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Protective Killed *Leptospira borgpetersenii* Vaccine Induces Potent Th1 Immunity Comprising Responses by CD4 and γδ T Lymphocytes

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Leptospira borgpetersenii **serovar hardjo is the most common cause of bovine leptospirosis and also causes zoonotic infections of humans. A protective killed vaccine against serovar hardjo was shown to induce strong antigen-specific proliferative responses by peripheral blood mononuclear cells (PBMC) from vaccinated cattle by 2 months after the first dose of vaccine. This response was absent from nonvaccinated control cattle. The mean response peaked by 2 months after completion of the two-dose vaccination regimen, and substantial proliferation was measured in in vitro cultures throughout the 7 months of the study period. Variations in magnitude of the response occurred among the vaccinated animals, but by 7 months postvaccination there was a substantial antigen-specific response with PBMC from all vaccinated animals. Up to one-third of the PBMC from vaccinated animals produced gamma interferon (IFN-γ) after 7 days in culture with antigen, as ascertained by flow cytometric analysis, and significant levels of IFN-γ were measured in culture supernatants by enzyme-linked immunosorbent assay. Two-color immunofluorescence revealed that one-third of the IFN-γ-producing cells were γδ T cells, with the remaining cells being $CD4^+$ T cells. The significance of this study is the very potent Th1-type immune response induced and sustained following vaccination with a killed bacterial vaccine adjuvanted with aluminum hydroxide and the involvement of γδ T cells in the response. Moreover, induction of this Th1-type cellular immune response is associated with the protection afforded by the bovine leptospiral vaccine against *L. borgpetersenii* serovar hardjo.**

Leptospirosis is a widespread zoonotic disease that affects virtually all mammals and is an important cause of reproductive failure and production losses in cattle throughout the world (14, 16, 18, 19, 23). The most common cause of leptospirosis among cattle in much of the world is infection with leptospires belonging to *Leptospira borgpetersenii* serovar hardjo. Cattle are the maintenance host for serovar hardjo and are responsible for the shed and spread of this pathogen in nature (18). Zoonotic infections of humans with serovar hardjo represent a significant public health problem (29). Two serologically indistinguishable but genetically distinct types of serovar hardjo have been identified. *Leptospira interrogans* serovar hardjo (type hardjoprajitno) is isolated primarily from cattle in the United Kingdom (17), while *L. borgpetersenii* serovar hardjo (type hardjo-bovis) is common in cattle populations throughout the world (17, 42).

Leptospiral vaccines used in cattle in the United States are inactivated whole-cell vaccines containing *L. interrogans* serovars hardjo (type hardjoprajitno), canicola, pomona, and icterohaemorrhagiae and *L. kirschneri* serovar grippotyphosa (21). These pentavalent vaccines provide adequate protection against disease caused by each of the serovars in the vaccine except serovar hardjo. That is, they failed to prevent abortion, stillbirth, and vertical transmission of infection when vaccinated cows were challenged with *L. borgpetersenii* serovar hardjo during pregnancy, and the infection rates for control and vaccinated cattle did not differ (7). Attempts to improve the protection against *L. borgpetersenii* serovar hardjo by including *L. borgpetersenii* serovar hardjo in a pentavalent vaccine (8) or by increasing the quantity of serovar hardjo antigen in a monovalent serovar hardjo vaccine (6) failed. In contrast to these results, recent studies by Bolin et al. (4, 5) and Ellis et al. (15) evaluating serovar hardjo monovalent vaccines formulated with a field isolate of *L. borgpetersenii* serovar hardjo and another formulated with *L. interrogans* serovar hardjo found that these vaccines prevented infection and tissue colonization following challenge with *L. borgpetersenii* serovar hardjo strains from the United States or Europe. Laboratory and field studies have shown that these vaccines decrease the incidence of infection, duration, and intensity of urinary shedding and the incidence of human leptospirosis in persons in contact with cattle (1, 28–30). The ability of these vaccines to protect against serovar hardjo may reflect differences in the serovar hardjo isolates used in the protective monovalent vaccines or the culture conditions used for preparing the vaccine bacteria. Such differences may affect the quality or type of immune response induced.

Because protective immunity to leptospirosis is serovar specific it was formerly believed to be almost exclusively humoral

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(18). While antibodies against leptospiral lipopolysaccharides (LPS) give passive protection in some animal models (26, 31), cattle vaccinated against serovar hardjo with pentavalent vaccines are vulnerable to infection with serovar hardjo despite the presence of high titers of anti-LPS antibody (6, 8). These studies were the first indicators that anti-LPS antibody was not sufficient for protection of cattle against serovar hardjo and evoked a reexamination of the paradigm that protective immunity is primarily humoral. Ellis et al. recently showed that peripheral blood mononuclear cells (PBMC) from cattle vaccinated with an *L. interrogans* serovar hardjo vaccine that provides protection against serovar hardjo proliferated in vitro in response to hardjo antigens (15). Thus, it has been suggested that a cell-mediated immune response to serovar hardjo may be necessary for protection and, therefore, a protective vaccine would be expected to stimulate this type of immune response (18). The purpose of this study was to evaluate the cellular immune response induced by a protective monovalent serovar hardjo vaccine.

MATERIALS AND METHODS

Animals and vaccination. Thirty-two 12- to 15-month-old heifers that lacked detectable serum antibodies against serovar hardjo as determined by the microscopic agglutination test (12) were used. Cattle were housed at the National Animal Disease Center in Ames, Iowa, and were divided into two groups: nonvaccinated control cattle ($n = 11$) and cattle vaccinated with a protective commercial monovalent serovar hardjo vaccine, Spirovac (CSL Ltd., Parkville, Victoria, Australia) ($n = 21$). Spirovac is a killed whole-cell vaccine formulated with a bovine isolate of *L. borgpetersenii* serovar hardjo and aluminum hydroxide. Two 2-ml doses of Spirovac were administered subcutaneously 4 weeks apart in the lateral aspect of the neck. All animal use complied with the relevant federal guidelines and institutional policies and was approved by Institutional Animal Care and Use Committee and was conducted in facilities approved by Association for Assessment and Accreditation of Laboratory Animal Care.

Collection of blood for analysis. Blood samples were collected from vaccinated and nonvaccinated control cattle prior to the administration of the first dose of vaccine (month 0), at the time of administration of the second dose of vaccine (month 1), and at the monthly intervals indicated thereafter.

Culture of lymphocytes. Blood was collected from jugular veins of cattle directly into anticoagulant and shipped by overnight courier mail arriving approximately 24 h after collection for processing. PBMC were isolated from blood by Ficoll-Paque Plus (Pharmacia, Piscataway, N.J.) density gradients following typical methods (20). PBMC were washed three times with Hanks' balanced salt solution with heparin at 0.5 U/ml, following which they were suspended in RPMI 1640 medium (Gibco-BRL, Rockville, Md.) containing 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, and 10 μg of gentamicin per ml (complete RPMI; cRPMI). For the proliferation assays, 5×10^5 PBMC were aliquoted into 96-well flat-bottom microtiter plates along with cRPMI only, concanavalin A (ConA) at a final concentration of 1 μg/ml, or serovar hardjo antigen sonicate (antigen was sonicated whole cells from *L. borgpetersenii* serovar hardjo-bovis clone RZ33) at a final concentration of 0.5 μg of protein/ml in cRPMI so that a total volume of 0.2 ml/well was achieved. All cultures were set up in quadruplicate. For cytokine evaluation, 5×10^6 PBMC/well were aliquoted into a 24-well tissue culture plate, and medium, ConA, or hardjo antigen was added as for microtiter plates, with a total volume of 2 ml/well.

Analysis of cell proliferation. To evaluate proliferation, cultures were incubated at 37°C with 5% CO_2 for 5 days, at which time 0.5 μCi of [^{3}H]thymidine was added to each well and the culture was incubated for an additional 12 h. Cultures were then harvested onto glass fiber filter paper using a cell harvester and incorporation of [^{3}H]thymidine was determined by liquid scintillation counting. Results are expressed as counts per minute (cpm) of [^{3}H]thymidine incorporated into DNA.

Cytokine evaluation by ELISA. PBMC cultures as described above were incubated for 5 days except at month 6, when the incubation was extended to 7 days. At the end of the culture period, PBMC were resuspended and allowed to settle, and supernatants were collected. Gamma interferon (IFN-γ) was measured in culture supernatants by enzyme-linked immunosorbent assay (ELISA) using a commercial kit (Biosource, Camarillo, Calif.) and a Dynex plate reader set to a wavelength of 450 nm.

Flow cytometry. To evaluate production of IFN-γ by flow cytometry, cultured cells were restimulated during the last 4 h by addition of phorbol myristate acetate (PMA) and ionomycin (at a final concentration of 0.5 μg/ml each) and monensin (final concentration, 2 μM) to facilitate accumulation of detectable amounts of IFN-γ inside the cell. After restimulation, cells were stained by indirect immunofluorescence for surface markers to assess T-cell subpopulations using monoclonal antibody (MAb) interleukin A12 (IL-A12) to identify CD4 (3), MAb MMCA837G to identify CD8 (Serotec, United Kingdom), and MAb GB21A to identify the δ chain of the γδ T-cell receptor (TCR) (VMRD, Pullman, Wash.). This was followed by goat anti-mouse fluorescein-conjugated isotype-specific secondary antibody (Southern Biotech, Birmingham, Ala.). Cells were washed and fixed with 1% paraformaldehyde for 10 min at room temperature. For intracellular staining, the cells were then permeabilized by incubating at 4°C overnight in a solution of 20% horse serum, 0.1% saponin, and 0.1% sodium azide in phosphate-buffered saline (PBS). Intracellular immunostaining was performed using anti-bovine IFN-γ MAb 7B6 or anti-bovine IL-4 MAb 1048, both kindly provided by J.-J. Letesson (45, 46). Cells were reacted with the anti-cytokine MAb in a solution of PBS containing 5% horse serum, 0.1% saponin, and 0.1% sodium azide for 45 min at 4°C, washed twice in PBS with 0.1% saponin, and then they were reacted with goat anti-mouse phycoerythrin-conjugated isotype-specific secondary antibody (Southern Biotech) for 30 min at 4°C, washed twice, and analyzed by flow cytometry using a FACS Calibur (Becton Dickinson, Palo Alto, Calif.). An isotype-matched control antibody for the anticytokine antibodies was used for intracellular staining in some experiments and found not to increase background fluorescence.

Purification of γδ T cells. γδ T cells were purified by magnetic bead sorting. Cells were stained with anti-WC1$^+$ MAb IL-A29 (11) for 20 min at 4°C, washed with a solution of PBS containing 2% heat-inactivated horse serum, and reacted with goat anti-mouse immunoglobulin (IgG) MACS secondary microbeads (Miltenyi Biotec, Auburn, Calif.). Cells were purified over a positive separation column (Miltenyi Biotec) and a sample was assessed by flow cytometry for enrichment. The remaining cells were cultured with antigen and assessed for proliferation and cytokine production as described above.

Statistical analyses. Responses by PBMC from vaccinated and nonvaccinated control cattle were compared by using Student's *t* test.

RESULTS

Proliferative response to antigen. There was a significant proliferative response by PBMC from vaccinated cattle when cultured with the *L. borgpetersenii* serovar hardjo antigen preparation by 2 months after cattle received the first dose of the vaccine (Fig. 1A). The response peaked by 2 months after the completion of the two-dose vaccine (i.e., month 3 of the study) and was sustained throughout the 7-month study period. Only low levels of spontaneous proliferation occurred in cultures without antigen (medium controls), and no significant proliferation above medium controls was ever observed when the PBMC from nonvaccinated animals were cultured with the antigen preparation (Fig. 1B). The PBMC from both vaccinated and nonvaccinated cattle had very similar proliferative responses to the mitogen ConA throughout the study. A suboptimal concentration of ConA was used to avoid overproliferation and cell death during the more prolonged culture times required to measure antigen-specific responses.

Variation in responses among vaccinated cattle. Variation in the proliferative responses by PBMC from individual vaccinated cattle to the antigen was apparent by 3 months after the first dose of vaccine (Fig. 2). PBMC from 5 of 19 animals exhibited proliferative responses to the antigen that were at least 3 standard errors less than the mean of all the vaccinated cattle (i.e., ≤ 49,000 cpm). However, the proliferative responses of PBMC from these animals continued to increase with time (Fig. 3) and by month 7 the magnitudes of the

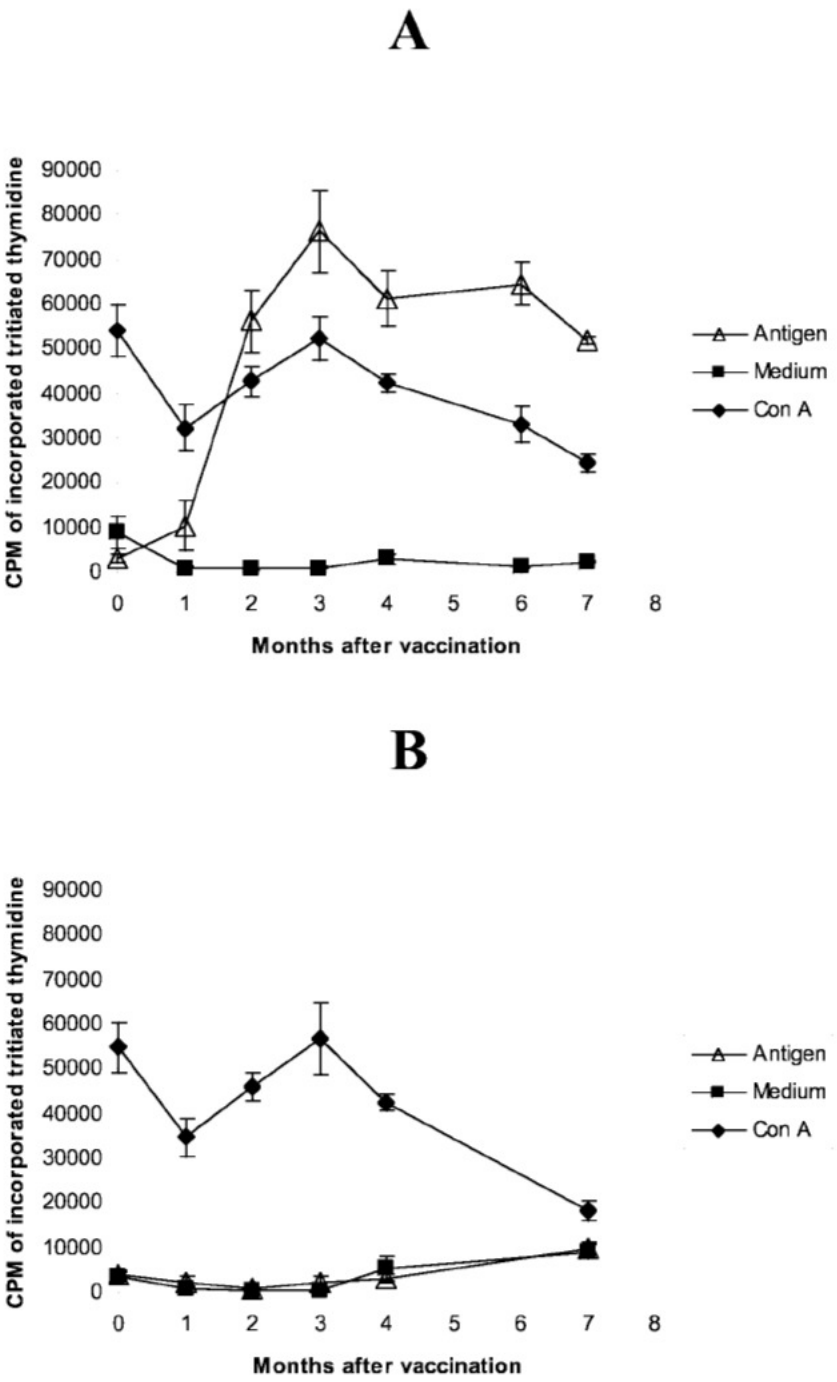

FIG. 1. Means and standard errors of proliferation by PBMC from vaccinated (A) or nonvaccinated (B) cattle, as determined by the incorporation of [^{3}H]thymidine in response to either *L. borgpetersenii* serovar hardjo-bovis antigen, medium, or a suboptimal concentration of ConA. Cattle were vaccinated with the first dose administered at month 0 and the second dose at month 1.

individual responses were similar to the mean of all the vaccinated cattle.

Production of IFN-γ. Once PBMC had shown a substantial proliferative response, IFN-γ production was evaluated. PBMC from vaccinated and nonvaccinated cattle were cultured with and without antigen and culture supernatants were assessed by ELISA (Fig. 4). Throughout the study, PBMC from vaccinated cattle produced more IFN-γ in antigen-stimulated cultures than in medium control cultures. Nonvaccinated cattle produced only minimal amounts of IFN-γ and generally not significantly more in antigen-stimulated cultures than in medium control cultures. At months 3 and 4, 15 and 20%, respectively, of the supernatants from cultures of PBMC from vaccinated cattle stimulated with antigen contained a quantity of IFN-γ higher than the sensitive range of the assay. Had those samples been taken to the endpoint, mean optical

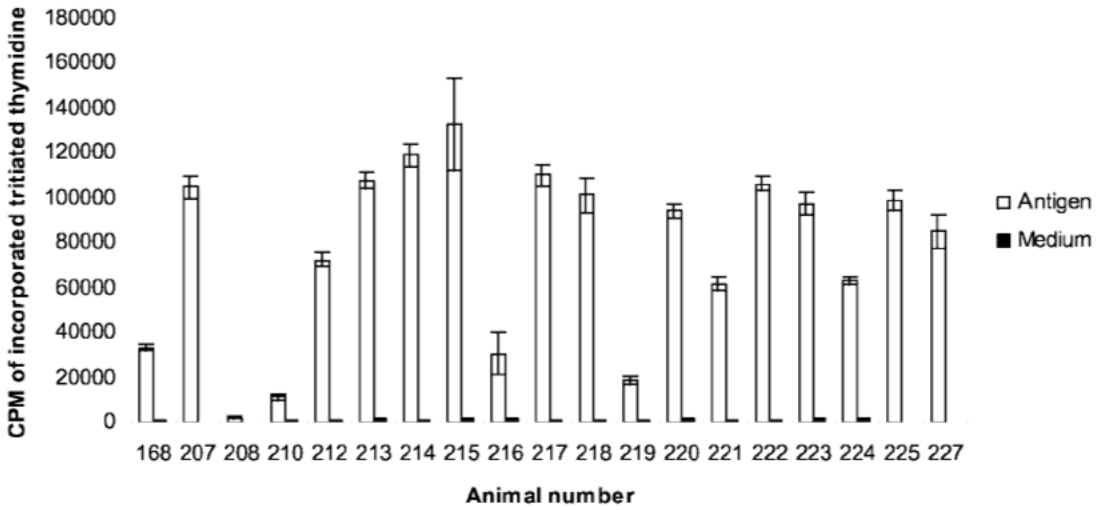

FIG. 2. Proliferation by PBMC from individual vaccinated animals in response to culture with either *L. borgpetersenii* serovar hardjo-bovis antigen or medium at 3 months after the first dose of vaccine. The means of incorporated [^{3}H]thymidine from quadruplicate wells are shown with the standard errors.

density (OD) values may have been higher for response to antigen. Supernatants in months 6 and 7 were diluted so that samples could be read within the sensitive range of the assay.

PBMC in cultures were also assessed for the percentage of cells producing IFN-γ by flow cytometry. The mean percentage of IFN-γ-producing cells in PBMC cultures from vaccinated cattle in response to antigen was significantly higher than that of nonvaccinated cattle (Fig. 5). The mean percentage of IFN-γ-producing cells from nonvaccinated cattle in response to antigen was similar to that in cultures without antigen. At month 4, PBMC from a sample of five vaccinated and five nonvaccinated cattle in antigen-stimulated cultures were stained for intracellular IL-4; none of the animals tested had IL-4-producing cells as determined by flow cytometry (data not shown).

Phenotype of IFN-γ-producing cells. Two-color flow cytometric analysis was done to determine the phenotype of IFN-γ^+ cells in cultures of PBMC from vaccinated cattle that were stimulated with antigen at months 5 and 6. An example of the two-color surface and intracellular immunofluorescence is shown (Fig. 6A). The results from vaccinated animals indicated that approximately two-thirds of IFN-γ^+ cells were within the CD4$^+$ T-cell population while the remaining one-third were γδ T cells (Fig. 6B). A few CD8 T cells were also shown to be producing IFN-γ in the antigen-stimulated cultures, but the percentage was not greatly higher than the low percentage of CD8$^+$/IFN-γ^+ cells detected in PBMC cultures with medium. That is, in the medium control cultures there were always a few IFN-γ^+ cells (<5%) as a result of restimulation with PMA and

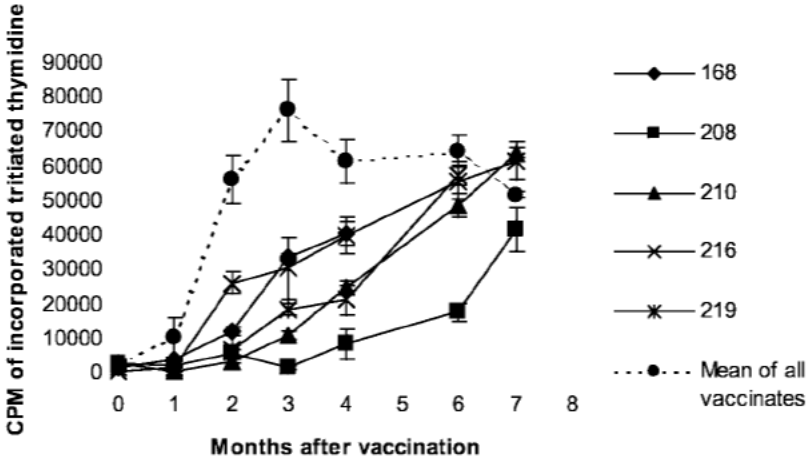

FIG. 3. The proliferation of PBMC from five vaccinated animals with low responses at 3 months after the first dose of the vaccine in response to *L. borgpetersenii* serovar hardjo-bovis antigen. The means for quadruplicate cultures are shown with the standard error. The dashed line indicates the mean values for the entire vaccinated group as a reference point.

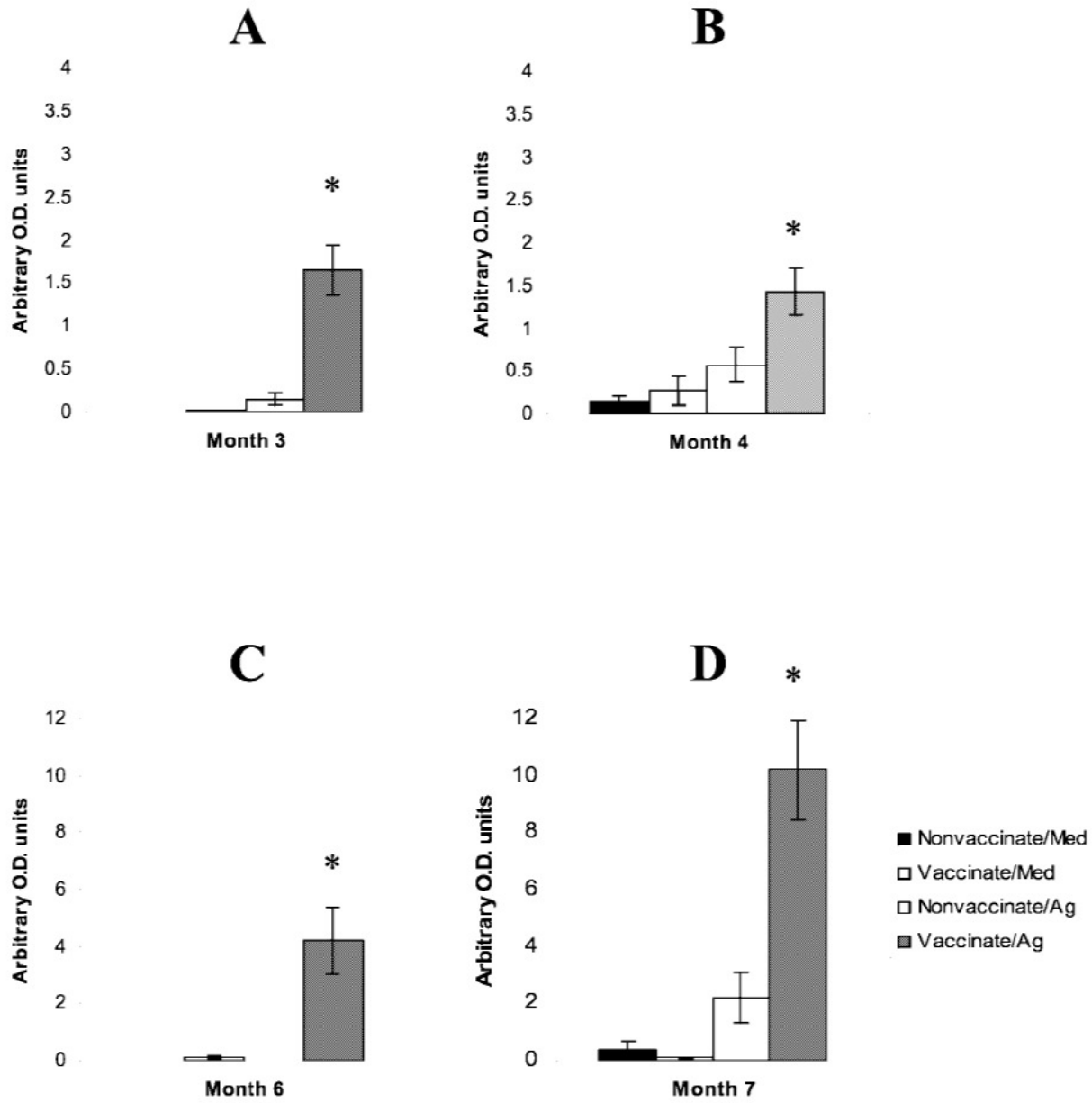

FIG. 4. The mean and standard error of IFN-γ produced in PBMC cultures from vaccinated or nonvaccinated cattle after culture with leptospira antigen (Ag) or medium (Med). PBMC were evaluated at 3 months (A), 4 months (B), 6 months (C), or 7 months (D). The culture supernatants were assessed by ELISA, and the units shown are OD units from an ELISA plate reader using a wavelength of 450 nm. At months 3 and 4, 15 and 20% of the samples, respectively, were beyond the range of the assay. At months 6 and 7, 18 and 68% of the samples, respectively, were beyond the range when tested undiluted; however, dilutions of the supernatants were tested and the OD units were adjusted according to the dilution. At month 6 only vaccinated cattle were sampled. An asterisk indicates a response by the vaccinated group cultured with antigen which is significantly higher than the response by the nonvaccinated cattle at each time point ($P < 0.01$).

ionomycin during the last few hours of culture necessary to detect intracellular IFN-γ (data not shown).

To further investigate the response of γδ T cells to the leptospira antigen, γδ T cells were enriched to 88 and 90% from PBMC by magnetic bead sorting using cells from two vaccinated animals that had previously shown significant proliferative responses and IFN-γ production in response to antigen. Enriched populations of γδ T cells from both animals proliferated in response to antigen (animal no. 214, 16,260 ± 3,216 cpm [mean ± standard deviation] with antigen and 3,249 ± 445 cpm in medium cultures; animal no. 223, 6,822 ± 1,730 cpm with antigen and 816 ± 133 cpm in medium cultures). Two-color immunofluorescence revealed that 73.5 and 74.2% of the γδ T cells from the two animals were IFN-γ^+ in cultures with antigen.

DISCUSSION

In the last decade it has been shown that a pivotal point in immune responses is the decision to make either a Th1-type

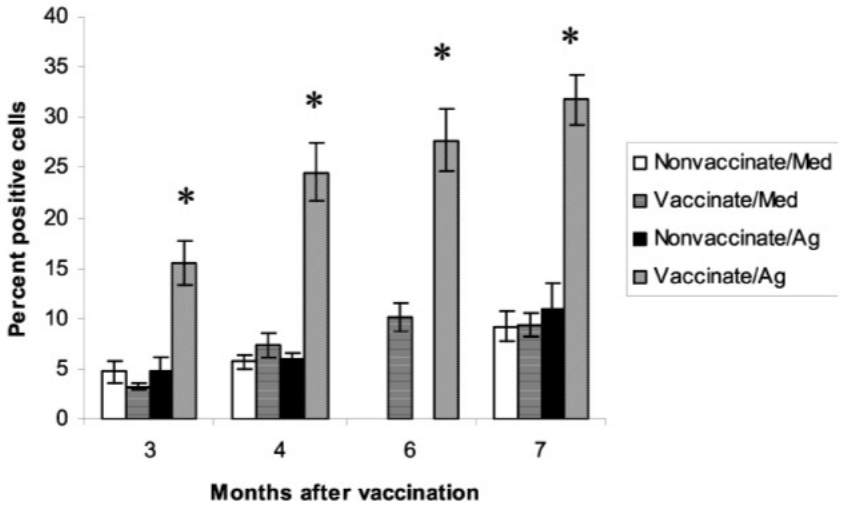

FIG. 5. Mean percentages of IFN-γ-positive cells from cultures of PBMC from vaccinated and nonvaccinated cattle. These cells were restimulated with PMA-ionomycin, permeabilized, and stained for intracellular IFN-γ using indirect immunofluorescence and flow cytometric analysis. The means and standard errors are shown. At month 6, only the vaccinated cattle were sampled. The asterisk indicates a significant difference in the response to antigen ($P < 0.03$).

response characterized by T-cell production of IFN-γ and a bias of the antibody response towards IgG2 or a Th2 response characterized by production of IL-4 by T cells and antibodies of the IgE, IgG1, and IgA isotypes. Like other mammals, cattle have Th1 and Th2 cells (10). The Th1-type response is a component of cell-mediated immunity and is generally considered necessary for resistance to intracellular microbial infections, while a Th2 response promotes a good humoral response and resistance to extracellular organisms. Thus, it was of interest to test the hypothesis that vaccines that protect against the extracellular bacterium *L. borgpetersenii* serovar hardjo induce cell-mediated immune responses. The results presented here indicated that the protective serovar hardjo vaccine did induce a strong, sustained Th1 or cell-mediated immune response since CD4 T cells from vaccinated cattle produced IFN-γ in response to stimulation by antigen. In a small pilot study, cattle given a reference vaccine typical of U.S. pentavalent leptospiral vaccines that fail to protect against hardjo did not develop such a response (B. M. Naiman, C. L. Baldwin, and C. A. Bolin, unpublished data). The lymphocyte proliferative response observed here was similar to that reported by Ellis et al. using PBMC from cattle vaccinated with another protective serovar hardjo vaccine (15). Further work will be required to determine which components of the Th1-type immune response are responsible for the protection against serovar hardjo. It is possible that IgG2 antibodies induced by IFN-γ may be singularly responsible for protection.

While the proliferative response peaked quickly in the majority of vaccinated animals, it was nevertheless sustained throughout the 7 months of the study. Even the response by the initially low responders continued to rise throughout the study period, and there were no differences in the phenotypes of cells that responded to antigen from the low responders and from the other vaccinated animals (data not shown). Why the kinetics of responses were so different in those five animals is unclear, but we speculate that there may be differences in the way the vaccine was deposited in vivo so that a slower release of antigen occurred in these animals. Some variation during the study period may have reflected a general decrease in proliferative responses since it has been shown that proliferation by bovine PBMC to mitogens decreases during winter months in the Northern hemisphere (39). The seventh month of this study was mid-January. The apparent seasonal effect was evident, for instance, in the responses to ConA with cells from both groups of cattle.

With a few exceptions, immunization with killed or inactivated bacteria or viruses is not generally associated with induction of a strong cell-mediated immune response. Thus, it was interesting that the killed whole-cell vaccine in this study induced such a strong Th1-type immune response. The response was not a mitogenic response since there was no significant proliferation in cultures of PBMC from the nonvaccinated control animals. Aluminum hydroxide, the adjuvant in the vaccine employed here, has been shown to potentiate either Th1 or Th2 immune responses (9, 27). Thus, the aluminum hydroxide adjuvant could facilitate the induction of a Th1 response; however, leptospira components in the vaccine strain may also be Th1-promoting adjuvants. For example, it is known that complete Freund's adjuvant which contains components of inactivated *Mycobacterium* produces a Th1 response (48) due in part to the unique lipid trehalose dimycolate in the bacterial cell envelope (37, 47). Other bacterial factors that may possess Th1 adjuvant properties include muramyl dipeptide (2) and CpG, a phosphate-containing bacterial DNA sequence. CpG elicits a strong Th1 immune response especially when used in conjunction with an alum adjuvant (32, 43).

With regard to the subpopulations of T cells that responded to the antigen in vitro, the lack of $CD8^+$ T-cell response was expected since the vaccine was killed, and thus, presentation by major histocompatibility complex class I would be unlikely. While some adjuvants such as saponin are known to promote major histocompatibility complex class I presentation (34), this

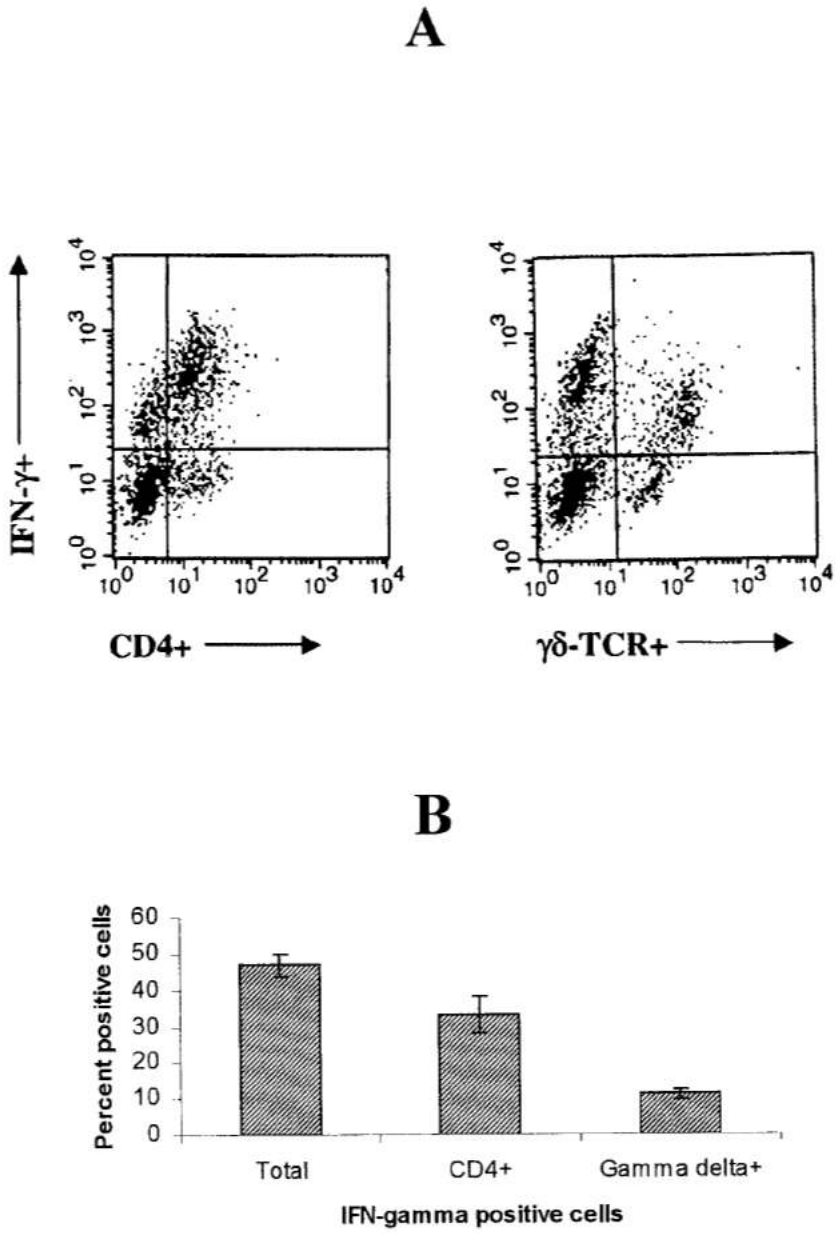

FIG. 6. Percentages of IFN-γ-producing cells in PBMC from six vaccinated animals with high proliferative responses at month 5 after stimulation with *L. borgpetersenii* antigen are shown. The PBMC were cultured for 7 days and restimulated with PMA-ionomycin, and two-color immunofluorescence was performed and analyzed by flow cytometry. (A) An example of two-color flow cytometric analysis shown by dot plot. Detection of IFN-γ is shown on the *y* axis, and either CD4 or γδ-TCR is shown on the *x* axis. Double positives are in the upper right quadrant. (B) Means and standard errors of results with PBMC from six vaccinated animals. Shown are the total percentage of IFN-γ^+ cells in the cultures and the percentage of cells double staining for CD4 and IFN-γ or for γδ-TCR and IFN-γ.

has not been reported for aluminum hydroxide. We speculate that the few $CD8^+$ T cells that were producing IFN-γ were responding as bystanders to cytokines made by the other T-cell populations, since we have shown that bovine $CD8^+$ T cells do proliferate in response to stimulation with recombinant IL-2 (36). Presumably IL-2 was present in the antigen-stimulated cultures since high levels of proliferation occurred. γδ T cells, however, were a major population-producing IFN-γ in addition to the CD4 T cells. Sorting of the γδ T cells by magnetic beads prior to exposure to antigen demonstrated that the response by this T-cell subpopulation is a direct response to antigen rather than a bystander effect in response to cytokines secreted by other cell types activated in the PBMC cultures. Interestingly, humans infected with another spirochete, *Borrelia burgdorferi*, also have a large proportion of γδ T cells that proliferate specifically to that organism (44). Production of IFN-γ by γδ T cells in this system is interesting especially in light of a recent report indicating that γδ T cells from *Mycobacterium bovis*-infected cattle proliferated to mycobacterial antigens but did not produce IFN-γ (38).

It may be the IFN-γ production by the γδ T cells that contributes to the strong CD4 Th1-type immune response induced by the vaccine since recent evidence suggests that IFN-γ activates CD4 T cells to express T-bet and differentiate to Th1 cells (40; S. Szabo, personal communication). It has also been shown that γδ T cells can selectively kill Th2 CD4 T cells through the apoptosis signal generated by Fas/Fas ligand interaction (25). It will be of particular interest to identify the components of the vaccine evaluated here that stimulate γδ T cells so effectively since identification of the γδ T-cell-stimulating component may reveal an interesting type 1-promoting adjuvant. While there is not a great deal known about the stimulatory ligands for γδ T cells, some antigens are derived from bacteria such as *Mycobacteria* heat shock protein (22) and the nonproteinaceous mycobacterial components known as isopentenyl pyrophosphate or TUBag1 to 4 (13, 41).

It has not been conclusively established whether γδ T cells are part of the adaptive or innate arm of the immune system. An early study found that the proportional representation of γδ T cells with various TCR differed between identical human twins (35), suggesting that the γδ T cell subpopulations expand as a result of antigenic experience. However, there are few studies of γδ T cells that evaluated a functional definition of memory, e.g., a more rapid response and/or effective control of a secondary infection. A recent study showed that people who are sensitized by *Mycobacteria* have a population of γδ T cells that rapidly proliferate in response to stimulation with defined mycobacterial components in vitro (24). In contrast, nonvaccinated people do not have this γδ T-cell response, suggesting that the vaccine expanded or sensitized the responsive γδ T cells in vivo, as occurred with the leptospira vaccine used here. Results from a study using mice that have been vaccinated with the microbial pathogen *Listeria monocytogenes* by an infection and antibiotic treatment protocol and then challenged with live listeriae show that γδ T-cell responses play a perceptible role in resistance to secondary challenge, although it was less than that of αβ T cells and only marginally greater than it was in primary infections (33). The γδ T cells in the studies reported here seemed to be responding specifically as part of an acquired immune response since γδ T cells from nonvaccinated cattle did not produce IFN-γ. It is possible that the participation of γδ T cells in the in vitro response reflected activation in vivo, although no response in medium cultures was observed. To determine if the γδ T-cell response is an adaptive immune response it will be necessary to determine whether they mature into a long-lived memory population. It will be of interest to use the leptospira system evaluated here for assessing this.

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