# Production and Characterization of Monoclonal Antibodies Against *Brucella abortus S (99)* Surface Antigens

Shohreh Farshad[1], Mohammad Javad Mehrabanpour[2], Mohammad Mehdi Namavari[2], Seyed Mohammad Hossein Hosseini[2], Akbar Tavakkoli[1] and Abbas Ghaderi*[3]

*[1]Dept. of Microbiology, Medical School, Isfahan University of Medical Sciences, Isfahan; [2]Razi Serum and Vaccine Research Institute, Shiraz; [3]Dept. of Immunology, Medical School, Shiraz University of Medical Sciences, Shiraz, Iran*

## ABSTRACT

**By immunizing mice with killed whole bacterial cells of *Brucella abortus S (99)*, a panel of six hybridomas producing monoclonal antibodies (mAb) specific for the surface antigens of this bacterium were produced. ELISA was used to screen the hybridoma supernatants. Immunoblots of the cell extract indicated that three mAb were specific for S-LPS (Ba-1, Ba-2, Ba-3) and three others were reactive with major outer membrane proteins (OMP) (Ba-4, Ba-5, Ba-6). The OMP recognized by these antibodies were the proteins with molecular masses of 25-27kDa (Ba-4, Ba-5) and 36-38kDa (Ba-6). None of the four mAb including Ba-3, Ba-4, Ba-5 and Ba-6 cross reacted with any other bacteria close to *Brucella abortus*, but Ba-1 and Ba-2 cross reacted with *B. melitensis 16M* and *B. suis*. By using cell extract and killed whole cell Ag in ELISA, it was indicated that all mAb except Ba-6 have better reactivity with cell extract Ag, but Ba-6 mAb reacted with killed whole cell Ag better than cell extract Ag.** *Iran. Biomed. J. 6 (1): 7-12, 2002*

*Keywords*: *Brucella*, Antigens, Monoclonal antibody

## INTRODUCTION

*Brucella* spp. are Gram-negative facultative intracellular bacteria that cause acute and chronic infection in man and animals [1]. Six species are recognized within the genus *Brucella*: *B. abortus*, *B. melitensis*, *B. suis*, *B. ovis*, *B. canis* and *B. neotomae*. The main pathogenic species worldwide are *B. abortus*, responsible for bovine brucellosis, and *B. melitensis*, the main etiologic agent of ovine and caprine brucellosis [2].

The *Brucella* cell envelope is a three-layered structure in which an inner or cytoplasmic membrane, a periplasmic space, and an outer membrane can be differentiated [3, 1]. *Brucella* cell walls consist of a peptidoglycan (PG) layer strongly associated with the outer membrane. The outer membrane contains LPS, proteins and phospho-lipids. The major *B. abortus* outer membrane proteins (OMP) have the molecular masses of 36-38 and 25-27kDa [3-6]. They are also called group 2 porin proteins and group 3 proteins respectively [7-10]. Cell wall of *B. melitensis* contains another major protein of molecular mass 31-34 kDa, which is minor in *B. abortus* strains. A lipoprotein covalently linked to PG has also been described as a major OMP [11, 12]. Other OMP identified so far are minor species with molecular masses of 10, 16.5, 19 and 89 kDa. OMP 10, 16 and 19 share antigenic determinants with bacteria of the family Rizobiaceae [13, 14]. The 89kDa OMP is probably a protein of group 1 with a molecular mass of 88-94kDa . All these OMP are surface-exposed as demonstrated by immunoelectron microscopy [3, 10, 12, 15].

The procedures most frequently used for the serodiagnosis of brucellosis are the rapid slide agglutination test (RSAT), standard tube agglu-tination test (SAT), 2- mercaptoetanol tube agglu-tination test (2ME), complement fixation test (CFT) and enzyme-linked immunosorbent assay (ELISA). One major problem in serodiagnosis is the crossreactions that occur between *Brucellae* and other bacteria. A second problem is that a

diagnosis of brucellosis cannot be established on the antibody titer alone, as healthy people connected with animal husbandry in endemic areas may show significant titers of *Brucella* antibodies [16].

This article describes production and characterization of several mAb against *Brucella abortus S (99)* surface antigens in order to identify different and/or common antigenic structures. These antigenic structures may be employed in serodiagnostic tests which currently utilize the whole cells bacteria, and consequently to increase the specificity and to decrease cross-reactions between *B. abortus* and the other bacteria.

## MATERIALS AND METHODS

***Reagents***. RPMI-1640 (BDSL KILMARNACK), HAT media supplement (HYBRI-MAX Sigma), PEG (HYBRI-MAX Sigma), FBS (HYBRI-MAX Sigma), PBS (NaCl 8 g, KCl 0.2 g, $Na_2HPO_4$ 1.15 g, $KH_2PO_4$ 0.2 g, DW up to 1 (L), pH 7.2), PBS/T (PBS containing 0.05% [vol/vol] Tween 20), PBS-2% BSA (PBS containing 2% [vol/wt] BSA), Conjucated Goat Anti mouse IgG (Sigma), Goat Anti mouse IgG1, IgG2a, IgG2b, IgG3, IgM and IgA (Sigma).

***Bacterial strains and cultures***. Bacteria of: Yersinia enterocolitica (RITCC-2016), *E. coli* (RITCC-1164), Vibrio cholerae (RITCC-2005), Salmonella urbana (RITCC1736), Salmonella typhimurium (RITCC-1735), Pseudomonas aeru-ginosa (RITCC-1474), Shigella sonnei (RITCC-1868), Staphylococcus epidermidis (RITCC-1898), *Brucella abortus S (99)*, *B. melitensis 16M* and *B. suis* were obtained from Razi Serum and Vaccine Research Institute, Hessarak, Iran. *B. abortus*, *B. melitensis* and *B. suis* were grown for 48 h at 37°C and harvested from *Brucella* medium supplemented with 0.1% yeast extract. The other bacteria were grown for 24 h at 37°C and harvested from trypticase soy agar.

***Whole cell antigen preparation***. The bacteria were harvested from their respective growth media and heat killed for 2 h at 60°C in PBS (pH 7.3). After washing, they were adjusted to a con-centration of $10^9$ organisms/ml in PBS containing 0.5% phenol, by optical density measurements at 600 nm in a spectrophotometer (OD = 0.165 for $10^9$ cells per ml for a 1-cm light path) and were stored at 4°C [3].

***Cell extract antigen preparation***. Cell extract of *B. abortus S (99)* was obtained by sonication. Cells were inactivated by heat at 65°C for 1 h, washed three times in 0.9% NaCl, and sonicated for 15 min in 1 mM EDTA-30mM Tris (pH 8). The sonicated cells were then centrifuged for 10 min at 4,000 × g, and the supernatant was recovered and stored at –70°C.

***Immunization***. Female BALB/c mice (6-8 week old) were injected intraperitoneally (i.p.) with 0.1 ml of a suspension containing $10^9$ organisms/ml of heat killed, phenol preserved *B. abortus S (99)* in PBS in days 0, 7, 21, 28 After 21 days, mice were boosted for two times by 3 weekly intraperitonial injections of $10^9$ heat killed *B. abortus cells*.

***Hybridoma production***. Four days after the last boost injection, spleen cells were fused with myeloma cells [P3-X63-Ag8.653 (NCBI C109, the Pasteur Institute of Iran)] at a ratio of 4:1. After fusion, cells were suspended in selective HAT medium and seeded in 96-well microtiter plates. Anti *Brucella* hybridomas were screened by ELISA and cloned by the limiting-dilution technique.

***Monoclonal antibodies screening assay***. Tissue culture supernatants were assayed for mAb activity by Indirect ELISA. The wells of a 96-well U-bottomed microtiter plate were coated with 100 µl of whole bacterial cells in a concentration of $10^9$ cell/ml in carbonate-bicarbonate buffer at 4°C overnight. After 3 times washing with PBS/T 100 µl of hybridoma supernatants were added to each well and incubated at 37°C for 1 h. Unbound mAb was removed by inverting and tapping the plate followed by 3 washes with PBS/T. A volume of 100 µl of a 1/1600 dilution of peroxidase conjugated goate anti-mouse immunoglobulin in PBS/T was added to each well and incubated at 37°C for 1 h. Then, the plate was inverted and washed 3 times with PBS/T and 100 µl of activated substrate (*O*-Phenylenediamine and $H_2O_2$) was added to each well. After about 10min, the color reaction was stopped by adding of 100 µl of 12.5% $H_2SO_4$. The ELISA titers were recorded at 490 nm. Then the positive hybridomas were cloned by limiting dilution method and the positive clones were selected.

***Monoclonal antibodies isotyping***. Monoclonal antibodies isotypes were determined by Double diffusion/Ouchterlony (DDO) method.

***Cross reaction assays***. Cross reactions of mAb with some bacteria close to *B. abortus* S (99) such as *E. coli, B. melitensis 16M, B . suis, Yersinia enterocolitica, Shigella sonnei, Vibrio cholerae,* Salmonella urbana*,* Salmonella typhimurium*, Pseudomonas aeruginosa, and Staphylococcus epidermidis* were performed by ELISA and their OD was compared to that of *B. abortus* S (99) at 490 nm.

***SDS-PAGE and immunoblotting***. The fractions of cell extract antigen were separated by SDS-PAGE in a 10% gel according to the method of Laemmli [17]. After electrophoresis, the proteins were transferred to nitrocellulose membranes. After blocking for 45 min in PBS 2% BSA at room temperature, the membranes were washed with PBS-T for 3-5 min. The membranes were then successively incubated at room temperature with hybridoma supernatants for 1 h, with peroxidase-conjugated goat anti-mouse immunoglobulin antiserum diluted 1/1000 for 45min . Washings between incubation periods were performed with PBS-T. After 3 washes, the blots were developed by incubation at room temperature in a solution of PBS containing 0.1% (wt/vol) 4-chloro-1-naphtol and 7-8 µl $H_2O_2$. The reaction was stopped by washing in distilled water.

***Comparison of reactivity of mAb with cell extract and whole cell Ag***. The wells of a 96-well U-bottomed microtiter plate were coated with 100 µl of the killed whole bacterial cells in a concentration of $10^9$ cell/ml. The wells of another plate were coated with 100 µl of cell extract (2 µg/ml). ELISA was performed using 1 to $10^{-5}$ dilutions of hybrodoma supernatants for each Ag. OD was read at 490 nm.

## RESULTS

After fusion between myeloma cells and spleen cells of BALB/c mice immunized against *B. abortus S (99)*, 15 hybridomas were produced . After screening, 6 clones Ba-1, Ba-2, Ba-3, Ba-4, Ba-5 and Ba-6 were found to produce antibody against *B. abortus S (99)*. Due to variations in antibody concentrations within the various mAb preparations, actual absorbance readings varied slightly among cultured hybridoma samples. The average absor-bance was more than 1.3 (data not shown), except Ba-4 that was 0.8. Isotypes of the 6 clones were identified by DDO method. By this method, the mAb of Ba-1, Ba-3, Ba-5, and Ba-6 were identified as IgG1 and Ba-2 and Ba-4 were identified as IgG2b isotype. Reactivity against some other bacteria close to *B. abortus S (99)* was also monitored by ELISA. Only Ba-1 and Ba-2 clones showed equal reactivity with *B. melitensis 16M* and *B. suis*, while others showed no cross reactivity with these bacteria, demonstrating the absolute specificity for *B. abortus S (99)*. To determine the specificity of the anti-*Brucella* mAb, immuno-blotting was performed using cell extract of *B. abortus S (99)* separated by SDS-PAGE (Fig. 1). Monoclonal antibodies Ba-1, Ba-2 and Ba-3 reacted with the band of 38-45 kDa. The mAb Ba-4 had a weak reaction with three bands of 25, 26 and 27 kDa, but mAb Ba-5 in addition to a good reaction with these three bands, also reacted with a band of 55.5 kDa. Ba-6 mAb had a weak reaction with the band of 36 kDa . By comparing ELISA on cell extract Ag and whole cell Ag, it was indicated that all mAb except Ba-6 have better reactivity with cell extract Ag (Fig. 2), but this mAb reacts with whole cell Ag better than cell extract Ag (Fig. 2).