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Production and Utilization of Monoclonal Antibodies against *Brucella melitensis* Rev1 Surface Antigens in Brucellosis Diseases

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By immunizing mice with killed whole bacterial cells of *Brucella Melitensis Rev1* a panel of four 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 tow mAb were specific for S-LPS (BOX-1, BOX-2, and tow others were reactive with major outer membrane proteins (OMP) (Box-3, Box-4). The OMP recognized by these antibodies were the proteins with molecular masses of 22-25 kDa (Box-3, Box-4) and 30-33 kDa (Box-1). None of the three mAb including Box-2, Box-3 and Box-4 cross reacted with any other bacteria close to *Brucella melitensis*, but Box-1 cross reacted with *B. abortus* S19 and *B. suis*. By using cell extract and killed whole cell Ag in ELISA, it was indicated that all mAb except Box-1 have better reactivity with cell extract Ag, but Box-4 mAb reacted with killed whole cell Ag better than cell extract Ag. We utilize the isolated antibodies (Box4) in lateral flow test.

Key words: *Brucella Melitensis,* Hybridomas, Monoclonal antibodies.

Brucellosis is a chronic zoonotic disease resulting in undulant fever in humans and abortion and /or infertility in affected animals[1]. *Brucellae* are small, non-motile, gram-negative cocobacilli, which cause disease in a variety of mammals[2]. There are six species of *Brucella* that are currently recognized based on their host-specificity. They include *Brucella abortus* (cattle), *B. melitensis* (goats), *B. suis* (hogs), *B. canis* (dogs), *B. ovis* (sheep), and *B.neotomae* (wood rat)[3]. Recently, *Brucella* has been recovered from a variety of marine mammals including cetaceans (e.g. dolphins), seals, and otters. Though the organisms recovered from these marine mammals do not fit into the nomen classification by the currently available laboratory identification scheme, they have been confirmed to be *Brucella*, but probably deserve a new species designation. There is at least one report of human exposure to *Brucella* isolated from a sea mammal[4].

Brucella organisms can be phenotypically categorized based on their colony morphology into smooth, rough, and intermediate/ mucoid[2]. Organisms characterized as smooth contain the O-antigen (O-polysaccharide composed of perosamine polymers) on their lipopolysaccharide (LPS); true rough organisms do not contain O-antigen. In general, smooth *Brucella* are more virulent than their rough counterparts[2]. *B. canis* and *B. ovis* are the only species of *Brucella* that naturally occur in the rough form, and yet are still pathogenic in their host species[2]. All other 4 nomen species naturally occur in the smooth form. The newly discovered marine isolates all appear to be smooth. Smooth *Brucella* organisms are better able to survive intracellularly than do their rough counterparts. Therefore, smooth lipopolysaccharide (S-LPS)

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probably plays a significant role in pathogenesis.

Experiments suggested that in *Brucella melitensis* strains, the expression of a fatty tissue called O-polysaccharides (OPS) on the outer membrane of the bacterium controls whether the bacterium will look smooth or round[5]. The absence of these O-polysaccharide chains turns the organism into a rough variant. This layer is important in identifying whether a pattern of species-specific flagellar gene inactivation's and flagellum gene clusters exist, because this would give a better understanding of host specificity and virulence. The need for these species to survive in a species-specific environment provides an explanation that the adaptation of the *Brucella* species requires an "intracellular life-style in a protected and more stable local environment or niche that provides a constant supply of nutrients[6]." Currently, little is known about their chromosomal exchange, and there is no evidence of plasmids or bacteriophages in these species. So product the specific antibodies against S-PLS of B. melitensis and utilizing it in lateral flow tests for detection of brucellosis causes B. melitensis in sheep and gout of remote villages can help to our, that we control of brucellosis. There is a report that it is possible that Brucella use as biological weapons.

MATERIALS AND METHODS

Bacterial strains

Brucella strains used were *B.melitensis* Rev. 1 (biovar 1, *ELISA* vaccine strain). They were from the *Brucella* Culture Collection, maintained at INRA,Nouzilly, France by J. M. Verger and M. Grayon. Bacterial cultures were grown on Trypticase Soy Agar (TSA) (bioMerieux, Marcy L'Etoile, France) supplemented with yeast extract (TSAYE medium) (Difco Laboratories, Detroit, MI) 0.1 % w/v. Strains were checked for purity, species and biovar characterisation by standard procedures.

Antigens

Cell envelope fraction (CEF), cell-wall fraction (C W), sodium dodecyl sulphate-insoluble(SDS-I) and -soluble (SDS-S) cell-wall fractions from *B. melitensis* Rev1 (S) and 0-polysaccharide (0-PS) from *B. melitensis* Rev1 were prepared as described previously [7-8]. Whole cell lysates of the *Brucella* biovars were prepared from 24-h slant cultures (c. 10'' bacteria) harvested in 3 ml of sterile H20 and centrifuged at 5000 *g* for 30 s. The pellets were lysed by heating for 10 min at 100°C in 500 µl of modified Laemmli sample buffer (62.5 mM Tris, SDS 2%, 100 mM DTT, glycerol lo%, pH 6.8) and dissolved proteins were further analyzed by SDS-PAGE and immunoblotting.

Mice, immunisations and hybridomas

Groups of five 8-week-old female BALB/c mice were inoculated intraperitoneally with lo7 cfu of *B.melitensis* strain Rev. 1. Mice were bled 5 weeks later to evaluate antibody responses to CW and CEF antigens. Three months after challenge mice infected with *B.melitensis* strain Rev1 were boosted intravenously with 30 µg of partially purified CEF and on the following day intraperitoneally with 300µg of CPE and intravenously with 40µg of CEF. Two days after the last booster injection, spleen cells were fused with cells of the SP2/0 myeloma cell line at a ratio of 5:l. After fusion, cells were suspended in selective hypoxanthine-aminopterin-thymidine-containing medium and seeded in 96-well microtitration plates at 10^5 splenocytes /well. Anti-*Brucella* hybridomas (tissue culture supernates diluted 1 in 3) were screened by ELISA with CW and CEF as antigens. Hybridomas of interest were cloned by the limiting-dilution technique.

ELISA

ELISA on CEF, CW, SDS-I, SDS-S and 0-PS antigens and on *B. melitensis* Rev1 whole or sonicated bacteria was performed as described previously[9, 10]. Antigens were coated overnight at room temperature on microtitration plates at a concentration of 1µg/well in phosphate-buffered saline (PBS) for all antigen preparations except for 0-PS which was at a concentration of 3 µg/ml. Binding of mouse polyclonal antibodies (sera serially diluted in PBS containing Tween20 ,0.05%; PBS-T) or MAbs (hybridoma supernates diluted 1 in 3 or serially diluted in PBS-T) was detected by HRP-conjugated goat anti-mouse immunoglobulins (BioRad, France) diluted 1 in 3000 in PBS-T. Substrate solution for detecting peroxidase activity was 4 mM H_20_2 and 1 mM TMB (3, 3', 5, 5'-Tetramethylbenzidine) in 50 mM sodium citrate, pH 4.2. Absorbance values at 450nm were recorded with an automatic ELISA reader (Phomo Autobio labtech, china) after add 1M H_2SO_4.

SDS-PAGE and immunoblotting techniques

SDS-PAGE and immunoblotting of CW, CEF (deposits of 60 µg), or whole cell lysates (deposits of 20 µg) were performed as described previously [7-8]. Binding of mouse polyclonal antibodies (sera diluted 1 in 100) or MAbs (1 in 3 or serially diluted hybridoma supernates) was detected by rabbit anti-mouse immunoglobulin antiserum (diluted 1 in 500) (Nordic Immunology, Tilburg, The Netherlands) and peroxidase-conjugated protein A (diluted 1 in 1000) (Sigma). Binding of sheep polyclonal antibodies (sera diluted 1 in 100) was detected by peroxidase-conjugated rabbit anti-sheep IgG immunoglobulin's (Jackson Immunoresearch Labs, West Grove, PA, USA). Peroxidase activity was revealed with the development kit from BioRad S.A., Paris, France, containing 4-chloro-1-naphtol, according to the manufacturer's instructions.

RESULTS

Antibody responses to CW in mice after infection

Antibody responses in BALB/c mice to CW induced by infection by *B. melitensis* Rev. 1 (S) were first measured by ELISA, 5 weeks after challenge, with CW as coating antigens (Fig. 1). The highest antibody titres to CW were observed in the mice infected with **S** *B. melitensis* strains Rev. 1 (Fig. la). The antibody titres were 50-100 times lower in the mice infected with R *B. melitensis* Rev1. The antibody responses to CW31 were also the highest in mice infected with *B. melitensis* Rev1. The higher antibody responses to CW induced by infection with **S** *B. melitensis* strains Rev. 1 were confirmed by immunoblotting of CW (Fig. 2). The antibody responses to CW were also the highest As antibody responses to CP were the highest in mice infected with *B. melitensis* Rev1, these mice were further used for MAb production.

Table 1. Brucella-specific hybridomas

Specificity	No. Obtaine	ELISA binding on CW	ELISA binding on CPE
12	1	++	+
24	8	+++	+++
31	22	+++	+
O-PS	3*	-	+++
Unknown	17#	-	+++

*MAbs to the O-PS protein were further shown to be specific for the CPE.

#Nine MAbs with unknown specificity reacted preferentially with CPE in ELISA, the others reacted preferentially with CPE.

MAbs

Tissue culture supernates from hybridomas were screened initially for the presence of antibody by ELISA with CW as coating antigens. Reactivity to 0-PS was also further monitored by ELISA. MAb specificity of ELISA positive hybridoma supernates was thereafter determined by immunoblotting with CW as antigens. Most hybridomas produced antibodies to CW (Table 1). The other CW specificities were CW12 and CW24. MAbs to the latter protein bound only to CW in ELISA and immunoblotting and were further shown to be specific for the CW with the *B. melitensis* Rev1. MAbs to CW31 showed high reactive-in

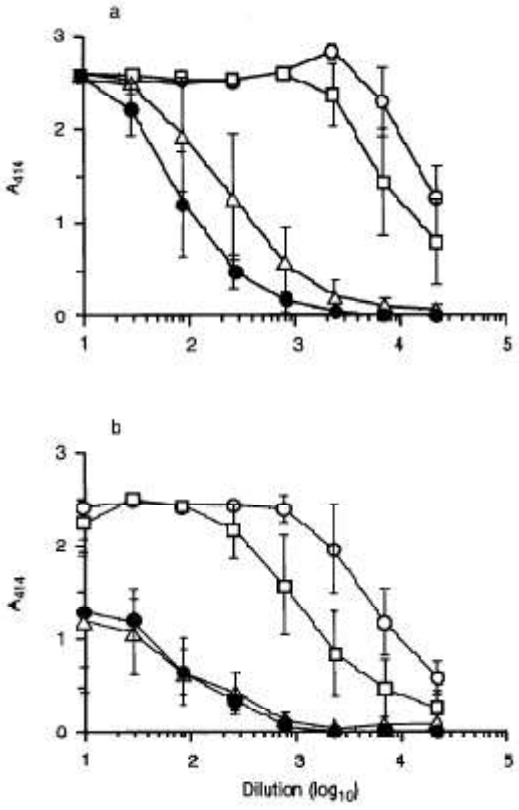

Fig. 1. Antibody reactivity measured by ELISA to B. Melitensis Rev1.CW(a) and CPE(b) antigens of sera from Balb/c mic infected with B. melitensis strains Rev1 (S)(O), H38(S)(□) or B115(R) (Δ) or B. Ovis strain 63/290(R) (•) (5 week after challenge)

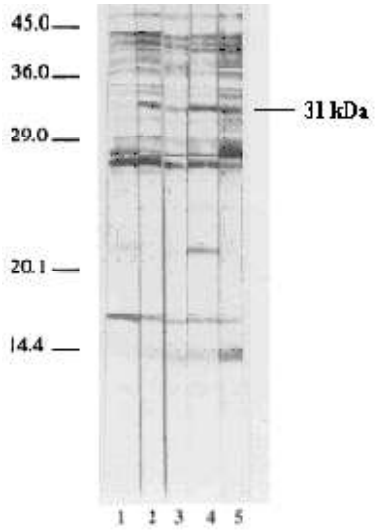

Fig. 2. Antibody responses detected by immunoblotting of *B. meliterzsis* B115 CPE antigens with sera (diluted 1 in 100) from BALB/c mice (five mice sera for each challenge) infected with *B. melitensis* strains H38 (S) (Len.2 and 3), Rev. 1 (S)(Len. 4and 5) or *B. ovis* strain 63/290 (R) (Len.1) *(5* weeks after challenge)

ELISA CW (most absorbance values were > 1.5) and reacted poorly with CPE (absorbance values < 0.5) (Table 1).ELISA results were confirmed by immunoblotting, i.e.

Twenty-one hybridomas were also obtained with supernates showing antibody reactivity in ELISA either on CW but which did not show antibody reactivity in immunoblotting. In addition, three hybridomas were shown by ELISA to secrete antibodies specific for 0-PS epitopes.

MAbs to CW reacted in immunoblotting to CEF bands of CW also recognised by serum antibodies of sheep infected naturally with *B. melitensis* and infected experimentally with strain Rev1 (Fig. 4).

Localization of CW31 in B.melitensis cells and fractions

Reactivity of MAbs with B. melitensis cells and subcellular fractions. MAbs to CW were further used to determine localisation and presence of these CEF in other subcellular fractions, i.e., CW, SDS-I and SDS-S fractions of B. melitensis

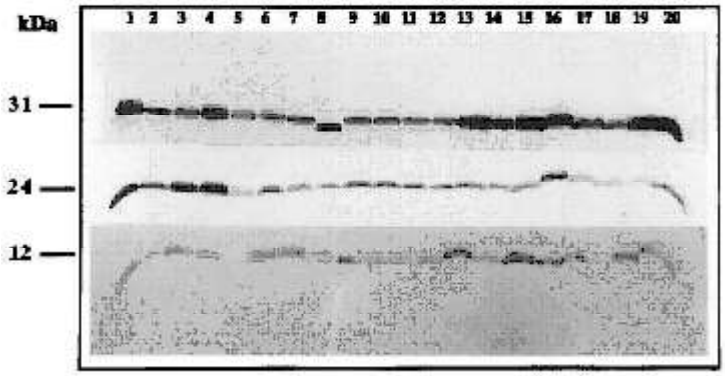

Fig. 3. Occurrence of CW12, CW24, and CW31 in *Brucella* species and biovars. Immunoblotting with anti-CW12 MAb V78/01B 1 O/G05, anti-CW24 MAb V78/04C 12/A12, and anti-CW31 MAb V78/05G03/H03 of whole-cell lysates from *B. melitensis* strains Rev.1 (lane l), 16M (2), 63/9 (3), Ether (4); *B. abortus* strains 544 *(5) ,* B19 (6), 86/8/59 (7), Tulya (8), 292 (9), B3196 (lo), 870 (ll), C68 (12); *B. suis* strains 1330 (13), Thomsen (14), 686 (15), 40 (16), 513 (17); *B.neotomae* strain 5K33 (18); *B. ovis* strain 63/290 (19); *B. canis* strain RM6/66 (20)

Rev1. CPE were added for comparison. None of the MAbs bound to whole B. melitensis Rev1 bacteria in ELISA, but bound to sonicated bacteria indicating an exclusively intracellular localisation of these CW. MAbs to CW31 bound only to CEF and in a limited manner to CEF.

Occurrence of CW31 in Brucella species and biovars

By immunoblotting of whole cell lysates with the MAbs, the presence of CW31 was observed in all *Brucella* species and biovars, including vaccine strains *B. melitensis* Rev. 1 and *B. abortus* B 19 (Fig. 3).

DISCUSSION

A previous study identified a number of protein bands by immunoblotting with CEF which discriminated antibody responses of sheep infected with *B. melitensis* from sheep vaccinated with Rev. 1 [5]. Among the CW, a 28-kDa CEF (CW31) was recognised by antibody of most infected sheep sera. As shown in the present study, antibody response patterns to *B. melitensis* CW in BALB/c mice infected by *B. melitensis* were similar to those observed in infected sheep. However, the anti- body response intensity depended on the challenge strains. The highest antibody responses to CW were observed in mice infected with **S** *B. melitensis* strains H38 (virulent) and Rev. 1 (vaccine). The antibody responses in BALB/c mice infected by R *B. melitensis* strain B115 and *B. ovis* strain 63/290 were appreciably lower. These observations are probably related to the pathogenicity and persistence of the strains used. **S** *Brucella* strains are considered to be more virulent than R strains and persist for longer in the host. Therefore, to produce MAbs of interest, spleen cells from mice infected with *B. melitensis* strain Rev1 were chosen for hybridoma production. In addition, these mice were boosted with CEF enriched fraction. The fact that only a limited number of hybridomas was obtained with 0-PS specificity may be due to the absence or limited content of 0-PS in the antigen preparations used for booster injections per- formed before the fusion experiment. The high immunogenicity of CW31 was reflected by the great number of CW31-specific hybridomas obtained. The other hybridomas obtained that secreted MAbs specific for CW15 and CW25 are also of interest, as CP8 and CP20 were also shown to be specific to infection status in sheep although antibody responses to these CEF were more heterogeneous than antibody responses to CW31.

In next step we will utilizing CW31 Mab in lateral flow base on a competition test that gold-Mab conjugate compare with native CEF anti-body in serum or milk infection gout or sheep.

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REFERENCES

1. Acha, P., and B. Szyfres., Zoonoses and communicable diseases common to man and animals. Pan American Health Organization, Washington, D.C, 1980.
2. Corbel, M. J., and W. J. Brinley-Morgan., Genus *Brucella*, p. 377-388. *In* N. R.Krieg (ed.), Bergey's manual of systematic bacteriology, vol. 1. Williams & Wilkins,Baltimore, 1984.
3. Corbel, M. J., Brucellosis: an overview. *Emerg Infect Dis*. 1997; **3**: 213-21.
4. Brew, S. D., L. L. Perrett, J. A. Stack, A. P. MacMillan, and N. J. Staunton., Human exposure to *Brucella* recovered from a sea mammal [letter]. *Vet Rec.* 1999; **144**: 483.
5. Fernandez-Prada C.M., Zelazowska E.B., Bhattacharjee A.K., Nikolich M.P., Hoover D.L., "Identification of smooth and rough forms in cultures of *Brucella melitensis* strains by flow cytometry". *Journal Immunology Methods.* 2006; **315**(1-2): 162-70.
6. Chain P.S., Comerci D.J., Tolmasky M.E., Larimer F.W., Malfatti S.A., Verquez L.M., Aquero F., Land M.L., Ugalde R.A., Garcia E., "Whole-genome analyses of speciation events in pathogenic *Brucellae,*", 2005.
7. Zygmunt MS, Debbarh HS, Cloeckaert A, Dubray G. Antibody response to *Brucella melitensis* outer membrane antigens in naturally infected and Rev1 vaccinated sheep. *Vet Microbiol*, 1994; **39**: 3346.
8. Zygmunt MS, Martin J-C, Dubray G. Analysis of immune response: comparison of immunoblots after isoelectric focusing and sodium dodecyl sulfate-polyacrylamide gel electrophoresis using cytoplasmic protein extract from *Brucella. FEMS Microbiol Lett* 1990; **70**: 263-268.
9. Cloeckaert A, de Wergifosse P, Dubray G, Limet JN. Identification of seven surface-exposed *Brucella* outer membrane proteins by use of monoclonal antibodies:immunogold labeling for electron microscopy and enzyme-linked immuno-sorbent assay. *Infect Immun* 1990; **58**: 398CL3987.
10. Zygmunt MS, Cloeckaert A, Dubray G. *Brucella melitensis* cell envelope protein and lipopolysaccharide epitopes involved in humoral immune responses of naturally and experimentally infected sheet. *J Clin Microbiol* 1994; **32**: 2514-2522.