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## Bovine Monoclonal Antibody Specific for *Brucella abortus* Lipopolysaccharide

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### ABSTRACT

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The development of a bovine monoclonal antibody against *Brucella abortus* smooth lipopolysaccharide (BM-8) by interspecies fusion of bovine peripheral lymphocytes from an immunized cow and a murine plasmacytoma cell line is described. The twice cloned cell line secreted bovine IgG<sub>1</sub> subclass antibody. Ascites fluid was prepared in pristane treated nu/nu mice by intraperitoneal injection. The pooled ascites fluid was purified by affinity chromatography and the functions of the antibody assessed in various serological tests. The BM-8 antibody did not agglutinate well at a neutral pH, however, under acid conditions it was efficient at agglutinating *B. abortus* cells. The antibody did not precipitate *B. abortus* LPS in double agar gel immunodiffusion but was very active in the direct complement fixation test and the indirect enzyme immunoassay, although it was unable to compete with a murine monoclonal antibody in a competitive enzyme immunoassay.

### INTRODUCTION

The production of heterologous monoclonal antibodies by fusion of murine plasmacytoma cells with lymphocytes from other species is particularly useful when no homologous myeloma cell lines are available for the species to be studied, such as cattle. Srikumaran et al. (1983a, 1983b, 1987) have produced several mouse-bovine hybrids that produce monoclonal immunoglobulins with the major characteristics of bovine immunoglobulins. These reagents are valuable in immunochemical studies and some cell lines were reported to secrete products that reacted with *Streptococcus* sp. or *Staphylococcus* sp. thus furnishing tools for antigen-antibody interaction studies with bovine immunoglobulins. Similarly, mouse × bovine hybrids have been developed which produce antibody to the K99 pilus of *E. coli* (Anderson et al., 1987) or to the bovine enteric coronavirus (Raybould et al., 1985). In addition, mouse × ovine monoclonal antibodies to testosterone have been made (Groves et al., 1987) and mouse × porcine hybridomas have been reported (Raybould et al., 1984).

While mouse  $\times$  mouse monoclonal antibodies to *Brucella abortus* have been prepared (Bundle et al., 1984; Holman et al., 1984; Quinn et al., 1984; Schurig et al., 1984; Bundesen et al., 1985; Greiser-Wilke and Moennig, 1987; Roop et al., 1987; Vendrell et al., 1987) and have proved to be useful reagents in Brucellosis serology, no mouse  $\times$  bovine hybrids with antibody activity to *B. abortus* have been reported. Such a reagent would be highly useful as a positive control in serological tests, such as enzyme linked immunosorbent assay (ELISA), the complement fixation test (CFT), agglutination and precipitation tests as well as for immunochemical studies. This report deals with the development and characterization of a murine  $\times$  bovine hybrid which produces bovine monoclonal IgG<sub>1</sub> to *B. abortus* lipopolysaccharide (LPS).

#### MATERIALS AND METHODS

*Bovine lymphocytes.* An adult Holstein Friesian cow was immunized intramuscularly with 1.0 ml of 8% *v/v* of heat-killed and washed *B. abortus* cells. Additional similar injections were given 42 and 62 days later. On day 67, blood was aseptically collected in EDTA and the lymphocytes were separated using Ficoll-paque.

*Hybridization.* Bovine lymphocytes ( $1 \times 10^7$  viable cells) were fused with mouse plasmacytoma cells (S/P 2,  $1 \times 10^7$  viable cells) using polyethylene glycol as described by Kennett et al. (1978). Two to 3 weeks after the fusion and removal of unfused S/P 2 cells by addition of HAT and several changes of the culture medium, the cell supernates were tested for antibody activity.

*Hybridoma screening.* An indirect ELISA procedure was used. Polystyrene 96-well plates were coated passively with 200 ng of *B. abortus* LPS in 200  $\mu$ l of carbonate buffer (0.06 M, pH 9.6) overnight at room temperature (RT). After washing four times with 0.1 M tris-HCl, pH 8.0, containing 0.15 M NaCl and 0.05% Tween 20 (tris/T), 200  $\mu$ l of culture supernate was added to each well for 1 h at RT. After four washes, 200  $\mu$ l of murine monoclonal antibody to bovine L-chain conjugated with horseradish peroxidase (HRPO, type VI from Sigma Chem., St. Louis, MO, U.S.A.), appropriately diluted in tris/T were added for 1 h at RT. Following four further washes in tris/T, 200  $\mu$ l of substrate-chromogen containing 1mM hydrogen peroxide and 4mM 2,2'-azino-bis(3-ethylbenz-thiazoline sulfonic acid) (ABTS) were added and incubated at RT with constant orbital shaking. After 10 min of development, optical densities were assessed with a spectrophotometer. An arbitrary OD value of 0.100 or greater, based on OD values of negative controls, was considered positive.

*Cloning.* Cells from wells giving a positive result on the screening test were

cloned twice by the soft agar technique. The clone producing the highest OD in the screening test was selected for propagation.

*Production of ascitic fluid.* Nu/nu mice, pristane treated 2 weeks previously by injection of 0.5 ml intraperitoneally (i.p.), were injected i.p. with  $3.3 \times 10^6$  viable hybridoma cells. Approximately 10 days later and every 2 days thereafter, ascites fluid was drawn from the peritoneal cavities. Usually three collections were taken from each mouse. All ascites aliquants were pooled, clarified by centrifugation ( $10\,000 \times g$  for 10 min), filter sterilized and frozen at  $-20^\circ\text{C}$ .

*Isotyping.* The bovine isotype of clones producing antibody to *B. abortus* LPS was tested by an indirect ELISA in which LPS was used as the antigen, as above, but murine monoclonal antibody to bovine IgM, IgG<sub>1</sub>, IgG<sub>2</sub> and IgA, conjugated with HRPO were used as detection reagents. The ELISA procedure was described above.

*Affinity purification.* Murine monoclonal antibody to bovine IgG<sub>1</sub> was covalently bound to agarose beads with cyanogen bromide using the technique of Johnson and Garvey (1977) and packed into a column which was run reverse to gravity with a peristaltic pump. Amounts of bovine monoclonal antibody were percolated through the column. Unbound materials were washed out with 0.1 M tris-HCl, pH 7.2, containing 1 mM EDTA and 0.15 M NaCl until the OD of the effluent was less than 0.01 at 280 nm. Bound materials were then eluted from the column with 0.1 M glycine-HCl buffer, pH 2.4 and neutralized immediately by addition of 0.5 M tris-HCl, pH 8.0.

Bound and unbound proteins eluted from the column were tested for antibody activity to *B. abortus* LPS by ELISA. Fractions containing antibody were pooled, dialyzed against 0.15 M NaCl and concentrated to 1.0 mg/ml.

IgG<sub>1</sub> was obtained by the same methods as the monoclonal antibody from serum from a cow infected with *B. abortus* which had a high complement fixation test titer. The affinity purified IgG<sub>1</sub> was further affinity purified with *B. abortus* LPS (Stiller and Nielsen, 1983). These reagents were used for serological testing.

*Serological tests.* The indirect ELISA was performed as described by Nielsen et al. (1984) using a timing procedure (Wright et al., 1985). A competitive ELISA for detection of antibody to *B. abortus* was used as described by Nielsen et al. (1987). In both cases, monoclonal detection reagents were conjugated with HRPO as per Henning and Nielsen (1987). The CFT as outlined by Samagh and Boulanger (1978) was used. Agglutination tests at a neutral or acid pH were done using doubling dilutions of the monoclonal antibody, mixing with appropriately diluted antigen (whole cell, 8% v/v, used at 1:200 or undi-

luted 12% *v/v* whole cells at a final pH of 3.65, respectively), incubating for 48 h or 10 min, respectively, and then assessing agglutination visually.

*Other tests.* Immuno-electrophoresis and double gel immunodiffusion were done using standard techniques using BM-8 and control IgG<sub>1</sub> at 1.0 and 0.1 mg/ml. Polyacrylamide gels containing sodium dodecyl sulfate (SDS-PAGE) were run with purified BM-8 preparations at 1.0, 0.1 and 0.01 mg/ml as described by Towbin et al. (1979).

## RESULTS

Screening hybrid cultures from several fusions resulted in one positive cell line, BM-8, which had antibody activity to *B. abortus* LPS. The ascites fluid produced from twice cloned cells was tested by ELISA and found to contain antibody of the IgG<sub>1</sub> subclass. No reactivity was apparent with antibody to mouse IgG (H and L) or mouse IgM. Antibody was prepared from pools of ascites fluid by affinity chromatography. Several batches were pooled, dialyzed and concentrated. A typical affinity chromatography run is presented in Fig. 1.

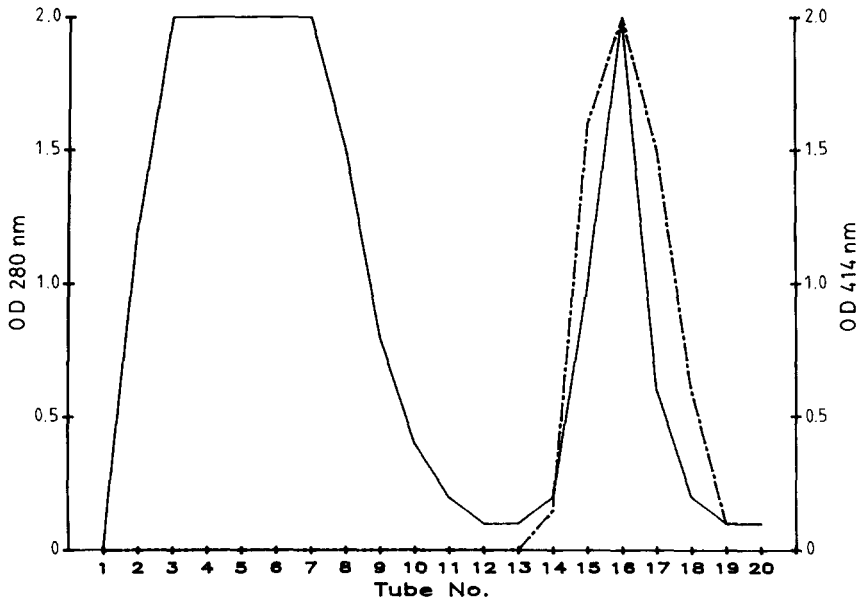


Fig. 1. Affinity chromatography purification of BM-8 bovine monoclonal antibody to *B. abortus* LPS using murine monoclonal antibody to bovine IgG<sub>1</sub> covalently attached to agarose. The right-hand Y-axis represents optical density readings at 414 nm of an indirect ELISA using *B. abortus* LPS as the antigen and anti-IgG<sub>1</sub> conjugated with HRPO as the detection reagent (solid line). The left-hand y-axis is the optical density readings at 280 nm (broken line). The x-axis represents 10 ml fractions eluted from the column.

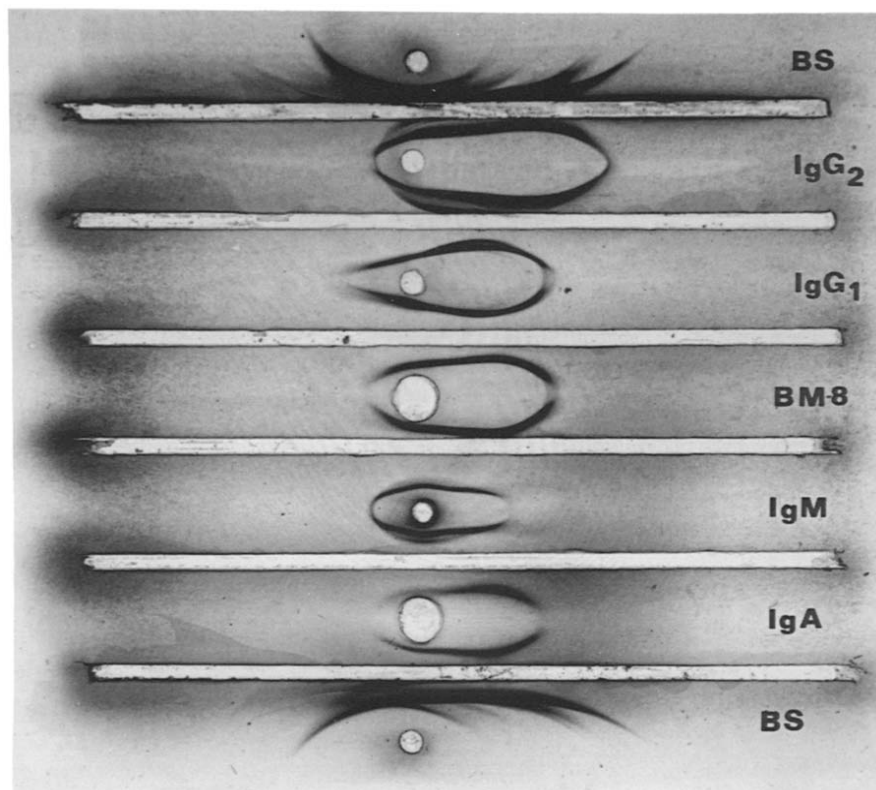


Fig. 2. Immunoelectrophoresis patterns obtained with bovine serum (top and bottom), IgG<sub>2</sub>, IgG<sub>1</sub>, BM-8, IgM and IgA and developed with a rabbit antibody to bovine gamma globulin.

Immunoelectrophoresis (Fig. 2) revealed a single precipitin arc when developed with rabbit anti-bovine gamma globulin. This arc was fairly long, indicating some heterogeneity, although not as extensive as IgG<sub>1</sub> prepared from serum (also by affinity chromatography). Double agar gel immunodiffusion with antisera specific for the heavy chains revealed reactivity with anti-IgG<sub>1</sub> only and anti- $\lambda$  antisera (results not illustrated).

SDS-PAGE gave two bands when stained with Coomassie blue, one at 55 000 D and one at 25 000 D, indicating the appropriate molecular weights for heavy and light chains (see Fig. 3).

The BM-8 antibody was not able to agglutinate *B. abortus* cells at a neutral pH at concentrations of antibody of less than 25  $\mu\text{g}/\text{ml}$ . Above 25  $\mu\text{g}/\text{ml}$  a very fine, "powdery" agglutinate could be visualized but no increase in clumping was seen with increased concentrations of antibody. Similarly, control IgG<sub>1</sub> antibody to *B. abortus* LPS did not cause agglutination at concentrations below 20  $\mu\text{g}/\text{ml}$ . Agglutination at pH 3.65 occurred with both BM-8 and control IgG<sub>1</sub>

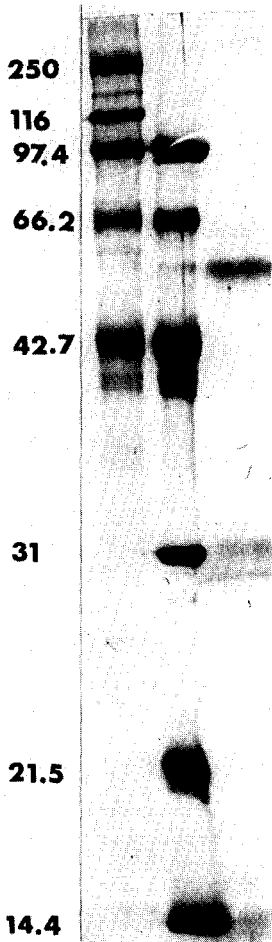


Fig. 3. SDS-PAGE gel of molecular weight markers in kD (lanes 1 and 2) and BM-8 in lane 3. The gel was stained with Coomassie blue.

to concentrations of 500 ng/ml in 10 min. The BM-8 antibody did not appear capable of precipitating *B. abortus* LPS in agar containing 1.0 M NaCl at the levels tested while the control IgG<sub>1</sub> preparation did give a diffuse precipitin band at 1.0 mg/ml.

The BM-8 antibody was capable of fixing guinea pig complement. In the diagnostic CFT, 200 ng/ml of the antibody gave 50% activation of three CH<sub>50</sub> units of complement (Fig. 4). Similarly, in the diagnostic indirect ELISA, using *B. abortus* LPS as the antigen and mouse monoclonal antibody to bovine IgG<sub>1</sub>, conjugated with HRPO, as the detection reagent, 25 ng/ml of BM-8 could be detected after 10 min of development at an OD of 1.0 at 414 nm. These data are presented in Fig. 5. In the competitive ELISA, the BM-8 antibody was unable to compete with the monoclonal antibody to *B. abortus* LPS.

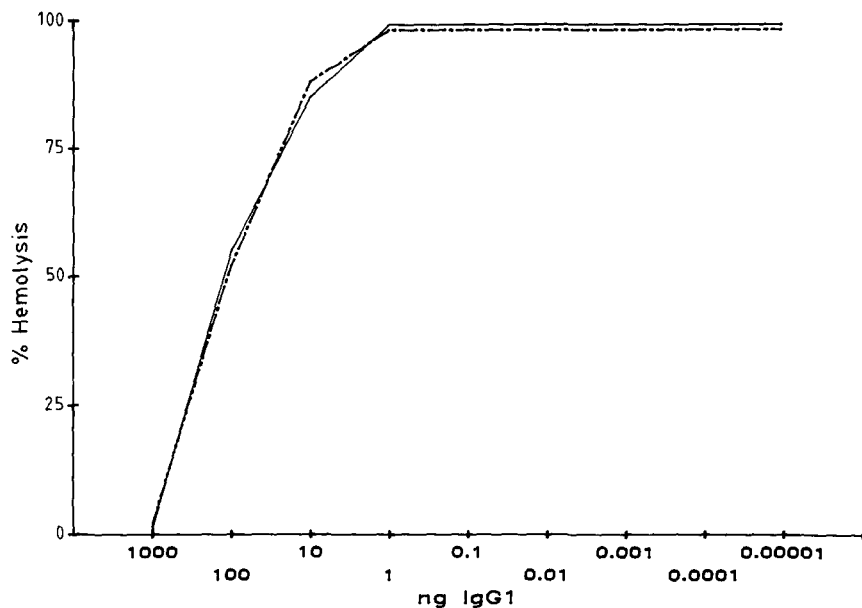


Fig. 4. Titration of bovine monoclonal antibody (BM-8) to *B. abortus* LPS (solid line) and polyclonal affinity chromatography purified IgG<sub>1</sub> (PC) (broken line) by the diagnostic complement fixation test. The y-axis represents the percent hemolysis of three CH<sub>50</sub> units of guinea pig complement. The x-axis is the concentration of monoclonal or polyclonal IgG<sub>1</sub> antibody.

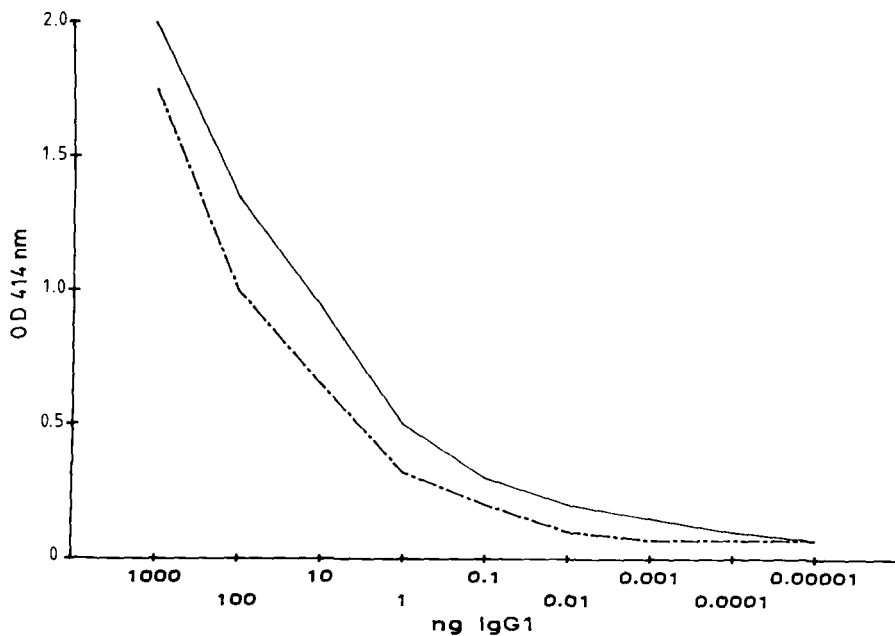


Fig. 5. Indirect (diagnostic) ELISA data for BM-8 (solid line) and affinity purified IgG<sub>1</sub> antibody to *B. abortus* LPS (PC) (broken line). The y-axis is the optical density readings at 414 nm. The x-axis is the concentration of monoclonal and polyclonal IgG<sub>1</sub> antibody added to the test.



## DISCUSSION

The development and characterization of a bovine monoclonal antibody to *B. abortus* LPS from fusion of bovine peripheral blood lymphocytes and a murine plasmacytoma cell line has been described. It is notable that, in spite of a high hybridization rate (approximately 60% of the wells contained growing hybrid cells), only one fusion product produced antibody of the desired specificity. This may reflect a lack of specific B-cells in the peripheral blood at a particular time, in spite of high levels of serum antibody. Thus the immunization schedule in relation to the time of bleeding for lymphocyte harvest is of importance. This is unlike the mouse system in which normally the spleen is used as a source of lymphocytes. Alternate approaches would be to biopsy lymph nodes that drain the immunization site (Tucker et al., 1984; Anderson et al., 1987) or spleen cells (Srikumaran et al., 1983a,b). Cell line stability with interspecies-fusion partners may be a problem as the murine chromosomes are lost and as a result immunoglobulin secretion ceases or diminishes with culture time. In the case of BM-8 this has not been a problem. The cell line appears stable in culture and several subcultures stored in liquid nitrogen prior to propagation have produced immunoglobulin in quantities comparable to the parent cell line. Production of ascitic fluid in large volumes alleviates some of the long term culture problems. An alternate approach to enhance stability of immunoglobulin production might be to re-fuse the hybridoma cell line with bovine lymph node cells obtained from immunized animals (Tucker et al., 1984).

While several reports of bovine  $\times$  mouse fusions are in the literature, including some reporting production of antibody of known specificity (see above for references), to our knowledge, none that produce antibody to *B. abortus* have been reported. This reagent could be valuable as a source for continuous quality control and standardization inherent to mass serological testing. In the past, individual sera or pools have been relied on as positive reagents in diagnostic serology, but in spite of large volumes on hand, these reagents eventually run out, necessitating the need for others which were never identical. An added problem has been the inability of laboratories to compare data to the lack of uniformity of reagents. The BM-8 monoclonal antibody could provide such a reagent for the CFT and ELISA. It is of interest to note that the BM-8 antibody fixes guinea pig complement as efficiently as does polyclonal affinity purified IgG<sub>1</sub> (Fig. 4); however, it is considerably more active in the indirect ELISA than polyclonal IgG<sub>1</sub> (Fig. 5).

There are some limitations to its use, including the use of mice for ascites production and the inability of BM-8 to compete for antigen epitopes in the competitive ELISA used to distinguish sera from *B. abortus* S19 vaccinated and field infected cattle (Nielsen et al., 1987). This discriminatory assay which uses the O-chain of *B. abortus* LPS as the antigen, appears to be based on at least three premises: (1) the antibody response of infected cattle differing from

that of S19 vaccinated cattle; S19 vaccinated animals lack antibody to the 'length' epitope of the O-chain while infected cattle develop antibody specific for both the 'tip' and the 'length' epitopes; (2) that the antibody affinity of vaccinated cattle is less than that of infected cattle and (3) that the antigen presented on the polystyrene 96-well plate has the 'tip' epitope occluded (Nielsen et al., 1988). The BM-8 antibody, derived from an immunized rather than infected animal would appear to lack specificity for the 'tip' determinant of *B. abortus* O-chain or perhaps a determinant not recognized by the murine monoclonal antibody. A third disadvantage is the price of nu/nu mice.

In spite of these problems, bovine monoclonal antibody to *B. abortus* LPS should be highly useful in standardizing the CFT and the indirect ELISA for detection of antibody to *B. abortus* in cattle and for immunochemical studies of immune complexes and their biological functions.

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