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Differential Immune Responses of Red Deer (*Cervus elaphus*) following Experimental Challenge with *Mycobacterium avium* subsp. *paratuberculosis*▽

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Immune responses of red deer (*Cervus elaphus*) that presented with different levels of paucibacillary pathology were profiled to detail immune changes during the progression of Johne's disease. Immune responses were monitored using an immunoglobulin G1 (IgG1) antibody enzyme-linked immunosorbent assay (ELISA), a gamma interferon (IFN-γ) ELISA, and flow cytometry. Animals in the study were divided into outcome groups postmortem according to disease severity. All animals mounted IgG1 antibody and IFN-γ responses to both the vaccination and experimental challenges. The *Mycobacterium avium* subsp. *paratuberculosis*-specific IgG1 antibody responses in the challenged group showed marked differences between infected and severely diseased animals. Slightly higher IFN-γ responses were seen in infected animals compared with severely diseased animals. No significant changes were seen in the phenotype of lymphocyte populations investigated. Vaccination with killed *M. avium* subsp. *paratuberculosis* in mineral oil adjuvant reduced the level of severe disease; however, it obscured immunological differences between the infected and severely diseased groups. This suggests protection is not exclusively mediated via the presence of a type 1 response and, furthermore, the presence of a type 2 response is compatible with protection. These profiles provide information on the different immune processes in Johne's disease progression.

Johne's disease (JD), also known as paratuberculosis, is a chronic, granulomatous enteritis of ruminants, the causative agent of which is *Mycobacterium avium* subsp. *paratuberculosis* (13, 25). It has been reported that up to 60% of dairy herds and 70% of sheep flocks in New Zealand have JD (3). In New Zealand deer, a reported 6% of herds have JD (10), although this number is likely to be a gross underestimate. In economic terms, JD has been estimated to cost the New Zealand agricultural sector $25 million per annum (3).

Studies of bovine models of JD have shown that $CD4^+$ T cells are the major cell subtype that expands early in *M. avium* subsp. *paratuberculosis* infection, and these cells are the major producers of gamma interferon (IFN-γ) (16, 21). This $CD4^+$ T-cell and IFN-γ response is observed within 6 months of infection (16), although *M. avium* subsp. *paratuberculosis*-specific antibody responses have been shown to precede this $CD4^+$ T-cell response in a calf model of JD (27). This early type 1 immune response, together with *M. avium* subsp. *paratuberculosis*-specific antibody, has been seen in many models of JD (2, 19, 21) and suggests that both type 1 and type 2 immune responses are generated following *M. avium* subsp. *paratuberculosis* infection. During the development of lesions characteristic of JD, many animals will change from the cell-mediated type 1 immune response to a predominantly humoral type 2 immune response mediated by immunoglobulin G1 (IgG1) antibody (8, 9). An antigen-specific T-cell anergy is observed in many of these animals (15, 28). In red deer, IgG1 immune responses have been associated with type 2 immune responses in a model of *Mycobacterium bovis* infection (11).

Current research into *M. avium* subsp. *paratuberculosis* infection and JD is focused on the development of improved diagnostic tools and vaccines to enable detection of subclinically infected animals and reduce the spread of disease. Both these goals are currently hindered by a lack of knowledge of how JD progresses from early-stage infection to clinical disease, as highlighted in a recent international meeting on JD animal models (14). While previous research has identified distinctly different immune responses during the early (9, 16) and late (5, 8) phases of JD, little work has profiled these changes as JD progresses.

In the current study, we obtained immunological data from an *M. avium* subsp. *paratuberculosis* vaccine efficacy trial which provided an opportunity to profile immune responses and identify potential immune differences indicative of different stages of JD progression. Results from the vaccine efficacy trial will be presented elsewhere. The impact of vaccination using Silirum (Pfizer) on the immune pathways was also studied as JD progressed in vaccinated animals.

MATERIALS AND METHODS

Animals. Ninety-two red deer from a herd with no history of JD were used in this trial; all animals were farmed on pasture at the AgResearch Invermay research facility under standard New Zealand deer farming conditions, with supplementary feeding during winter. All animals were female and 5 months of age at the start of the trial. Animals were separated into four groups that were managed separately: 6 animals were unvaccinated and unchallenged controls, 6 animals were vaccinated and unchallenged controls, 40 animals were vaccinated and challenged, and 40 animals were unvaccinated and challenged. These animals were used for IgG1 enzyme-linked immunosorbent assays (ELISAs). A

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subgroup of 24 animals (4 unvaccinated and unchallenged controls, 4 vaccinated and unchallenged controls, 8 vaccinated and challenged, and 8 unvaccinated and challenged) were randomly selected, and this subgroup was used for IFN-γ ELISA and flow cytometry analysis. The trial was approved by the AgResearch Invermay Animal Ethics Committee.

Animals were vaccinated with a killed, whole-cell *M. avium* subsp. *paratuberculosis* (316F) vaccine, with mineral oil as adjuvant, registered as Silirum (Pfizer), subcutaneously in the neck at week zero. At week 6 animals were challenged daily for 4 days with an oral suspension of $\sim 10^9$ virulent *M. avium* subsp. *paratuberculosis* of the bovine strain type isolated from *M. avium* subsp. *paratuberculosis*-infected deer tissues without in vitro culturing, as described previously (17). For all sampling, blood was collected into 10-ml heparinized Vacutainers (Becton Dickinson) by jugular venipuncture with a single draw of blood. Animal health was monitored throughout the course of the experiment, and as part of the ethics requirements four animals were euthanized humanely using a captive bolt gun due to clinical JD, which was characterized by >5% weight loss over 2 weeks, loss of muscle mass over the loins, and diarrhea. Three other animals died of unrelated natural causes during the course of the experiment.

Necropsy. At week 56 remaining animals were euthanized humanely using a captive bolt gun and processed in a registered deer slaughter plant under statutory regulations. Gut mucosa and lymph nodes from euthanized animals were examined visually for gross lesions. Tissue samples from the jejunal lymph nodes, ileocecal lymph nodes, hepatic lymph nodes, jejunum, ileum, and ileocecal valve were taken at necropsy for histological examination and mycobacterial culture from individual tissue samples to confirm JD. Similar samples were taken for both the elective and enforced necropsies.

Fresh tissue samples were submitted to the Infectious Disease Laboratory (Wallaceville Research Centre, Upper Hutt, New Zealand) for culture of *M. avium* subsp. *paratuberculosis*. For histological examination, tissues were fixed in 10% buffered formalin and tissue sections were stained with hematoxylin and eosin and Ziehl-Neelsen stains. Microscopic pathology was graded from 0 to 11 using criteria for JD in red deer as previously described (17) and was also classified as either paucibacillary or multibacillary. The classification as either paucibacillary or multibacillary was based on previously published work in sheep (6), with paucibacillary animals showing <10 acid-fast organisms per macrophage and multibacillary animals showing >10 acid-fast organisms per macrophage. Using the culture status and histological grading, animals were divided into either infected (*M. avium* subsp. *paratuberculosis* culture positive in one or two tissue samples, histopathology scores below 4, and paucibacillary) or severely diseased groups (*M. avium* subsp. *paratuberculosis* culture positive in >3 tissue samples, histopathology scores above 8, and paucibacillary).

IgG1 ELISA. The methods used for the IgG1 antibody ELISA were as described previously (11, 12). This work used cross-reactive antibodies to detect both cervine IgG and IgG1 specific for *M. avium* subsp. *paratuberculosis* and *M. bovis* antigens (11, 12). Briefly, serum samples were obtained from red deer and stored at −20°C prior to assay. Ninety-six-well flat-bottomed microtiter plates (Maxisorp Immuno plate; Nunc, Denmark) were coated with *M. avium* purified protein derivative (PPDa; Biocor) at 12.5 μg/ml, *M. avium* subsp. *paratuberculosis* purified protein derivative (PPDj; CIDC Lelystadt, The Netherlands) at 12.5 μg/ml, and protoplasmic antigen (PPA; Allied Monitor) at 2 μg/ml. Following plate washing, serum diluted 1:100 was added to duplicate wells. Serum from a JD-positive deer was used as a positive control, and serum from an unvaccinated and noninfected deer was used as a negative control. Plates were incubated for 1 h at 37°C and then washed. Antibody specific for cervine IgG1 (9f-98) at 100 μg/ml was added to each well. Plates were incubated at 37°C for 30 min and then washed. A detector goat anti-mouse IgG–horseradish peroxidase–conjugated antibody (Biosource) at 205 ng/ml was added to each well. Plates were incubated at 37°C for 30 min and then washed. One hundred microliters of substrate solution (*o*-phenylenediamine dihydrochloride) was added to each well, and plates were incubated in the dark at room temperature for 20 min. Plates were read in an ELISA reader (Bio-Rad model 3550 microplate reader; Japan) at 490 nm with results expressed as ELISA units.

IFN-γ ELISA. Blood samples were obtained from red deer, and within 12 h, four 1.5-ml aliquots of whole blood were placed in a 24-well sterile plate (multiwell Falcon; Becton Dickinson). One hundred microliters of phosphate-buffered saline as a negative control, PPDa (Biocor) at 300 μg/ml, *M. bovis* purified protein derivative (PPDb; Biocor) at 300 μg/ml, or PPDj at 300 μg/ml was added to each well. Plates were incubated at 37°C in 5% CO_2 for 48 h before plasma was separated and stored at −20°C until assay. Ninety-six-well flat-bottomed microtiter plates (Maxisorp Immuno plate; Nunc, Denmark) were coated with IFN-γ capture antibody (Serotec) at 1.67 μg/ml and incubated at room temperature overnight. Plates were washed, and plasma diluted 1:2 in wash buffer was added to duplicate wells. Wash buffer only was added as a negative control, and recombinant cervine IFN-γ (expressed from the cervine IFN-γ sequence cloned into an *Escherichia coli* expression vector [20]) was added as a positive control. Plates were incubated for 1 h at room temperature and then washed. Biotinylated detection antibody (Serotec) at 0.125 μg/ml was added to each well, and plates were incubated for 1 h at room temperature. Plates were washed, and streptavidin-horseradish peroxidase conjugate (0.5 units/μl; Roche, Germany) diluted 1:2,000 was added to each well. Plates were incubated in the dark for 20 min at room temperature and then washed. One hundred microliters of 3,3′,5,5′-tetramethylbenzidine solution (Invitrogen) was added to each well, and plates were incubated in the dark at room temperature for 30 min before the reaction was stopped. Plates were read in an ELISA reader (Bio-Rad model 3550 microplate reader; Japan) at 450 nm with reference at 650 nm. Results are expressed as optical densities.

Flow cytometry. Anticoagulated blood was mixed 1:1 with phosphate-buffered saline and layered over Histopaque 1083 (Sigma-Aldrich). The Histopaque-blood preparation was centrifuged, and the lymphocyte layer was removed, washed, and resuspended at 2.5×10^6 cells per ml in RPMI (Gibco, Invitrogen) supplemented with 10% deer serum (untreated pooled serum from mixed-age red deer hinds). The methods used for flow cytometry were adapted from methods described previously (1, 16). Briefly, isolated peripheral blood mononuclear cells suspended at 2.5×10^6 cells/ml in RPMI were aliquoted into 96-well sterile plates (Microwell Nunclon; Nunc, Demark). Either RPMI (nonstimulated) or PPDj at 100 μg/ml (stimulated) was added to each well, and plates were incubated at 37°C in 5% CO_2 for 72 h. The cells were centrifuged, the supernatant was removed, and the pellet was resuspended. Primary antibody or appropriate isotype controls (Serotec) were then added. Primary antibodies used were 17D1 (anti-CD4; Washington State University), ST8 (anti-CD8; Washington State University), GB112A (anti-CD25; Washington State University), BAQ4A (anti-WC1; Washington State University), and BAQ155A (anti-B-B4; Veterinary Medical Research and Development, Inc.). Unbound antibodies were removed by washing, and secondary antibody labeled with fluorescein (donkey anti-mouse IgG–fluorescein isothiocyanate or donkey anti-mouse IgM–fluorescein isothiocyanate; Jackson Immunochemicals) at 17.5 μg/ml was added to the appropriate wells. Following incubation the cells were washed and then resuspended in 1% formaldehyde solution and left in the dark at 4°C until analysis in a FACScan (Becton Dickinson) using CellQuest software (Becton Dickinson).

Analysis of data. All statistical analyses were carried out using InStat version 3.0b for Macintosh from GraphPad Software. Data from different groups were analyzed using the Kruskal-Wallis test (nonparametric analysis of variance) and Dunn's multiple comparison test as a post test. Data from the same group over time were analyzed using the Friedman test (nonparametric repeated measures analysis of variance) and Dunn's multiple comparison test as a post test. Significance is expressed at a P level of either <0.05, <0.01, or <0.001, as indicated below.

RESULTS

Necropsy. Two animals in the vaccine efficacy trial presented with multibacillary disease and, due to the previously reported differences between paucibacillary and multibacillary JD (5, 6) and the small number of multibacillary animals, these animals were excluded from further group analysis. All remaining challenged animals were grouped into infected or severely diseased groups as described in the Materials and Methods section. Infected animals showed no blunting of the intestinal villi, with the occasional small granulomas usually seen in lymph nodes. Severely diseased animals showed extensive blunting of the intestinal villi, and numerous granulomas were seen throughout the mucosa and submucosa.

Challenged animals that presented with culture results and histology results that were between the infected and severely diseased classifications were excluded from further group analysis in order to polarize the extremes of *M. avium* subsp. *paratuberculosis* infection and JD. The unchallenged unvaccinated control group showed no histopathology characteristic of JD, and baseline immune responses to mycobacterial antigens were seen in this group (Fig. 1D; see also Fig. 3D, below). Two animals from the unchallenged control groups were mycobacterial culture positive and were excluded from further analysis.

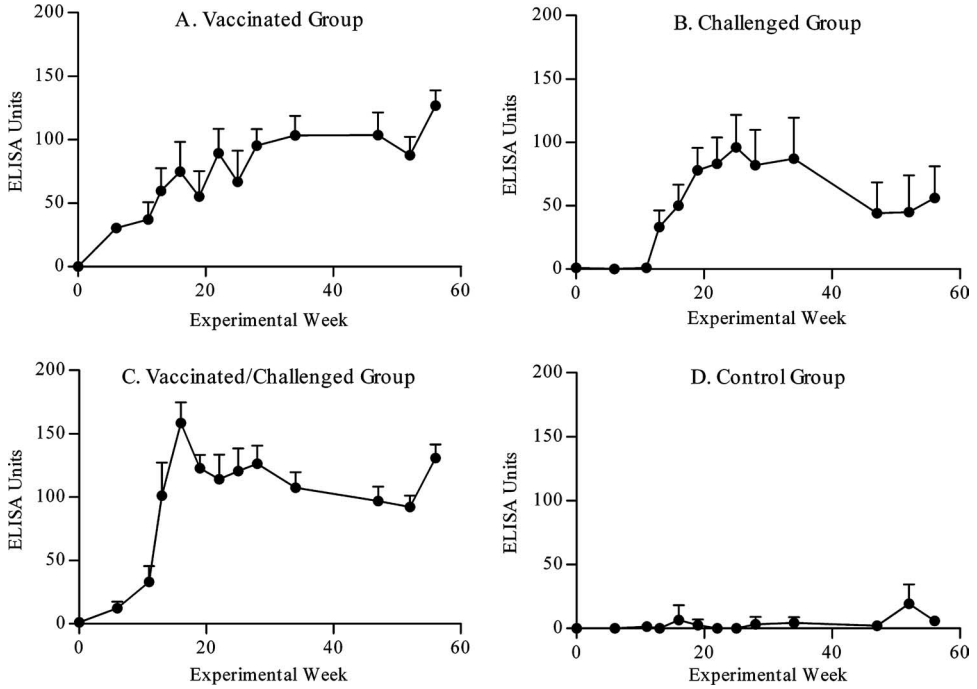

FIG. 1. Protoplasmic antigen-specific IgG1 levels (expressed as ELISA units) in treatment groups (means + standard errors of the means). (A) Vaccinated unchallenged group ($n = 3$); (B) unvaccinated challenged group ($n = 8$); (C) vaccinated challenged group ($n = 8$); (D) unvaccinated unchallenged control group ($n = 3$).

One animal was *M. avium* culture positive but showed no immunological signs of infection; therefore, this was considered environmental contamination, and data were used in group comparisons. Vaccination reduced the severity of disease but did not reduce the level of *M. avium* subsp. *paratuberculosis* infection after experimental challenge; these results will be presented elsewhere (C. Mackintosh, unpublished data).

IgG1 ELISA. As shown in Fig. 1, PPA-specific IgG1 responses were generated within 6 weeks of vaccination in the vaccinated unchallenged group ($n = 3$) and within 7 weeks of experimental challenge in the unvaccinated challenged group ($n = 8$). Baseline *M. avium* subsp. *paratuberculosis*-specific IgG1 responses were seen in the unvaccinated unchallenged control group ($n = 3$) over the course of the experiment (Fig. 1D). In both the vaccinated unchallenged and unvaccinated challenged groups the PPA-specific IgG1 responses generated were above the diagnostic threshold of 50 ELISA units for extended periods of time (Fig. 1A and B). When experimental challenge was added on top of vaccination in the vaccinated challenged group, an earlier peak was seen in the IgG1 responses (Fig. 1C). This returned to the level of the vaccinated unchallenged group by 28 weeks postchallenge (Fig. 1A and C). Similar patterns were seen when looking at PPDj-specific IgG1 responses or PPDa-specific IgG1 responses, although in the case of the latter the overall levels of antibody present were much lower (data not shown).

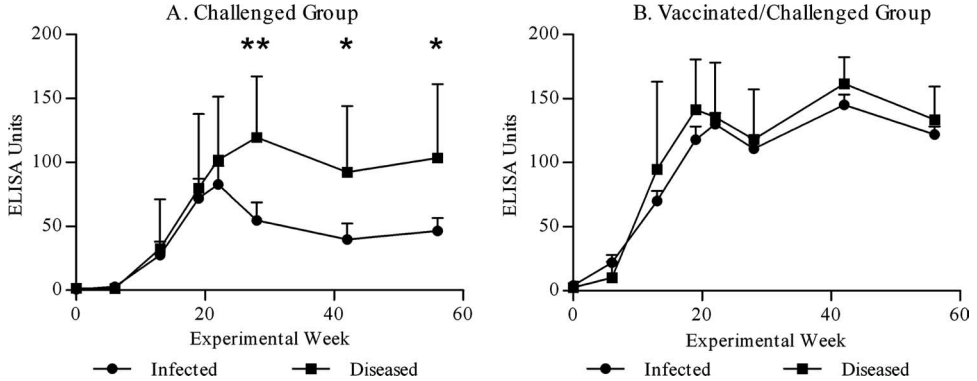

FIG. 2. Protoplasmic antigen-specific IgG1 levels (expressed as ELISA units) in the infected versus severely diseased subgroups of both the unvaccinated challenged (A) and vaccinated challenged (B) groups (means + standard errors of the means). Both unvaccinated challenged subgroups (infected or severely diseased) had 13 animals, and the vaccinated challenged subgroups had 21 (infected) and 8 (severely diseased) animals, respectively. *, $P < 0.05$; **, $P < 0.01$.

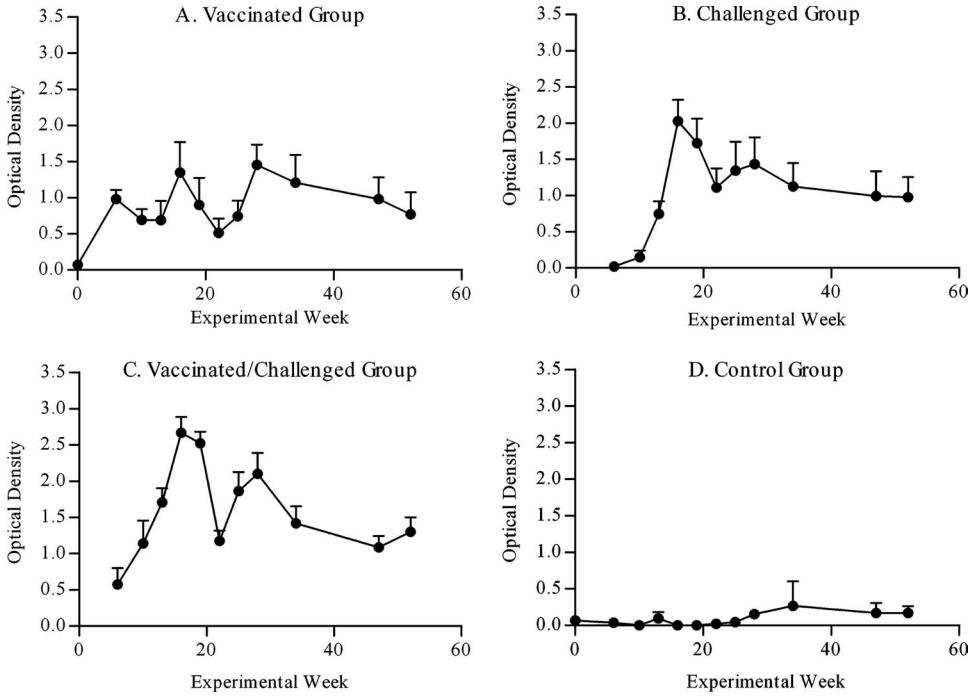

FIG. 3. IFN-γ responses to johnin purified protein derivative (expressed as optical densities) in treatment groups (means + standard errors of the means). (A) Vaccinated unchallenged group (n = 3); (B) unvaccinated challenged group (n = 8); (C) vaccinated challenged group (n = 8); (D) unvaccinated unchallenged control group (n = 3).

Both the unvaccinated challenged infected group (n = 13) and the unvaccinated challenged severely diseased group (n = 13) had similar IgG1 responses until 16 weeks postchallenge. By 22 weeks postchallenge, however, the IgG1 response of the infected subgroup had declined (Fig. 2A). Significant differences in the levels of IgG1 between the infected and severely diseased subgroups were seen for the remainder of the experiment (Fig. 2A). This significant difference was also seen when looking at PPDj-specific IgG1 responses or PPDa-specific IgG1 responses (data not shown). When challenged animals had been vaccinated previously, as in the vaccinated challenged group, these differences were not seen (Fig. 2B). The infected and severely diseased subgroups of the vaccinated challenged animals had identical IgG1 responses over the course of the experiment (Fig. 2B).

IFN-γ ELISA. PPDj-specific IFN-γ responses were generated within 6 weeks of vaccination in the vaccinated unchallenged group (Fig. 3A) and within 7 weeks of experimental challenge in the unvaccinated challenged group (Fig. 3B). Baseline IFN-γ responses were seen in the unvaccinated unchallenged control group over the course of the experiment (Fig. 3D). When experimental challenge was added on top of vaccination in the vaccinated challenged group, an earlier peak in the IFN-γ response was seen (Fig. 3C). This returned to the level of the vaccinated unchallenged group by 28 weeks postchallenge (Fig. 3A and C). Similar patterns were seen when

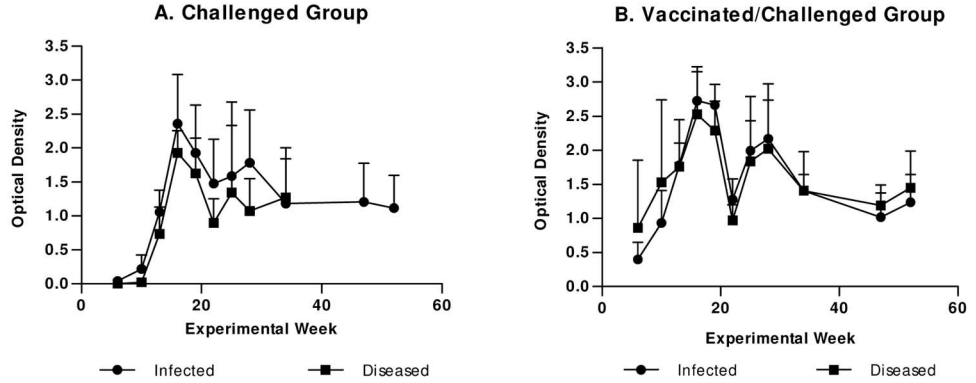

FIG. 4. IFN-γ responses to johnin purified protein derivative (expressed as optical densities) comparing the infected and severely diseased subgroups of both the unvaccinated challenged (A) and vaccinated challenged (B) groups (means + standard errors of the means). For all subgroups, n = 3.

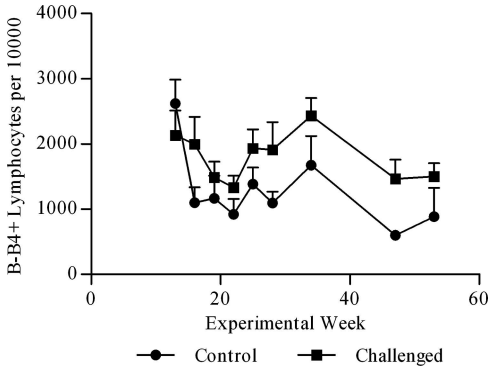

FIG. 5. Total numbers of B-B4$^+$ lymphocytes per 10,000 lymphocytes in the nil-stimulation samples of the unvaccinated unchallenged control and unvaccinated challenged groups (means + standard errors of the means). For the unvaccinated unchallenged control group, n = 3; for the unvaccinated challenged group, n = 8.

looking at PPDa-specific IFN-γ responses or PPDb-specific IFN-γ responses, although the overall levels of IFN-γ present were much lower than the PPDj-specific responses (data not shown).

There were no significant differences in the IFN-γ responses between the infected and severely diseased subgroups (Fig. 4). There was a general trend in the unvaccinated challenged group, in which the infected animals tended to exhibit higher IFN-γ responses to PPDj compared to the severely diseased animals (Fig. 4A). This trend was not present in the vaccinated challenged group (Fig. 4B). These trends were also seen when looking at the IFN-γ responses to either PPDa or PPDb (data not shown).

Flow cytometry. No significant differences were observed in the total numbers of CD4$^+$, CD8$^+$, WC1$^+$, and B-B4$^+$ cells present per 10,000 circulating lymphocytes between any of the treatment groups over the course of the experiment. This was regardless of whether the lymphocytes were stimulated with PPDj or left unstimulated (data not shown). A trend toward an increase in the total numbers of B-B4$^+$ cells present in the unstimulated lymphocytes from the unvaccinated challenged group compared to unvaccinated unchallenged controls was observed (Fig. 5). The expression of the cell activation marker CD25 in response to PPDj stimulation did show a large increase over time in both the unvaccinated challenged and vaccinated challenged groups which peaked 19 weeks postchallenge (Fig. 6). No differences were seen between the infected and severely diseased subgroups with any of the cell surface markers studied (data not shown).

DISCUSSION

The animals in this study all presented with paucibacillary disease, which was classified into either infected or severely diseased groups. Two animals with multibacillary disease were excluded due to previously identified immunological differences between paucibacillary and multibacillary JD in sheep (5, 6). While the small numbers of animals in this study excluded comparing multibacillary and paucibacillary JD, knowledge of these disease states in deer is needed. The animals presenting with intermediate paucibacillary pathology would also be interesting to study; however, once again limited numbers of animals precluded this option in the current study.

Immune profiles can be used to distinguish between different stages of *M. avium* subsp. *paratuberculosis* infection and JD, with IgG1 responses distinguishing between paucibacillary infected and severely diseased animals. These *M. avium* subsp. *paratuberculosis*-specific IgG1 immune response differences may assist in estimating disease severity antemortem in future experimental trials. The *M. avium* subsp. *paratuberculosis*-specific IgG1 responses also raise two other important points. Firstly, the large peak in IgG1 responses after challenge, even in the infected group with little pathology, will interfere with accurate disease diagnosis. It suggested that at best current diagnostic methods are a measure of *M. avium* subsp. *paratuberculosis* exposure and can explain the current diagnostic tests' lack of specificity when looking for JD (2, 7, 24). Secondly, these results suggested that while higher IgG1 levels correlate with severe disease, an IgG1 response in itself does not preclude a protective immune response to *M. avium* subsp. *paratuberculosis* infection and does not play a direct role in the pathological endpoint of JD.

When mycobacterial challenge occurs following vaccination with a killed whole-cell *M. avium* subsp. *paratuberculosis* vaccine and a mineral oil adjuvant, immune profile differences between infected and severely diseased animals are masked. Previous work with vaccines that used a mineral oil adjuvant showed that this type of vaccine evokes strong cellular and humoral immune responses (18, 26), as has been shown in this

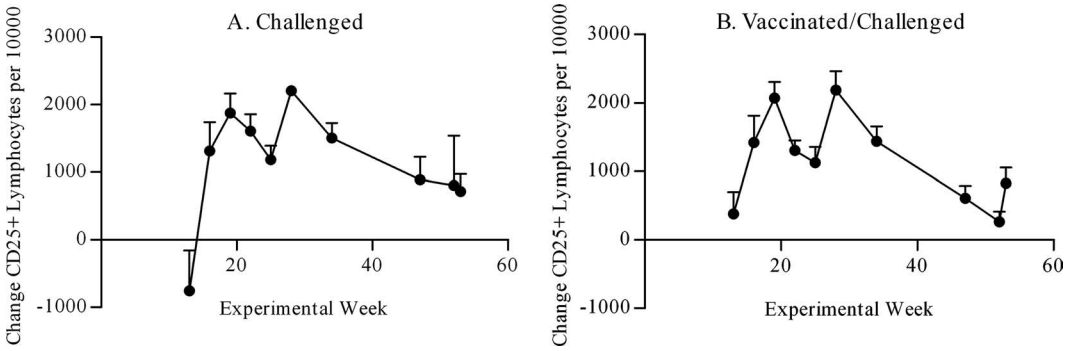

FIG. 6. Change in CD25-positive lymphocytes per 10,000 lymphocytes following stimulation with johnin purified protein derivative (means + standard errors of the means). (A) Unvaccinated challenged group (n = 8); (B) vaccinated challenged group (n = 8).

study. These large immune responses to vaccination obscure any differences in the immune responses of the infected and severely diseased subgroups and also have significant implications for diagnostic testing in vaccinated animals. All the vaccinated animals studied had IgG1 and IFN-γ responses to *M. avium* subsp. *paratuberculosis*-specific antigens that would be considered positive diagnostic results for JD.

The IFN-γ assay and flow cytometry technique for measuring lymphocyte subpopulations were limited by the small group sizes ($n = 3$). This limitation of the study meant any differences were less likely to be statistically significant. Both the infected and severely diseased groups showed large IFN-γ responses to *M. avium* subsp. *paratuberculosis*-specific antigens. While these were slightly higher in the infected group, the large IFN-γ responses in the severely diseased group suggested that the presence of IFN-γ responses alone is not enough to constitute a protective response. Previous work looking at IFN-γ has produced conflicting results that correlate it with either protection or pathology (4, 5, 15, 19, 22). The results reported here support the idea that IFN-γ plays a role in both protection and pathology. These roles of IFN-γ in both protection and disease have previously been shown in white-tailed deer infected with *Mycobacterium bovis* (23). Once again, vaccination with a killed whole-cell *M. avium* subsp. *paratuberculosis* vaccine and a mineral oil adjuvant masked any differences between infected and severely diseased animals, further supporting the idea that IFN-γ responses alone are not predictive of the outcome of *M. avium* subsp. *paratuberculosis* infection and JD.

No significant differences in the total numbers of lymphocyte subpopulations were seen in this experiment. While previous work showed differences in both the numbers of CD4-, WC1-, and CD25-positive cells, these studies looked at either noninfected versus infected or low versus high shedders (15, 16, 27). Very few studies have looked at differences within paucibacillary diseased animals, and this study suggested that while there may be differences they are likely to be at the level of cell activation status rather than individual cell populations. In addition, the current study did not look specifically at the proliferating subpopulation of lymphocytes, as previous studies have done (16, 27). CD25, also known as the interleukin-2 receptor, is upregulated on activated cells, and increases in expression of CD25 were used as a measure of cellular activation. The expression of CD25 upon stimulation with PPDj did increase in both the unvaccinated challenged and vaccinated challenged groups, with the peak of CD25 expression seen at 13 weeks postchallenge. This early peak in CD25 expression has been previously observed in cattle, although not as early following challenge, and this demonstrates the rapid progression of JD in the red deer model (16).

The immune responses of red deer were profiled following experimental challenge with virulent *M. avium* subsp. *paratuberculosis*, and immune differences indicative of infection and severe disease in paucibacillary animals were identified. These differences were only present when animals had no previous exposure to *M. avium* subsp. *paratuberculosis* antigens and, indeed, vaccination served to mask these immunological differences. These results have important implications for diagnostic testing of red deer for JD and also suggest that the roles of IgG1 and IFN-γ responses in *M. avium* subsp. *paratuberculosis* infection and JD need to be assessed in light of more complete immunological profiles.

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