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Mitogenic Activity of Cell Wall Components from Smooth and Rough Strains of *Brucella abortus*

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On the basis of [^{3}H]thymidine incorporation by normal mouse spleen cell cultures, cell wall preparations from a smooth (45/0) strain and a rough (45/20) strain of *Brucella abortus* were strongly mitogenic. On the other hand, none of several subcomponents extracted from the cell wall preparations, including aqueous and phenolic lipopolysaccharides, was active. These results contrast with the marked mitogenic activity of lipopolysaccharides isolated from other gram-negative bacteria such as *Salmonella typhimurium*.

A variety of substances, including lipopolysaccharides isolated from gram-negative bacteria, have been shown to activate some mammalian lymphocytes (7) and have proven useful for identifying the role of subgroups of lymphocyte in the immune response (8, 12, 17). The host response to *Brucella* infection is both humorally and cellularly mediated, with the latter more important in clearing the infection and conferring resistance (6). Thus, the host defensive response is similar to that observed with other facultative intracellular parasites and may involve activation of both T and B lymphocytes. Since *Brucella* has a mosaic of surface components common to gram-negative bacteria yet is distinct from the acid-fast tubercle bacillus and *Listeria monocytogenes*, it was of interest to determine whether cell walls of *Brucella* could activate lymphocytes and whether any surface components could be correlated with the activation of subgroups of lymphocytes.

A smooth strain of *Brucella abortus* (45/0) and a non-smooth, isogenic variant (45/20) were used throughout these experiments. The two strains differ with respect to virulence, colonial morphology, agglutinability, and phase adsorption. The present studies demonstrate that crude cell wall preparations from both strains of *B. abortus* induced significant [^{3}H]thymidine incorporation by cultured mouse spleen cells, but isolated constituents of these cell wall preparations (e.g., lipopolysaccharides, mucopeptide-lipoprotein) did not. These data differ from those obtained with isolated wall components from other gram-negative bacteria, some of which are mitogens for B lymphocytes (11).

MATERIALS AND METHODS

Preparation of cell walls and cell wall fractions. *B. abortus* strains 45/0 and 45/20 were grown and harvested as described by Robertson and McCullough (16). Cell walls were isolated from both strains of *B. abortus* by a modification of the procedure of Braun (3). Briefly, bacterial cells were suspended in distilled water (absorbancy at 620 nm of 40), and 1.0 mg of deoxyribonuclease was added per 50 g of cells. The cells were then disrupted by using a Bronwill MSK cell homogenizer (Bronwill Scientific Inc., Rochester, N.Y.) as described by Robertson and McCullough (15), and the cell homogenate was centrifuged at 9,000 × *g* for 1 h. The pellet was washed three times with distilled water, lyophilized, and stored at −20°C.

Complexes of murein-lipoprotein were isolated from both strains of *B. abortus* by the procedure of Braun (3). One gram of cell walls was suspended in 1.0 ml of deionized water, followed by the addition of 6 volumes of boiling 4% sodium dodecyl sulfate. The suspension was stirred overnight at room temperature and then centrifuged at 78,000 × *g* for 20 min. The pelleted complex of murein-lipoprotein was washed three times with distilled water and stored at −20°C until use.

Lipopolysacchrides (LPS) were isolated from both strains of *B. abortus* by the phenol-water procedure of Westphal as modified by Raff and Wheat (13). LPS were purified from both the aqueous and phenolic extraction phases of *B. abortus* 45/0, but only aqueous LPS were isolated from *B. abortus* 45/20. Phospholipids and fatty acids were extracted from both strains of *Brucella* by the procedure of Nelson and Buller (10).

Mice. Male and female CF-1 mice (20 to 25 g) were obtained from Carworth Farms (Boston, Mass.) and were maintained on Purina lab chow and water ad libitum.

Cell culture procedures. Culture medium was prepared by addition of 5% heat-inactivated fetal calf serum, sodium pyruvate, nonessential amino acids, antibiotics, and L-glutamine to RPMI 1640 (Grand Island Biological Co., Grand Island, N.Y.). Mice were sacrificed with ether, and the spleens were removed and placed in cold Hanks balanced salt solution. Single cell suspensions were prepared

by homogenization in a sterile Ten Broeck glass homogenizer, and the cells were collected by centrifugation (150 × g). The cell pellet was washed once with cold 0.83% ammonium chloride, to lyse contaminating erythrocytes, and once with Hanks balanced salt solution, and then it was suspended in supplemented RPMI 1640. The cell suspension was then passed through sterile, fine-mesh nylon cloth to remove large debris and cell aggregates. Cell suspensions were assayed for viability within 5 min by trypan blue exclusion and were adjusted to a final density of 1×10^7 viable cells per ml. Finally, cell cultures were established by seeding 1.0 ml (10^7 cells) of the spleen cell suspension into prewashed, sterile glass scintillation vials (petite vials, Demuth Glass Co., Rockaway, N.J.). Experimental cultures received varying amounts of the bacterial preparations being tested for mitogenic activity. Control cultures received no additions. The spleen cell cultures were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2 for 48 h.

Mitogenicity assays. The mitogenic activity of the various preparations on spleen cell cultures was measured by the incorporation of [^{3}H]thymidine. Twenty-four hours prior to harvest, each culture received 1.0 μCi of [^{3}H]thymidine (2.0 Ci/mol; Amersham/Searle Corp., Arlington Heights, Ill.). Incubations were terminated by the addition of 5.0 ml of cold 5% trichloroacetic acid, and the resulting precipitate was washed twice with cold trichloroacetic acid and twice with absolute methanol. The trichloroacetic acid-insoluble precipitate was solubilized with 0.2 ml of NCS solubilizer (New England Nuclear Corp., Boston, Mass.), followed by the addition of 4.0 ml of liquid scintillation cocktail consisting of 2,5-diphenyloxazole and 1,4-bis-(5-phenyloxazolyl)benzene in toluene (all from New England Nuclear Corp.). Precipitating, washing, and radioactivity counting of the cultures were performed in the vials used for the spleen cell cultures. LPS isolated from *Salmonella typhimurium* were used as positive mitogenicity controls throughout these studies (11). The mitogenic response of cultured mouse spleen cells to the various *B. abortus* cell wall preparations and *S. typhimurium* LPS was based on a mitogenic index for triplicate cultures. The index, *E/C*, was calculated as:

$$E/C = \frac{\text{cpm in experimental culture}}{\text{cpm in control culture}}$$

Indexes greater than three were considered significant.

Biochemical assays. The protein content of the bacterial cell wall preparations was determined by the microbiuret method (2), and carbohydrate content was specified by the phenol-sulfuric acid assay (5). 2-Keto-3-deoxyoctonate and dideoxyaldoses were measured by the method of Ashwell (1). Muramic acid was determined by the procedure of Hadzija (9).

Reagents. All reagents and solvents were analytical grade. *S. typhimurium* LPS was obtained from Difco Laboratories (Detroit, Mich.), and fetal calf serum was purchased from International Scientific Industries (Cary, Ill.). Deoxyribonuclease was purchased from Sigma Chemical Co. (St. Louis, Mo.).

RESULTS

Chemical analysis of isolated cell walls. Cell wall preparations isolated from the two strains of *B. abortus* contained similar levels of carbohydrate, 2-keto-3-deoxyoctonate, and muramic acid (Table 1). Cell walls of *B. abortus* 45/0 had a higher level of dideoxyaldoses, which are usually found in the polysaccharide moiety of LPS. The differences in protein levels and dry-weight percentages between the two strains are of questionable significance.

Effect of *Brucella* cell wall preparations on incorporation of [^{3}H]thymidine by spleen cells. Cell wall preparations isolated from both strains of *B. abortus* were strongly mitogenic for CF-1 mouse spleen cells (Table 2). The responses had similar dose dependencies, with an optimal incorporation of [^{3}H]thymidine at cell wall concentrations of 50 to 100 μg per culture. Suppression of [^{3}H]thymidine incorporation was observed using 250 to 500 μg of either cell wall preparation. Treatment with Pronase or boiling did not affect the mitogenicity of the cell wall preparations.

S. typhimurium LPS displayed optimal mitogenic concentrations between 10 and 250 μg per culture, with stimulation indexes ranging between four and eleven.

Isolated components. The mitogenic activity of several isolated cell wall components was

TABLE 1. *Chemical properties of cell walls isolated from B. abortus 45/0 and 45/20*[a]

Strains	Cell walls (mg/100 mg of whole cells)	Cell wall carbohydrate (hexose) (μg/mg of cell wall)	KDO[b] (μmol/mg of cell wall)	Dideoxyaldose (cell wall)[c] (μmol of KDO[b] [cell wall])	Muramic acid (μg/mg of cell wall)	Cell wall protein (μg/mg of cell wall)
45/0	21.0	140.0	0.012	7.0	4.6	370.0
45/20	14.0	129.0	0.013	1.8	4.0	475.0

[a] All values expressed as dry weight.
[b] KDO, 2-Keto-3-deoxyoctonate.
[c] Relative absorbancy at 532 nm.

examined in an attempt to identify the mitogenic factor(s) present in cell walls of *B. abortus*. Neither aqueous nor phenolic LPS extracts isolated from either strain of *B. abortus* had mitogenic activity when used at levels of 1.0 to 500 μg per culture (Table 3). In fact, in some experiments LPS of *B. abortus* 45/0 sharply suppressed [^{3}H]thymidine incorporation. Likewise, mucopeptide-lipoprotein complexes and lipid fatty acids from cell walls of either strain had no activity (Tables 4 and 5).

DISCUSSION

Cell wall preparations from a smooth, intermediate strain (45/0) and a rough strain (45/20) of *B. abortus* contained a factor(s) that stimulated the uptake of [^{3}H]thymidine into mouse spleen cells. It is not known yet whether more than one mitogenic factor exists in these preparations. Isolated subcomponents of *Brucella* cell walls were not mitogenic when tested over a wide dose range of one-tenth to 1,000-fold the concentration of cell walls that yielded optimal stimulation.

The lack of mitogenic activity in these isolated subcomponents may have resulted from a destruction of the active factor(s) by the extraction procedures used to isolate the individual components. It is also possible that the activity of the cell wall preparation is due to multiple surface components, which individually are not mitogenic but are mitogenic when present collectively in a unique structural conformation (e.g., perhaps a critical association of LPS, protein, and phospholipids). Although not tested here, reconstitution of the mitogenic activity might be achieved by appropriate mixing and reassociation of the isolated cell wall components.

In any case, it is curious that LPS from either strain of *Brucella* were not mitogenic for cultured mouse spleen cells, which contrasts sharply with the positive activity demonstrated for LPS isolated from other gram-negative bac-

TABLE 2. *Mitogenic response of mouse spleen cells to cell walls isolated from B. abortus 45/0 and 45/20*[a]

Dose[b] (μg)	45/0 cell walls			45/20 cell walls		
	1[c]	2	3	1	2	3
0	1.0	1.0	1.0	1.0	1.0	1.0
1	0.6	1.1	1.5	1.3	3.6	2.0
10	2.5	1.4	2.7	3.3	7.1	2.8
50	6.3	7.7	4.0	6.7	ND[d]	2.5
100	7.0	6.4	4.4	5.9	14.9	3.0
250	7.0	2.3	3.6	5.4	2.0	3.0

[a] All values expressed as *E/C*.
[b] Micrograms of dry weight per spleen cell culture (10^7 viable cells).
[c] Number of experiment run.
[d] ND, Not determined.

TABLE 3. *Mitogenic response of mouse spleen cells to LPS isolated from B. abortus 45/0 and 45/20*[a]

Dose[b] (μg)	45/0 WL[c]		45/0 PL[d]		45/20 WL	
	1[e]	2	1	2	1	2
0	1.0	1.0	1.0	1.0	1.0	1.0
1	1.0	0.4	1.0	0.6	0.9	0.5
10	0.9	0.6	1.0	0.6	1.1	1.2
50	1.2	0.6	1.0	0.6	0.8	1.7
100	1.4	0.4	1.1	0.4	0.8	1.7
250	1.2	0.1	0.7	0.2	1.3	1.0
500	ND[f]	0.1	ND	0.1	ND	0.6

[a] All values expressed as *E/C*.
[b] Micrograms of dry weight per spleen cell culture (10^7 viable cells).
[c] Water-Light extraction phase.
[d] Phenol-Light extraction phase.
[e] Number of experiment run.
[f] ND, Not determined.

TABLE 4. *Mitogenic response of mouse spleen cells to mucopeptide-lipoprotein complexes isolated from B. abortus 45/0 and 45/20*[a]

Dose[b] (μg)	45/0 MULP[c]			45/20 MULP		
	1[d]	2	3	1	2	3
0	1.0	1.0	1.0	1.0	1.0	1.0
1	1.0	0.6	0.9	0.8	1.4	0.8
10	1.1	1.7	1.0	0.8	1.9	0.8
50	1.2	1.5	1.3	1.0	2.7	0.9
100	1.5	1.3	1.5	1.3	1.8	1.0
250	ND[e]	0.4	1.7	1.1	0.6	1.2

[a] All values expressed as *E/C*.
[b] Micrograms of dry weight per spleen cell culture (10^7 viable cells).
[c] MULP, murein-lipoprotein.
[d] Number of experiment run.
[e] ND, Not determined.

TABLE 5. *Mitogenic response of mouse spleen cells to lipid fatty acids isolated from B. abortus 45/0 and 45/20*[a]

Dose[b] (μg)	45/0		45/20	
	1[c]	2	1	2
50	ND[d]	1.8	ND	1.9
100	ND	1.8	ND	1.9
200	0.7	1.9	0.5	1.9
400	0.7	1.9	0.6	1.9
1,000	0.6	0.7	0.6	1.1
2,000	0.4	ND	0.2	ND
10,000	0.3	0.2	0.1	0.1

[a] All values expressed as *E/C*.
[b] Micrograms of dry weight per spleen cell culture (10^7 viable cells).
[c] Number of experiment run.
[d] ND, Not determined.

teria (7, 8, 11). In a related study, Renoux (14) demonstrated that *Brucella* LPS (endotoxins) did not increase the numbers of antibody-forming cells in the spleens of mice injected with sheep erythrocytes (i.e., no adjuvant activity). Nevertheless, the cell walls of *Brucella* are mitogenic, though by some structure(s) other than LPS alone, and that activity may trigger host defense mechanisms, probably those involving cell-mediated mechanisms (6). Release of lymphokines by sensitized lymphocytes or those activated by the mitogen(s) in the *Brucella* cell wall could result in an activation of macrophages to increase their brucellicidal activity. An analogous theory has been proposed for this mechanism involving the gram-positive intracellular parasite *Listeria monocytogenes* (4). In this context, it may be important to appreciate that the LPS isolated from the virulent strain of *B. abortus* 45/0 sometimes suppressed [^{3}H]thymidine incorporation by spleen cells below control values, whereas the LPS isolated from the genetically related 45/20 strain (which has lowered virulence) did not. The mechanism of this suppression is not known. But this activity of the LPS of the virulent *Brucella* strain 45/0 may be a possible virulence factor in *Brucella* infections.

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