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Surface Antigens of Smooth Brucellae

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Surface antigens of smooth brucellae were extracted by ether-water, phenol-water, trichloroacetic acid, and saline and examined by immunoelectrophoresis and gel diffusion with antisera from infected and immunized rabbits. Ether-water extracts of *Brucella melitensis* contained a lipopolysaccharide protein component, which was specific for the surface of smooth brucellae and was correlated with the M agglutinogen of Wilson and Miles, a polysaccharide protein component devoid of lipid which was not restricted to the surface of smooth brucellae and was not correlated with the smooth agglutinogen (component 1), and several protein components which were associated with internal antigens of rough and smooth brucellae. Immunoelectrophoretic analysis of ether-water extracts of *B. abortus* revealed only two components, a lipopolysaccharide protein component, which was correlated with the A agglutinogen, and component 1. Component 1 from *B. melitensis* and *B. abortus* showed identity in gel diffusion tests, whereas component M from *B. melitensis* and component A from *B. abortus* showed partial identity with unabsorbed antisera and no cross-reactions with monospecific sera. Attempts to prepare monospecific sera directly by immunization of rabbits with cell walls or ether-water extracts were unsuccessful. Absorption of antisera with heavy fraction of ether-water extracts did not always result in monospecific sera. It was concluded (as has been described before) that the A and M antigens are present on a single antigenic complex, in different proportions depending upon the species and biotype, and that this component is a lipopolysaccharide protein complex of high molecular weight that diffuses poorly through agar gel. Components 1, A, and M were also demonstrated in trichloroacetic acid and phenol-water extracts. With all extracts, *B. melitensis* antigen showed greater diffusibility in agar than *B. abortus* antigens. After mild acid hydrolysis, *B. abortus* ether-water extract was able to diffuse more readily.

At least 24 antigenic components were demonstrated in a water extract from *Brucella suis* analyzed by immunoelectrophoresis; the number of components was dependent upon the antigenic preparation and the immune serum employed (7). No relationship was observed between the components in the water extract and the agglutinogen of *B. suis* or *B. abortus* (8, 20). In contrast, one band of precipitation developed by ether-water extract of *B. abortus* and *B. melitensis* when the gel diffusion technique was used could be related to the smooth agglutinogen (8, 10). Leong et al. (13) showed that this component carried the endotoxic activity.

M. S. Redfearn (Ph.D. Thesis, Univ. of Wisconsin, Madison, 1960) found that a phenol extract from *B. melitensis* developed a band when reacted with monospecific *B. melitensis* serum but not with monospecific *B. abortus* serum. The same preparation of *B. abortus* did not react with monospecific serum until it was partially hydrolized with acetic acid, when it produced a clear band with monospecific *B. abortus* serum.

In this study, we have tried to localize the surface antigen or antigens of *B. abortus* and *B. melitensis* and to study their relationship with the A and M antigens postulated by Wilson and Miles (22) and to clarify the differences between smooth and rough *Brucella*.

MATERIALS AND METHODS

Antigenic preparations. Strains of brucellae employed in this study have been described previously (1, 9, 10). Unless otherwise stated, the *B. melitensis* (M) culture was smooth 16M, and the *B. abortus* (A) culture was smooth 2308. Ether-water extracts (EW-M and EW-A) were prepared according to the method of Ribi et al. (19). Ether-water extracts were centrifuged at 60,000 × *g*, as described by Leong et al. (13), and the pellets were designated "heavy fraction" of endotoxin (EW-M-HF and EW-A-HF) in the present study. Soluble antigen (SA) and crude cell walls (CW) from ultrasonically treated cells were

prepared as described previously (13), except that cell walls were not treated with trypsin and nuclease unless designated as CW-ENZ. Trichloroacetic acid extracts were prepared by the method of Boivin and Mesrobeanu (5) as employed by Baker et al. (2). Hot phenol-water extracts were prepared by the method developed for *Brucella* by M. S. Redfearn (Ph.D. Thesis, Univ. of Wisconsin, Madison, 1960) and described by Baker et al. (2). Saline extracts were prepared by harvesting agar grown cells in saline, treating cells at 60 C for 2 hr with continuous shaking, centrifuging, dialyzing the supernatant fluid against distilled water, and lyophilizing.

Preparation of immune sera. Sera from infected rabbits were taken each week for 5 weeks after intravenous inoculation of 5×10^9 viable organisms of smooth *B. melitensis* 16M, smooth *B. abortus* 2308, rough *B. melitensis* B115, or rough *B. ovis*. Two rabbits were injected with each strain, and the sera were pooled at each bleeding time.

Immune sera against cell walls were prepared by suspending lyophilized CW-M or CW-A in saline (1 mg/ml) and injecting 1 ml intravenously, followed by two injections of 1 ml each given intraperitoneally, within 1 week. The rabbits were bled 1 week after the last injection.

Immune sera against EW-M-HF and EW-A-HF were prepared by injecting 10-lb (4.5-kg) rabbits intravenously with 1 mg of lyophilized antigen in saline. No deaths occurred after injection, but the rabbits showed toxic reactions. They were bled at 3, 7 and 15 days.

Monospecific sera were prepared, as described by Jones (11), by absorption of serum obtained 7 and 9 days after infection with living *B. abortus* or *B. melitensis*. Heat-killed, whole cell antigens were employed to absorb the heterologous antibodies; i.e., killed *B. abortus* cells were mixed with sera from rabbits infected with *B. melitensis*, and killed *B. melitensis* cells were mixed with sera from rabbits infected with *B. abortus*. Proportions of 1 ml of packed cells to 10 ml of undiluted serum were employed. The mixtures were maintained at 37 C for 2 hr with frequent shaking and centrifuged to remove the cells. Sera with agglutinin titers of more than 1:320 for the homologous antigen and less than 1:20 for the heterologous antigen were pooled, Seitz-filtered, and lyophilized in 2 ml volumes. They were reconstituted in 0.5 ml for gel diffusion tests.

Immunological methods. The methods employed for agglutination, gel diffusion, and immunoelectrophoresis have been described previously (9). For experiments, sera were absorbed with 0.5 ml of packed cells per ml of serum, or 50 mg of lyophilized SA or CW-ENZ per ml of serum (Tables 1 and 2). The mixture was placed on a shaker at 37 C for 2 hr and then centrifuged at 5,500 $\times$ *g*. The supernatant fluid was tested in gel diffusion and agglutination tests. For experiments (Tables 3 and 4), lyophilized EW-A-HF and EW-M-HF were suspended in distilled water and diluted, giving the concentrations shown. These were added to equal volumes of serum which had been lyophilized and reconstituted in one-half the original volume. After 2 hr of absorption at 37 C on a shaker, the mixtures were centrifuged and the supernatant liquid was tested in gel diffusion with EW-A and EW-M and in agglutination tests with *B. abortus* and *B. melitensis* whole cell antigens.

Staining procedures. Thiazine Red R was employed as described by Kagan et al. (12) for the staining of proteins. Oil Red O was employed for staining of lipoprotein, and the periodic-acid-Schiff reaction was used for staining glycoproteins and polysaccharides according to the methods described by Uriel et al. (21).

TABLE 1. *Correlation of absorption of precipitins with absorption of agglutinins from various antisera*

Antigen for absorption	Serum from rabbits infected with *B. melitensis*					Serum from rabbits infected with *B. abortus*			Anti-CW-M sera		Anti-CW-A sera	
	Precipitation lines with EW-M				Agglutination titer[a]	Precipitation lines with EW-A		Agglutination titer[a]	Precipitation line with EW-M / M	Agglutination titer[a]	Precipitation line with EW-A / A	Agglutination titer[a]
	M	1	2	3		A	1					
None	+	+	+	+	1:5,120	+	+	1:5,120	+	1:1,280	+	1:1,280
Living *B. melitensis* smooth	−	−	+	−	<1:20	−	−	<1:20	−	<1:20	−	<1:20
Living *B. melitensis* rough	+	+	−	−	1:5,120	+	+	1:5,120	+	1:1,280	+	1:1,280
Living *B. abortus* smooth	−	−	+	−	<1:20	−	−	<1:20	−	<1:20	−	<1:20
Living *B. abortus* rough	+	+	+	−	1:5,120	+	+	1:5,120	+	1:1,280	+	1:1,280
Living *B. ovis*	+	+	−	+	1:5,120	+	+	1:5,120	+	1:1,280	+	1:1,280
Living *B. canis*	+	+	+	−	1:5,120	+	+	1:5,120	+	1:1,280	+	1:1,280

[a] Standard *B. abortus* tube test antigen was employed.

RESULTS

Immunoelectrophoretic analysis of ether-water extracts from B. melitensis and B. abortus. Figure 1 is a diagrammatic illustration of the precipitin lines produced in immunoelectrophoresis with EW-M and various antisera. Monospecific *B. melitensis* serum produced a line (M) near the antigen well which was not produced with monospecific *B. abortus* serum. Line M was produced by unabsorbed anti-cell wall sera and sera from rabbits infected with *B. melitensis* or *B. abortus*. An additional line (component 1) was produced by sera from rabbits infected with *B. abortus*. Sera from rabbits infected with *B. melitensis* produced M and 1 (strong lines) and lines 2 and 3 which were weaker.

When EW-A was examined with these same antisera (Fig. 2), a precipitation line was produced by monospecific *B. abortus* serum but not by monospecific *B. melitensis* serum. This component was designated A. Unabsorbed anti-cell wall sera and sera from infected rabbits developed line A, and the latter sera also developed an additional line designated component 1.

Staining reactions showed that all lines were stained with Thiazine Red R, indicating the presence of protein. Lines M and 1 from EW-M and lines A and 1 from EW-A were stained with Schiff's reagent, indicating the presence of polysaccharide. The lipid stain Oil Red R was taken up only by lines A and M. This suggested that A and M were lipopolysaccharide protein complexes, whereas component 1 was a polysaccharide protein complex.

TABLE 2. *Absorption of precipitins*[a]

Antigen for absorption	Serum from rabbits infected with *B. melitensis*				Serum from rabbits infected with *B. abortus*	
	M	1	2	3	A	1
None	+	+	+	+	+	+
SA, *B. melitensis* smooth 16M	−	−	−	−	−	−
SA, *B. melitensis* rough B115	+	−	−	−	+	−
SA, *B. abortusis* smooth 2308	−	−	−	−	−	−
SA, *B. abortus* rough 2308	+	+	−	−	+	+
SA, *B. ovis*	+	+	−	−	+	+
SA, *B. canis*	+	+	−	−	+	+
SA, *B. abortus* smooth 11	−	−	−	−	−	−
CW, ENZ *B. abortus* smooth 11	−	−	+	+	−	−

[a] Results show presence or absence of precipitation lines with EWM or EWA for serum from rabbits infected with *B. melitensis* or *B. abortus*, respectively.

TABLE 3. *Sera from rabbits infected with B. melitensis absorbed with different amounts of the heavy fraction of ether-water extract from B. abortus or B. melitensis and examined in gel diffusion and agglutination tests*

Amt of absorbing antigen	Absorbed with EW-A-HF					Absorbed with EW-M-HF				
	Agglutination titer with antigens[a]		Precipitation lines[b]			Agglutination titer with antigens[a]		Precipitation lines[b]		
	A	M	A	M	1	A	M	A	M	1
(*mg/ml*)										
20	—	160	−	+	−	ND[c]			ND	
10	—	160	−	+	−	20	—	−	−	−
5	−	320	−	+	−	80	40	−	−	−
2.5	80	640	−	+	−	160	160	−	−	−
1.25	320	2,560	±	+	−	320	640	+	+	−
0.625	1,280	5,120	+	+	−	640	1,280	+	+	−
0.312	1,280	5,120	+	+	−	1,280	1,280	+	+	−
0.155	2,560	5,120	+	+	+	1,280	2,560	+	+	−
0.078	2,560	5,120	+	+	+	2,560	2,560	+	+	+
0.039	2,560	5,120	+	+	+	2,560	2,560	+	+	+
0.019	ND			ND		2,560	2,560	+	+	+
0	2,560	5,120				2,560	5,120			

[a] Whole cell antigens prepared from *B. abortus* or *B. melitensis*, respectively.

[b] EW-A was employed for production of line A, and EW-M was employed for production of line M. Component 1 could be produced with both antigens.

[c] Not determined.

TABLE 4. *Sera from rabbits infected with B. abortus absorbed with different amounts of the heavy fraction of ether-water extracts from B. abortus or B. melitensis and examined in gel diffusion and agglutination tests*

Amt of absorbing antigen	Absorbed with EW-A-HF					Absorbed with EW-M-HF				
	Agglutination titer with antigens[a]		Precipitation lines[b]			Agglutination titer with antigens[a]		Precipitation lines[b]		
	A	M	A	M	1	A	M	A	M	1
(*mg/ml*)										
20	40	–	–	–	–	320	20	+	–	–
10	160	80	–	–	–	320	160	+	±	–
5	320	320	+	+	–	1,280	320	+	+	–
2.5	640	640	+	+	–	1,280	1,280	+	+	–
1.25	2,560	1,280	+	+	–	2,560	1,280	+	+	–
0.625	2,560	2,560	+	+	–	2,560	2,560	+	+	–
0.312	2,560	2,560	+	+	–	2,560	2,560	+	+	–
0.155	5,120	2,560	+	+	–	5,120	2,560	+	+	–
0.078	5,120	2,560	+	+	–	5,120	2,560	+	+	–
0.039	5,120	2,560	+	+	+	5,120	2,560	+	+	+
0	5,120	2,560	+	+	+	5,120	2,560	+	+	+

[a] Whole cell antigens prepared from *B. abortus* or *B. melitensis*, respectively.
[b] EW-A was employed for production of line A, and EW-M was employed for production of line M. Component 1 could be produced with both antigens.

Components M, A, and 1 were resistant to treatment of the extracts with 0.25 N NaOH for 1 hr at 65 C or 1% acetic acid at 100 C for 0.5 hr, but components 2 and 3 did not appear following these treatments. The acetic acid treatment of EW-A resulted in improved diffusibility of lines A and 1.

The amount of diffusible antigen in EW-A seemed to be much less than that present in EW-M since concentrations of 25 to 50 mg of EW-A per ml were required for the appearance of lines A and 1, whereas 3 mg of EW-M per ml was sufficient for the appearance of lines M and 1. Antibodies to the different antigenic components appeared at various times after the infection of rabbits. Antibodies to components A and M were present in rabbit sera 1 week after infection, antibodies to component 1 appeared 2 weeks after infection, and antibodies to components 2 and 3 were observed 5 weeks after infection. Antibodies to component 1 were not revealed in hyperimmune anti-CW or SA sera.

Analysis of ether-water extracts by gel diffusion method. To determine the antigenic relationship of components A and M in EW-A and EW-M, double gel diffusion was carried out with anti-CW and monospecific sera (Fig. 3). A line was produced near the antigen well of EW-A (well 5) with all sera except monospecific *B. melitensis* serum (well 3), which did not produce a line although several combinations of antigen and antibody were employed. The lines produced by anti-

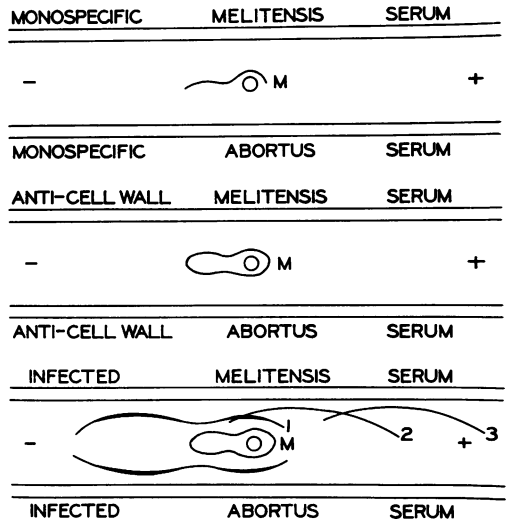

FIG. 1. *Diagram of precipitation lines produced in immunoelectrophoresis with EW-M and various antisera.*

CW sera (wells 1 and 2) showed total identity with the lines produced by monospecific *B. abortus* serum (well 4). When EW-M was examined (well 6), all sera except monospecific *B. abortus* serum (well 4) produced lines which showed complete identity with the line formed by monospecific *B. melitensis* serum (well 3). Sera from

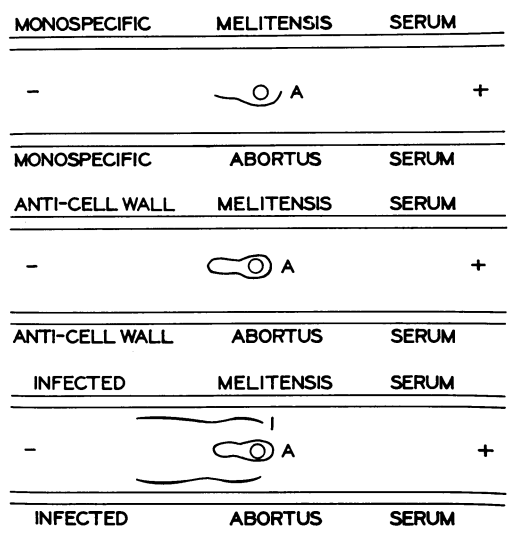

FIG. 2. *Diagram of precipitation lines produced in immunoelectrophoresis with EW-A and various antisera.*

infected rabbits showed the same reactions as anti-CW sera.

These results show that unabsorbed sera from immunized or infected rabbits, whether given *B. abortus* or *B. melitensis*, contain antibodies to both the A and M antigens. This means that both species carry both A and M antigens. Since it is possible to prepare monospecific serum by careful agglutinin absorption, the distribution of the antigens on the two species must differ. The location of these antigens is still unknown.

When EW-A and EW-M were tested with unabsorbed serum, line A gave a reaction of partial identity with line M. The degree of identity was dependent upon the serum. Serum taken early in infections showed less identity (Fig. 4) than serum taken later in infection. This indicates that the component M must carry some antigenic determinant for A, and component A must carry some antigenic determinant for M. Component 1 from EW-A showed identity with component 1 from EW-M with all sera containing antibodies to this component. This is shown in Fig. 5 in which component 1 is the line closer to the antiserum well in each case.

Absorption of antisera. Sera from rabbits infected for 5 weeks with *B. melitensis* or *B. abortus* and anti-CW sera were absorbed with living brucellae and tested in gel diffusion and agglutination (Table 1). Living smooth *B. melitensis* and *B. abortus* cells were able to remove precipitins to components M and 1 from EW-M, and precipitins to components A and 1 from EW-A, and agglutinins to smooth brucellae. The rough cul-

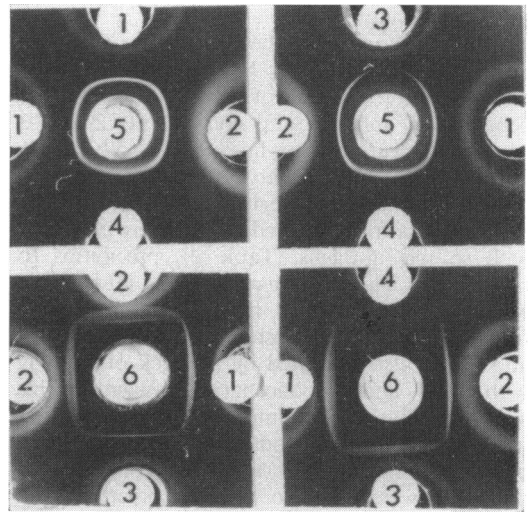

FIG. 3. *Antigenic relationship of components A and M in EW-A (wells 5) and EW-M (wells 6) tested with anti-CW-A serum (wells 1), anti-CW-M serum (wells 2), monospecific melitensis serum (wells 3), and monospecific B. abortus serum (wells 4).*

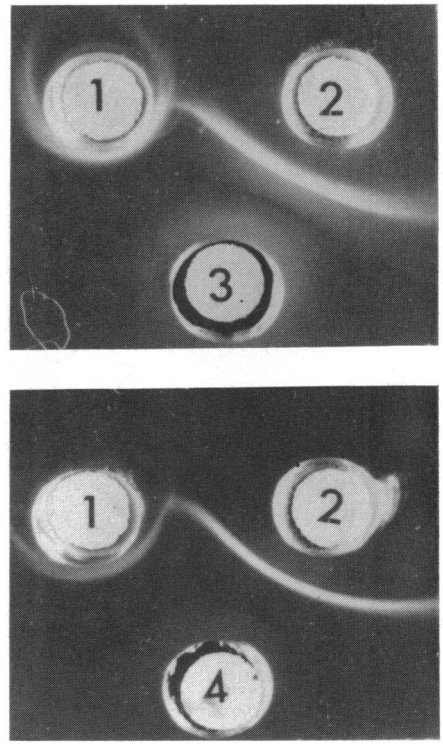

FIG. 4. *Antigenic relationship of components A and M in EW-A (well 1) and EW-M (well 2) tested with sera taken from rabbits 1 week after infection with B. abortus (well 3) and B. melitensis (well 4).*

tures, including *B. ovis* and *B. canis*, did not remove antibodies to these antigens. This indicated that components M, A, and 1 were associated with the surface of smooth brucellae. The behavior of antibodies to components 2 and 3 was not as clear, as these lines tended to be weak. Some cells were unable to remove precipitins to these components (Table 1).

When sera from infected rabbits were absorbed with soluble antigens (Table 2), precipitins to components 2 and 3 were removed by all antigens except cell walls which had been treated with trypsin. The protein nature of components 2 and 3 was also indicated by the lack of staining with lipid and polysaccharide reagents. This suggested that these components are present in internal antigens of smooth and rough brucellae of several species. All antigens from smooth brucellae were able to remove precipitins to components M, A, and 1, and agglutinins, as had been shown with absorption by living smooth brucellae in Table 1. Precipitins to component 1 were, however, also removed by SA from rough *B. melitensis* without affecting precipitins to component A or M, or agglutinins.

An attempt was made to isolate a polysaccharide component from the rough *B. melitensis* by the ether-water, phenol-water, and trichloroacetic acid extraction. Only the trichloroacetic acid extract yielded an ethyl alcohol-precipitated material which produced a line in immunoelectrophoresis similar to component 1. When sera were absorbed with the trichloroacetic acid extract from rough *B. melitensis*, antibodies to component 1 were removed, whereas those to components M and A remained, as did the agglutinins. Figure 6 is a photograph of these reactions.

Analysis of heavy fraction of ether-water extracts. Leong, Diaz, and Wilson (13) centrifuged EW-A at 60,000 × *g* for 12 hr at 5 C in a Spinco L (rotor 40) ultracentrifuge, and the pellet was tested in gel diffusion with hyperimmune *B. abortus* serum which produced a single line near the antigen well. Later the pellet was tested with monospecific serum, and the line was identified as component A. The possibility of producing monospecific sera with the heavy fraction from *B. abortus* and *B. melitensis* was then examined. Rabbits were given 1 mg of lyophilized EW-A-HF in saline by the intravenous route and were bled

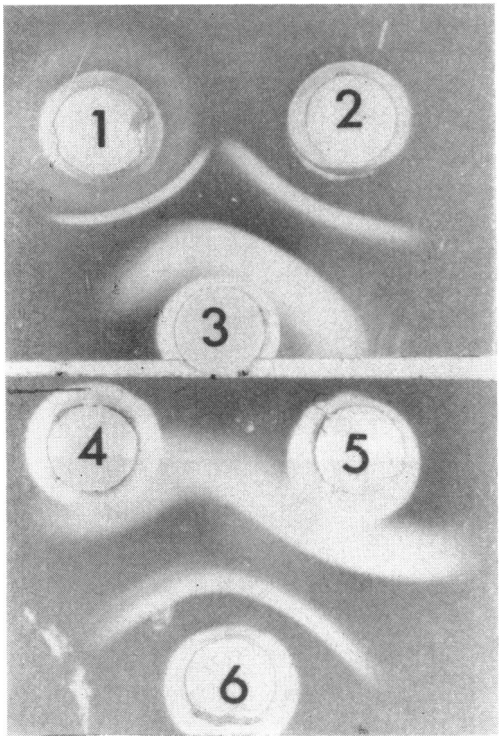

FIG. 5. *Antigenic relationship of components A, M, and 1 in EW-A (well 1) and EW-M (wells 2 and 6) tested with sera taken from rabbits 5 weeks after infection with B. abortus (well 4) and B. melitensis (wells 3 and 5). This picture was taken 12 hr after preparation.*

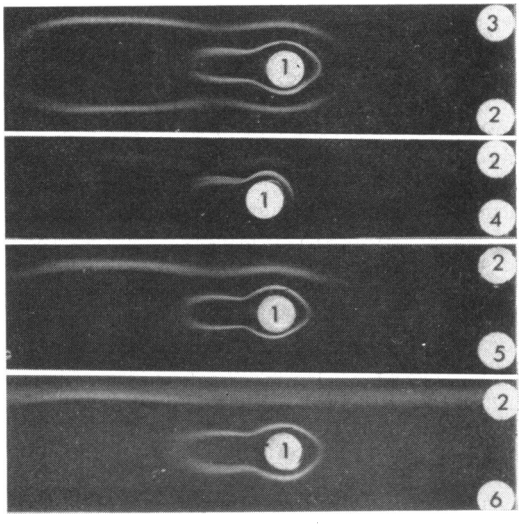

FIG. 6. *Absorption of sera from rabbits taken 5 weeks after infection with smooth B. melitensis. Sera tested in immunoelectrophoresis with EW-M (well 1). Sera unabsorbed (trough 2) and absorbed with soluble antigen from B. ovis (trough 3), CW-ENZ (trough 4), soluble antigen from rough B. melitensis (trough 5), and trichloroacetic acid extract from rough B. melitensis (trough 6).*

3, 7, and 15 days later. Another group was given 1 mg of EW-M-HF in a similar manner. All antisera reacted with both *B. abortus* and *B. melitensis* antigens in agglutination and gel diffusion; thus, it was apparent that monospecific sera could not be obtained without absorption by the heterologous antigen.

Antigenic component 1 was not revealed in EW-HF when tested with the antisera described above. When tested with sera from rabbits infected with *B. abortus* or *B. melitensis*, component 1 was present in EW-HF. Serum from rabbits infected for 3 weeks with *B. melitensis* was absorbed with various amounts of both EW-A-HF and EW-M-HF and retested in the agglutination test with two antigens and in gel diffusion with two antigens (Table 3). Serum from rabbits infected for 3 weeks with *B. abortus* was similarly tested (Table 4).

Antiserum monospecific for *B. melitensis* in agglutination and gel diffusion could be prepared from sera of rabbits infected with *B. melitensis* when it was absorbed with EW-A-HF (Table 3). Antisera monospecific for *B. abortus* in gel diffusion could be prepared from sera of rabbits infected with *B. abortus* when it was absorbed with EW-M-HF, but low titer agglutinins to *B. melitensis* remained in this serum (Table 4). When the sera were absorbed with homologous antigen, antibodies to both A and M components were removed more or less uniformly, and monospecific sera were not obtained. The amount of EW-A-HF antigen needed to remove heterologous antibodies from anti-*B. melitensis* serum was much less (2.5 to 5 mg) than the amount of EW-M-HF antigen needed to remove heterologous antibodies from anti-*B. abortus* serum (20 mg was insufficient).

Precipitins to component 1 were removed by very small amounts of either absorbing antigen. The removal of these precipitins did not affect the agglutination titer, whereas the removal of precipitins to A or M was accompanied by a reduction in agglutination titer.

Presence of A and M components in other antigenic preparations. The M and 1 components were also revealed in trichloroacetic acid and phenol-water extracts of *B. melitensis*. Similar preparations of *B. abortus* produced A and 1 lines if sufficient concentrations of extract (25 to 50 mg per ml) were employed. Saline extract of *B. melitensis* produced the M line. Saline extract of *B. abortus* produced the A line when 50 to 100 mg per ml was employed. SA from *B. melitensis* also developed the M component (Fig. 7), but SA from *B. abortus* did not reveal a diffusible A component even when the highest possible concentrations were used.

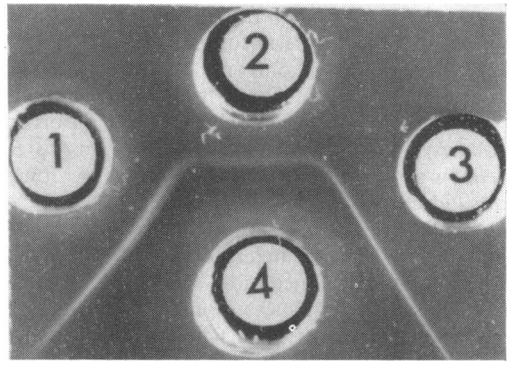

FIG. 7. *Demonstration of M component in EW-M (well 1), SA-M (well 2) and saline extract of B. melitensis (well 3) with monospecific B. melitensis serum (well 4).*

DISCUSSION

These studies have shown that ether-water extracts from *B. melitensis* and *B. abortus* when analyzed by immunoelectrophoresis with sera from infected rabbits contain two components that are specific for the surface of smooth brucellae. Absorption of infected serum with living smooth brucellae removed the antibodies for these two components, whereas absorption with living rough brucellae did not. Differences could be shown in the two components by the use of monospecific sera. EW-A produced a single line, designated A, with monospecific A serum, and EW-M produced a single line, designated M, with monospecific M serum. When EW-A and EW-M were tested in gel diffusion against an unabsorbed antisera, whether anti-A or anti-M, reactions of partial identity were obtained, thus indicating that both A and M components were present on both antigens, although in different proportions.

Miles and Pirie (14, 15) failed to separate by chemical means the A and M antigens which Wilson and Miles (22) had postulated were present in different proportions on cells of *B. abortus* and *B. melitensis*. They concluded that the two antigens must be present in a single antigenic complex. But this does not explain how it is possible to obtain monospecific A and M sera by absorption with whole cells (11) or, in some cases, with extracts (M. S. Redfearn, Ph.D. Thesis, Univ. of Wisconsin, Madison, 1960).

Attempts were made to prepare monospecific sera with antisera from rabbits infected for 3 weeks with *B. abortus* or *B. melitensis* by absorption with the heavy fractions of ether-water extracts. Antisera monospecific for *B. melitensis* in agglutination and gel diffusion tests could be

prepared from sera of rabbits infected with *B. melitensis* when it was absorbed with EW-A-HF. Antisera monospecific from *B. abortus* in gel diffusion could be prepared from sera of rabbits infected with *B. abortus* when it was absorbed with EW-M-HF, but low titer agglutinins to *B. melitensis* remained in this serum. L. M. Jones (*unpublished data*) also found that it was easier to remove heterologous A antibodies from anti-M sera than to remove heterologous M antibodies from anti-A sera. Since more antibody molecules are required to demonstrate precipitation in agar gels than agglutination of whole cells, it is not surprising that less absorbing antigen is required for preparation of serum monospecific for gel diffusion tests than for agglutination tests.

Component 1, which was identical in ether-water extracts of *B. melitensis* and *B. abortus*, apparently does not play an important role in agglutination reactions as its antibodies can be removed by absorption without affecting the agglutination titer. In addition, antibodies to this component were removed by SA and trichloroacetic acid extracts of a rough *B. melitensis* culture, without removing precipitins to A or M components or agglutinins. This component appears to be a polysaccharide protein complex devoid of lipid. Antibodies to this component appeared in sera of infected rabbits after 2 weeks, but it was not present in other antisera, although the killed antigens employed in the immunization of these rabbits were known to contain component 1 by absorption experiments. This suggests that there are some differences in immunogenicity in preparations of living versus killed cells.

On the basis of staining properties, antigenic component 1 was a polysaccharide protein complex, whereas A and M components were lipopolysaccharide protein complexes.

We also found components 1, A, and M in trichloroacetic acid and phenol-water extracts. Baker and Wilson (1) observed no chemical or biological differences among tricholoroacetic acid, phenol-water, and ether-water extracts of *B. abortus*.

Ether-water extract from *B. melitensis* showed two additional components when analyzed with serum from rabbits taken 5 weeks after infection with *B. melitensis*. Antibodies to these components could be absorbed by SA from rough or smooth brucellae. These two components were destroyed by treatment with acetic acid and NaOH, indicating that they were protein in nature. Components 1, A, and M were stable to acid and alkali treatment.

Ether-water extract from *B. melitensis* had much greater diffusibility in agar gel than EW-A, suggesting a difference in molecular weight or particle size. Whereas 3 mg ml of EW-M per ml was sufficient concentration to develop components 1 and M, 25 to 50 mg of EW-A per ml was required to develop 1 and A. After mild acid hydrolysis, 6 mg of EW-A per ml was sufficient, which suggested that this treatment resulted in particles of lower molecular weight.

It has been reported with many trichloroacetic acid extracts of *Enterobacteriaceae* (3, 4) that the higher molecular weight material contains all biological activity, but this material is very nonhomogenous with respect to sedimentation rate in sucrose density gradient studies. Previously (13), we reported that the pellet from centrifugation of EW-A at 200,000 $\times$ *g* contained nearly all the biological activity. Centrifugation at 60,000 $\times$ *g* (*unpublished data*) did not sediment all biological activity since the supernatant fluid contained toxicity for mice, and examination in immunoelectrophoresis showed it contained component A. This means that in EW-A there are at least two sizes of particles. When sufficient concentration of EW-A is employed in immune diffusion, there are enough small particles to diffuse through agar and give a precipitation line. After mild acid hydrolysis of EW-A, more particles of low molecular weight are present, and consequently less antigen is required to give a precipitation line.

It has been reported previously (6, 18) that trichloroacetic acid, saline, and phenol extracts of *B. melitensis* produced a precipitation line that could be correlated with the M agglutinogen, but similar extracts from *B. abortus* did not give a precipitation line. Our results show that if sufficient concentrations of trichloroacetic acid, saline, phenol, and ether-water extracts from *B. abortus* are employed, a precipitation line will develop which can be correlated with the A agglutinogen.

Other investigators have observed a precipitation line which is characteristically close to the antigen well in preparations from cultures of *B. melitensis* (17), or *B. abortus* biotypes 4 and 5 that are *B. melitensis* serologically (16). They referred to this as "M" line, but did not correlate it with the M agglutinogen. Redfearn and Berman (18) examined phenol extracts of 145 *Brucella* cultures in gel diffusion and observed that extracts which produced a precipitation line with unabsorbed antisera were obtained from all cultures which were agglutinated by monospecific *B. melitensis* serum, whereas cultures which were agglutinated by monospecific *B. abortus* serum did not produce an agar diffusible antigen in the concentrations employed. They suggested that the gel diffusion test would be a simple, accurate method of serological classification without the necessity of preparing monospecific sera.

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