Antigen-Specific B-Cell Unresponsiveness Induced by Chronic
*Mycobacterium avium* subsp. *paratuberculosis* Infection of Cattle

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*Mycobacterium avium* subsp. *paratuberculosis* infection of cattle results in a chronic granulomatous enteritis. Clinical disease (i.e., cachexia, diarrhea, and high fecal bacterial counts) is preceded by a lengthy subclinical stage of disease. The immunologic mechanisms associated with the progression of infected cattle from subclinical to clinical disease are unclear. In this study, a cell proliferation assay was used in combination with flow cytometry to compare peripheral blood lymphocyte responses of cattle with subclinical paratuberculosis to responses of cattle with clinical paratuberculosis. B cells from cattle with subclinical disease proliferated vigorously upon stimulation with *M. avium* subsp. *paratuberculosis* antigen, with up to 12.4% of the total B cells responding. However, B cells from cattle with clinical disease did not proliferate upon antigen stimulation despite good proliferation in response to concanavalin A stimulation. In addition, these animals had high percentages of peripheral blood B cells. B cells from noninfected animals did not proliferate upon *M. avium* subsp. *paratuberculosis* antigen stimulation. Thus, it appears that B-cell proliferation is a sensitive indicator of subclinical Johne's disease. Furthermore, the immunologic mechanisms responsible for the antigen-specific unresponsiveness of peripheral blood B cells may be significant in the eventual progression from subclinical to clinical Johne's disease in cattle.

Paratuberculosis, or Johne's disease, is caused by the intracellular bacterium *Mycobacterium avium* subsp. *paratuberculosis* (6, 21). In ruminants, the bacterium infects macrophages within the intestinal mucosa and mesenteric lymph nodes, inducing a chronic granulomatous enteritis (9, 27). Ruminants are usually infected at an early age through ingestion of *M. avium* subsp. *paratuberculosis*-contaminated milk or feces (6). In field conditions, animals may be infected for ≥3 years without developing clinical signs of disease. During this subclinical stage of disease, the animals generally have undetectable levels of *M. avium* subsp. *paratuberculosis*-specific serum antibody and increasing gamma interferon (IFN-γ) responses to *M. avium* subsp. *paratuberculosis* and shed undetectable to low numbers of bacteria in feces (2, 19). Clinical disease is characterized by chronic diarrhea, cachexia, and eventual death, with abundant specific serum antibody, decreasing IFN-γ responses, and high numbers of bacteria shed in feces (2, 6, 8, 9, 19). Thus, it appears that cell-mediated immune responses keep bacterial shedding under control, and a switch to a humoral immune response is associated with the progression of cattle to clinical disease and increased bacterial shedding. Similar paradigms have been extensively characterized for other mycobacterial infections (15, 23).

T-cell immune responses are essential in limiting the severity of paratuberculosis infection (1, 7, 14, 24). Clearly, antibody production affords little, if any, protection against this intracellular pathogen. However, B cells can provide support for T-cell responses through antigen presentation and costimulation function (10, 24). In the present study, we examined in vitro antigen-specific proliferative responses of peripheral blood lymphocyte subsets isolated from *M. avium* subsp. *paratuberculosis*-infected or noninfected cattle. Surprisingly, proliferative responses of B cells from cattle with subclinical disease were as much as 6.5 times greater than proliferative responses of T cells from the same animals. Furthermore, animals with clinical signs of disease had severely depressed B- and T-cell proliferative responses and abnormally high percentages of peripheral blood B cells. These findings suggest that the progression of paratuberculosis in cattle from subclinical to clinical disease is associated with peripheral blood lymphocyte unresponsiveness, which is most remarkable within the B-cell subset.

**MATERIALS AND METHODS**

**Animals, bacterial culture, and antigen.** The animal groups used consisted of three noninfected and six *M. avium* subsp. *paratuberculosis*-infected Holstein cows. Infection was determined by a standard fecal culture method and following a previously described procedure (20). All animals were housed in American Association for Accreditation of Laboratory Animal Care-accredited facilities (National Animal Disease Center, Ames, Iowa) in temperature- and humidity-controlled rooms.

**Lymphocyte blastogenesis.** Peripheral blood mononuclear cells (PBMC) were isolated from buffy coat fractions of peripheral blood collected in 2× acid citrate dextrose by standard procedures (5). Wells of 96-well round-bottom microtiter plates (Falcon, Becton Dickinson, Lincoln Park, N.J.) were seeded with 2 × 10⁵ PBMC in a total volume of 200 μl/well. The medium was RPMI 1640 (Fisher Scientific, Pittsburgh, Pa.) supplemented with 100 U of penicillin/ml, 0.1 mg of streptomycin/ml, 5 × 10⁻³ M 2-mercaptoethanol (Sigma, St. Louis, Mo.), and 10% fetal bovine serum (Atlanta Biologics, Atlanta, Ga.). The cells contained either concanavalin A (Con-A) (5 μg/ml; Sigma), *M. avium* subsp. *paratuberculosis* antigen (10 μg/ml; whole-cell sonicate), or medium alone (no stimulation). The plates were then incubated at 37°C in a 5% CO₂ humidified atmosphere for 4 days. After 4 days, 0.5 μCi of [methyl-³H]thymidine (specific activity, 6.7 Ci mmol⁻¹; Amersham Life Science, Arlington Heights, Ill.) in 10 μl of medium was added to each well, and the plates were incubated for an additional 20 h. The well contents were harvested onto glass fiber filters with a PHD cell harvester (Cambridge Technology, Cambridge, Mass.), and incorporated radioactivity was measured by liquid scintillation counting. Treatments were run in triplicate, and...
Fluctuations in the concentration of M. avium PCU/mL, as measured by the PKH2 assay, were consistent with previous observations that animals with subclinical disease, animals were examined for fecal shedding of M. avium paratuberculosis and clinical signs of disease, including diarrhea and cachexia. At the time of the study, animals 167, 5247, and 323 were shedding at least 50 CFU of M. avium subsp. paratuberculosis organisms per g of feces. Prior to the study, animals 10, 107, and 1072 shed small amounts (<10 CFU/g of feces) of M. avium subsp. paratuberculosis organisms, yet several fecal cultures obtained during the study failed to detect any organisms from these three animals. This was consistent with previous observations that animals with subclinical disease are often negative for M. avium subsp. paratuberculosis growth upon fecal culture. Control animals (420, 423, and 477) were negative upon biannual fecal cultures for M. avium subsp. paratuberculosis taken over a 5-year period prior to and including the time of the study. Animals 5247 and 167 had clinical signs of Johne’s disease (e.g., cachexia and diarrhea). Animal 5247 was subsequently euthanized due to clinical deterioration at the conclusion of this study. No signs of clinical Johne’s disease were noted in the other animals.

Proliferative responses and phenotype. The PKH2 proliferation assay is a new procedure for examining proliferation of PBMC. We compared the results of this assay to results obtained with a standard [3H]thymidine uptake proliferation assay (Fig. 1). With both assays, PBMC from animals 10, 1072, and 323 had strong proliferative responses upon stimulation with M. avium subsp. paratuberculosis antigen. Weak antigen-specific responses were detected for animals 107, 167, and 5247.
TABLE 1. Mean number of cells proliferating in response to stimulation with either Con-A or M. avium subsp. paratuberculosis antigen

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Untreated</th>
<th>CD3&lt;sup&gt;+&lt;/sup&gt;</th>
<th>CD4&lt;sup&gt;+&lt;/sup&gt;</th>
<th>CD8&lt;sup&gt;+&lt;/sup&gt;</th>
<th>B</th>
<th>γδ T</th>
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<tbody>
<tr>
<td>Con-A stimulated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Controls (n = 3)</td>
<td>2,033 ± 417</td>
<td>773 ± 92</td>
<td>186 ± 67</td>
<td>210 ± 46</td>
<td>913 ± 209</td>
<td>423 ± 40</td>
</tr>
<tr>
<td>Infected (n = 6)</td>
<td>2,417 ± 308</td>
<td>947 ± 245</td>
<td>182 ± 52</td>
<td>202 ± 52</td>
<td>916 ± 50</td>
<td>714 ± 231</td>
</tr>
<tr>
<td>Antigen stimulated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls (n = 3)</td>
<td>0 ± 0</td>
<td>6.4 ± 5.8</td>
<td>5.4 ± 5.4</td>
<td>4.2 ± 4.2</td>
<td>6.1 ± 6.1</td>
<td>14.4 ± 9.8</td>
</tr>
<tr>
<td>Infected (n = 6)</td>
<td>*233 ± 88</td>
<td>19.1 ± 7.9</td>
<td>13.5 ± 8.2</td>
<td>11.5 ± 4.3</td>
<td>62.2 ± 34.5</td>
<td>20.9 ± 6.6</td>
</tr>
</tbody>
</table>

* PBMC were labeled with PKH2 and incubated in round-bottom 96-well tissue culture plates for 5 days at 37°C and 5% CO<sub>2</sub>, with or without Con-A (5 µg/mL) or antigen (10 µg/mL) stimulation. The cells were then harvested and analyzed by flow cytometry for expression of cell surface molecules and PKH2 staining. The data represent the mean number of cells proliferating ± SEM in response to either Con-A or antigen stimulation per 5,000 PBMC for ungated or gated (i.e., CD3<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup>, B, and γδ-T-cell) samples as analyzed by ModFit Proliferation Wizard software. The mean number of cells proliferating was calculated by subtracting the number of cells proliferating in nonstimulated wells from the number of cells proliferating in either Con-A- or antigen-stimulated wells. *, significantly greater (P < 0.05) than controls.
erated upon antigen stimulation had a concurrent γδ-T-cell proliferative response, suggesting nonspecific proliferation of γδ T cells in response to antigen stimulation. Infected animals had detectable levels of CD4⁺⁻, CD8⁺⁻, and γδ-T-cell proliferation in response to antigen stimulation (Table 1). Animals 323, 167, and 5247 had the lowest percentages of CD4⁺ cells (data not shown); interestingly, these three animals had similar PKH2 staining patterns in nonstimulated and antigen-stimulated cultures. The decreased intensity of PKH2 staining (as detected in antigen-stimulated cultures from subclinical animals) indicates cell division, since daughter cells have reduced PKH2 staining within their membranes compared to that of their parent generation. Animals with clinical disease (E and F) had higher percentages of B cells compared to noninfected animals (A and B) and animals with subclinical disease (C and D). PE, phycoerythrin.

**DISCUSSION**

The immunologic mechanisms associated with the progression of paratuberculosis from subclinical to clinical disease have not been determined. Animals with clinical signs of disease generally have high levels of antigen-specific serum antibody in conjunction with increased bacterial replication and fecal shedding of *M. avium* subsp. *paratuberculosis* (8, 17). Increased serum antibody associated with clinical deterioration and increased bacterial replication suggest that a switch to a
humoral immune response signals progression towards clinical disease. The most intriguing finding in this study was that animals with subclinical Johne’s disease demonstrated antigen-specific B-cell proliferative responses while animals with clinical Johne’s disease had minimal to no antigen-specific B-cell proliferative responses. Interestingly, animals with clinical disease had higher percentages of peripheral blood B cells while animals with subclinical disease had percentages of peripheral blood B cells similar to those of control animals. Thus, it appears that progression from subclinical to clinical Johne’s disease is accompanied by increases in peripheral blood B cells and decreases in peripheral blood antibody-specific B-cell proliferative responses.

It is surprising that antigen-specific B-cell proliferative responses are weak in animals with clinical Johne’s disease, since significant levels of serum M. avium subsp. paratuberculosis-specific antibody are detected in these animals. These results imply that antigen-responsive B cells are no longer present in the peripheral blood of clinically affected animals; yet, elsewhere in the body, terminally-differentiated antigen-specific plasma cells are producing antibody. Thus, it is possible that antigen-specific lymphocytes have trafficked to effector sites, resulting in diminished peripheral blood B-cell proliferative responses through lack of peripheral antigen-specific B cells. It is also possible that T cells necessary for B-cell proliferation have redistributed to sites other than the peripheral blood.

B-cell unresponsiveness in bovine paratuberculosis appears to be a sensitive indicator of M. avium subsp. paratuberculosis infection as well as clinical progression of paratuberculosis. Cattle with subclinical infection have strong B-cell proliferative responses and normal numbers of peripheral blood B cells, whereas animals with clinical disease have weak B-cell proliferative responses and abnormally high percentages of peripheral blood B cells. The mechanisms of B-cell unresponsiveness in animals with clinical disease remain unclear.

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