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Early Diagnosis of Johne's Disease in the American Bison by Monoclonal Antibodies Directed against Antigen 85

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ABSTRACT: **Several monoclonal antibodies derived from hybridomas from mice that had been immunized with recombinant *Mycobacterium bovis* antigen 85 (Ag85) were tested for reactivity against antigen 85 (Ag85) from *M. bovis* and against sera from 100 bison inoculated with *M. paratuberculosis* and from 100 control bison from a disease-free herd. Monoclonal antibodies mAb85.1, mAb85.44.1, mAb85.44.9, and mAb85.96 reacted against three or four 30–33-kDa bands of the Ag85 complex of *M. bovis*. Importantly, these mAbs also reacted with bands of similar molecular weight in the sera of bison inoculated with *M. paratuberculosis*. Additionally, when sera from 198 bison in four herds were reacted against mAb85.1 and mAb85.96, 26 bison reacted positively for the presence of Ag85 by either mAb or by both. These preliminary results indicate that monoclonal antibodies may eventually lead to a reliable diagnostic test for the early detection of *M. paratuberculosis* infections in ruminants as well as to a means for identifying contaminated dairy products.**

KEYWORDS: **Johne's disease; *Mycobacterium paratuberculosis*; *Mycobacterium bovis*; monoclonal antibodies; antigen 85**

INTRODUCTION

Johne's disease, caused by *Mycobacterium paratuberculosis*, affects numerous ruminants, including the American bison (*Bison bison*), in which it is manifested as a chronic wasting disease exhibiting clinical signs only in older animals. In bison, the disease usually appears in animals 6–10 years old.[1–3] Although diagnostic assays are available for *M. paratuberculosis*, they are time-consuming, labor intensive, and usually incapable of detecting infections in young animals.[4] Antibodies against *M. paratuberculosis* are generally not present in young animals, nor do young infected

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animals consistently shed the bacterium in their feces.[5] Antigen 85 (Ag85) is a highly conserved complex of proteins that is secreted by *Mycobacterium*-infected macrophages.[6] The Ag85 protein complex consists of fibronectin-binding proteins that are secreted early during infection by various species of *Mycobacterium*, including *M. tuberculosis*, *M. bovis,* and *M. paratuberculosis.*[7] Additionally, Ag85 has been shown to be important in cell wall biosynthesis in *M. tuberculosis*.[8] Thus, diagnostic assays that are based on the detection of Ag85 might provide a means of early detection of *Mycobacterium* infections.

Recently, antigen 85 has been the focus of much work in a variety of settings. A current study has shown that recombinant bacillus Calmette-Guérin (BCG) vaccines expressing the *Mycobacterium tuberculosis* 30-kDa major secretory protein induce greater protective immunity against tuberculosis than conventional BCG vaccines.[9] Additionally, a DNA vaccine encoding Ag85A from *M. bovis* demonstrated protection against Buruli ulcer.[10] Antigen 85C has also been crystallized, and the data from that study have suggested potential drug and vaccine targets that may aid in the fight against mycobacterial infections.[11]

Diagnostic assays are also now beginning to use antibodies and combinatorial strategies to detect antigen 85 from *M. tuberculosis* in humans and orangutans, and it is likely that this approach will also work well with bison.[12,13] The mAbs that we have produced in this study may be the first step toward such an aim. Additionally, the fact that these mAbs were generated against *M. bovis* Ag85 and reacted with Ag85 in the sera of bison inoculated with *M. paratuberculosis* suggests that global screening of herds may be accomplished to test for mycobacterial infections, a tool that would help guide management decisions.

MATERIALS AND METHODS

Animals

Balb/c mice were housed in the Animal Resource Center at Montana State University. American bison were housed on various private ranches in western Montana.

Immunizations

Female Balb/c mice aged 6–8 weeks were immunized i.p. with purified *M. bovis* antigen 85-MBP fusion protein generously provided by Dr. Gerhardt Schurig at Virginia Tech School of Veterinary Medicine. For the first immunization, 50 µg of the antigen was mixed with TiterMax™ (Sigma Chemical Co., St. Louis, MO) according to the manufacturer's instructions. Additional immunizations of 50 µg in PBS (Sigma) were carried out every three weeks until antibody titers were sufficiently high: greater than 1:1000 dilution by Western blot analysis. A final boost was administered i.p., and the spleen cells harvested four days later for production of monoclonal antibodies.

Generation of Monoclonal Antibodies

A fusion was performed on spleen cells to produce mAbs as described.[14] Briefly, spleen cells were harvested by tissue emaciation in a tissue grinder. The cells were

counted in the presence of Trypan blue (Sigma) at a 1:1 ratio, and viable cells noted. SP2/0 myeloma cells were previously grown to confluence in T-175 flasks (Fisher Scientific Co., Pittsburgh, PA), counted as above, and viable cells noted. Spleen and SP2/0 cells were fused at a ratio of 2:1 in 50% PEG (Sigma) over two minutes, centrifuged, and added to a 1× and ½× flask containing peritoneal feeder cells and HAT. The cells from each flask were plated onto 48-well plates at a final volume of 500 µL and incubated without disturbance for 5–7 days at 37°C and 10% CO_2. The wells were then visually checked for the presence of clones.

Testing of Clones

Supernatant fluid (~100 µL) from wells with clones was aspirated, and antibody reactivity tested by Western blot. Briefly, purified antigen 85, generously provided by Dr. Gerhardt Schurig, was suspended in 1× running buffer, loaded onto a precast 8% polyacrylamide gel (BioRad, Hercules, CA), and electrophoresed. The proteins were transferred to nitrocellulose (Sigma), blocked for 45 min with equine serum (Sigma), and rinsed with TBST. The nitrocellulose was placed in a miniblot apparatus (BioRad), sealed, and 80 µL of the supernatant fluid added to each lane and incubated for 1 hr at room temperature. After incubation, the blot was washed with 200 ml of TBST, and 80 µL of second-stage goat anti-mouse IgG and IgM alkaline phosphatase (1:250) was added to each lane. The blot was then incubated again for 45 min at room temperature. It was washed again with 200 mL of TBST and developed by the addition of 133 µL of Nitroblue (Sigma) and 67 µL of 5-bromo-4-chloro-3-indolyl phosphate (Sigma) in 20 mL of AP buffer. Positive clones were identified by their reactivity with the specific antigen 85 bands seen from ~30–35 kDa.

Subcloning of Positive Clones

Once positive clones were identified, they were transferred to 24- or 6-well plates, depending on their confluency, and immediately subcloned. Viable cells were counted as above, and peritoneal feeder cells collected. Dilutions were made from a 1×10^4 stock solution of hybridoma cells and plated into flat-bottomed 96-well plates as follows: One plate was plated at a density of 50 cells/mL, two plates were plated at 5 cells/mL, and a final two plates were plated at 2.5 cells/mL. The plates were then incubated at 37°C and 10% CO_2 for 3–4 days, at which point the wells were visually screened for single, clonal colonies. Once identified, supernatant fluid from these colonies was collected and tested for reactivity as above. Positive clones were then grown to confluency in 5-liter roller bottles; and mAbs isolated, purified, and characterized again for their ability to recognize specific antigen 85 bands by Western analysis.

Screening of Inoculated American Bison Serum

American bison sera were collected from bison calves inoculated with 1×10^8 *Mycobacterium paratuberculosis* organisms and from bison known to be free of Johne's disease. The sera were electrophoresed as above and probed for the presence of antigen 85 by Western analysis with the mAbs generated as described above. The presence of antigen 85 in the sera was noted by specific reactivity of the antigen 85 protein (~30–35 kDa) with the anti-antigen 85 mAbs.

American Bison Herd Screening

Bison sera samples were obtained from peripheral blood, electrophoresed, transferred to nitrocellulose, and probed for Ag85 reactivity by mAbs Ag85.1 and Ag85.96. The presence of antigen 85 was noted by reactivity with either of these mAbs, and the results reported in table format. A total of 198 samples from four separate herds was tested.

RESULTS AND DISCUSSION

Several mAbs were generated against recombinant *M. bovis* Ag85 that specifically recognize Ag85 (consists of three or four bands at 30–33 kDa) in Western blots of the purified antigen and in the serum of bison experimentally inoculated with *M. paratuberculosis* (FIGS. 1–3). Eighty 2- to 4-month-old bison were inoculated five times with *M. paratuberculosis*: 40 of them received high doses of 10^8 bacteria, and 40 received low doses of 10^6. Additionally, 20 yearling bison were inoculated with the high dose. One hundred bison from a disease-free herd were used as controls. At 180 days after the last inoculation, sera obtained from these bison and purified Ag85 as a control were tested in Western blots with Ag85-specific mAbs, principally mAb85.1, mAb85.44.1, mAb85.44.9, and mAb85.96. All mAbs reacted with recombinant Ag85 (FIGS. 1–3), and mAb85.1 and mAb85.96 (the only ones tested) reacted with Ag85 complexes in the sera from animals inoculated with *M. paratuberculosis*; no reactions occurred with sera from control animals (FIG. 3).

In an additional study, mAb85.1 and mAb85.96 were used in Western blots to test for the presence of Ag85 in the sera of 198 bison selected from four large herds. One hundred and one, 71, 20, and 6 bison were tested from the four herds, of which 5,

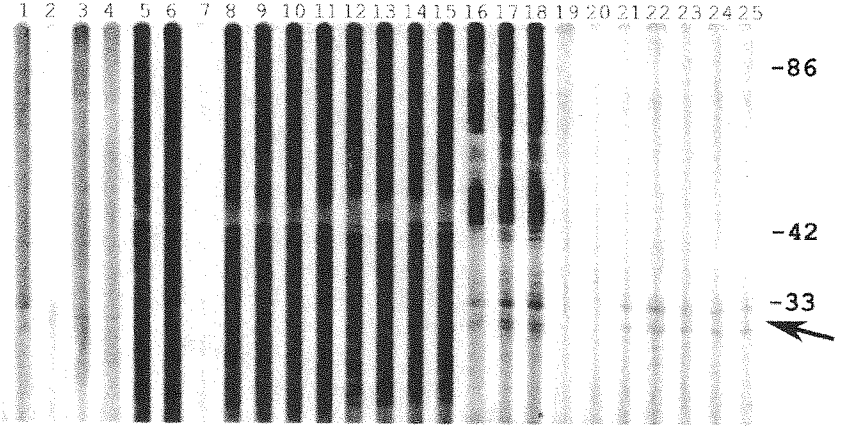

FIGURE 1. Western blot of subclones of two hybridomas generated against purified Ag85 from *Mycobacterium bovis*. *Lanes 1–4* show reactivity of supernatant fluid obtained from four wells containing subclones of mAb85.1; *lanes 5–9* show reactivity of supernatants of five wells containing subclones of mAb85.96. *Arrow* indicates location of Ag85, which consists of three or four primary bands ranging from 30 to 33 kDa. Approximate molecular weights are shown at the right side of the figure.

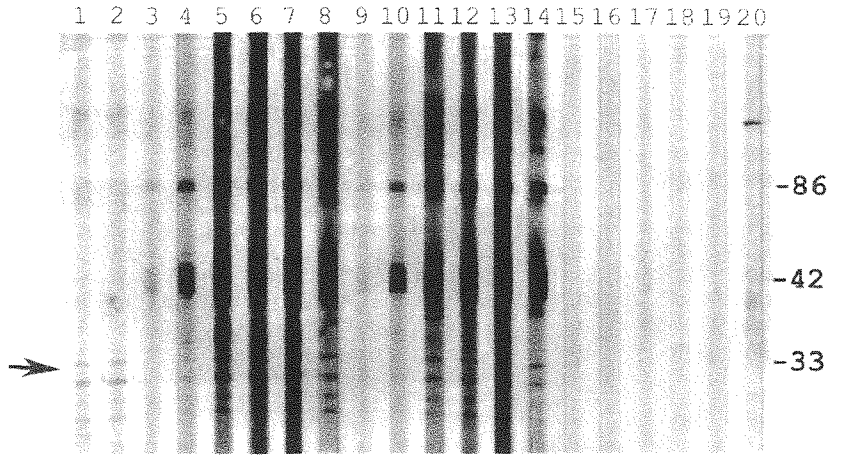

FIGURE 2. Western blot of purified mAbs reacting against purified *M. bovis* antigen 85. *Lanes 1* and *2* were reacted with 50 and 100 µg/mL of mAb85.96; *lanes 3–8* contain 10, 50, 100, 250, 500, and 1000 µg/mL of mAb85.44.9; lane 9 is supernatant fluid from unpurified mAb85.44.9; *lanes 10–13* contain 50, 100, 500, and 1000 µg/mL of mAb85.44.1; *lane 14* is supernatant fluid from unpurified mAb85.44.1; *lanes 15–19* contain 50, 100, 500, and 1000 µg/mL of mAb85.1. *Lane 20* is supernatant fluid from unpurified mAb85.1. *Arrow* shows location of Ag85 bands. Molecular weight markers are shown on the right.

15, 6, and 0, respectively, reacted positively for the presence of Ag85 with mAb85.1 or 85.96 or both (TABLE 1).

Antibody-based tests to detect Ag85 early during mycobacterial infections need to be developed that specifically differentiate between various species of *Mycobacterium*. These preliminary results indicate that monoclonal antibodies against Ag85 from *M. bovis* and *M. paratuberculosis* might be used to provide a general screen of public and private bison herds across the United States. Any positive animals may then be given extra attention both by further visual diagnosis and other diagnostic assays available. The combinatorial approach may eventually lead to a more reliable diagnostic assay that could be used to detect early infections with *M. paratubercu-*

TABLE 1. Screening of American bison herds for reactivity with serum Ag85 by mAb85.1 and mAb85.96

Animals tested	No. positive	mAb85-1	mAb85-96	Both
FZ herd–71	15	6	2	7
AY herd–20	6	0	2	4
VZ herd–101	5	5	0	0
#'s herd–6	0	0	0	0

NOTE: total samples tested = 198; total samples positive = 26. American bison sera samples were tested for reactivity with Ag85-specific mAbs by Western blot analysis. Samples were categorized as either positive by mAb mAb85.1 reactivity, mAb mAb85.96 reactivity, or by reactivity with both mAbs.

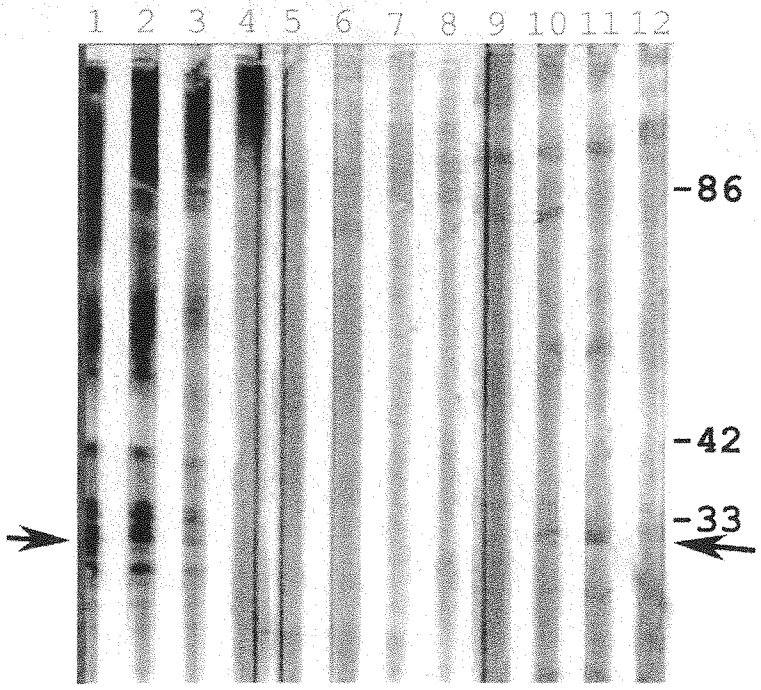

FIGURE 3. Western blot analysis of mAb85.96 and mAb85.1 reacted against purified Ag85 from *M. bovis* and sera from control bison and bison inoculated with *M. paratuberculosis*. *Lanes 1–4* show reaction of 500, 100, 50, and 1 μg/mL of purified mAb85.96 with Ag85; *lanes 5* and *6* show nonreactivity of 10 and 100 μg/mL of mAb85.96, respectively, with serum from noninoculated bison; *lanes 7* and *8* show nonreactivity of 10 and 100 μg/mL of mAb85.1, respectively, with serum from noninoculated bison; *lanes 9–12* show positive reactions of serum from *M. paratuberculosis*-infected bison with mAb85.96 (lanes 9 and 10 contain 10 and 100 μg/mL serum, respectively) and mAb85.1 (lanes 11 and 12 contain 100 and 10 μg/mL serum, respectively). *Arrows* show location of Ag85 bands; molecular weight markers are on the right side.

losis in the American bison as well as other ruminants. Such tests might also be used to detect *Mycobacterium* in dairy products including milk and cheese, which are suspected sources of *M. paratuberculosis* infections in humans associated with Crohn's disease.[15,16]

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