Iranian Biomedical Journal 6 (1): 7-12 (January 2002)

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

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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 25-27 kDa (Ba-4, Ba-5) and 36-38 kDa (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. cants* 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 phospholipids. The major *B. abortus* outer membrane proteins (OMP) have the molecular masses of 36-38 and 25-27 kDa [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 89 kDa OMP is probably a protein of group I with a molecular mass of 88-94 kDa. All these OMP arc 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 agglutination test (SAT), 2- mercaptoetanol tube agglutination test (2ME), complement fixation test (CPT) 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

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[DOR: 20.1001.1.1028852.2002.6.1.8.9]

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 (I-IYBRI-MAX Sigma), PEG (FIYBRI-MAX Sigma), FBS (HYBRI-MAX Sigma), PBS (NaCl 8 g, KCI 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), Conjugated 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. coil* (RITCC-1164), Vibrio cholerae (RITCC-2005), *Salmonella urbana* (RITCC1736), *Salmonella typhimurium* (RITCC-1735), *Pseudomonas aeruginosa* (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 concentration of $l0^9$ organisms/m1 in PBS containing 0.5% phenol, by optical density measurements at 600 nm in a spectrophotometer (OD = 0.165 for $l0^9$ 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-30 mM Iris (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 $l0^9$ organisms/m1 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 intraperitoneal injections of $l0^9$ heat killed *B. abortus* cells.

Hybridoma production. Four days alter 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 µ1 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 µ1 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 I-H_2O_2) was added to each well. After about 10 min, the color reaction was stopped by adding of 100 µl of 12,5% H2SO4. 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 difflusion/Ouchterlony (DDO) method.

Cross reaction assays. Cross reactions of mAb with some bacteria close to B, *abortus* S (99) such as *E. coil, 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

[DOR: 20.1001.1.1028852.2002.6.1.8.9]

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 45 min. 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-l-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 10^9cell/ml. The wells of another plate were coated with 100 µl of cell extract (2 µl) ELISA was performed using 1 to 10^{-5} dilutions of hybridoma 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 absorbance 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 lgG2b 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, immunoblotting 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).

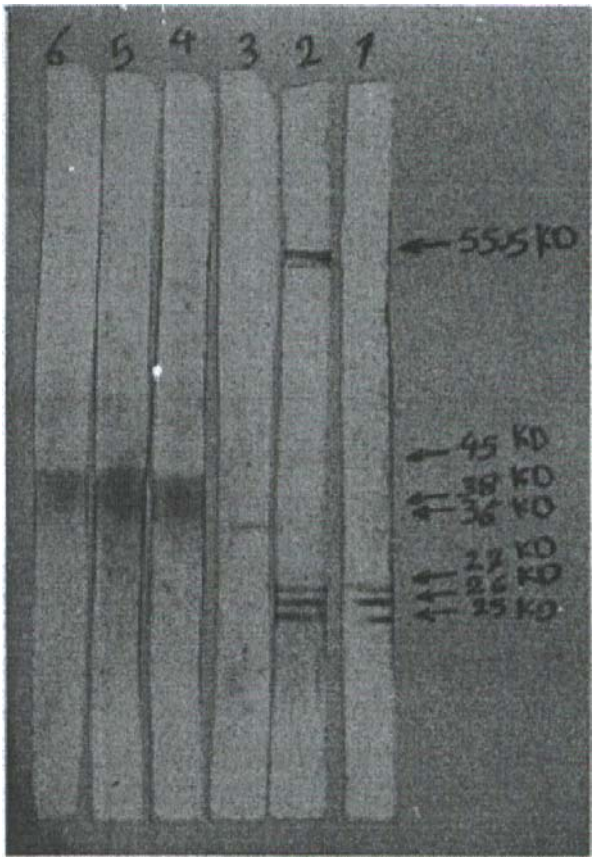

Fig. 1. Western inununoblotting of sonicated cell extract of *B. abortus(99)* with mAb: lane 1, (Ba-1) (38-45 kDa); lane 2, (Ba-2) (38-45 kDa); lane 3, (Ba-3) (38-43 kDa); lane 4, (Ba-4) (25-27 kDa), lane 5. (Ba-5) (25-27 and 55.5 kDa); lane 6. (Ba-6) (36 kDa).

DISCUSSION

The data presented here demonstrate the specificity of 6 different hybridoma clones producing monoclonal antibodies for the surface antigens of *B. abortus S (99).* Three clones of Ba-1, Ba-2 and Ba-3 produce antibodies which recognize a diffuse band in the region of 38-45 kDa of cell extract of *B. abortus S (99).* As described by others [16, 18], the band of 36-45 kDa is comprised the region that contains S-LPS of smooth *B. abortus.*

[DOR: 20.1001.1.1028852.2002.6.1.8.9]

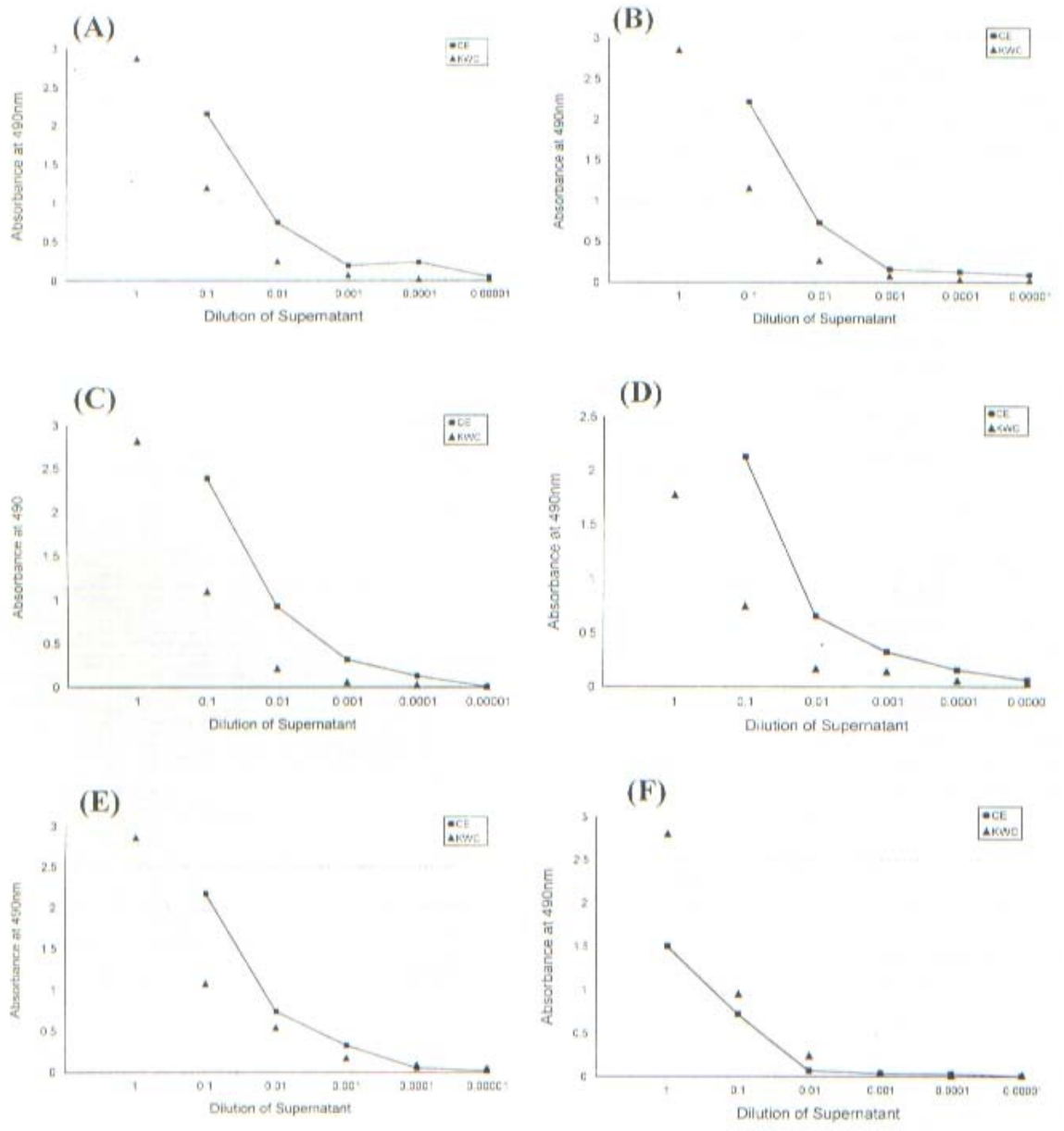

Fig. 2. Comparison of the reaction of hybridoma supernatants with cell extract Ag and the killed whole cell Ag (KWC) of *B. abortus S (99)* in ELISA: Ba-1 (A); Ba-2 (B); Ba-3 (C); Ba-4 (D); Ba-5 (E); Da-6 (F). OD in dilution of 1 for A, B, C, D and E is out of range.

In this study, we showed that this band might correspond to the band of 38-45 kDa. According to this observation, we assume that these three clones produce antibodies with reactivity against S-LPS. Antibodies produced by clones of Ba-1 and Ba-2 reacted with *B. melitensis 16M* and *B. suis* as well as *B. abortus S (99).* As the cross-reacting epitopes among smooth *Brucella* strains supposedly reside the O-chains, it can be postulated that mAb produced by these clones may recognize a common epitope shared in S-LPS of these three bacteria.

Up to now seven specific epitopes on the O-chains of *Brucella* species have been reported that contain M, A, C (M>C), C (M = A), C/Y(M>A), C/Y(M = A), C/Y(A>M). The epitopes of M and A present on M and A dominant *Brucella* strains, respectively. The C (common) epitope is strictly specific for smooth *Brucella* spp. either A or M dominant, while the C/Y epitopes have been reported to be common for smooth *Brucella* spp. and *Y. enterocolitica O.9* [18].

Accordingly, the clones of Ba-1 and Ba-2 may have

[DOR: 20.1001.1.1028852.2002.6.1.8.9]

specificity for A and M dominant S-LPS which is regarded as being specific for the properties of C epitope. This recognized epitope may be the common antigenic determinant among smooth strains of *Bruce/fa* that correlate with the reactivity with whole cell antigen of Wright strain or *B. abortus S (99)* in serodiagnostic tests [19, 20]. On the other hand, the clone of Ba-3 was specific for *B. abortus S (99),* with no cross reactivity for other bacteria and thus was shown to be highly specific for A dominant S-LPS.

Culture supernatants of these clones reacted more significantly with cell extract than with whole cell Ag. It is known that O-chains are immunodominant epitopes of S-LPS. Since S-LPS biosynthesis takes place in the cytoplasmic membrane, more free O-chain is expected to be accessible in cell extract preparation, as it is explained by Cloeckaert *et al.* [21].

IgG2b antibody produced by clone of Ba-4 recognized three bands in regions of 25, 26 and 27 kDa and Ba-5 antibody with IgG1 isotype recognized one band of 55.5 kDa in addition to these three bands. This multiple band pattern has already been described by others [22]. It has been shown that by using anti peptidoglycan and anti 25-27 kDa mAb, large amounts of these proteins are tightly associated, presumably covalently to the PG. Also the high-molecular-mass band ranging from 50 to 55 kDa reacting with the anti 25-27 kDa mAb probably represent dimeric form of OMP.

On the other hand, these multiple bands in nonlysozyme-treated sonicated cell extracts were also reactive with the anti 25-27 kDa antibody. It has been suggested that association of PG subunit is not the only cause of OMP heterogeneity observed in Western blotting. It may be due to variation in the size of the oligosaccharide portion [22, 23]. Based on these explanations and as we used nonlysozyme-treated cell extract, clones Ba-5 and Ba-4 may have specificities for 25-27 kDa major OMP.

The difference between reactivity of these two clones in immunoblotting may reflect the presence of two different epitopes or may be due to a common epitope but with different expressions. Lack of reactivity of Ba-4 clone with 55.5 kDa band may be explained by the possibility that the corresponding epitope for Ba-4 was not accessible in dimmer, as a result of variation in conformational structure of dimmeric form of 25 kDa OMP,

The specific epitopes of Ba-5 and Ba-4 clones were more accessible in cell extract than whole cell Ag. Our data is consistent with those of Van Aert *et al.* [20] in which they explained that S-LPS probably hinders the binding of anti-OMP mAb such as anti 25-27 kDa antibody on epitopes of the OMP close to the S-LPS on the surface of whole cell.

The Ba-6 clone (IgG1I isotype) reacted weakly with a putative epitope in the region of 36 kDa. Cloeckaert et *al.* [22] reported that in sonicated cell extracts not treated with lysozyme, only one major band at the region of 36 kDa is observed reacting with the anti 36-38 kDa mAb. They postulated that the multiple banding patterns observed for the major 36-38 kDa OMP in lysozyme-treated sonicated cell extracts of *Brucella,* are essentially due to PG subunit residues associated with this protein. Our result corresponds to this finding, since we did not treat the cell extract with lysozyme and single band observed for Ba-6 clone might be a representative of the 36-38 kDa OMP. In ELISA assay, this mAb had a good reactivity with whole cell Ag in comparison with cell extract Ag.

As mentioned previously, the reactivity of mAb produced by this clone was weak in immunoblotting. These results are consistent with the findings of Cloeckaert *et al.* [3, 23]. They reported contradictory results for mAb to 36-33 kDa OMP in competitive ELISA when compared with immunoblotting. The contradictory is due to the possibility that the antibody recognizes conformational epitopes with low or no binding activity on the denatured form of protein in SDS-PAGE.

None of the Ba-4, Ba-5 and Ba-6 clones cross reacted with other bacteria used in cross reaction assays, and were exclusively specific for *B. abortus S (99).* The potential applications of these three species-specific mAb for characterization and biological properties of the recognized epitopes on *Brucella* surface structure are currently under investigation.

ACKNOWLEDGMENTS

This work was supported financially by a grant (No. 74-0450408105) from Razi Serum and Vaccine Research Institute, Shiraz, Iran. The authors wish to thank Dr. Hayati, and Mr. Shirazi for their technical assistant.

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[DOR: 20.1001.1.1028852.2002.6.1.8.9]