitude of the blastogenesis response in many of the vaccinated animals exceeded that observed in naturally or experimentally infected cattle, in which maximum Δcpm rarely exceed 20,000 (Baldwin, Ph.D. dissertation). In infected cattle,

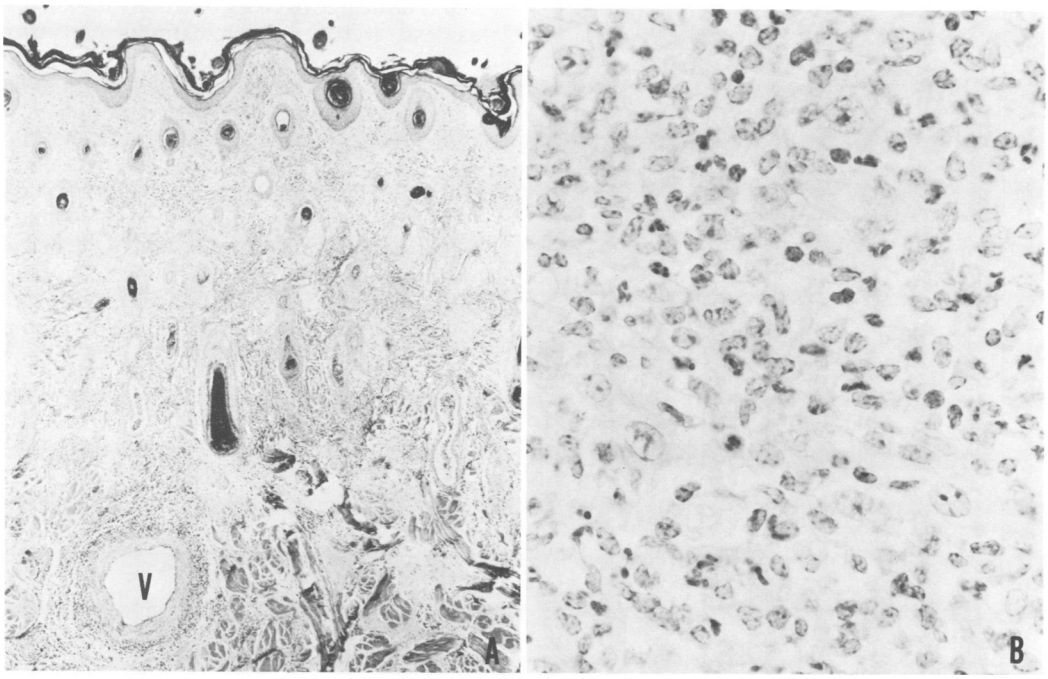

FIG. 3. Skin from a heifer immunized 6 weeks previously and biopsied 48 h after intradermal injection of porin. (A) Dense aggregates of inflammatory cells throughout the superficial and deep, edematous dermis and in perivascular location (V). Magnification, ×29. (B) Dermal infiltrates composed predominantly of macrophages with interspersed lymphocytes, neutrophils, and eosinophils. Magnification, ×461. Hemotoxylin and eosin stain.

antibodies to the porin-LPS complex fail to develop (Baldwin, Ph.D. dissertation; R. H. Jacobson and A. J. Winter, unpublished data), and double skin thickness to 100 μg of porin measures no more than 5 to 7 mm (A. J. Winter and W. L. Castleman, unpublished data; P. Nicoletti, personal communication, 1983). It is therefore possible that with the correct combination of antigens and an appropriate schedule of immunization, such vaccines may confer better protection than is acquired by convalescence from the disease (16).

Soper et al. (18) reported a seasonal fluctuation in blastogenesis of bovine lymphocytes in response to phytohemagglutinin and concanavalin A, with mean counts per minute 17.8 and 15.9 times greater, respectively, in the peak month of August than in the low month of February. We have noted that lymphocyte responses to antigens are also much lower during the winter months (C. L. Baldwin and A. J. Winter, unpublished data). This may account to an important extent for the much larger blastogenesis response in the pilot study after the second immunization (on 1 July) than after the third (on 12 January) (Fig. 2). It is notable that in these heifers, DTH reactions were intense, and antibody titers reached their highest levels after the third immunization (Fig. 2, Table 2).

It was demonstrated with WC subcellular fragments and extracted proteins that 5 mg of a given antigen produced antibody and blastogenesis responses equivalent in duration and intensity to those produced by 25 mg. Since this occurred with three physical forms of antigen, it is likely that with these adjuvants a dosage of 5 mg of any *Brucella* antigen with reasonably good immunogenicity would be sufficient for cattle.

Two concentrations of oil were tested with fixed quantities of antigen and TDM in hopes that a higher ratio of oil would permit a larger proportion of antigen to be incorporated into the oil phase (6). Vaccines containing the larger quantity of oil, regardless of their antigen content, produced antibody responses which were successively greater at each time interval (Fig. 5B). The basis for this effect is unexplained, but the difference was great enough to warrant use of the larger amount of oil in vaccination-challenge trials.

Better antibody responses to porin were produced with OMP than with OM vaccines. The reverse held true for blastogenesis responses, although this would not have been so had only 6- and 10-week responses been taken into account (Fig. 5A). Mean responses to WC vaccine were generally lowest (Fig. 5A and C). Several factors may account for the differences observed. The

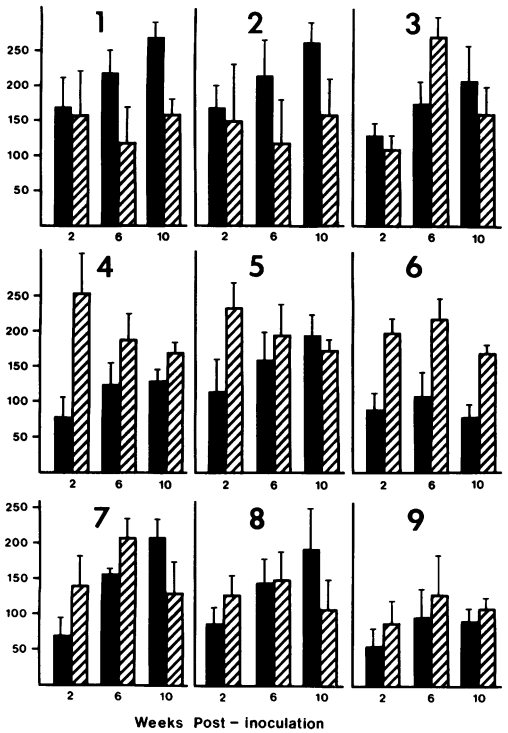

FIG. 4. Principal study: response in ELISA and blastogenesis of 9 treatment groups (see Table 1) at 2, 6, and 10 weeks after vaccination. ELISA data (■) are expressed as means ± standard errors of percent increases in slopes in comparison with the prevaccination values. Blastogenesis responses (▨) are expressed as means ± standard errors of the square roots of the maximum Δcpm.

quantity of porin per unit weight of vaccine was OMP > OM > WC. Blastogenesis measured the response to porin alone, whereas antibodies specific for LPS as well as for porin were detected in the ELISA. Besides this, extracted porin may have been more readily accessible to B cells than porin on WC or OM, or it may have been slightly modified through the extraction procedure. If the latter is true, antibodies to native porin induced by OM or WC would have been measured less effectively in the ELISA, in which extracted porin served as antigen. This would not have been a factor in blastogenesis, in which T cell responses were measured by denatured antigens (20).

Advantages in the use of vaccines derived from rough strains (2) require that relevant rough-phase antigens be closely related to those of virulent smooth strains. Prior studies in rabbits had indicated close antigenic relatedness among porins of *B. abortus* (21), but it was necessary to ensure cross-reactivity in responses of cattle. Overall, the magnitude of response in blastogenesis to smooth strain 2308 was about 25% lower than to the homologous rough strain 45/20. The degree of cross-reactivity in skin tests was less, if based upon extrapolations from dose-response studies performed on two of the heifers (Table 2) which suggest that 20 μg or less of homologous antigen produced responses requiring 100 μg of heterologous antigen. More extensive studies will be required to confirm these preliminary findings.

Pregnant cattle are much more susceptible to brucellosis (11), and there is some evidence for an alteration in their humoral immune response to *B. abortus* (5). Conclusions regarding an influence of pregnancy on the immune response

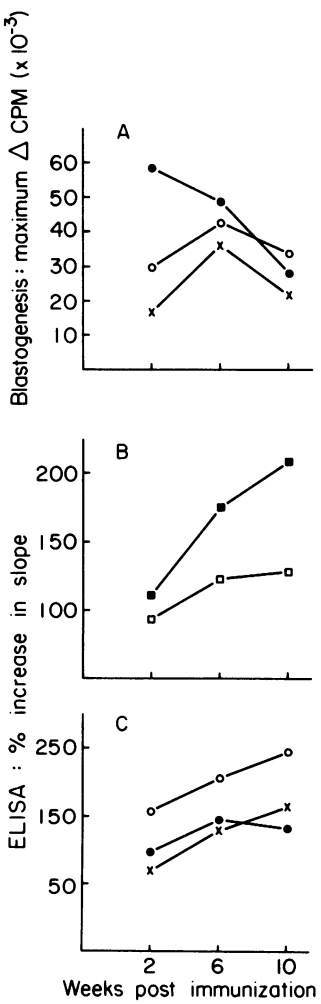

FIG. 5. Principal study: means of responses at 2, 6, and 10 weeks after vaccination. (A) Blastogenesis (maximum Δcpm × 10^{-3}) of combined groups immunized with OMP (○), OM (●), or WC (×). (B) ELISA (percent increase in slope values) of combined groups vaccinated with large quantities of oil (■; groups 1, 2, 4, 5, 7, and 8) and little oil (□; groups 3, 6, and 9). (C) ELISA of combined groups immunized with OMP (○), OM (●), and WC (×).

cannot be drawn from this study, which was not designed to test this point. No explanation is presently possible for the positive correlation between stage of pregnancy and response in blastogenesis which occurred at one time period. Experiments dealing with qualitative and quantitative effects of pregnancy on the immune response of cattle, and the schedule of immunization best suited to achieve protection from brucellosis during the second and third trimesters, are currently under way.

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