

Monitoring responses by use of five-color flow cytometry in subsets of peripheral T cells obtained from cattle inoculated with a killed *Mycobacterium avium* subsp *paratuberculosis* vaccine

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Objective—To monitor by use of 5-color flow cytometry the antigen-specific responses of subsets of peripheral T cells in cattle inoculated with a killed *Mycobacterium avium* subsp *paratuberculosis* (MAP) vaccine and to compare results with those for 2 established cell-mediated immunity assays.

Animals—45 female Holstein cattle with negative results for MAP in skin tests conducted at time of inoculation with MAP.

Procedures—Cattle were allocated to 4 groups. Cattle of group 1 (n = 12) were 0 to 3 months old and inoculated with a killed MAP vaccine. The 10 cattle of group 2 were the same age as those in group 1 but were not inoculated with MAP vaccine. The 11 cattle of group 3 were 9 to 12 months old and inoculated with killed MAP vaccine. The 12 cattle of group 4 were the same age as those in group 3 but were not inoculated with MAP vaccine.

Results—Flow cytometry identified T-cell subsets that responded specifically to the recall antigen. Results of assays for CD25 expression and whole-blood interferon- γ had the strongest correlation with results for skin tests as well as results with each other. Intracellular expression of interferon- γ was not correlated as well with results for the other tests.

Conclusions and Clinical Relevance—Flow cytometry can be useful for characterizing the immune response after administration of MAP vaccine and should be evaluated with regard to its sensitivity and specificity when used in detecting cattle naturally infected with MAP. (*Am J Vet Res* 2006;67:2050–2058)

| ABBREVIATIONS | |
|------------------|---|
| MAP | <i>Mycobacterium avium</i> subsp <i>paratuberculosis</i> |
| CMI | Cell-mediated immunity |
| PPD | Purified-protein derivative |
| IFN- γ | Interferon- γ |
| WB IFN- γ | Whole-blood IFN- γ |
| CD25 EI | CD25 expression indices |
| OD | Optical density |
| TCR | T-cell receptor |
| PBMC | Peripheral blood mononuclear cell |
| PBS++ | PBS solution containing 0.5% bovine serum albumin and 0.1% sodium azide |
| IFN- γ + | Interferon- γ positive |

marked reduction in the incidence of clinical disease and fecal shedding after administration of killed MAP cells in oil.²⁻⁴ However, definitive information is not available on the protective host-defense mechanisms.

The conventional CMI test to detect animal exposure to MAP antigen is a delayed-type hypersensitivity skin test that uses ID injection of MAP-PPD. However, there may be cross reactions with other *Mycobacterium* spp, which could cause the skin test to have false-positive results.⁵ An in vitro CMI assay based on detection of IFN- γ production in samples of whole blood (ie, the WB IFN- γ assay) was originally developed for diagnosis of tuberculosis in cattle^{6,7} and was further modified for diagnosis of MAP infection.⁸ The test initially provided promising results for use in differentiating experimentally and naturally infected cattle and sheep during the early stages of infection.⁸⁻¹² However, a study¹³ conducted in young calves to evaluate diagnostic tests for paratuberculosis yielded positive results for IFN- γ with that assay in samples obtained from control calves.

Another CMI assay, flow cytometry, has been used to evaluate specific proliferation of MAP-activated lymphocyte subsets.¹⁴ In addition, an ELISA for IFN- γ content in the culture supernatant was evaluated in a concurrent study.¹⁵ In those studies, proliferation of B cells was a sensitive indicator of subclinical paratuberculosis,¹⁴ whereas CD4+ cells were the major cells that produced IFN- γ .¹⁵ In another study¹⁶ that used whole blood obtained from infected goats, CD25, which is the α chain of the interleukin-2 receptor, was expressed on T and B cells after the cells were activated by contact with antigen they recognized, and CD25 expression was used as a CMI marker in an MAP-activated assay.

Paratuberculosis (Johne's disease) is caused by MAP and causes important economic losses to dairy and beef producers in widespread areas of the United States and other countries.¹ The losses to cattle producers are related to decreased market value of slaughter cows as a result of emaciation, reduced income related to the inability to sell replacement breeding stock, and decreased milk production of affected cows. There is a

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Kinetics of CD25 expression as a percentage of CD25+ cells in whole blood were further evaluated in subsets of CD4, CD8, and $\gamma\delta$ T cells by use of dual- or triple-color analysis.¹⁷ Flow cytometry has been used successfully by our laboratory group to evaluate T-cell responses to various infectious agents of cattle through detection of upregulation of CD25,¹⁸⁻²⁴ intracellular expression of IFN- γ by use of multicolor flow cytometry,^{23,24} or both. Five-color flow cytometry simultaneously identifies T-cell subsets that express CD25 or intracellular IFN- γ (or both) from the same samples.²⁴ Upregulation of CD25 was reported as the CD25 EI, and intracellular IFN- γ content was calculated as the net percentage increase in intracellular IFN- γ production, compared with IFN- γ production by unstimulated cells of the same T-cell subset from the same cattle.

The specific objectives of the study reported here were to monitor by use of 5-color flow cytometry responses of peripheral T-cell subsets in naive cattle inoculated with killed MAP cells in oil at 2 ages and to compare the results with those for 2 established CMI assays. The hypothesis tested was that 5-color flow cytometry could be used to detect specific memory responses of T-cell subsets to MAP-PPD in cattle injected with a killed MAP vaccine.

Materials and Methods

Animals—The study involved 45 female Holstein cattle on a commercial dairy farm in Iowa. Historically, paratuberculosis had been diagnosed in this herd. Cattle represented 2 populations of animals (22 were 0 to 3 months old at time of vaccination, and 23 were 9 to 12 months old at vaccination). All cattle had negative results for a skin test against MAP-PPD. All cattle were involved in the study at the same time. The study was conducted in accordance with a protocol approved by the Iowa State University Committee on Animal Care and Use.

Study design—Cattle were allocated to 4 groups. Two groups were inoculated with a USDA-licensed killed-cell MAP vaccine^a containing oil adjuvant. Vaccine (1 mL) was administered SC in the brisket, as recommended by the manufacturer. Cattle of group 1 (n = 12) were 0 to 3 months old at the time of vaccine administration. The 10 cattle of group 2 were the same age as those of group 1 but were not vaccinated. The 11 cattle of group 3 were 9 to 12 months old at time of vaccine administration, whereas the 12 cattle of group 4 were the same age as those of group 3 but were not vaccinated.

At 10.25 months after vaccine administration, a blood sample (10 mL) was collected in 1.5 mL of 2X acid citrate dextrose (0.15M sodium citrate, 0.076M citric acid monohydrate, and 0.287M dextrose) from each of the cattle for use in flow cytometry. At 11 months after vaccine administration, another set of blood samples was collected for use in a WB IFN- γ assay and flow cytometry, and all cattle were subjected to a skin test. Blood samples for a WB IFN- γ assay (10 mL) were collected in evacuated blood collection tubes^b that contained sodi-

um heparin; blood samples for flow cytometry were collected as described previously. All blood samples were transported at ambient temperature to the laboratory for processing during the same day on which they were collected.

The MAP-PPD (National Veterinary Services Laboratory lot No. 9801), prepared as described elsewhere,²⁵ was used as the recall antigen in all tests (skin test, WB IFN- γ assay, and 5-color flow cytometry). Cattle in groups 3 and 4 were pregnant at the time testing was conducted 11 months after vaccine administration. A mean value for each animal was calculated from the 2 flow cytometry results. Three cattle in group 2 and 4 cattle in group 4 had positive results for the skin test performed 11 months after vaccine administration; these 7 cattle were analyzed as a separate group.

Skin test—A delayed-type hypersensitivity test was conducted on all 45 cattle by an experienced veterinarian (RLR). Testing involved ID injection of MAP-PPD in the cervical region of each animal. Thickness of the skin was measured before and 72 hours after MAP-PPD injection. The increase in skin thickness for each animal was determined. An increase in skin thickness of ≥ 3 mm was considered a positive test result.

WB IFN- γ assay—The WB IFN- γ assay was performed by personnel at the National Animal Disease Center in Ames, Iowa. The assay was conducted as described elsewhere.¹² Briefly, 1 mL of heparinized whole blood was added to each of 3 wells of a 24-well tissue culture plate. One well was treated by the addition of 10 μ g of MAP-PPD, a second well was treated by the addition of 1 μ g of *Staphylococcus aureus* enterotoxin A^c (positive control well), and the third well was left as an unstimulated control well. Plates were incubated

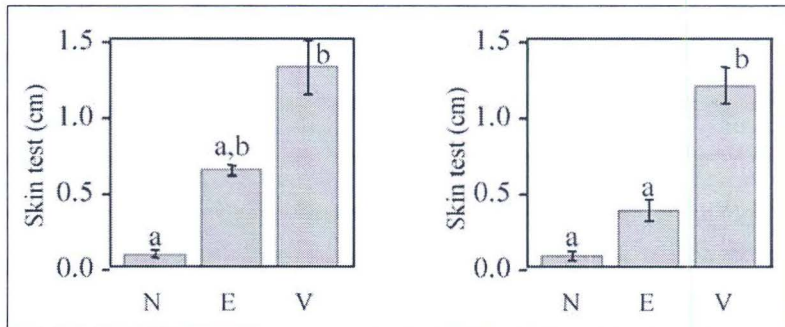


Figure 1—Mean \pm SEM net increase in skin thickness in 3 groups of cattle tested 11 months after they were administered a killed MAP vaccine. Cattle were inoculated when they were 0 to 3 months of age (left) or 9 to 12 months of age (right). The groups represent nonvaccinated cattle that were not naturally exposed to MAP (N), nonvaccinated cattle that were naturally exposed to MAP and subsequently had positive results for the skin test (E), and vaccinated cattle (V). a,b Within an age group, values with different letters differ significantly ($P < 0.05$).

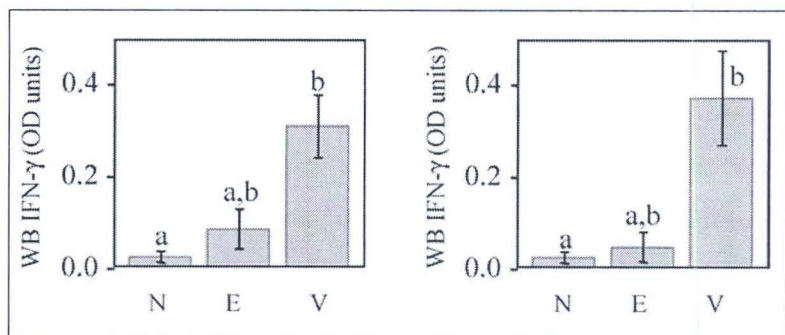


Figure 2—Mean \pm SEM net OD for the WB IFN- γ assay measured after 24 hours of incubation with MAP-PPD. See Figure 1 for remainder of key.

for 24 hours at 37°C in a humidified 5% carbon dioxide environment. After incubation, plates were centrifuged at 1,200 X g for 5 minutes. Plasma was harvested and stored at -80°C until assayed.

IFN- γ ELISA—A commercial IFN- γ ELISA kit^d was used in accordance with the manufacturer's instructions. Plasma

samples were assayed in duplicate, and OD of wells was measured at 450 nm.¹² Positive and negative control samples from the kit were included in all plates.

The net OD was obtained by subtraction of the OD of the unstimulated well from the OD of stimulated wells for samples obtained from the same animal. Results were considered positive when the net OD was ≥ 0.1 . For valid results, the net OD between the OD for the well containing *S aureus* enterotoxin A and the OD of the negative control well had to be ≥ 0.1 .

Five-color flow cytometry—Upregulation of surface expression of CD25 and production of intracellular IFN- γ in response to MAP-PPD were evaluated in CD4+, CD8+, and $\gamma\delta$ TCR+ lymphocytes. Buffy coats were collected from blood samples that contained 2X acid citrate dextrose, and PBMCs were isolated as described elsewhere.²⁴ Cells (n = 500,000) of each PBMC suspension were added to each of 3 wells of a 96-well flat-bottomed tissue culture microtiter plate. One well was treated by the addition of 50 μ L of MAP-PPD (final concentration, 5 mg/mL). A second well was treated by the addition of 50 μ L of concanavalin A (0.01 mg/well; mitogen control well). The third well was treated by the addition of 50 μ L of RPMI containing 15% fetal bovine serum and 1.5% penicillin-streptomycin solution (nonantigen stimulated control well). Plates were incubated for 6 days at 37°C in a humidified 5% carbon dioxide environment.

After incubation for 6 days, 50 μ L of protein-transport inhibitor^e was added to all wells at the concentration recommended by the manufacturer. The PBMCs were then transferred to 96-well round-bottomed tissue culture plates, which were then incubated for 4 hours. Cell pellets were washed 3 times with PBS++ solution. The antibody mixtures included monoclonal anti-bovine T-cell antigens and activation marker CD25 and goat or rat anti-mouse isotype secondary antibodies (Appendix).^f All monoclonal antibodies, anti-bovine surface antigen antibodies, and goat or rat anti-mouse isotype secondary antibodies were diluted in PBS++ solution to achieve previously determined optimum dilutions (data not shown). The monoclonal antibody to bovine IFN- γ was biotinylated,^g and streptavidin conjugate^h was used as the secondary reagent. The monoclonal antibody and secondary reagent conjugate for detection of bovine IFN- γ were diluted in wash solution^p to achieve previously determined optimum dilutions (data not shown).

Monoclonal antibody mixture for surface antigens was added to all test wells. Plates were incubated at 25°C for 15 minutes. After 3 wash cycles with PBS++ solution, the secondary antibody conjugate mixture was added to all test wells (Appendix). Plates were then incubated at 25°C for an additional 15 minutes. After 3 wash cycles with PBS++ solution, 100 μ L of cell-fixing and permeabilizing solution^q was added to each well, and the plates were incubated at 25°C for 1 hour. Plates were washed twice with wash solution,^p and biotinylated mouse anti-bovine IFN- γ ^r (50 μ L) was added

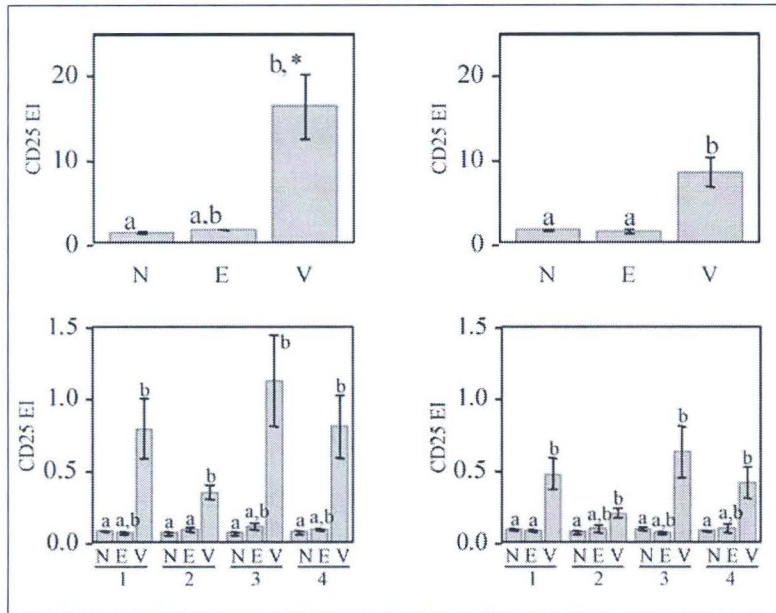


Figure 3—Mean \pm SEM CD25 EI for all PBMCs (top) and specific T-cell subsets (bottom) obtained from calves inoculated with MAP vaccine at 0 to 3 months of age (left) or 9 to 12 months of age (right) and measured after 6 days of incubation with MAP-PPD. The specific subsets were as follows: 1, CD4+ cells; 2, CD8+ cells; 3, $\gamma\delta$ TCR+ cells; and 4, non-T cells. a,b Within an age group (top) or within each T-cell subset (bottom), values with different letters differ significantly ($P < 0.05$). *Value differs significantly ($P < 0.05$) from value for the same cattle in the other age group. See Figure 1 for remainder of key.

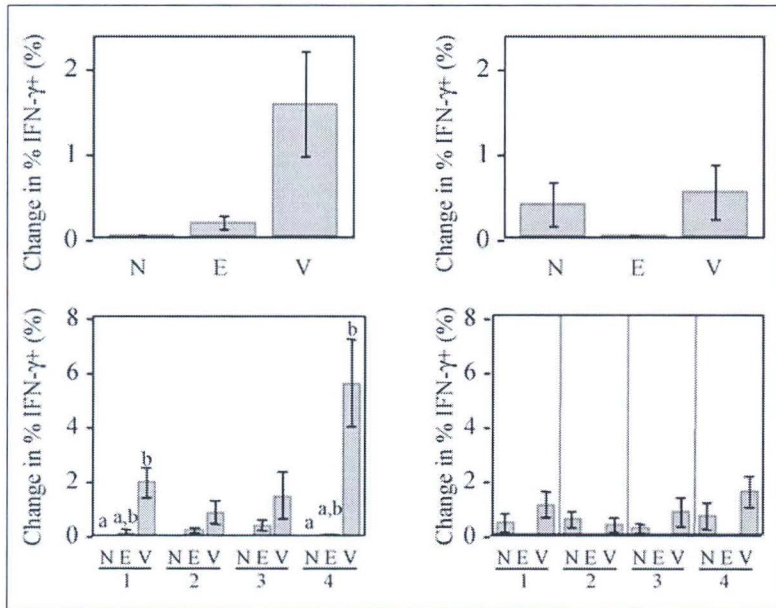


Figure 4—Mean \pm SEM change in percentage of IFN- γ + for all PBMCs (top) and specific T-cell subsets (bottom) obtained from calves inoculated with MAP vaccine at 0 to 3 months of age (left) or 9 to 12 months of age (right) and measured after 6 days of incubation with MAP-PPD. See Figures 1 and 3 for key.

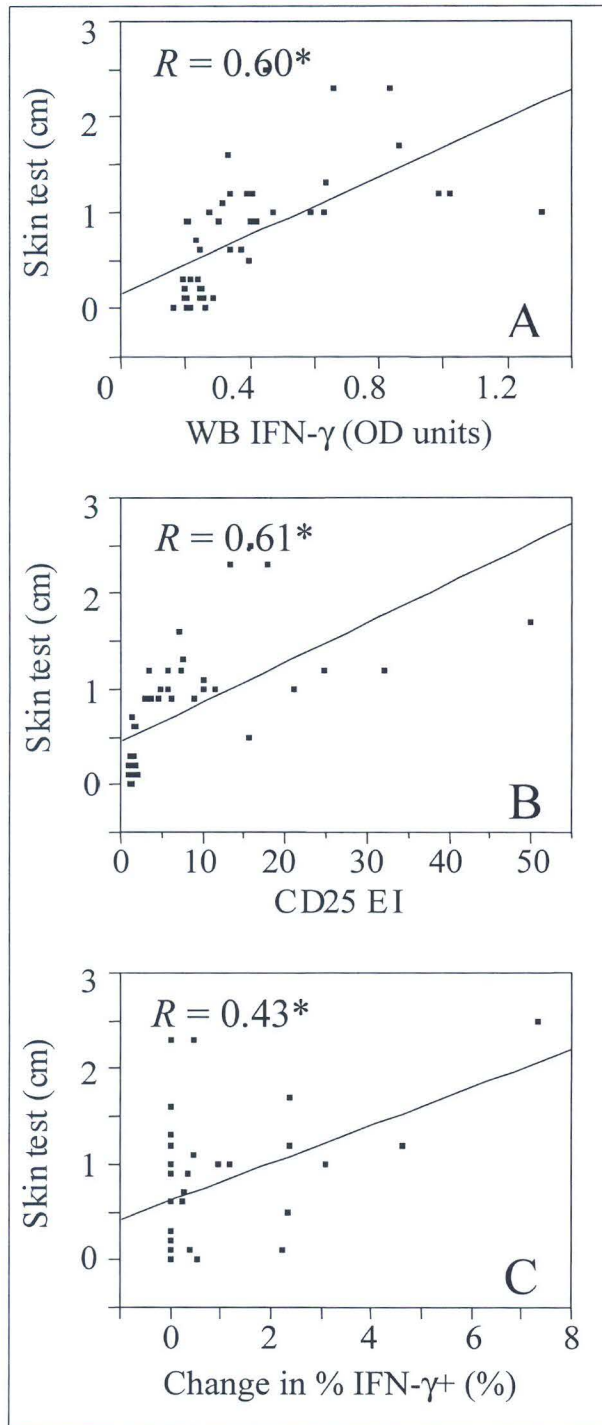


Figure 5—Correlation between results of a skin test and results of the WB IFN- γ assay (A), CD25 EI (B), and change in percentage of IFN- γ^+ (C) for evaluation of T-cell responses to incubation with MAP-PPD. *Tests are significantly ($P < 0.05$) correlated.

to each well. Plates were again incubated at 25°C for 15 minutes, which was followed by 3 wash cycles with wash solution. Streptavidin-conjugate^o (50 μ L) was then added to each well, and plates were incubated at 25°C for 15 minutes. Three more wash cycles were performed with wash solution, and 150 μ L of 1% ultrapure formaldehyde^a in PBS solution was added to each well. The stained PBMCs were transferred to flow tubes

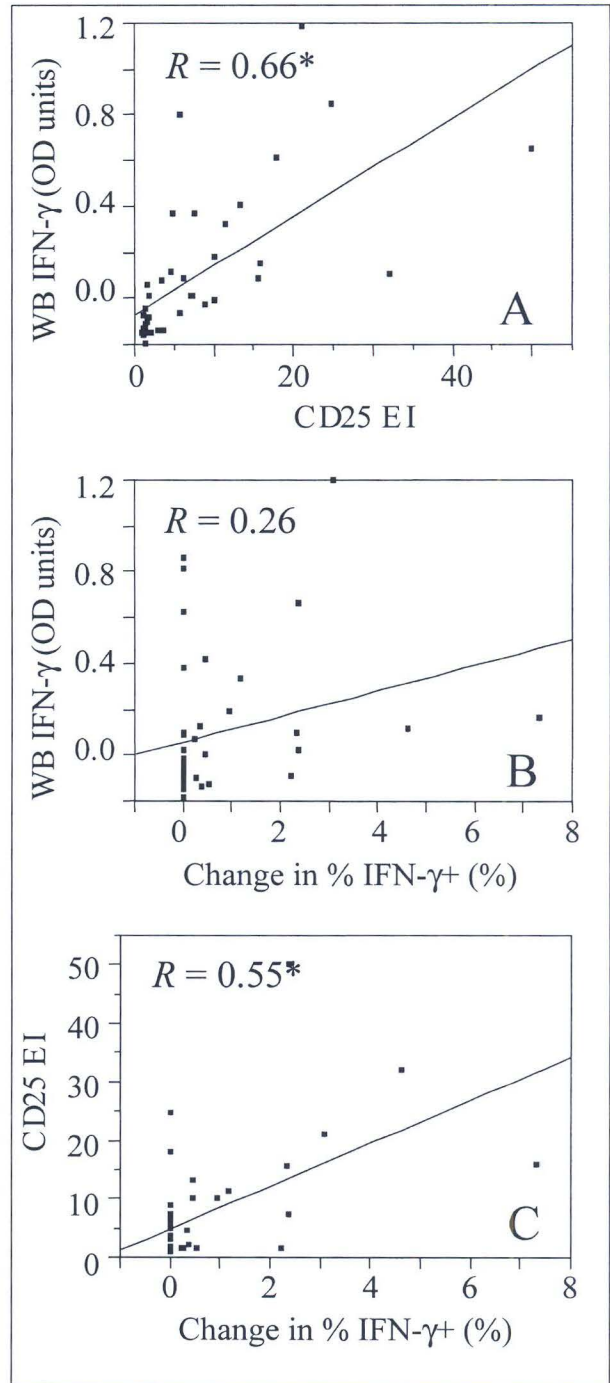


Figure 6—Correlation between results of the WB IFN- γ assay with the CD25 EI (A) and change in percentage of IFN- γ^+ (B) and between the CD25 EI and change in percentage of IFN- γ^+ (C) for evaluation of T-cell responses to incubation with MAP-PPD. See Figure 5 for remainder of key.

and stored in the dark at 4°C until flow cytometric analysis was performed.

Flow cytometric analysis—Five-color flow cytometric analysis was performed by personnel of a university laboratory facility¹ by use of a cytometer.⁵ Data were collected from at least 10,000 live cells of each sample. Cell analysis computer software¹ was used to analyze flow data. Four major

subsets of bovine T cells were identified. Mean percentage prevalence in all MAP-PPD-stimulated samples after incubation for 6 days was as follows: CD4+ CD8- $\gamma\delta$ TCR- (ie, CD4+), 21.2%; CD4- CD8+ $\gamma\delta$ TCR- (ie, CD8+), 23.5%; CD4- CD8+ $\gamma\delta$ TCR+ (ie, CD8+ $\gamma\delta$ TCR+), 2.4%; and CD4- CD8- $\gamma\delta$ TCR+ (ie, $\gamma\delta$ TCR+), 32.8%. In addition, cells with no T-cell marker (ie, non-T cells, 14.9%) were also identified.

The percentage of each T-cell subset that expressed CD25 and the geometric mean fluorescent intensity were determined. The CD25 EI of each subset was calculated by multiplying (for antigen-stimulated cells) the percentage of each T-cell subset that expressed CD25 by the geometric mean fluorescent intensity and dividing that value by the product obtained by multiplying the percentage of each T-cell subset that expressed CD25 by the geometric mean fluorescent intensity for non-antigen-stimulated cells of the same subset from the same cattle.

The change in percentage in IFN- γ + was calculated by subtracting the percentage of IFN- γ + for non-antigen-stimulated cells from the percentage of IFN- γ + for antigen-stimulated cells of the same subset from the same animal. When the value for the change in percentage in IFN- γ + was negative, it was adjusted to 0 before being used in additional analyses. The T-cell subset CD4- CD8+ $\gamma\delta$ TCR+ had extremely low numbers of CD25+ and IFN- γ + cells, and we did not analyze that subset to determine differences among groups.

Statistical analysis—Seven nonvaccinated cattle (3 cattle in group 2 and 4 cattle in group 4) had positive results on the skin test conducted 11 months after the start of the study. These cattle were considered to have been naturally exposed to MAP during the study period. Thus, data from these 7 cattle were analyzed as a separate group from the other nonvaccinated cattle.

Statistical analysis was performed by use of computer software.¹⁴ Data were tested for normality by use of the Shapiro-Wilk W test for goodness of fit, and the equality of variances was tested by use of the Levene test. When the variances were equal, mean differences among groups were tested by use of a 1-way ANOVA. When the variances were not equal, the nonparametric Wilcoxon-Kruskal-Wallis test was used. Significant differences for all group comparisons were determined by use of the Tukey honestly significant different test. To analyze the correlation between any 2 tests, the Pearson product-moment correlation coefficient and significance were obtained by use of multivariate analysis. Values of $P < 0.05$ were considered significant for differences among groups for all tests.

Results

Skin test—Skin thickness after ID injection of MAP-PPD in vaccinated cat-

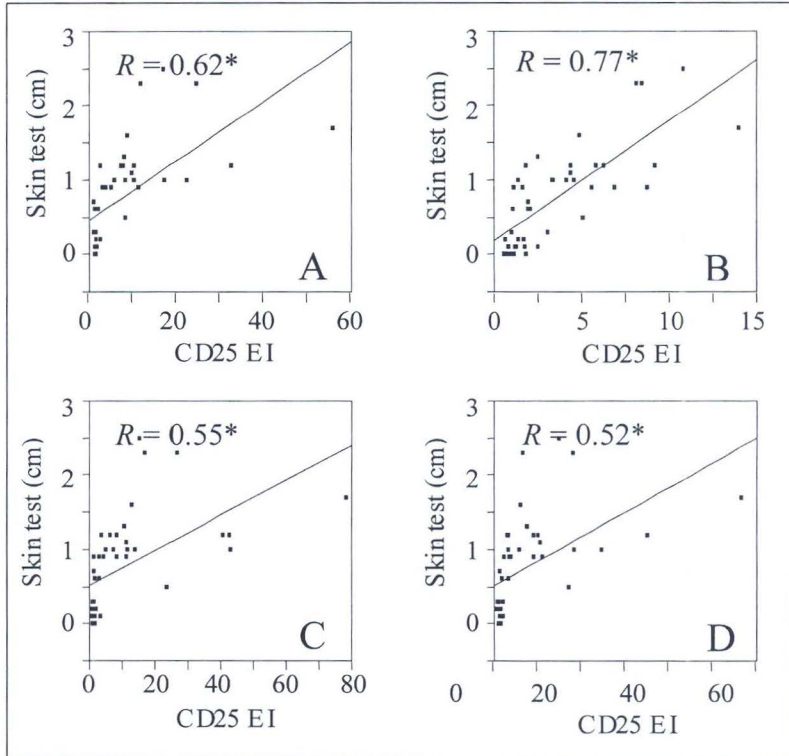


Figure 7—Correlation between results of a skin test and results for CD25 EI for responses of CD4+, (A), CD8+ (B), $\gamma\delta$ TCR+ (C), and non-T cells (D) to incubation with MAP-PPD. *Tests are significantly ($P < 0.05$) correlated.

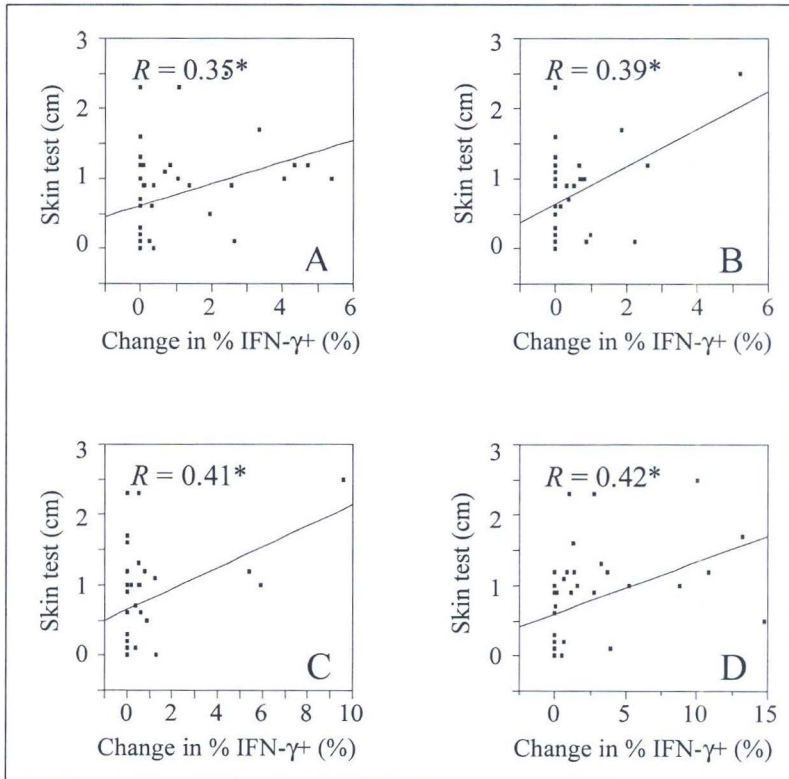


Figure 8—Correlation between results of a skin test and results of the change in percentage of IFN- γ + for responses of various subsets of PBMCs to incubation with MAP-PPD. See Figure 7 for key.

tle of each age group was significantly greater than skin thickness of nonvaccinated cattle that were not natu-

rally exposed to MAP during the study, except for the nonvaccinated, naturally exposed cattle of group 2 (Figure 1). There was no significant difference in skin thickness between vaccinated cattle from the 2 age groups. All 3 nonvaccinated, naturally exposed cattle of group 2 had high values for skin thickness (0.6 to 0.7 cm) that were not significantly different from skin thickness of vaccinated cattle of group 1 or nonvaccinated cattle of group 2 that were not naturally exposed to MAP. The 4 nonvaccinated, naturally exposed cattle of group 4 that had positive results on the second skin test had moderate to high values (3 with a skin thickness of 0.3 cm and 1 with a skin thickness of 0.6 cm) that were not significantly higher than the values for the nonvaccinated cattle of group 4 that were not naturally exposed to MAP, but which were significantly lower than values for the vaccinated cattle of group 3.

WB IFN- γ assay—Mean net ODs of the ELISA for the vaccinated cattle of both age groups were significantly greater than the values for the corresponding nonvaccinated cattle in those age groups that were not naturally exposed to MAP (Figure 2). There was no significant difference between mean net OD of vaccinated cattle from the 2 age groups. The mean net ODs from the nonvaccinated cattle of each age group which were naturally exposed to MAP were not significantly different from values for the corresponding vaccinated cattle or nonvaccinated cattle that were not naturally exposed to MAP.

Five-color flow cytometry—Vaccinated cattle expressed significantly higher CD25 EI from all PBMCs than the nonvaccinated cattle of the same age that were not naturally exposed to MAP (Figure 3). Cattle that were 0 to 3 months old at the time of vaccination had significantly higher responses for CD25 EI than cattle vaccinated when they were 9 to 12 months of age. The CD25 EI responses of nonvaccinated, naturally exposed cattle of group 2 did not differ significantly from responses for vaccinated cattle of group 1 or nonvaccinated cattle of group 2 that were not naturally exposed to MAP, whereas responses for nonvaccinated, naturally exposed cattle of group 4 were significantly lower than the responses for age-matched vaccinated cattle of group 3. When analyzed on the basis of specific T-cell subsets, the CD25 EI of vaccinated cattle was significantly higher than the value for nonvaccinated cattle that were not naturally exposed to MAP in all T-cell

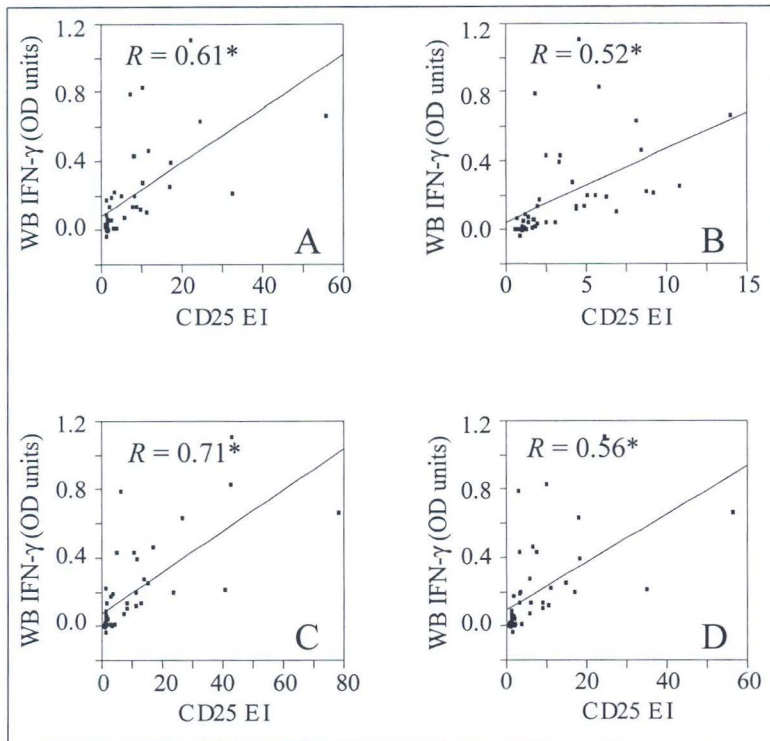


Figure 9—Correlation between results of a WB IFN- γ assay and results for CD25 EI for responses of various subsets of PBMCs to incubation with MAP-PPD. See Figure 7 for key.

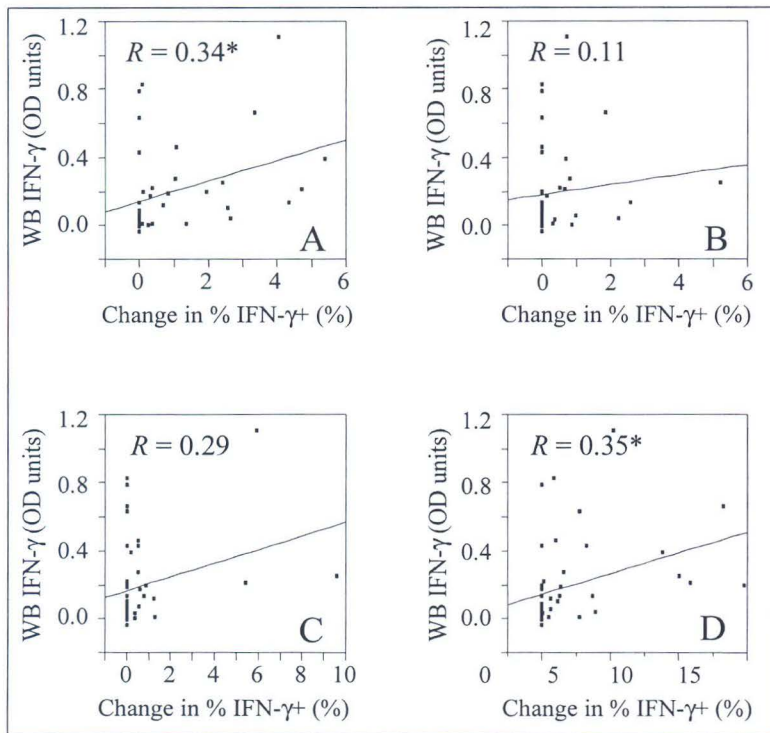


Figure 10—Correlation between results of a WB IFN- γ assay and results of the change in percentage of IFN- γ + for responses of various subsets of PBMCs to incubation with MAP-PPD. See Figure 7 for key.

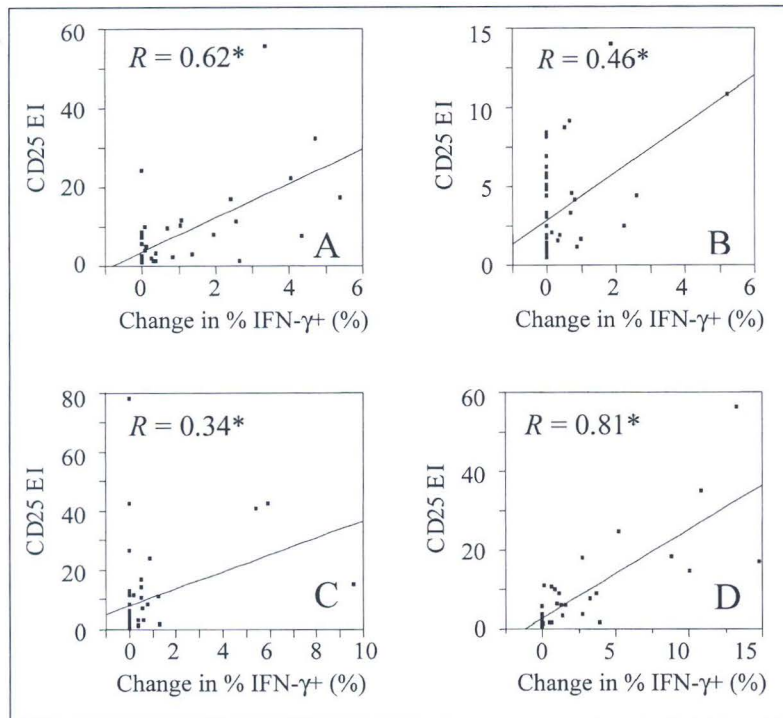


Figure 11—Correlation between results for CD25 EI and results of the change in percentage of IFN- γ + for responses of various subsets of PBMCs to incubation with MAP-PPD. See Figure 7 for key.

subsets of both age groups. However, CD25 EI values for only the CD8 subset of the younger age group and the CD4 subset of the older age group differed significantly between nonvaccinated cattle that were and were not exposed naturally to MAP.

Results for the change in percentage of IFN- γ + as a proportion of all PBMCs did not differ significantly among any of the groups (Figure 4). There was no significant difference in the change in percentage of IFN- γ + for specific T-cell subsets between nonvaccinated cattle that were or were not naturally exposed to MAP. The only significant differences were between vaccinated cattle of group 1 and the nonvaccinated, naturally exposed cattle of group 2 for the CD4 and non-T-cell subsets.

Assay correlation—Correlation analysis was performed to compare results among tests (skin thickness, ELISA net ODs from WB IFN- γ assay, and CD25 EI and change in the percentage of IFN- γ + of all PBMCs from flow cytometry), regardless of cattle group. Skin thickness was significantly correlated with results for each of the other tests (Figure 5). The CD25 EI and the WB IFN- γ assay had the strongest correlation ($R = 0.61$ and $R = 0.60$, respectively) with results for the skin test, whereas results for the change in the percentage of IFN- γ + had the weakest correlation ($R = 0.43$) with results for the skin test.

Results for the WB IFN- γ assay for all PBMCs were significantly correlated with results for CD25 EI but not with the change in the percentage of IFN- γ + (Figure 6). Results were significantly correlated between CD25 EI and the change in the percentage of IFN- γ + for all PBMCs.

Correlation analysis of the various tests was performed on the basis of T-cell subsets determined by use of flow cytometry. There was a significant correlation ($R = 0.52$ to $R = 0.77$) of skin test results with results of CD25 EI for all T-cell subsets (Figure 7). Similarly, skin test results were significantly correlated ($R = 0.35$ to $R = 0.42$) with results for the change in percentage of IFN- γ + for all T-cell subsets (Figure 8). Significant correlations were detected between results for the WB IFN- γ assay and CD25 EI for all T-cell subsets ($R = 0.52$ and $R = 0.71$, respectively; Figure 9). However, results for the WB IFN- γ assay were significantly correlated with results for the change in percentage of IFN- γ + only in the CD4 and non-T-cell subsets (Figure 10). Results for CD25 EI and the change in percentage of IFN- γ + were significantly correlated ($R = 0.34$ to $R = 0.81$) for all T-cell subsets (Figure 11).

Discussion

In the study reported here, 3 CMI tests were compared for their use in monitoring the CMI responses to injection of MAP-PPD in naïve cattle that had been vaccinated at 0 to 3 months of age or 9 to 12 months of age. We determined that 5-color flow cytometry was useful for detecting memory responses of specific T-cell subsets to MAP-PPD in vaccinated cattle. The MAP vaccine induced in vitro responses to MAP-PPD in all 3 T-cell subsets (CD4+, CD8+, and $\gamma\delta$ TCR+) by upregulating surface expression of CD25 in vaccinated cattle of both age groups. This is an indication that CD4+, CD8+, and $\gamma\delta$ TCR+ memory T cells were induced by administration of the vaccine and were still detectable 11 months after inoculation. It also indicates that older cattle can respond to the MAP vaccine. Non-T cells were responsive to MAP-PPD in vitro as determined by upregulation of CD25 and intracellular expression of IFN- γ . Non-T cells are probably a mixture of B cells and natural killer cells. It would be expected that CD25 expression would be upregulated in MAP-PPD-specific memory B cells in response to MAP-PPD. Natural killer cells are likely to increase surface expression of CD25 and intracellular IFN- γ content in response to interleukin-2 in supernatant obtained from antigen-specific T cells.^{26,27}

To compare results for all tests, blood samples were collected for the WB IFN- γ assay and 5-color flow cytometry and processed on the same day that the skin test was performed. Flow cytometry was performed on samples obtained twice within a 3-week interval, and the corresponding mean value was calculated to provide the best estimate of responsiveness for T-cell subsets. The 5-color flow cytometry was able to detect significant differences between MAP-vaccinated cattle and nonvaccinated cattle that were not naturally exposed to MAP (ie, had negative results for the skin test). The

skin test is the conventional *in vivo* test used to detect delayed-type hypersensitivity of cattle exposed to MAP. It is simple to perform and can be conducted in on-farm settings with minimal equipment. However, there can be false-positive reactions as a result of cross-reaction with other environmental *Mycobacterium* spp.⁵ This may also be true for the other assays used in our study. Some nonvaccinated cattle had positive results on the skin test performed 11 months after onset of the study. This was probably attributable to infection after natural exposure because paratuberculosis had historically been diagnosed in this herd. The WB IFN- γ assay has been used as an *in vitro* assay to indicate specific CMI to MAP. It requires a commercial ELISA kit and does not allow the determination of which T-cell subset is producing IFN- γ . Five-color flow cytometry is more labor intensive and expensive than the other tests, but it permits the determination of the specific T-cell subset or subsets that are responding. Five-color flow cytometry has been used successfully by our laboratory group¹⁸⁻²⁴ to measure specific responses of cattle to pathogens. It is a novel technique to test responses of bovine T-cell subsets to MAP-PPD antigen.

The major advantage of 5-color flow cytometry over the other tests is the ability to identify specific T-cell subsets that express the activation marker CD25, intracellular IFN- γ , or both. Flow cytometry requires only a single microtiter well for each antigen to obtain all the information, which reduces the number of PBMCs needed, the time required for the entire process, and the cost of all antibodies. Stained cells are from the same well, which eliminates well-to-well variation, and results for each T-cell subset are all obtained from the same cell population. A potential disadvantage is that the optimum time to detect CD25 EI and the change in percentage of IFN- γ + may differ substantially, such that the sensitivity may be compromised when cells are stained on the same day.

The value for CD25 EI in the study reported here increased substantially in vaccinated cattle and was a more consistent marker of activation than was the change in percentage of IFN- γ +. When comparisons were made regardless of cattle group or natural MAP exposure, results from all tests were significantly correlated with each other, except for the WB IFN- γ assay and the change in percentage of IFN- γ +. The intracellular IFN- γ assay had the lowest correlation with the other assays. For the WB IFN- γ assay, blood samples were incubated with MAP-PPD for 24 hours, and the total amount of IFN- γ released into the supernatant was determined. The IFN- γ content obtained by use of the WB IFN- γ assay can best be detected at day 3 of incubation.¹⁶ In the intracellular IFN- γ assay, the amount of intracellular IFN- γ in the cytoplasm at day 6 of incubation of PBMCs with MAP-PPD was determined. The intracellular IFN- γ is trapped in the cell for the last 4 hours of incubation *in vitro* by inhibiting protein transport; this enables researchers to determine the cells that are synthesizing IFN- γ . It is possible that analyzing the change in percentage of IFN- γ after a shorter *in vitro* incubation would have improved the correlation of this assay with the other assays.

The CD25 EI was the only result that had significantly higher responses for the group of cattle that was vaccinated at 0 to 3 months of age, compared with responses for the group of cattle vaccinated at 9 to 12 months of age. This suggests that the CD25 EI had higher sensitivity for measuring subtle differences in the magnitude of T-cell reactivity among groups. The higher responses in the group vaccinated at 0 to 3 months of age may imply a better response to vaccine administration for calves vaccinated at a younger age. Alternatively, the T-cell responses of the group vaccinated at 9 to 12 months of age may have been compromised by physiologic changes attributable to pregnancy when samples for testing were obtained from the cattle at 20 to 23 months of age.²⁸

The CD25 EI for the CD8+ T cells had the strongest correlation ($R = 0.77$) with the skin test results, compared with correlations among tests for the other T-cell subsets or the combined PBMCs. This suggests that antigen-specific upregulation of CD25 expression by the CD8+ subset may be a more sensitive predictor of a memory CMI response and should be investigated further.

The pathogenesis of MAP infection and the host responses at various stages and for various types of infection are complex. There is no single test suitable for use as a criterion-referenced standard. The results reported here reveal that 5-color flow cytometry is a research tool useful in characterizing the responses of specific T-cell subsets in cattle administered MAP vaccine. This assay should be evaluated to determine sensitivity and specificity for use in detecting cattle naturally infected with MAP. Future investigations will need to determine the optimal *in vitro* antigen concentration and incubation time in cattle naturally exposed to MAP and other mycobacterial species.

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- a. Mycopar, Fort Dodge Animal Health, Fort Dodge, Iowa.
 - b. BD vacutainer, Becton-Dickinson & Co, Franklin Lakes, NJ.
 - c. *Staphylococcus aureus* enterotoxin A, Toxin Technology, Sarasota, Fla.
 - d. Bovigam, CSL Animal Health, Victoria, Australia.
 - e. GolgiPlug (Brefeldin A), BD Biosciences Pharmingen, San Diego, Calif.
 - f. CACT138A, VMRD, Pullman, Wash.
 - g. M 32017, Caltag Laboratories, Burlingame, Calif.
 - h. BAQ111A, VMRD, Pullman, Wash.
 - i. A-21238, Molecular Probes, Eugene, Ore.
 - j. GB21A, VMRD, Pullman, Wash.
 - k. A-21141, Molecular Probes, Eugene, Ore.
 - l. CACT108A, VMRD, Pullman, Wash.
 - m. 1150-09, Southern Biotech, Birmingham, Ala.
 - n. MCA1783B, Serotec, Raleigh, NC.
 - o. S-12383, Molecular Probes, Eugene, Ore.
 - p. Perm and wash solution, BD Biosciences Pharmingen, San Diego, Calif.
 - q. Ultrapure formaldehyde (16%), Polyscience, Warrington, Pa.
 - r. Flow Cytometry Facility, The Office of Biotechnology, Iowa State University, Ames, Iowa.
 - s. Canto cytometer, Beckman Coulter Inc, Fullerton, Calif.
 - t. FlowJo, Tree Star Inc, San Carlos, Calif.
 - u. JMP, version 5.1, SAS Institute Inc, Cary, NC.
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Appendix

Antibodies and reagents used in 5-color flow cytometry of bovine T-cell subsets and IFN- γ assay.

| Primary monoclonal antibodies | Isotype or label | Secondary antibody or reagent conjugates |
|---|------------------|--|
| Mouse anti-bovine CD4 ^f | IgG1 | Goat anti-mouse IgG1, Phycoerythrin-Texas red ^g |
| Mouse anti-bovine CD8 ^h | IgM | Goat anti-mouse IgM, Alexa Fluor 647 ⁱ |
| Mouse anti-bovine $\gamma\delta$ TCR ^f | IgG2b | Goat anti-mouse IgG2b, Alexa Fluor 488 ^h |
| Mouse anti-bovine CD25 ^f | IgG2a | Rat anti-mouse IgG2a, Phycoerythrin ^m |
| Mouse anti-bovine IFN- γ ^f | Biotin | Streptavidin, Alexa Fluor 700 ^o |