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Study of T-Lymphocyte Subsets of Healthy and *Mycobacterium avium* subsp. *paratuberculosis*-Infected Cattle

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The relative contributions of T-lymphocyte subsets to host defense in cattle infected with *Mycobacterium avium* subsp. *paratuberculosis* is reported. The subsets were purified with appropriate monoclonal antibodies and a magnetic bead column separation system, and their purity was verified by flow cytometry. Biological activity of each subset, expressed as lymphoproliferation and gamma interferon (IFN-γ) production, was measured in response to phytohemagglutinin (PHA) and an *M. avium* antigen preparation (A-PPD). IFN-γ was measured by antibody capture enzyme-linked immunosorbent assay. The results showed a correlation between proliferation and IFN-γ production in response to A-PPD but not to PHA. In response to PHA, $CD4^+$ lymphocytes were the most prolific producers of IFN-γ. $CD8^+$ lymphocytes produced IFN-γ to a lesser extent, whereas $\gamma\delta^+$ T lymphocytes produced little or no IFN-γ. Differences observed between the amount of IFN-γ produced by $CD4^+$ versus $CD8^+$ cells and $CD4^+$ versus $\gamma\delta^+$ cells were significant ($P < 0.01$), but those between peripheral blood mononuclear cells (PBMC) and $CD4^+$ T cells were not. Similar responses to A-PPD were observed except that PBMC produced higher levels of IFN-γ than did $CD4^+$ T cells. These data for cattle are similar to observations made for other animal species, where $CD4^+$ cells are the major type of T lymphocytes producing IFN-γ. They further suggest that whatever the role $\gamma\delta^+$ T cells may play in paratuberculosis, it is not likely to be mediated by IFN-γ production.

Mycobacterium avium subsp. *paratuberculosis* is the causative agent of the chronic enteritis of ruminants called paratuberculosis (Johne's disease), which is characterized by untreatable chronic diarrhea, emaciation, and the eventual death of the animal (5). Cattle are most susceptible to infection within the first 6 months of life, but disease manifestation is typically evident at 3 to 5 years of age (16). Infection occurs by ingestion of contaminated manure, colostrum, or milk from infected cows. Fetal infections also occur, particularly in pregnant cows with advanced disease (7). As in other mycobacterial infections, cell-mediated immunity is critical to host defense, but the specific roles of T-lymphocyte subsets are not well understood. Besides $CD4^+$ and $CD8^+$ cells, T cells that are $CD4^-$ $CD8^-$ but that express the γδ T-cell receptor (TCR) form a major subpopulation of circulating lymphocytes in bovines—particularly in calves, where they constitute more than 25% of peripheral blood mononuclear cells (PBMC) and more than 40% of all T lymphocytes (13). The number of TCR $\gamma\delta^+$ cells steadily declines to only about 5% of adult peripheral blood lymphocytes (6). A minor subpopulation of $CD8^+$ cells also expresses the γδ TCR (14). $CD4^+$ and $CD8^+$ T lymphocytes represent 25 to 35 and 15 to 25%, respectively, of PBMC of adult cattle (10, 19).

The pivotal role of gamma interferon (IFN-γ) in cell-mediated immune response in mycobacterial infection has been demonstrated (1). In mice and humans, much of the IFN-γ is produced by the Th1 subset of $CD4^+$ T lymphocytes following antigen challenge in vitro (18). $CD8^+$ T cells (9) and other cell types, such as NK cells and TCR $\gamma\delta^+$ cells, also produce IFN-γ (12). We report here the results of a study on the contributions of T-lymphocyte subsets to the production of IFN-γ in both normal and *M. avium* subsp. *paratuberculosis*-infected cows, with particular focus on the contribution of $\gamma\delta^+$ T cells, given their abundance when calves are most susceptible.

Animal infection. Two 1-month-old Holstein calves were fed milk with 10^8 CFU of *M. avium* subsp. *paratuberculosis* on three consecutive days and were monitored monthly for infection. Infection was confirmed 23 months postchallenge by radiometric culture (BACTEC system [8]) of feces and tissue (ileum and lymph node biopsy specimens) and by detection of serum antibodies and plasma IFN-γ with commercially available kits (IDEXX Laboratories, Inc., Westbrook, Maine). Two age-matched uninfected calves served as controls. Each cow was sampled two times at intervals of at least 2 weeks. The results from both samplings were sufficiently similar and were therefore combined in this presentation. For both proliferative responses and IFN-γ production, experiments were carried out in triplicate and the means of the three wells were used for comparisons.

Mononuclear cells were isolated (13) from heparinized blood collected from all the cows by way of the jugular vein. T-lymphocyte subsets were separated by using a magnetic bead column separation system (MiniMACS; Miltenyi Biotec Inc., Sunnyvale, Calif.). Anti-CD4 and anti-CD8 antibodies were prepared from supernatants of cultured IL-A11 and IL-A51 cell lines (American Type Culture Collection, Rockville, Md.). Anti-γδ monoclonal antibody (MAb) (TcR1-N24) was purchased from VMRD (Pullman, Wash.) and used at a final concentration of 5 μg/ml. The secondary antibody was goat anti-mouse immunoglobulin G (IgG) conjugated to magnetic beads. The cells were thoroughly washed and loaded on the columns. Labeled cells were retained on the column within a magnetic field. The rest were washed through and collected as negatively selected cells. The column was removed from the magnetic field, and the positively selected cells were eluted.

Flow cytometric analysis. Both the proportion of each T-cell subset in whole PBMC and the effectiveness of subset deple-

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tion were checked by flow cytometry on EPICSC (Coulter Electronics, Hialeah, Fla.) after incubation with appropriate antibodies. Briefly, 10^6 cells in 80 μl of Hanks balanced salt solution were incubated at 4°C for 30 min with anti-CD4 or anti-CD8 MAb (prepared as earlier stated) or anti-CD3 (MMIA) or anti-γδ (TcR1-N24 [GB21A]) MAbs (obtained from VRMD) at the equivalent of 5 μg per ml. The control cells were incubated without primary antibody. The secondary antibody was fluorescein (DTAF)-conjugated [$F(ab')_2$] goat anti-mouse IgG (H+L) at a final concentration of 10 μg/ml (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pa.). The cells were washed, resuspended in Hanks balanced salt solution with 0.02% azide, and fixed with 4% formaldehyde for flow cytometric analysis. The specificity of the antibodies is very well documented (17), and our depletion of the target subset was generally 100%.

Lymphoproliferation assay. Whole-PBMC proliferation assay was done as described elsewhere (2) with slight modifications. Briefly, cells were cultured at 2.5×10^5 per 100 μl of RPMI 1640 (Gibco BRL, Gaithersburg, Md.) with 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and 2 mM glutamine supplement per well in 96-well flat-bottom culture plates (Costar Corp., Cambridge, Mass.). Soluble antigens (100 μl) were added to give a final volume of 200 μl/well and a final antigen concentration of 6.25 μg/ml. Cells were incubated for 3 days at 39°C in a humidified 5% CO_2–air mixture, pulsed for 18 h with 0.5 μCi of [^{3}H]thymidine/well (DuPont NEN Products, Boston, Mass.), harvested on glass fiber filter strips (Cambridge Technology Inc., Watertown, Mass.), and prepared for liquid scintillation counting (TriCARB; Packard Instrument Co., Meriden, Conn.). Results were expressed as the stimulation index (SI), calculated as the mean disintegrations per minute (of triplicate wells) for antigen- or phytohemagglutinin (PHA)-stimulated cells divided by the mean disintegrations per minute for unstimulated cells. An SI of >3 was considered indicative of a specific response.

For T-lymphocyte subset assays, 10^5 antigen-presenting cells (prepared from irradiated [2,000 rads] adherent autologous PBMC) were incubated for 72 h with 2×10^4 cells of each purified subset of T lymphocytes and appropriate antigens in triplicate wells. Negatively selected (column effluent) cells were simply cultured at 2.5×10^5 per well. In preliminary experiments, the optimum concentration of *M. avium* purified protein derivative (A-PPD) (IDEXX) and PHA (Murex Diagnostics, Dartford, United Kingdom) for peak lymphoproliferation and IFN-γ production was determined to be between 0.4 and 0.625 μg/100 μl (4 to 6.25 μg/ml).

IFN-γ assay. Cultures for estimation of IFN-γ production were not pulsed with [^{3}H]thymidine. Instead, culture supernatants were employed in the IFN-γ antibody capture enzyme-linked immunosorbent assay (ELISA) (IDEXX). Briefly, 100 μl of culture supernatant was introduced into triplicate wells of a flat-bottom 96-well microtiter plate precoated with anti-bovine IFN-γ MAb. The plate was incubated at room temperature for 1 h and then washed thoroughly. Anti-bovine IFN-γ conjugated to horseradish peroxidase was added, and the plate was incubated for 30 min at room temperature. The plates were washed, and 100 μl of freshly prepared substrate mix (tetramethylbenzidine with hydrogen peroxide in citrate phosphate buffer) was added to each well. Following a further incubation for 30 min, the reaction was stopped with dilute hydrofluoric acid and the optical density (OD) was read at 650 nm with a Biokinetics ELISA reader (Bio-Tek Instruments Inc., Winooski, Vt.). The IFN-γ concentration was derived from the ELISA value (EV) calculated from OD values as follows: EV = [(sample OD − negative control OD)/(assay positive control OD − negative control OD)] × 100. In preliminary experiments, we determined the duration of antigen and lymphocyte incubation for optimum proliferation and IFN-γ production to be 72 h (results not shown). Other workers have reported the death of irradiated feeder cells when they were incubated beyond 3 days (4).

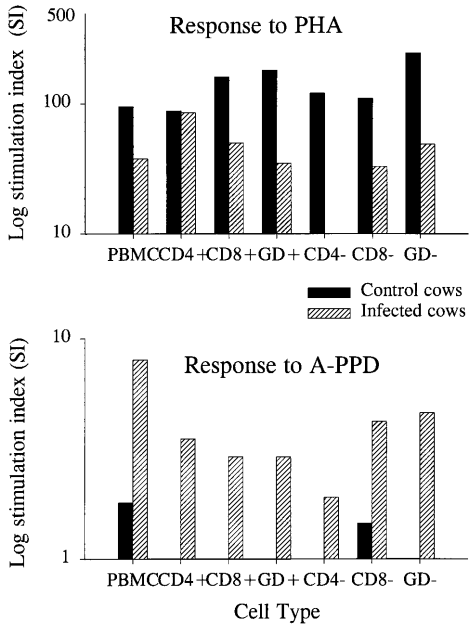

FIG. 1. Mean proliferative response of whole PBMC and T-cell subsets from control and infected cows to PHA (top) and A-PPD (bottom).

IFN-γ standard curve derivation. IFN-γ concentrations in experimental samples were derived from a standard curve prepared with recombinant bovine IFN-γ (Genentech, Inc., San Francisco, Calif.) by using the formula [IFN] (in picograms per milliliter) = 4.838 × EV. Statistical analysis and comparison of proliferative and IFN-γ responses between animal groups and between cell types was performed by unpaired *t* test, one-way analysis of variance, and the Tukey-Kramer multiple comparison test with the InStat (San Diego, Calif.) software package. ([^{3}H]thymidine incorporation was measured and the disintegrations per minute were transformed for statistical analysis rather than using the SIs.) Regression analysis was used to establish the IFN-γ standard curve and to correlate lymphoproliferation with IFN-γ production (Lotus 1-2-3; Lotus Development Corp., Cambridge, Mass.).

Lymphoproliferative responses. As expected, PBMC and T-cell subsets of all cows proliferated in response to PHA. Although there were variations in responses within the groups, the patterns of proliferative responses to PHA were different for control and infected groups, particularly in the population depleted of $CD4^+$ cells (Fig. 1). Except for $CD4^+$ fractions, the mean proliferative responses of PBMC and the other subsets of T lymphocytes from control cows were higher than those of corresponding cell fractions from the infected group ($P < 0.05$). Individual subsets from infected cows showed higher proliferative responses than did combined subsets. This depressed response of subset combinations was most prominent in the cell fractions depleted of $CD4^+$ and $CD8^+$ and less evident in those depleted of $\gamma\delta^+$. In response to A-PPD, PBMC and subsets of T lymphocytes from controls did not proliferate (SI < 3). Cells from the infected group, on the other hand, did proliferate, and fractions depleted of $CD8^+$

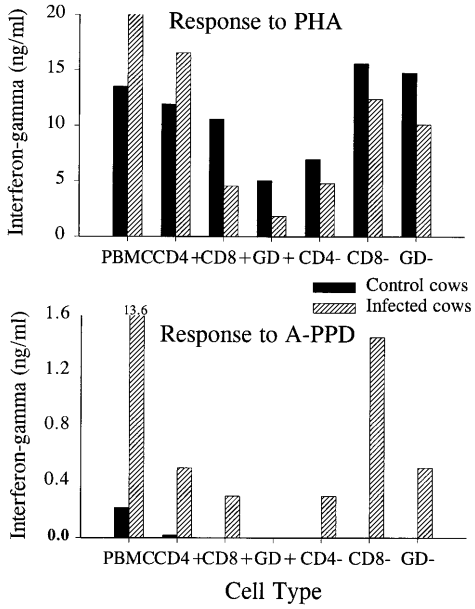

FIG. 2. Results of mean IFN-γ production of whole PBMC and T-cell subsets from control and infected cows in response to PHA (top) and A-PPD (bottom). The complementary profiles of positively and negatively selected subsets serve to further verify the contribution of each subset to IFN-γ production.

and $\gamma\delta^+$ showed higher responses than did the individual subsets. Whether this represents synergism between the cell populations that were left and/or the absence of some suppressive effect that would have been exerted by the subset that was removed is not clear. $CD4^+$-lymphocyte depletion greatly reduced the response to stimulation.

IFN-γ production in response to PHA. PBMC, $CD4^+$, and $CD8^+$ T cells of both control and infected cows produced IFN-γ in response to PHA, albeit to different levels. $\gamma\delta^+$ T cells did not produce significant levels of IFN-γ. There was no significant difference in response to PHA ($P > 0.05$) between control and infected subjects when their corresponding PBMC and subsets were matched. However, a pattern was obvious: $CD4^+$ T cells, for instance, produced very high levels of IFN-γ, while $CD4^-$ cells produced very low levels. In a reverse order, $\gamma\delta^+$ cells produced little or no IFN-γ while $\gamma\delta^-$ fractions produced very high levels of IFN-γ—a further confirmation of the contribution of each cell type (Fig. 2). Tests for significance of IFN-γ production between subsets showed that while there was no significant difference in the levels of IFN-γ produced by whole PBMC and $CD4^+$ cells in response to PHA ($P > 0.05$), differences in the levels produced by $CD4^+$ versus $CD8^+$, $CD4^+$ versus $\gamma\delta^+$, and PBMC versus $\gamma\delta^+$ cells were significant at P values of <0.05, <0.001, and <0.001, respectively.

IFN-γ production in response to A-PPD. Generally, IFN-γ production in response to A-PPD was lower than that in response to PHA. Control cows produced virtually no IFN-γ in response to A-PPD. The mean concentrations of IFN-γ produced by PBMC of infected and control cows were 13,366 pg/ml and 304 pg/ml of sample, respectively. Within the infected group, the levels of IFN-γ produced by PBMC were very much higher than those produced by all T-cell subsets ($P < 0.001$), an observation we have no real explanation for. Levels of IFN-γ produced by $CD4^+$ T cells were higher than those for $CD8^+$ and $\gamma\delta^+$ T cells ($P < 0.05$).

Contributions of the subsets to IFN-γ production. The results suggest that $CD4^+$ T lymphocytes are the predominant lymphocyte producers of IFN-γ, producing up to 101 and 87% of that produced by whole PBMC from control and infected cows, respectively. Lymphocytes that were $CD8^+$ produced less IFN-γ, releasing 71 and 21% of that produced by PBMC and 80 and 27% of $CD4^+$ T cell levels in control and infected cows, respectively. The $\gamma\delta^+$ T cells did not contribute to IFN-γ production to any significant degree. Generally, high levels of IFN-γ production by fractions depleted of $CD8^+$ and $\gamma\delta^+$ lymphocytes were observed.

Correlation between lymphoproliferation and IFN-γ production. Regression analysis showed a strong correlation between lymphoproliferation and IFN-γ production for PBMC ($r^2 = 0.96$) and $CD4^+$-T-lymphocyte ($r^2 = 0.93$) responses to A-PPD. Conversely, there was a low correlation in PBMC response ($r^2 = 0.058$) or $CD4^+$ response ($r^2 = 0.72$) to PHA. This observation is important because it shows the limitations of the use of nonspecific mitogens for defining biological activity of T lymphocytes (11).

Equal numbers of T-lymphocyte subsets were employed in these experiments. However, the subsets are not equally distributed in the peripheral blood and tissues, which adds significance to the results expressed here. Correcting for actual subset proportions in the peripheral blood can only serve to highlight the critical role of $CD4^+$ lymphocytes in IFN-γ production. Changes in the ratio of $CD4^+$:$CD8^+$ over the duration of Johne's disease has been attributed only to variations in the $CD4^+$-cell concentration (reference 4 and unpublished results). Depletion of $CD4^+$ cells or prevention of their proper activation could therefore conceivably shut down the production of IFN-γ, with an attendant failure of host defenses. Such a shutdown may well occur in late-stage Johne's disease, when the IFN-γ levels drop drastically (data not shown) immediately preceding further weight loss, diarrhea, recumbency, and death. Further studies contrasting the responses of the peripheral blood cells and those in the intestine and lymphoid tissues, where the subsets are distributed differently, will help our overall understanding of their role.

As stated earlier, $\gamma\delta^+$ T cells did not contribute to any significant degree to IFN-γ production. What low amounts were detected may be attributable to the minor subset of $CD8^+$ cells known to express the γδ TCR (14). A sequential subset subtraction of all $CD8^+$ and then all $\gamma\delta^+$ cells would be one way of verifying this hypothesis. The abundance of $\gamma\delta^+$ T lymphocytes in cattle when they are most susceptible to *M. avium* subsp. *paratuberculosis* and their peculiar distribution to mucosal tissues, which are the preferred point of entry for mycobacterial pathogens, are intriguing. In humans, a role as a first line of defense and in genesis of a primary immune response has been suggested for $\gamma\delta^+$ T cells, based on their relatively faster kinetics of mobilization compared with the other subsets (3). Reports that they are not restricted by classical (class I or II) or nonclassical major histocompatibility complex molecules and that they undergo apoptosis following an initial encounter with antigen (15) would suggest that they are not only unregulated in their responses, at least not in ways properly understood, but also lack memory components thought to be very important for sustained immunity. In bovine studies, $\gamma\delta^+$ T cells have actually been reported to prevent [^{3}H]thymidine incorporation by antigen-primed $CD4^+$ T cells (4), suggesting an ominous immunoregulatory function in paratuberculosis, particularly in the newborn calf.

Much of what is known about bovine T-lymphocyte response to infection with mycobacteria has been, to a large extent, extrapolated from human and mouse studies. This work specifically addresses the bovine response to infection with mycobacteria. Defining the contribution of each T-lymphocyte sub-

set is the first step toward manipulating the immune response in Johne's disease in favor of the host.

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