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THE CELL WALL AS THE ANTIGENIC SITE FOR ANTIBODIES STIMULATING INGESTION (MSF) AND INHIBITION (BIF) OF BRUCELLA IN MACROPHAGES FROM NORMAL AND IMMUNE ANIMALS

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SUMMARY.—Following extraction with hot trichloracetic acid and digestion with trypsin, deoxyribonuclease and ribonuclease, cell wall residues of *Brucella melitensis* strain Rev I contained an antigenic moiety capable of removing antibodies responsible for stimulation of ingestion, inhibition of Brucella growth by macrophages, and agglutination of Brucella. The preparation was dermonecrotic for both normal and immune rabbits and, when injected in oil, stimulated antibody production. The peptidoglycan-containing structure was degraded by egg-white lysozyme and enzymes from normal and immune macrophages with an accompanying loss of dermonecrotic and serum-absorbing capacity.

In studies of the serum and cellular changes accompanying immunization of the rabbit by the attenuated Rev I strain of *Brucella melitensis* we have shown that soluble factors active in promoting rapid ingestion and protection against streptomycin (macrophage-stimulating factors, MSF) and inhibiting brucella growth in macrophage cultures (Brucella-inhibiting factors, BIF) appear in high titre (Ralston and Elberg, 1968*a*, 1969*a*). Some of these factors, present in heated serum, are active in dilutions exceeding 10^{-5}, suppressing growth for prolonged periods. Separation by gel filtration has revealed both 19S and 7S molecular classes with these biological activities, as well as low molecular weight material (Ralston and Elberg, 1971). The antibodies are induced primarily by the smooth strain of Rev I, to a much less extent by a rough isolate (unpublished), and then only after injection of at least 10-fold greater numbers of cells. Antiserum to the smooth variant shows little MSF or BIF effect on infection of macrophages by the rough strain. Since changes from smooth to rough have been shown to involve materials on the cell wall (Leong, Diaz and Wilson, 1968) the previous data suggested that the attachment site of the antibody is a cell wall component.

We have examined a peptidoglycan (mucopeptide) containing cell wall residue remaining after extraction with hot trichloracetic acid (TCA) and enzyme digestion. The method of extraction, originally described for the preparation of staphylococcal wall mucopeptide had also been applied to the Rev I strain and shown to yield coccoid wall " ghosts ", sensitive to the action of crystalline egg white lysozyme (Ralston and Elberg, 1961*a*). In the present study wall residues were prepared from heat-killed smooth Rev I. Antiserum rich in MSF and BIF activity was absorbed with these walls and tested for residual activity to determine whether it

was possible to separate the 2 activities. The relationship of the antigen(s) to portions of the peptidoglycan of the wall were investigated by testing for residual absorbing capacity after extensive digestion of the walls with egg white lysozyme or with macrophage extracts containing lysozyme-like activity in addition to other enzymes.

Rabbits were tested for their responses to the purified wall preparation with respect to (1) cutaneous responses, (2) the induction of antibodies by walls, and (3) the cutaneous effects of enzyme-digested wall residues and filtrates of fragments dissolved by enzymes on normal and immune rabbits.

MATERIALS AND METHODS

Bacterial strain.—Br. melitensis Rev I was used throughout these experiments. Its origin and maintenance have been described (Alton and Elberg, 1967). For preparations of heat-killed cells, growth from trypticase soy (Baltimore Biological Laboratories, Baltimore, Md.) agar slants was inoculated on TSA for 5 days at 37°. For tests of macrophage infection the strain was cultured on Brucella agar (Albimi Laboratories, Brooklyn, N.Y.) and prepared as described in other reports (Ralston and Elberg, 1968*a*, 1969*a*). The smoothness of the strain was determined by the usual crystal violet and the acriflavine techniques.

Preparation of TCA-extracted, enzyme-digested wall residues (CW).—Smooth Rev I cells were harvested from the surface of TSA agar in saline and steamed for 1 hr at 95°–100°. The cells were washed 6 times in sterile deionized, glass distilled water (GDW). Washed bacilli (65 ml., $5{\cdot}4 \times 10^{11}$/ml.; dry weight = 121 mg./ml.) were mixed with 100 per cent TCA (w/v) to make 5 per cent TCA and heated at 95° for 20 min. The TCA-treated bacilli were washed several times in GDW and finally were suspended in 350 ml. 0·5M phosphate buffer (PB), pH 7·0, and exposed to crystalline ribonuclease 30 μg./ml. and desoxyribonuclease 30 μg./ml. (Worthington Biochemicals, Freehold, N.J.) for 24 hr at 37°, after which $MgCl_2$ was added to 0·001M for a second 24-hr period. At 48 hr, crystalline trypsin (Worthington), 50 μg./ml., was added. The brucella walls were digested for 6 days at 37° until the turbidimetric reading had fallen from a reading of 800–168 on the Klett photometer, representing 7·8 per cent of the original turbidity, as calculated from standard curves derived from direct counts of the unextracted heat-killed bacilli. The walls were washed 6 times (1 hr at 16,000 × *g*) in GDW, 440 ml. The optical densities (O.D.) of the supernatant from the 6th wash were < 0·001 at nm. 280, 260, 240, and 0·065 at 220 nm. The walls were suspended in 100 ml. GDW. Five ml. samples were reheated for 20 min. and stored at 4°. (Dry weight = 4·9 mg./ml.; approximately 6·3 per cent of the starting material by weight).

Digestion of walls (C–W) with lysozyme and lysozyme-like enzymes from macrophages.—Walls were exposed to lysozyme (3·8 μg./ml. 2 × crystallized egg white lysozyme, Worthinton) or macrophage extract (1/100 dilution) in 0·02M KH_2/K_2HPO_4 buffer, pH 7·2. Mixtures containing 0·6 ml. CW, 5·0 ml. buffer, 0·015 ml. lysozyme (1000 μg./ml.) or 0·06 ml. macrophage extract (IE = immune, NE = normal extract) were incubated for 25 hr at 37° in sterile tubes and then at 20° for 28 days. At the end of this period all samples had been reduced approximately 50 per cent in turbidity. Macrophage extracts were prepared from 2×10^8/ml. cells in 0·85 per cent NaCl by sonifying under $N_2 + CO_2$ (Ralston and Elberg, 1969*a*). Immune extracts were made from macrophages collected in the 4th week after primary immunization of rabbits. They were active in lysis of *Micrococcus lysodeikticus* at dilutions of 1/100.

To stain for mucopeptide, Sepraphore electrophoresis strips were wet in Na–acetate, pH 3·9, and towel dried. A 5 mm. loop of undiluted digest was applied and dried. The strip was submersed in Alcian Blue dye (0·5 ml. of a 1 per cent solution Alcian Blue 8 GX added to 15 ml. 0·1N sodium acetate, pH 3·9) for 3 min., then rinsed in 0·1N sodium acetate followed by rinsing in GDW, and dried. The strips were examined for stained areas.

Ultraviolet absorption spectra.—Samples of walls were washed 3 times in 5 ml. GDW and sedimented at 36,000 *g* at 4°. They were diluted 1 : 10 in GDW and read over the range 210–340 nm in a quartz cell, 1 cm. light path, in a model DU Beckman spectrophotometer. There was no peak of absorption at 260 or at 280, indicating that the TCA–enzyme digestion mixture had removed a high percentage of proteins and nucleic acids.

Cutaneous responses in rabbits.—Normal and immunized rabbits (4–5 weeks after primary immunization) were injected i.d. with 0·1 ml. washed walls and wall residues in amounts equivalent to $3{\cdot}3 \times 10^9$ and $3{\cdot}3 \times 10^8$ untreated bacilli. Skin responses were read at 4, 24, and 48 hr with a vernier caliper. Filtrates of walls and wall digests (lysozyme and macrophage extracts) were prepared by passage through 0·22 μ membranes, and injected i.d. in a similar manner.

Serological responses to brucella-wall residues.—Rabbits were given s.c. injections of 0·2ml. untreated cells walls in 2 ml. sterile Klearol, or 2 ml. saline. Serum was collected after 4 weeks by cardiac bleeding. Tests were made for presence of agglutinating antibodies, using the standard *Br. abortus* antigen. The presence of BIF was measured on infected macrophages fixed-to-glass, as previously described (Ralston and Elberg, 1969*a*).

Absorption of antibody with brucella walls (C–W) and wall residues from lysozyme treatment (L–W) and from treatment with extracts of normal macrophages (NE–W) and immune macrophages (IE–W).—Walls ($1{\cdot}75 \times 10^{11}$ in 0·01M PBS + 0·001 $MgCl_2$, pH 7·2) were mixed with 0·05 ml. anti-Rev I serum, 4th week after injection of vaccine (IRS_4) incubated 18 hr at 37° and 48 hr at 4°. One ml. buffer was added and the mixtures were centrifuged at 36,000 *g* for 1 hr at 4°. The supernatant fluids were filtered through 0·45 μ membranes, and the pellets were washed in GDW, resuspended in 0·2 ml. A second absorption of the serum was carried out in a manner similar to the first: $IRS_{absIE-w}$ and IRS_{absC-w} were mixed with IE–W and C–W, respectively, using $1{\cdot}65 \times 10^{11}$ walls/ml. at 1/80 final serum dilution in 0·01M PBS, pH 7·2. The IE–W for this procedure was obtained from a pool of walls lysed in 0·0166M versene in 0·005M PB, pH range 6·5–8·0, until lytic endpoints (40 per cent) had been reached. Determinations of the residual BIF and MSF titres of the absorbed sera were made with brucella-infected normal rabbit macrophages (NRM). Throughout this work dilutions of immune sera were made in heat-inactivated NRS. The residual antibodies were calculated in terms of resistance index or protection index as explained in Table I.

TABLE I.—*Residual Antigenic Sites for MSF and BIF after Digestion of Brucella Walls with Lysozyme and Lytic Enzymes from Normal and Immune Macrophages, as Determined by Tests of Absorbed Sera plus Infected Macrophages*

Absorption of IRS 4 week prior to test	(MSF) Protection Index $\Delta Log_{10}B_{IRS}-\Delta Log_{10}B_{NRS}$* in presence of 50 μg. DHSM		Per cent of unabsorbed control		(BIF) Resistance Index $\Delta Log_{10}B_{NRS}-\Delta Log_{10}B_{IRS}$†		Per cent of unabsorbed control	
	After absorption		Absorbed		After absorption		Absorbed	
	1×	2×	1×	2×	1×	2×	1×	2×
C–W	0·082	0·000	3	0	0·760	0·000	52	0
IE–W	2·068	0·000	82	0	1·353	0·410	92	22
NE–W	1·338	nt	52	nt	1·038	nt	71	nt
L–W	1·362	nt	53	nt	0·924	nt	63	nt
Untreated IRS	2·555	2·459	100	100	1·477	1·868	100	100

* $\Delta Log_{10}B_{IRS}$ designates the net log_{10} increase in brucella (ml. after 3 days, 37°) in IRS at a test dilution equivalent of 1 : 1000. $\Delta Log_{10}B_{NRS}$ designates a similar calculation for infection in NRS.

† $\Delta Log_{10}B_{NRS}$ designates a calculation made as under *, but after 2 days, 37°; similarly for $\Delta Log_{10}B_{IRS}$, at a test dilution of 1 : 1000.

In vitro *tests of effects of walls and wall-digests on macrophages.*—NRM, 5×10^5/ml., were planted on the floor of vials in the presence of 0·1 ml. ($3{\cdot}3 \times 10^9$ wall equivalents) of C–W, IE–W, and L–W in Tyrode solution + heated IRS_3. After 24 hr, the unattached macrophages were removed and $1{\cdot}5 \times 10^6$ brucella were added for 1 hr at 37°. The free brucella were removed and the cell layer was washed; the medium was replaced with corresponding wall and serum mixtures. Survival of macrophages and growth of brucella were tested at 0 hr (1 hr post-infection) and on days 1, 2, by procedures already described (Ralston and Elberg, 1968*a*, 1968*b*), with the exception that intracellular bacilli were released by sonic oscillation, 60 sec. at 100 W, in a Branson sonic disintegrator, Model W1850 (Branson Co., Stamford, Conn.).

RESULTS

Preparation and properties of brucella walls.—A method of hot trichloracetic acid extraction (Ralston and Elberg, 1961*a*) to prepare Rev I wall mucopeptide was modified by additional digestion with deoxyribonuclease and ribonuclease, accompanied by trypsin treatment. Lysis of the TCA-extracted Rev I resulted in a decrease to approximately 7·8 per cent of the original turbidity (heat-killed whole bacilli). Microscopic observation under phase contrast at 1500× revealed barely visible, translucent coccoid bodies. These were stained with Alcian Blue 8 Gx, a dye with affinity for the wall mucopeptide. The walls agglutinated in 1 : 10 IRS prepared against living bacilli, but the rate of visible clumping was much slower than with whole bacilli or with TCA-extracted residues prior to digestion with the above-mentioned enzymes, and was seen most easily by slide agglutination tests. In the absence of serum, 0·001M $MgCl_2$ also caused clumping.

Digestion with lysozyme and macrophage enzymes.—Lysozyme dissolved approximately 40 per cent of the walls in 24 hr and 50 per cent when incubation was extended as long as 28 days. The IE and NE preparations dissolved about 50 per cent within 24 hr. This value was not increased upon further incubation. The wall residues, L–W, IE–W, and NE–W, no longer stained with Alcian Blue, and slightly more material absorbing between 210–250 nm. was released from IE–W than L–W and NE–W.

IE–W no longer showed visible clumping in IRS, whereas NE–W and L–W still reacted, but to a diminished degree, suggesting that the immune extract had removed more of the antigen(s) concerned.

Absorption of Rev I inmune serum with Rev I walls.—A single absorption with C–W removed all antibody responsible for agglutination of *Br. abortus* and reduced the titre of MSF and BIF. A single absorption with IE–W failed to remove much of the antibody concerned in MSF or BIF, as indicated by the activity of the residual serum in macrophage tests (Table I) amounting to 82–92 per cent of the indices for unabsorbed controls. When serum $IRS_{absIE-W}$ was reabsorbed with a fresh preparation of IE–W, most of the remaining activity was removed, indicating that although some of the receptor sites had been digested away by the macrophage enzymes, a significant portion had remained and that all of the MSF and BIF activity in the serum could ultimately be absorbed onto this portion. Single absorptions with L–W and NE–W produced reductions in activity of BIF and MSF but to lesser degrees, indicating that these enzyme preparations also were able to remove a portion of the antigenic sites of antibody attachment. Since egg white lysozyme is known to act on the mucopeptide portion of the bacterial cell wall, the data suggest that solution of this polymer is associated with loss of the receptor sites for MSF and BIF antibodies. However, we also do consider the possibility that the dissolved fragments merely carry away reactive side groups. Cell wall material prepared from the Rev I strain over 10 yr previously still retained the properties described above, indicating the great chemical stability.

In vitro *action of walls on infected macrophages.*—When cell walls were added to macrophages previously infected in immune or normal serum, the walls had no toxic effect on the survival of attached macrophages in normal sera and, in fact, even enhanced the number of viable cells recovered at 48 hr. Immune serum in 1 : 10 dilution was toxic and the addition of L–W and C–W seemingly decreased

this toxicity (Table II). Immune serum remained inhibitory in the presence of IE–W walls, whereas the control walls, C–W, decreased the BIF resistance index approximately 50 per cent. The L–W, like the IE–W, had little effect (Table III). These results confirm the earlier results, suggesting that BIF receptors were partially removed by the enzymes.

TABLE II.—*Effect of Brucella Wall Fractions on Macrophages Infected with Rev I in Heated NRS and IRS*

Sample of walls	Serum source	Macrophage survival cells/ml. × 10^5, day 0	Macrophage survival cells/ml. × 10^5, day 2	Per cent day 2
L–W	NRS	0·84	1·20	143
IE–W	NRS	1·34	1·58	117
C–W	NRS	1·06	1·16	109
No walls	NRS	0·62	0·62	100
L–W	IRS*	0·60	0·44	74
IE–W	IRS	1·92	0·78	41
C–W	IRS	1·45	1·04	72
No walls	IRS	1·28	0·58	46

* 3 week IRS diluted 1/10 with NRS used in this experiment.

TABLE III.—*Effect of Rev I Walls (C–W, L–W, IE–W) on Infection in NRM in Presence of NRS and IRS*3 week

Wall sample ($3·3 \times 10^9$/ml.)	NRM + IRS (BIF) resistance index, Day 2	NRM + IRS (BIF) resistance index, Per cent of IRS	NRM + NRS (BIF) resistance index day 2
C–W	0·6828	51	<0·0000
IE–W	1·2484	94	0·0060
L–W	1·0812	82	0·3573
IRS	1·3162	100	—

Cutaneous responses to brucella walls (C–W, IE–W, NE–W, and L–W).—The methods of extraction produced toxic residues from strain Rev I. Injection of rabbits i.d. with C–W produced erythematous lesions in 4 hr which persisted for several days. Both immune and normal animals were sensitive. There was no indication of a delayed hypersensitivity response.

After treatment with lysozyme or the macrophage extracts, approximately 50 per cent of the turbidity was removed together with the bulk of the toxicity. Most of the residual toxicity was found in the larger-sized sedimentable fragments, which showed approximately 1/10th the activity of C–W (Table IV). The enzyme treatments dissolved fragments able to pass through 0·22 μ filters. These produced small sized erythematous reactions that disappeared within 48 hr. It may be significant that all the wall digest residues produced slightly larger-sized lesions on immune animals than on normal animals. In tests with IE–W, and especially with C–W, the erythema in immune animals increased between 24–48 hr, in contrast to all others. The C–W suspensions did not contain detectable amounts of soluble toxic material since their filtrates produced no lesions, which also serve as indication that the aqueous medium used to suspend the walls contained no toxic materials.

TABLE IV.—*Cutaneous Responses of Rabbits to Rev I Walls (C–W), to Enzyme-Digested Wall residues (L–W, IE–W, and NE–W), and to Soluble Materials from Wall Residue*

Rabbit used for test	Skin test antigen treatment	fraction	Response to dose 1 ($3{\cdot}3 \times 10^9$ wall equivalents) Erythema* at hr 24 (mm.² × 0·785)	Erythema at hr 48	Induration at hr 24 (mm.)	Induration at hr 48	Response to dose 2 ($3{\cdot}3 \times 10^8$ wall equivalents) Erythema at hr 24 (mm.² × 3·14)	Erythema at hr 48	Induration at hr 24	Induration at hr 48
IRM	L	Residue	90	59	0	0	0	0	0	0
		Filtrate	72	0	0	0	0	0	0	0
NRM	L	Residue	81	67	0	0	0	0	0	0
		Filtrate	36	0	0	±	0	0	0	0
IRM	IE	Residue	90	100	0·25	0	0	0	0	0
		Filtrate	64	0	0	0	0	0	0	0
NRM	IE	Residue	81	22	0·10	0	0	0	0	0
		Filtrate	64	0	0·50	±	0	0	0	0
IRM	NE	Residue	132	40	0	0·25	0	0	0	0
		Filtrate	63	0	0	0	0	0	0	0
NRM	NE	Residue	48	25	0	0	0	0	0	0
		Filtrate	36	0	0	0	0	0	0	0
IRM	Control	Residue	144	250	1·50	1·50	168	271	0·50	0·25
		Filtrate	0	0	0	0	0	0	0	0
NRM	Control	Residue	405	208	2	1·25	196	56	0·75	0·25
		Filtrate	0	0	0	0	0	0	0	0

* Erythema calculated from: Area, sq. mm. = 3·14 × (D − d)/4, where D and d represent diameters of the greatest and smallest dimensions, respectively.

We found that induration was most marked in response to C–W walls. Following enzyme treatment the capacity to produce induration was greatly decreased.

Injection of $3{\cdot}3 \times 10^{10}$ C–W only produced agglutinating antibodies when incorporated in sterile mineral oil. Following i.d. injection of walls in 2 ml. Klearol, antiserum inhibited brucella growth at greater than 1/1000 dilution and agglutinated *Br. abortus* at titres exceeding 80.

DISCUSSION

Hot TCA removed more endotoxic material from brucella cells than is removed by the Boivin extraction technique, which is usually carried out in the cold (Boivin and Mesrobeaunu, 1935). A toxic residue remained in the insoluble fraction of wall structures sedimenting at 16,000 × *g* in 60 min. (almost all the wall material in the TCA extract could be sedimented in 15 min. at 10,000 *g*, except for a faintly turbid fraction which was brought down at the higher speed).

The wall residue, part of which may be dissolved by lysozyme, may consist of a mucopeptide linked with some other non-protein substrate. We have made no efforts to remove lipids or 100 per cent of the lipopolysaccharides so that their existence in the Rev I wall residue remains a distinct possibility. Recent studies of *Mycobacterium tuberculosis* described a lipopolysaccharide-peptidoglycan polymer, resisting destruction with hot phenol at 68°, and digestion with pronase. It was dissociated with difficulty, requiring refluxing with 2·5 per cent KOH in methanol benzene solvent (Kanetsuna, 1968).

Since purified egg white lysozyme is an endo-N-acetylmuramidase hydrolysing the β1–4 linkages between N-acetylmuramic acid and N-acetylglucosamine of the glycan structure common to many bacterial species, its lysis of the Rev I material may be expected to release fragments of glycan polymers broken at sites available to the enzyme. These in turn may carry other structures, some or all of which may be involved in producing the toxic responses of the 0·22 μm. filtrates. Recent studies of radioactively labelled *Br. abortus* endotoxin has identified activity with a lipopolysaccharide-containing, trypsin resistant, slow-diffusing portion of the smooth wall (Leong, *et al.*, 1968).

Cell walls prepared from other bacteria so as to retain mucopeptide layers have also been shown to produce toxic skin reactions (Abdulla and Schwab, 1966) and to inhibit phagocytosis and growth of tissue culture cells (Jones and Schwab, 1970). In the case of streptococci, the toxicity was increased with the removal of specific polysaccharide bound to mucopeptide (Jones and Schwab, 1970). Whether a similar structure is involved in the Rev I wall remains to be investigated. It is assumed to contain at least 5 typical components of gram-negative wall mucopeptide now reported in brucella: glucosamine, muramic acid, alanine, glutamic acid and 2,6-diaminopimelic acid (Mardarowicz, 1966).

A number of reports have ascribed immunogenic properties to brucella walls (Keppie, Witt and Smith, 1963; Markenson, Sulitzeanu and Olifzki, 1962; Rasooly, Boros and Gerichter, 1968) including a certain degree of protection against virulent challenge. Unfortunately chemical analysis of the brucella wall is very fragmentary. Consequently, until comparative studies are available, it is impossible to be certain what structures pertain to the wall, become absorbed to the wall during extraction, or result from reaction with materials used for their preparation. Our report merely describes a method for producing a mucopeptide-containing wall fragment, which carries sites of attachment of antibodies whose biological activity includes (a) the capacity to enhance ingestion of brucella to streptomycin-protected loci within macrophages, (b) an ability to restruct brucella growth, and (c) the ability to produce agglutination of smooth standard abortus antigen. Absorption of smooth antisera made against living Rev I with the wall preparation removed antibody responsible for agglutination of whole *Br. abortus* as well as substances responsible for macrophage stimulation and brucella inhibition (MSF and BIF).

Exposure of the wall suspension to extracts of immune macrophages (IE) removed a receptor for MSF and BIF more effectively than lysozyme or extracts from normal macrophages (NE). The immune extract had been prepared from rabbits immunized for 4 weeks and was growth-inhibitory for Brucella under anaerobic conditions, when combined with serum (Ralston and Elberg, 1969*b*). In the rabbit a minimal generalized macrophage activation is detectable at this time (unpublished), suggesting that the IE contained slightly more active enzyme, or possibly that a second enzyme had been increased.

In the past we have speculated that one mode of attack on brucella in the immune defence might be a step-wise degradation of the cell wall, first by agents rendering the wall (sites) available to lytic enzymes, and secondly by the action of macrophage enzymes on the wall (Ralston and Elberg, 1961*a*, *b*). While still in the realm of speculation, this theory remains compatible with the present data and is in accord with the recent studies of *Br. suis* in immune macrophages (McGhee and Freeman, 1970).

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