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ISOLATION OF THREE *BRUCELLA ABORTUS* CELL-WALL ANTIGENS PROTECTIVE IN MURINE EXPERIMENTAL BRUCELLOSIS

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Résumé

ISOLEMENT DE TROIS ANTIGÈNES PROTECTEURS DE LA PAROI DE *BRUCELLA ABORTUS* DANS LA BRUCELLOSE EXPÉRIMENTALE DE LA SOURIS. — La fraction insoluble (fraction SDS-I) obtenue par traitement des parois de brucella par le dodécylsulfate de sodium (SDS) bouillant est capable de protéger efficacement la souris contre les brucella. Cette fraction, hydrolysée par le lysozyme et analysée par électrophorèse en gel de polyacrylamide en présence de SDS (EGPA-SDS) contient trois bandes majeures, de nature protéique, dont les poids moléculaires ont été estimés : 37000 (I), 25000 (II), 15000 (III). Les trois bandes majeures ont été séparées par EGPA-SDS puis isolées en découpant les gels et en les éluant. Ces trois bandes ainsi que les témoins négatifs (placebo) et positifs (vaccin référence *Brucella melitensis* H 38, la fraction totale) ont été mélangés à l'adjuvant incomplet de Freund et injectés sous la peau à des groupes de six souris. Un mois plus tard, les souris sont éprouvées par la souche *Brucella abortus* 544 injectée par voie intrapéritonéale. Quinze jours après l'injection d'épreuve, les souris sont autopsiées, les rates sont prélevées et les brucella sont dénombrées. Les bandes isolées, I, II, III sont capables de protéger la souris à un niveau équivalent à celui du vaccin efficace de référence *B. melitensis* H 38.

Protective antigens against brucella infection were found in either complex soluble or insoluble fractions extracted from the brucella cell-wall (Roux, 1972 ; Dubray and Plommet, 1976). An insoluble cell-wall fraction (SDS-I fraction) extracted by boiling cell-walls in sodium dodecyl sulfate solution (SDS) was shown to induce reasonable immunity in guinea-pigs (Bosserey *et al.*, 1978) and a good immunity in mice (Dubray *et al.*, 1974 ; Plommet and Bosserey, 1977 ; Bosserey, 1978).

In the murine experimental brucellosis, the protective capacity of the SDS-I fraction is approximately that of living or killed whole cells vaccines, which are known to be fairly protective in farm animals (Plommet and Bosserey, 1977). This fraction was characterized morphologically as a sacculus (Dubray, 1973) and biochemically as containing proteins and polysaccharides strongly bound to the cell wall peptidoglycan (Dubray, 1973 ; Dubray and Plommet, 1976).

The present investigation describes the isolation of three cell-wall antigens of molecular weight 37000, 25000, 15000 and their protective effects in mice against a *Brucella abortus* challenge.

Materials and Methods

1. Culture of organisms

B. abortus 99 (S : smooth phase) was grown at 37 °C in broth (Alton *et al.*, 1977) until the end of the exponential phase. The cells were harvested by centrifugation and washed in distilled water. *B. abortus* 544 (S) was used as the challenge strain.

2. Preparation of the SDS-I fraction (Dubray, 1973 ; Bosseray *et al.*, 1978)

Briefly, cells of *B. abortus* were inactivated by heating at 65 °C for 1 h and broken with glass beads in a Braun MSk homogeniser. After removal of glass beads by sedimentation, cell-walls were recovered by centrifugation (53 000 × g, R max = 13.3 cm, 1 h at 4 °C).

The cell-wall fraction was suspended in distilled water and added dropwise to boiling 4 % SDS (Serva, Heidelberg, West Germany). The insoluble fraction (SDS-I fraction) was recovered by centrifugation, washed three times with distilled water and lyophilized. The fraction was then delipided twice by chloroform-methanol extraction (2/1, v/v). The dry weight yield was 10 % of the dry weight of cells. Absence of cytoplasmic membrane and cytoplasmic components was estimated by electron microscopic observation of ultra-thin sections (Dubray and Bezard, 1980).

3. Lysozyme degradation of the SDS-I fraction

Delipided SDS-I fraction (5 mg) was added to a 1 ml solution of lysozyme (2 % w/w of fraction) in 0.1 M ammonium acetate buffer (pH 6.25). After incubation (24 h at 37 °C) with a few drops of toluene, the suspension was lyophilized.

4. Protein determination

Protein concentrations were determined (Lowry *et al.*, 1951) using bovine serum albumin (British Drug Houses, Poole, England) as the standard and percentages of protein in

each band were estimated by scanning Coomassie-blue-stained polyacrylamide gels at 600 nm with a Vernon PHI 5 densitometer (Vernon, Paris, France).

5. Polyacrylamide gel electrophoresis in presence of SDS (SDS-PAGE)

Electrophoresis in slabs of polyacrylamide gel (10 %) in the presence of SDS (0.1 %) was carried out as described by Laemmli (1970). Samples (40 µl) at different SDS-I fraction concentrations (0.5 to 5 mg per ml) were solubilized 5 min at 100 °C in 0.0625 M Tris buffer (pH 6.8), 2 % SDS, 5 % β-mercaptoethanol, 10 % glycerol and 0.001 % bromophenol blue (tracking dye). Pharmacia low molecular weight proteins kit (Pharmacia, Fine Chemicals, Le Chesnay, France) was used as a molecular weight standard. Slab gels were stained either with 0.05 % Coomassie blue R 250 (Gurr, London, England) for proteins or with the cationic carbocyanine dye (Eastman Kodak Company, Rochester, USA) for acid glycoproteins, lipids (King and Morrison, 1976) and lipopolysaccharides (Tsang *et al.*, 1976) and then with Coomassie blue R 250.

For preparative electrophoresis, four slabs (3 mm thick) were run at the same time. Two lateral guides were then cut out from the slab and stained. After destaining, the areas corresponding to the major bands were determined on the unstained gel and cut out, then eluted three fold with 0.25 % SDS in water. The eluents were pooled, concentrated under vacuum and dialysed against distilled water containing 0.25 % SDS.

6. Preparation of vaccines

Doses of vaccines were expressed as the equivalent of brucella cells (1 mg, dry weight = 10¹⁰ cells) containing the quantity of fraction or band produced, taking into account the yield for SDS-I fraction, and the protein percentage of each band in the cells for the bands I, II, III.

Each band and the hydrolyzed SDS-I fraction were prepared in 0.25 % SDS in distilled water (0.1 ml) at three 10-fold dilutions. They were mixed volume to volume with Freund's incomplete adjuvant (FIA) (Difco laboratories, Detroit, USA) and homogenised just prior to injection. SDS distilled water mixed with FIA was injected as negative control (placebo), *Brucella melitensis* H38 vaccine (10⁸ of killed whole cells per dose = 10 µg) was prepared

and injected as positive control (Plommet and Bosseray, 1977).

7. Protection assay in mice

The mouse challenge test used for controlling the potency of brucella vaccines was employed (Plommet and Bosseray, 1977). Briefly, groups of six OF1 five-week-old mice (Iffa Credo, Lyon, France), were injected subcutaneously with one of the vaccines or placebo. One month later, the challenge strain *B. abortus* 544 (S) was prepared so that 0.2 ml injected intraperitoneally contained 2×10^5 colony forming units. Fifteen days later, mice were sacrificed and the spleens were removed and ground in saline. Dilutions were made and each dilution spread onto two petri plates and incubated five days at 37 °C. Brucella were counted and the total number (x) of brucella per spleen was expressed as $y = \log(x/\log x)$. With this transformation, the experimental error is distributed near normality making classical statistical analysis valid (Bosseray and Plommet, 1976). Response means were compared to the mean of negative (placebo) and positive (reference H38 vaccine) control groups by the Dunnett's procedure (Steel and Torrie, 1960).

8. Immunization of rabbits

Rabbits were immunized by intramuscular injection of 1 mg of SDS-I fraction in presence of Freund's complete adjuvant (FCA) (Difco laboratories, Detroit, USA) for the first injection and FIA for the others, once a week for six weeks. The available quantities of bands I (250 µg), II (370 µg) and III (125 µg) mixed with FIA were given intradermally on days 0, 7 and 14. Antisera were collected seven days after the last injection and stored at -20 °C in 1 ml aliquots.

9. Polysaccharide immunodiffusion analysis

The polysaccharide antigen of smooth specificity (PS (S) antigen) utilised here was a gift from Dr L.M. Jones (Jones and Berman, 1975). It was prepared from *B. abortus* strain 1119-3 by trichloroacetic extraction of the washed whole cells followed by acetic acid hydrolysis to remove lipids and proteins. It contained 85 % carbohydrate (phenol method) and 1.4 % protein.

Capillary precipitin technique : unabsorbed, undiluted antisera were first drawn into the

capillary (2 mm inner diameter by 40 mm long) followed by an equal volume of PS(S) antigen solution (5 mg/ml). The precipitin reaction was read after incubation at 20 °C, several times, for one day.

Double diffusion test in tube (Oakley and Fulthorpe, 1953) was performed with unabsorbed, undiluted antisera in 0.3 % agarose and PS(S) antigen solution (5 or 10 mg/ml). The precipitin reactions were followed for fifteen days at 20 °C.

Results

The lysozyme hydrolyzed SDS-I fraction when analyzed by SDS-PAGE showed a characteristic pattern (fig. 1a) with three major-protein bands : band I, apparent molecular weight 37000 (37 K) ; band II, apparent molecular weight 25000 (25 K) ; band III, apparent molecular weight 15000 (15 K). When the gel was loaded with different amounts of fraction and scanned after Coomassie blue stain-

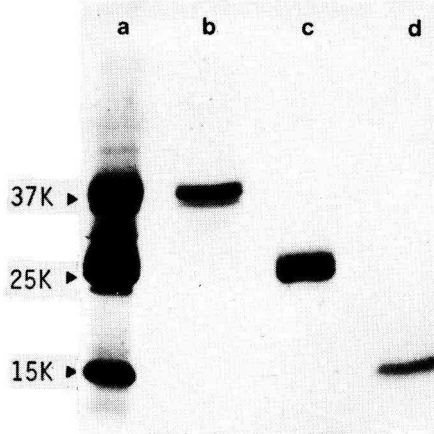


Fig. 1. — SDS-PAGE patterns of hydrolyzed SDS-I fraction and isolated bands after Coomassie blue staining of the gel.

- SDS-I fraction hydrolysed by lysosyme is composed of three major bands. To visualize the minor bands, 300 µg (150 µg of protein) was applied to the gel. The apparent molecular weights of the more intense subband are : 37 K, 25 K, 15 K.
- Isolated band I (30 µg of protein).
- Isolated band II (50 µg of protein).
- Isolated band III (30 µg of protein).



Fig. 2. — Ultrathin section of SDS-I fraction of *B. abortus* 99 (S). The dense layer (5 nm) is well observed with a thin layer (2.5 nm) at 2.5 nm apart. X 124 000.

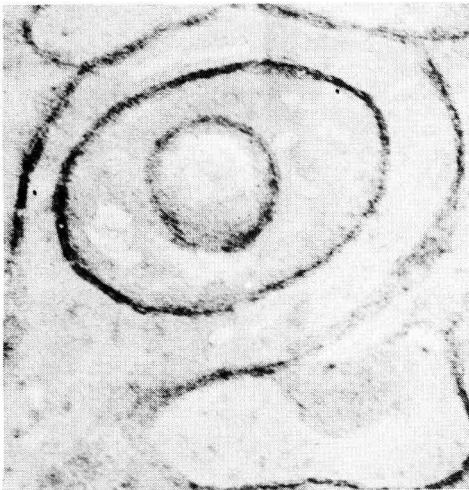


Fig. 3. — Ultrathin section of the DSD-I fraction of *B. abortus* 99 (S) hydrolyzed by lysozyme. A three layered membrane structure is seen with the disappearance of the dense layer. X 150 000.

ning, the bands I and II were in fact composed of subbands 34 K, 37 K, 38 K, 40 K and 23 K, 25 K, 26 K, 27 K respectively. When the gel was overloaded, a small band (31 K) was seen between I and II. The percentages of each major band were : 27 (I), 40 (II), 9 (III, after correction for lysozyme). They accounted for 76 % of the 50 % protein content of the fraction.

Among the 24 % of the other proteins of the SDS-I fraction, a part could be cytoplasmic membrane proteins or cytoplasmic proteins. Nevertheless these are minor ; indeed electron microscopic observation of ultra-thin sections of the SDS-I fraction showed only dissymmetrical three-layered structures (fig. 2) with sometimes few cytoplasmic areas. After lysozyme hydrolysis, the dense layer disappeared and we saw a symmetrical membrane structure (fig. 3).

The carbocyanine dye stained band I red (protein) ; band II, blue (acidic glycoprotein) and band III, yellow with a central purple line (lipoprotein or lipid + protein). Under band III, an additional yellow-orange broad band representing lipid and/or lipopolysaccharide (LPS) was observed in the bromophenol blue region.

The three major bands were separated and eluted from cut gel slabs. They were reanalyzed on SDS-PAGE (fig. 1b, c, d) and no other proteic band was seen. Contamination of each band with other proteins was estimated by densitometry of Coomassie-blue-stained gel as 5 % or less. The recovery (quantity of protein from eluted bands I + II + III / quantity of protein I + II + III layered on the gel) was 25 %.

The intensity of splenic infection used as an index of protective capacity is high in the negative control mice (4.66) and low in vaccinated mice (table 1). The vaccines had a significant effect as measured by variance analysis ($F = 7.82$, $F_{2,5}^* = 2.78$, $P = 0.001$). All the vaccine means were different from the negative control mean. The means of only two vaccines (largest dose of band I : 1.287 ; smallest dose of band III : 3.76) were different from the positive control (2.59). This indicated that isolated bands I, II, III at convenient doses were able to protect mice at a level corresponding to the killed whole-cells *B. melitensis* H38 reference vaccine.

In immunodiffusion analysis with the two techniques, we observed a strong reaction with the SDS-I fraction antiserum and the PS(S) antigen which indicates the presence of

Table 1. — Evidence of protective activity of *Brucella* cell-wall antigens.

Treatment	Doses ^a (μ g)				
	0	1	10	100	1 000
Water-SDS (negative control)	4.667 ± 0.806*
Killed whole cells H.38 vaccine (positive control)	2.597 ± 0.382
SDS-I fraction	2.275 ± 0.356	...
SDS-I fraction hydrolyzed by lysozyme	...	2.782 ± 0.240	2.056 ± 0.608	2.104 ± 0.307	...
Band I	3.028 ± 0.861	2.816 ± 0.377	1.287 ± 0.522*
Band II	3.042 ± 0.930	2.677 ± 0.564	2.740 ± 0.362
Band III	3.760 ± 1.215*	3.338 ± 1.077	2.175 ± 0.620

a : doses are expressed as the equivalent of *Brucella* cells containing the quantity of injected fraction.

b : mean ± s.d. of *Brucella* per spleen of six mice.

* Significantly different from reference H.38 vaccine with the Dunnett's test ($P \leq 0.05$).

PS(S) determinant in the SDS-I fraction. Antisera raised against the bands I, II, III respectively did not give any precipitin reaction with the PS(S) antigen solution. However there were reactions in the capillary precipitin test between the three bands and their homologous antiserum.

Discussion

The protective capacities of the negative control (SDS-water) and the positive control (killed whole-cell H 38 vaccine) were in agreement with the values obtained with CD1 mice (Plommet and Bosseray, 1977) and OF1 mice (N. Bosseray, personal communication). The data presented here show that the three isolated major protein bands of the SDS-I fraction of *B. abortus* 99 (S) were protective in mice after challenge with *B. abortus* 544 S.

The biochemical characterization of these antigens is in progress in spite of the small quantities available. However, the carbocyanine dye reveals what is probably a protein for band I and an acidic glycoprotein for band II. Band III may be a lipoprotein like Braun's lipoprotein of *Escherichia coli* (Braun *et al.*, 1976 ;

Hirashima *et al.*, 1973). The presence of LPS (Geyer *et al.*, 1979 ; Tsang *et al.*, 1976) and lipids (Hirashima *et al.*, 1973) in the front of the gel is in agreement with the insolubility of the hydrolysed SDS-I fraction in water and its three-layered membrane structure and the positive precipitin reaction with the PS(S) determinant carried on LPS(S) molecules (Jones and Berman, 1975). In spite of the presence of LPS in SDS-I fraction, an important LPS(S) contamination of the isolated bands was ruled out by the negative result obtained in immunodiffusion analysis of antisera I, II, III with the PS(S) antigen.

Several hypotheses could explain the heterogeneity of bands I and II in SDS-PAGE : firstly, lysozyme when splitting the glycan chain of peptidoglycan left one, two, or more monomers of an approximate molecular weight of 1000 and 2000, ... This may also depend on the molecular weight of the linked proteins because band III did not show this heterogeneity. Secondly, glycan moiety other than monomer of peptidoglycan may also introduce heterogeneity. Thirdly, these proteins may differ by modification of amino acid residue like the protein I of *E. coli* (Ichira and

Mizushima, 1978). Fourthly, in *Salmonella typhimurium* major outer membrane proteins have close molecular weights : 34000, 35000, 36000 (Ames *et al.*, 1974).

These results indicate that isolated bands I, II, III after heating in SDS were immunologically active. Immunologically active proteins have also been isolated after SDS treatment in other bacterial species. Proteins I and II of *E. coli* when reheated at 100 °C in 0.2 % SDS prior to SDS-PAGE retained antigenicity in crossed immunoelectrophoresis (Van Tol *et al.*, 1979). Porin proteins of *Salmonella typhimurium* isolated in the presence of 2 % SDS at 20-37 °C were also found immunogenic in rabbits and antibodies were directed against porins in their native state (Smit and Nikaido, 1978). The lipoprotein of *E. coli* isolated from boiled cell-walls in SDS, when reheated with boiling 4 % SDS and precipitated by acetone reacted strongly with antisera against the native lipoprotein (Braun *et al.*, 1976). In the present study, the isolated bands which were precipitated by acetone were still able to inhibit antibodies against SDS-I fraction in the ELISA test. This indicates that after SDS treatment, either conformational antigenic

sites are recovered or there is a preponderance of sequential antigenic sites.

Various surface components of gram-negative bacteria have been shown to be protective as vaccines. Capsular polysaccharides are now well established as vaccines (Robbins, 1978). Outer membrane proteins of group B meningococci (Craven and Frasch, 1979) and of *S. typhimurium* (Kuusi *et al.*, 1979) have shown promise against experimental infection. In the present study, the three major protein antigens were also protective against experimental infection in mice.

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Summary

The insoluble fraction, SDS-I, obtained by boiling cell-walls of *Brucella abortus* in sodium dodecyl sulfate (SDS) was hydrolyzed by lysozyme. Three major bands of apparent molecular weight 37000 (I), 25000 (II), 15000 (III) were isolated by gel slicing after polyacrylamide gel electrophoresis in the presence of SDS. The three bands were injected subcutaneously to mice using Freund incomplete adjuvant and immunity was tested one month later by intraperitoneal administration of a virulent strain of *B. abortus*. Fifteen days after challenge, numbers of viable brucella within the spleens were determined. The isolated bands I, II and III were able to protect mice at a level corresponding to the killed whole-cells *Brucella melitensis* H38 reference vaccine.

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