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Animal Brucellosis

Edited by

Klaus Nielsen, J. Robert Duncan

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PREFACE

Animal brucellosis is a disease that encompasses six bacterial species and principal hosts: *B. abortus* (cattle), *B. melitensis* (goats), *B. suis* (swine), *B. canis* (dogs), *B. ovis* (sheep), and *B. neotoma* (desert rat). As a generalization, the principal manifestations of brucellosis are reproductive failure: that is, abortion or birth of unthrifty offspring in the female and orchitis, epididymitis with frequent sterility in the male. Persistent (lifelong) infection is common with this facultative intracellular parasite with shedding in reproductive and mammary secretions. Man, although considered an end host, often develops a persistent infection characterized by intermittent influenza-like disease termed "undulant fever". Because of its economic impact on animal health and the risk of a debilitating human disease, support has been found in most countries for programs to control and eradicate the disease from domestic animal populations. These programs have employed two principal tools: vaccination of young or mature animals and the slaughter of infected/exposed animals, usually on the basis of a reaction in a serological test. Vaccination has been widely used, with the best example being *B. abortus* strain 19 used in cattle. Normally calves would be vaccinated before 8 months of age and would not be tested serologically for brucellosis until 18 months of age when an allowance for elevated agglutination titers would be applied. In the past decade, vaccination of adult cattle with a reduced dosage of *B. abortus* strain 19 has been used as a control measure in heavily infected areas. While vaccination has been demonstrated to protect up to 75% of cattle, serological reactions for reasons that include persistent *B. abortus* strain 19 infection, have interfered with the application of serological tests and therefore the test and cull program.

Definitive diagnosis is by bacteriological culture of the causative organism. However, this is an expensive and time-consuming procedure and as a result, presumptive tests most frequently involving the measurement of antibody in body fluids have been developed. Initially, agglutination tests with a whole cell antigen were used. It was soon realized that nonspecific reactions were frequent, and as a result numerous modifications of the agglutination test were devised. These modifications included acidification of the antigen-serum mixture, heat treatment of serum, treatment with reducing agents such as 2-mercaptoethanol or dithiothreotol, precipitation with Rivanol (6,9-diamino-2-ethoxyacridine lactate) or addition of chelating agents such as ethylene diaminetetraacetic acid. All of these treatments resulted in various levels of inactivation antibody of the IgM class, the main cause of nonspecific reactions. While the agglutination tests have been used successfully in several countries for eradication of brucellosis in animals, these tests have major drawbacks in terms of their very high sensitivity and therefore low specificity and their inability to distinguish antibody resulting from vaccination from that induced by virulent bacteria. A complement fixation test, using a whole-cell antigen and guinea pig serum as a source of complement was also devised and for many years served as the diagnostic standard. This test is only slowly being replaced by primary binding assays in the diagnostic laboratory. While the sensitivity and specificity of the complement fixation test are excellent, its major drawbacks are the technical difficulties in its performance and its inability to distinguish the antibody response of vaccinated from infected animals. Precipitation tests have been developed and were the first serological procedures to employ more purified antigens and in, the case of *B. abortus* strain 19 vaccination, could actually be used for differentiation of vaccine-induced antibody from antibody to field strains. The major problem with precipitin tests is their relative lack of sensitivity. The considerable drawbacks of the classical serological tests have led to the application of primary binding assays to the serodiagnostics. While radioimmunoassay proved very useful in detection of human antibody, the amount of radioisotope required for national animal testing programs would be too difficult to handle and to dispose of. Therefore, primary binding assays utilizing enzymes or fluorochromes are more applicable and a variety of test procedures using both detections systems have been described. These

tests are very sensitive, and by selecting the appropriate antigenic components and a suitable specificity for the detection system can be made very specific as well. Other advantages include the ability to distinguish vaccinal antibody from that of true infection, relative ease of automation and data handling, and the ability to manipulate each step of the procedure to suit requirements. The main disadvantages are the expense of the equipment and the standardization requirements.

In this volume we have attempted to include up to date knowledge on brucellosis of animals. Since *B. abortus* has been the topic of most published reports, this species occupies a prominent role in comparison to the other species of *Brucella*. It is clear that a great deal of the research on *B. abortus* is applicable to the other species and it is equally clear that other findings do not apply.

While we currently have most of the tools to efficiently diagnose brucellosis in animals, test and slaughter programs to eradicate this disease are not realistic in large areas of the world and as a result, human brucellosis will continue to be a problem.

A number of notable achievements have occurred over the years through research efforts in brucellosis. These include the development of live attenuated bacterial vaccines for cattle and goats (*B. abortus* strain 19 and *B. melitensis* Rev. 1) and the discovery of poly B with the subsequent demonstration by chemical and immunochemical means of the unique properties of *B. abortus* O polysaccharide. The O polysaccharide of *B. abortus* lipopolysaccharide has been shown to be immunodominant and it induces a readily detectable and discernible antibody response, principally of complement fixing IgG_1. This resulted in the development of a highly specific and sensitive diagnostic complement fixation test that relied on the measurement of IgG_1 antibody rather than IgM, which does not fix guinea pig complement. IgM Fc receptors were demonstrated on this Gram-negative facultative intracellular parasite and these, possibly along with agalactosylated IgG, were shown to be a cause nonspecific agglutination. In a similar vein, it was shown that bovine IgG_1 was capable of agglutinating *B. abortus* cells only at an acid pH, resulting in the development of agglutination tests of increased specificity. *Brucella* was one of the first cases demonstrating reversion from L-forms to intact bacteria. *Brucella* has also been used extensively in basic research, initially as the classical T-independent antigen and to contrast *E. coli* lipopolysaccharide in T and B cell regulation. Subsequently, it has been used in the study of cell-mediated immunity mechanisms and genetic resistance to infection. *Brucella* has also been used as an interferon inducer and in the study of the regulation of polyclonal stimulation, particularly of IgG_{2A} isotype (similar to anti-delta chain antibody) via gamma-interferon. Thus, *Brucella* is a useful model in the delineation of the role of interleukins in the antibody isotype switching/regulation mechanism.

In order to make further inroads into the elimination of this disease, a great deal of further research is required in areas such as diagnostic and protective antigens, production of worthy antigens in a cheap, efficient and safe manner, and possibly most important of all, what constitutes a protective immune response in the host.

THE EDITORS

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Over the past 15 years, Dr. Nielsen has been interested in the immune response of ruminants to infectious disease, using brucellosis and paratuberculosis as models, as well as the interaction of complement in the initiation of the antibody response. Dr. Nielsen is a member of the American Association of Immunologists, the American Association of Veterinary Immunologists, and the British Society for Immunology.

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Chapter 1

CURRENT CONCEPTS IN THE TAXONOMY OF THE GENUS *BRUCELLA*

Margaret E. Meyer

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I. DEVELOPMENT AND STRUCTURE OF THE GENUS

The isolation of the causal agent of Malta fever by Bruce[1] a century ago had a decisive and permanent impact on clinical medicine as well as on systematic bacteriology. Malta fever had been difficult to differentiate symptomatically and clinically from other fevers, i.e., the so called typhoid-malarial complex, then endemic in countries of the Mediterranean littoral. Thus, to clinicians, the isolation of these organisms separated and defined Malta (undulant) fever as a distinct clinical entity. Since we have the advantage of historical hindsight, we now know that when Bruce[2] later named the causal organisms *Micrococcus melitensis*, he created the first species of *Brucella*.

Interestingly, the realization that undulant fever of man and brucellosis of animals were different manifestations of the same infection led to the creation of the genus *Brucella*. Evans[3] established that *M. melitensis* was, in fact, a small rod (coccobacilli) rather than a coccus and that it was morphologically, culturally, and biochemically essentially indistinguishable from *Brucella abortus*. Because these two organisms shared the distinctive *in vivo* capabilities of producing abortion in animals and undulant fever in man, Meyer and Shaw[4] found unacceptable Evans[1] suggestion that they be classified in the genus *Bacterium*, which included the typhoid-dysentery group of intestinal organisms. To accommodate the distinctive features of *M. melitensis* and *B. abortus* and to commemorate the work of David Bruce, the researchers gave them separate rank as the genus *Brucella*.

From its formation in 1920 to 1963, an additional species, *B. suis* was incorporated into the genus,[5,6] as were several biotypes.[6-11] During these 43 years, there were various critical assessments as to the naming and numbering of biotypes and as to whether these were aberrant, atypical, and/or transitional strains of brucellae.[7,12-17] Nonetheless, the genus membership remained stabilized with the three species of *B. abortus*, *B. suis*, and *B. melitensis*, now frequently referred to as the three classical species.

Since 1966, three additional species have been added to the genus: *B. neotomae*, *B. ovis*, and *B. canis*, now appropriately referred to as the three new species. *B. neotomae* was accepted without controversy[18] as it has an essentially smooth colonial morphology, fits other criteria by which *Brucella* organisms can be identified,[19] and also has a distinctive metabolic pattern.[20]

The flow of thought that prevailed concerning both the structure of the genus and the pedigree required for admission into it was abruptly interrupted with the descriptions of *B. ovis*[21] and the accompanying suggestion that it was a *Brucella* organism, and that it should be considered a new species.[22] In fact, for 18 years, doubt and controversy[23] reigned regarding the true identity of *B. ovis* before it was ultimately admitted into the genus *Brucella*. *B. ovis* had not previously been assigned to a taxonomic niche because it differed markedly from the existing criteria for generic recognition of brucellae and because the manifestations of infection it caused in individual animals, as well as in flocks of sheep, did not fit the classic disease pattern associated with brucellosis. Further, it contradicted the conventional wisdom that only smooth brucellae were virulent and could long maintain themselves in populations of host/reservoir animals. The same circumstances initially clouded the identity of *B. canis*.[24-27]

Hoyer and McCullough[28] ushered in the "high tech" era in this genus in 1968 by being the first to explore species relatedness at the genome level. The results of their DNA-DNA hybridization experiments established that the then four accepted species *(B. abortus, B. suis, B. melitensis,* and *B. neotomae)* had 100% homology among them in their polynucleotide sequences, that *B. ovis* had 94% homology with the other species, and that the base composition of G + C of 56 to 58 mol% was the same in all five species. In a subsequent paper,[29] they established that *B. canis* had DNA homology with the three classical species and by reciprocal DNA-DNA hybridization established that the difference in *B. ovis* was

not due to a rearrangement of 6% of the sequences, but that they were actually missing from the genome. On the basis of these results, they concluded that *B. ovis* was a deletion mutant of one of the classical species and that all the species are closely related.

Recently, Verger et al.[30] examined the DNA homologies of the polynucleotide sequences in 51 strains of *Brucella* which included representatives of the six species and several strains of biotypes within each of the classical species (there are no reported biotypes within the three new species). In DNA-DNA reassociation experiments using labeled DNA strands from *B. melitensis* 16M to determine its homology with the other 50 strains, they reported relative binding ratios (percent homology) of from 84 to 100%. In their results on reciprocal DNA-DNA relatedness, they reported percentages ranging 87 to 104%. Even though their 23% range in percentages of binding ratios and 17% range in reciprocal ratios considerably exceeded the reported standard error of 3% in DNA relatedness results,[31,32] they nonetheless denied the validity of Hoyer and McCullough's finding concerning the 6% difference between *B. ovis* and the other species. However, by using a different molecular genetic technique, De Ley et al.[33] established with certainty the genetic similarity of the six species. These investigators previously had found that genome sizes (i.e., molecular complexes) are similar among different strains within a single, well-defined species (standard deviation of a group of averages is less than 14.5%). When the same techniques were applied to the six species of *Brucella*,[34] they found genome molecular complexities of 2.37×10^9, with standard deviation of 8%, indicating an intimate genetic relationship. Their data on DNA ribosomal RNA hybridization also shows, via a similarity map, there to be but little measurable differences among the species. Thus, by all available molecular genetic techniques for ascertaining relatedness at the genome level, it is clear that the relationship among all brucellae is exquisitely close.

II. CURRENT TAXONOMIC STATUS OF THE GENUS

What, then, is the current taxonomic status of this genus? Taxonomy seems to mean different things to different people. However, in its purest sense, it means having a scheme of hierarchical classification that reflects and reveals the evolutionary relatedness of the organisms, ideally at all taxon levels, i.e., biotypes, species, genus, and family. As distinct from the evolutionary relatedness imbued in a taxonomy, the taxonomic process obviously includes a workable identification key and a system of nomenclature.

For many years the genus *Brucella* was sequestered in the family *Brucellaceae*,[35] which also included many other genera (i.e., *Bordetella, Pasteurella,* etc.). Through DNA-DNA hybridization studies and G + C base ratio determinations, the genus *Brucella* was found to be unrelated to the other family members and, in fact, most of the genera in the family were found to be unrelated to each other. In the most recent edition of *Bergey's Manual,*[36] the genus is not subsumed to any family but is free floating in a group of Gram-negative rods and cocci.

However, based on recent results calculated from DNA-ribosomal RNA reciprocal hybridizations, De Ley et al.[34] reported that the genus *Brucella* and plant pathogens in the *Agrobacterium-Rhizobium* complex of organisms have a "rather close phylogenetic origin and they sprang from the same ancestor". These investigators also commented that this unique finding would have to be examined further with other sophisticated genetic techniques. Nonetheless, the genus may now have a family affiliation and a very unexpected one at that.

Because of the exquisite closeness of the genetic relationship among all brucellae, there is no question but what the boundaries of this genus are elegantly defined. Based on all these genetic lines of evidence, the suggestion has been made that all *Brucella* strains are biotypes (biovars) of a single species and should be renamed to reflect this fact.[30,37] This

may well be true, but all these sophisticated molecular techniques have revealed only genome similarities, and no attempt has been made to account for the discrete and substantial differences known to exist among these organisms. Additionally, these techniques have revealed only two evolutionary clues, i.e., *B. ovis* is probably a deletion mutant of one of the classical species, and the genus may be descended from some plant pathogens.

III. DIFFERENCES AMONG THE SPECIES

Differences between and among the species that could be useful in ascertaining evolutionary pathways include the following:

Differences in the number of biotypes within a species — Even though the current classification scheme recognizes but 8 biotypes (biovars) within the species *B. abortus*, at least 22 have been reported.[38-41] In the species *B. suis*, there are four recognized biotypes, and a fifth has been reported.[42] In the species *B. melitensis*, there are no reported biotypes, but there are three serotypes. There are no reported biotypes or serotypes within the species *B. neotomae*, *B. ovis*, or *B. canis*. Thus, the species *B. abortus* is genetically labile, while the others are relatively stable with respect to the numbers of biotypes.

Differences in colonial morphology at time of initial isolation — Each of the three classical species and *B. neotomae* are smooth on initial isolation. *B. ovis* and *B. canis* are nonsmooth (i.e., mucoid), but not fully rough.

Differences in metabolic patterns — Each of the six species has a characteristic and definitive pattern of oxidation on an array of 14 amino acid and carbohydrate substrates. In addition, in the species *B. suis*, the oxidative pattern also is discrete for the biotypes.[14-17,20,27,43,44]

Patterns of growth on appropriate concentrations of the dyes basic fuchsin and thionin — Even though these dyes are man-made products derived from coal tar and are substances the organisms are unlikely to encounter in nature, nonetheless, there is a consistent pattern of growth that recurs throughout the species and their biotypes. Possibly the ring structures of these dyes are mimetic of substrates naturally occurring in mammalian tissues.

Need for CO_2 and serum for growth, especially on initial isolation — While either or both serum and CO_2 are required only by some of the biotypes of *B. abortus* and by *B. ovis*, nevertheless, it is an inherent environmental pabulum unneeded by other genus members and is important in considering lines of descendancy both of the species and of the biotypes.

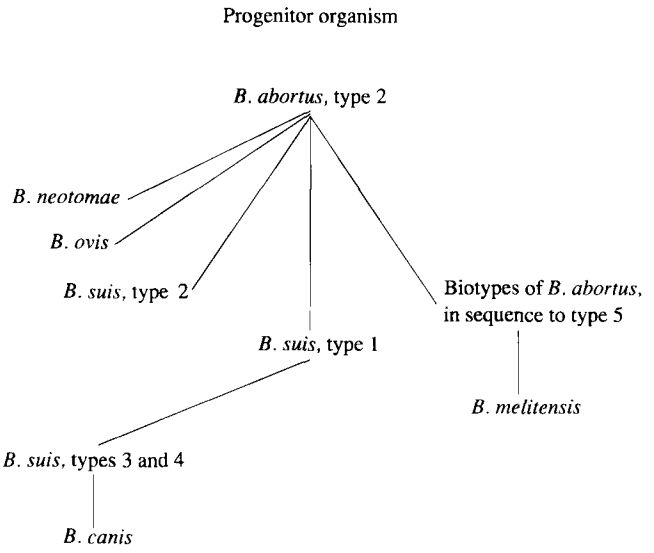
Susceptibility to *B. abortus* bacteriophage, strain 3, also known as the Tbilisi phage — Several strains of phage have been found that lyse various species of *Brucella*,[45] but strain 3 has consistently provided the most precise results and also is highly correlated to the metabolic patterns.[46] As such, it may offer clues to evolutionary descendancy.

Biological behavior in nature — Each of the three classical species has a preferential reservoir of infection, and each differs in its host range.[47,48] Certainly, each occasionally infects other animals, but they do not perpetuate themselves indefinitely in nonreservoir, nonpreferential hosts. In contrast to the host ranges exhibited by the three classic species, each of the three new species of *B. neotomae*, *B. ovis*, and *B. canis* has a very limited host range. As far as is known, each is restricted, respectively, to wood rats, sheep (especially rams), and dogs. Also, of probable significance is that all the biotypes of *B. abortus* have the same host range, while in *B. suis*, hosts of the biotypes differ. On the other hand, the host ranges of *B. suis* biotypes may reflect the geographic range of the hosts.

Additionally, no disease in man has been attributed to *B. suis, type 2*, *B. neotomae*, or *B. ovis*. Also, brucellosis in man is but rarely caused by either *B. canis* or *B. abortus*, type 5.

It is, thus, abundantly clear that there are marked and substantial differences in nature in the biological behavior of the organisms in this genus. Even though classification purists and numerical taxonomists insist that host not be considered when ordering a classification hierarchy, there is experimental evidence to indicate, at least in the genus *Brucella,* that such behavior can provide clues to the lines of evolutionary descent.

MODEL FOR EVOLUTIONARY DERIVATION OF *BRUCELLA* ORGANISMS WITH SUPPORTING EVIDENCE



Inasmuch as it has been established by several lines of evidence that all organisms in this genus share an exquisitely close genetic relationship, it is biologically reasonable to assume as a working hypothesis that they arose from a common "stem" or progenitor organism. Also, since "new species" are of recent origin, it is reasonable to assume that the progenitor is an extant organism. The only genus member than can serve in this role is *B. abortus,* type 2. It is sensitive to both basic fuchsin and thionin and requires both CO_2 and serum for growth. Thus, its environmental demands are the strictest among the three classical species, and, except for *B. ovis,* all derivative organisms are less environmentally demanding. In addition, all other *Brucella* organisms either share the pattern of dye sensitivity of *B. abortus,* type 2 or are less sensitive.

There is strong evidence to support considering *B. ovis* a derivative organism of *B. abortus,* type 2.

In fact, an organism essentially indistinguishable from *B. ovis* in its colonial morphology, environmental requirements, and metabolic pattern was derived *in vitro* from a culture of *B. abortus,* type 2. The mechanism of derivation was the induction of L-forms by steroid hormones and the subsequent incomplete reversion of these L-forms to their parental forms. There are substantial reasons to believe that the laboratory lineage of *B. ovis* parallels its natural lineage. *B. abortus,* type 2 is the only extant *Brucella* organism that requires both CO_2 and serum for growth and, thus, is the only species and/or biotype that could transmit these characteristics to a derivative. Hoyer and McCullough[29] hypothesized that the 6% deletion in polynucleotide sequences occurred as a result of multiple, small stepwise losses. Under laboratory conditions, the derivative occurred following a single exposure to the inducing agent, and the resultant derivative was, at the most, two generations removed from its parent — one generation on progesterone and one generation for reversion. The same

TABLE 1
Biotypes of *Brucella abortus* Taxonomically Ordered According to the Sequential Flow of Their Characteristics

Brucella abortus Current Biotype Designation	Line Number	Number of Strains Examined Total 613	Characteristics By Conventional Determinative Methods							
			Serum Required for Growth ■=Yes □=No	Growth on Dyes: Basic Fuchsin ■=growth; Thionin □=No growth; Micrograms Dye/ML Medium: Basic Fuchsin 10 20; Thionin 10 20 50		H_2S Produced ■=Yes □=No	CO_2 Required for Growth ■=Yes □=No	Lysis by Bacteriophage ■=Yes □=No RTD=Routine Test Dilution: RTD	10⁴xRTD	Agglutination in Monospecific Antiserums ■=Yes □=No: A(abortus)	M(melitensis)
2	1	38	■	□ □	□ □ □	■	■	■	■	■	□
−*	2	35	■	□ □	□ □ □	■	■	■	■	□	■
2	3	18	■	□ □	□ □ □	■	□	■	■	■	□
2	4	11	□	□ □	□ □ □	■	■	■	■	■	□
2	5	9	□	□ □	□ □ □	■	□	■	■	■	□
2	6	8	□	□ □	□ □ □	■	■	■	■	□	■
1	7	163	□	■ ■	□ □ □	■	■	■	■	■	□
1	8	108	□	■ ■	□ □ □	■	□	■	■	■	□
−*	9	3	□	■ ■	□ □ □	■	□	■	■	■	■
4	10	43	□	■ ■	□ □ □	■	■	■	■	□	■
3	11	20	□	■ ■	■ □ □	■	■	■	■	■	□
3	12	21	□	■ ■	■ □ □	■	□	■	■	■	□
9	13	13	□	■ ■	■ ■ □	■	■	■	■	□	■
9	14	5	□	■ ■	■ ■ □	■	□	■	■	□	■
−*	15	2	□	■ ■	■ ■ □	■	■	■	■	■	■
6	16	2	□	■ ■	■ ■ □	■	□	■	■	■	□
6	17	17	□	■ ■	■ ■ □	□	□	■	■	■	□
7	18	2	□	■ ■	■ ■ □	□	□	■	■	■	■
5	19	95	□	■ ■	■ ■ □	□	□	■	■	□	■

* Strains with combinations of characteristics not previously described.

brevity of time and events must have existed in nature. If the loss of polynucleotide sequences happened over a period of time with many small losses, then strain variants of *B. ovis* should be found. Such variants have not been found. In fact, the characteristics of all reported isolates of *B. ovis* are of such uniformity as to have prompted investigative comment[51] and a separate report on the matter.[52] Further, this uniformity has been confirmed at the genome level by O'Hara et al.,[57] who, after analyzing 33 strains of *B. ovis* DNA with 11 different restriction endonucleases, concluded that *B. ovis* is a very homogenous species and that *B. abortus, B. melitensis,* and *B. canis* are more closely related to each other than each is to *B. ovis.* One lesson learned from this derivation of *B. ovis* is that the order of descendancy from a progenitor is not necessarily linear.

A. DERIVATION OF THE BIOTYPES OF *BRUCELLA ABORTUS*

Table 1 shows the taxonomic ordering of the biotypes of *B. abortus* according to the sequential flow of their characteristics by the conventionally measured characteristics. Table 2 shows those biotypes arranged in six groups according to loss of a characteristic. *B. abortus,* type 2 has the greatest environmental demands (need for serum and CO_2) and is the most sensitive to basic fuchsin and thionin, other dyes,[41] and a variety of antibiotics.[53]

TABLE 2
Biotypes of *Brucella abortus* Divided into 6 Groups

Line Numbers on Table I	Characteristics By Conventional Determinative Methods ■=Yes □=No					
	Needs Serum for Growth	Sensitive to Basic Fuchsin	Sensitive to Thionin	Produces Hydrogen Sulfide	Needs Carbon Dioxide for Growth	Suseptible to Phage
Group I Lines 1,2	■	■	■	■	■	■
Group 2 Lines 3,4,5,6	1st Loss	■	■	■	□■*	■
Group 3 Lines 7,8,9,10		2nd Loss	■	■	□■	■
Group 4 Lines 11,12			3rd Loss ■	■	□■	■
Group 5 Lines 13,14,15,16				4th Loss ■	□■	■
Group 6 Lines 17,18,19				5th Loss	□**	■

* Need variable.
** Loss becomes permanent.

The greatest change in biotype characteristics occurs between biotypes 1 and 2, wherein there is a loss of the environmental demands and a concurrent loss in sensitivity to numerous dyes and antibiotics. Since these changes cannot be attributed to a massive occurrence of simultaneous and multiple mutations, this change in sensitivities may well indicate an alteration in permeability and/or structure of the cell surface, wall, or membranes. In any event, the change is dramatic and shared by all subsequent biotypes of *B. abortus*. Even though the metabolic pattern of all biotypes is identical, including type 2, there are changes that are substantial and can be measured by alterations in the sensitivity to basic fuchsin and thionin. When arranged in sequential order, it can be seen (Table 1) that *B. abortus*, type 2 is the only possible progenitor of the biotypes in *B. abortus* and that *B. abortus*, type 5 is, so to speak, the end of the line.

B. POSSIBLE DERIVATION OF *BRUCELLA MELITENSIS*

By all the conventional determinative methods, *B. melitensis* is indistinguishable from *B. abortus*, biotype 5. However, in its metabolic pattern, it differs on three carbohydrate substrates (arabinose, galactose, and ribose). There is evidence, however, that utilization of arabinose and galactose are interdependent, i.e., all species and biotypes oxidize either both or neither of these substrates. Additionally, it is known that one of the by-products of arabinose oxidation is galactose.[54] Thus, alteration in oxidation of one of these two substrates may reflect only one change, the second difference being oxidation of ribose.

Another difference that separates *B. melitensis* from *B. abortus*, biotype 5 is susceptibility to *B. abortus*, phage strain 3. In view of the fact that there is now extant a strain of *B. abortus* that is known to be resistant to this phage and that many strains of *B. melitensis* show lysis from without on exposure to this phage, differences in phage susceptibility may not mark as decisive a difference as previously believed. Nonetheless, there is essentially a permanent change in the phage susceptibility of *B. melitensis*, perhaps reflecting only a change in the phage receptor sites on the cell surface. Differences in cell surface may also be involved in the differing host responses (i.e., the preferential reservoir situation) to *Brucella* organisms and may be related to both host susceptibility and lack of maintenance in nature among the nonpreferential hosts. In any event, according to all available measures of similarity, *B. melitensis* is a close relative of *B. abortus*, type 5. This biotype is rarely pathogenic for man (a characteristic which may be a good indicator of the impending differences between the preferential hosts of *B. abortus* and *B. melitensis*).

C. DERIVATION OF *BRUCELLA SUIS*

The first steps in the derivation of *B. suis* type 2, i.e., loss of need for CO_2 and serum, are identical to the first steps in derivation of *B. neotomae* and of the biotypes of *B. abortus*. Thereafter, all of the biotypes of *B. suis* are derived in the same pattern of sequential flow of characteristics as are the biotypes of *B. abortus*, except the loss of sensitivity to basic fuchsin by the biotypes of *B. suis* is the mirror image of the loss of sensitivity to thionin by the biotypes of *B. abortus*. In the species *B. suis*, type 3 and 4 (type 4 quantitatively has some M antigen) are apparently the "end of the line". The essential difference in metabolic pattern between *B. abortus* and *B. suis* is the oxidation of amino acids in the urea cycle by *B. suis*.

It appears that whatever happens in nature to spark the initiation of altering the characteristics in *B. abortus*, type 2, the critical change can be measured by whether the first loss occurs as a loss of sensitivity to thionin, in which event, the organism becomes characteristic of what we recognize as the species of *B. suis* and its descendant biotypes. On the other hand, if this first loss is measured as a loss of sensitivity to basic fuchsin, the organisms become characteristic of what we recognize as the species *B. abortus* and its descendant biotypes. After this first critical alteration, the biotypes then occur in a sequential and essentially linear descent for each successive biotype.

The change that is measured by the dyes is only mimetic and not duplicative of the changes that occur in nature. By using the techniques of providing selective environments, it is not difficult to obtain mutants with altered dye sensitivities. However, other changes, such as altered sensitivities to other dyes, antibiotics, or alterations in metabolic pattern, do not accompany changes in growth on these two dyes. Obviously, elucidation of the nature of this first critical alteration in *B. abortus,* type 2 is crucial for the recapitulation of the evolution of members in this genus.

D. ORIGIN OF *BRUCELLA CANIS*

There is strong circumstantial evidence to indicate that *B. suis,* type 3 is the progenitor organism of *B. canis,* and that it arose by the same natural phenomenon as did *B. ovis.* By all the conventional determinative methods, it is indistinguishable from *B. suis,* type 3, and it also has the metabolic pattern that characterized the species *B. suis.* Further, it has the identical colonial morphology (i.e., mucoid) to *B. ovis* and, similarly, has a very restricted host range.

E. DERIVATION OF *BRUCELLA NEOTOMAE*

The characteristics of *B. neotomae* indicate that this species also could have been derived from *B. abortus,* type 2, in a fashion similar to *B. suis,* type 2, i.e., loss of the need for CO_2 and serum and loss of sensitivity to thionin. It also is similar to *B. suis* in that it is susceptible only to high concentrations of *B. abortus* bacteriophage, strain 3 and oxidizes one of the major substrates in the urea cycle. As with other of the "new species," it is not pathogenic for man and has a limited host range.

Thus, the model accounts for the known and possible lineages of all organisms in the genus *Brucella* and provides a structural working hypothesis for future investigations.

IV. MECHANISMS THAT COULD ACCOUNT FOR ALTERATIONS IN CHARACTERISTICS OF MEMBERS OF THE GENUS *BRUCELLA*

A. INCOMPLETE REVERSION OF L-FORMS, PRODUCING STRAINS WITH ALTERED CHARACTERISTICS

This phenomenon has already been confirmed experimentally in the case of *B. ovis* and may well occur with other organisms within this genus, especially in consideration of the fact that L-forms can be induced by steroid hormones. Since the preferred habitat of brucellae is the reproductive tract, especially the gravid uterus, no doubt exists about the organisms encountering these hormones *in vivo.*

In fact, a naturally occurring cell wall-defective variant of *B. abortus* has been isolated from bovine tissue.[59] Significantly, this organism required serum for growth, the colonies were nonsmooth, and the animal had a history of having been treated with stilbestrol.[59,60]

The phenomenon of incomplete reversion in the genus *Brucella* was first encountered in a culture of *B. melitensis,*[49] so the mechanism certainly is not confined to the species *B. abortus.* Nor is it confined to this genus. It has been reported to occur in *Streptococcus faecalis*[58] and is responsible for changes in features of *Escherichia coli,*[61] *Salmonella typhimurim,*[62] and β-hemolytic streptococci.[63]

There is no evidence to indicate that all incomplete and/or altered revertants undergo a full 6% loss in polynucleotide sequences, and the possibility certainly exists that nucleotide changes could be more subtle, i.e., less than 3% and thus not detectable by current DNA-DNA techniques.

B. ACTIVITY OF PLASMIDS

There is an increasing body of evidence accumulating to indicate that brucellae do not

contain plasmids, or they cannot be liberated by the methods used in other genera and species.

In the brucellosis laboratory at the University of California, Davis, we have examined 600 strains of *Brucella,* which included strains of all species and biotypes. Use of the Kado and Liu,[64] Guerry et al.,[65] and Clewel and Helenski[66] methods, each on essentially equal numbers of strains, found no evidence of plasmids. Simon[67] also reported negative results in his search for plasmids.

C. CONVENTIONAL MUTATIONAL EVENTS INVOLVING DISCRETE CHARACTERISTICS

In view of the repeatedly expressed speculations on the mutability of the *Brucella* species (see review by Meyer in Reference 55), surprisingly little has been reported on the mutation of discrete characteristics. It is known that under laboratory conditions, mutations are responsible for the change from smooth to rough colonial morphology,[68] for loss of CO_2 requirement in *B. abortus* type 1,[69] and for alterations in sensitivities to various dyes,[70,71] erythritol,[72] and penicillin[73] and in hydrogen sulfide production.[74]

However, no mutants of mucoid colonial morphology comparable to the morphology of colonies of *B. ovis* and *B. canis* have been found to occur under laboratory conditions, and we now know that laboratory-selected mutants with altered sensitivities to basic fuchsin and/or thionin are not comparable to the wild-type mutants. However, the essentially minute changes among the species biotypes could easily be accounted for by one step or several one-step sequential mutations.

D. ACTIVITY OF BACTERIOPHAGES

The potential impact that bacteriophages may have on causing alterations in strain characteristics is under active investigation. There are several approaches that can be used. One is to "cure" strains of any indigenous phage and check the before and after results. Another is to infect noninfected strains using a variety of phages and ascertain the before and after characteristics. Both these and other avenues for exploring the molecular biology of *Brucella* have been reported by Rigby et al.[75]

Another avenue of approach in the investigation of the relationship of phages to alterations in strain characteristics is to further explore the possible role of sticky white *Brucella* phage carrier colonies. These types of colonies and their associated lytic activity have been reported by Renoux and Suire,[76] McDuff et al.[77] and Meyer,[49] who also commented that carrier phage may be involved in the incomplete reversion process since sticky white colonies and lytic activity invariably accompany this phenomenon.

E. FURTHER USE OF RESTRICTION ENDONUCLEASES

Restriction endonuclease analysis has shown that there is a very close relatedness among all the *Brucella* species and that *B. canis, B. suis,* and *B. melitensis* are more closely related to each other than to *B. ovis.* However, we should now be looking for a technique that will help account for the observed differences.

F. ACTIVITY OF PORINS

The existence of porin channels in the outer membrane of brucellae was first suggested by Verstreate et al.[78] and further elucidated by Douglas et al.,[79] Verstreate and Winter,[80] and Santos et al.[81] The relative sizes of the porin channels differ among the species, being wide in *B. canis,* medium in *B. abortus,* and narrow in *B. melitensis.* These findings certainly may partially account for the differing permeability of the dyes basic fuchsin and thionin and be reflected in the differing patterns of organism sensitivity to these dyes. However, before any conclusions can be drawn about porins, the critical strains remain to be examined, i.e., *B. abortus,* types 2 and 5, *B. neotomae,* and *B. suis,* type 2.

G. USE OF GAS CHROMATOGRAPHY TO ASCERTAIN DIFFERENCES IN CONSTITUENT FATTY ACIDS

Tanaka et al.[82] examined the 16 *Brucella* reference strains and 66 field isolates by gas chromatography for quantitative comparisons of 15 cellular fatty acids. Using the resultant data, they did a numerical taxonomic analysis to determine interspecies similarity matrices and intraspecies matrices on the species *B. abortus, B. melitensis, B. suis,* and *B. canis.* Since only one strain each of *B. ovis* and *B. neotomae* were included in the study, the same type of analysis could not be done on the latter two species. These investigators found that *B. abortus* and *B. melitensis* are quite similar to each other, that *B. ovis* and *B. neotomae* are more similar to *B. abortus* than to the other species, that *B. suis* had overall similarity but was a distinctive group, and that *B. ovis* had greater similarity to *B. suis* than to the other species. Within the species, there was great similarity among biotypes within *B. abortus* and *B. melitensis* and complete homogeneity among strains of *B. canis.* In the *B. suis* species, *B. suis* biotype 2 was distinctly different.

In a later study on the fatty acid content of *B. canis* and *B. suis* by Dees et al.[83] large amounts of 19-carbon cyclopropane acid were present in all strains of *B. suis* and absent in all strains of *B. canis.*

The significance of these findings on the fatty acids lies in the following: (1) if *B. canis* is a derivative of *B. suis* via the route of incomplete reversion of L-forms, then one of the losses must be 19-carbon cyclopropane acid; (2) the marked difference observed in *B. suis* type 2 goes hand in hand with the fact that its behavior in nature is different, i.e., it has a restricted host range and is noninfectious in man. Additionally, *B. suis* type 2 colonies differ subtly, but perhaps significantly, from the classic fully smooth forms. They are neither fully smooth nor fully mucoid. Other investigators have also commented on the colonial morphology of *B. suis,* type 2.[84] This organism may well be from an L-form that has not completely reverted, but has reverted more fully than *B. canis* and *B. ovis.* Since *B. suis,* type 2 is so remarkably different from *B. suis* biotypes 1, 3, and 4, the model shows these biotypes arising independently rather than sequentially.

H. EXPLORATION OF THE RIBOSOMES

One of the newer and significant additions to the elucidation of the process of evolution in prokaryotic cells is the concept that RNA, i.e., ribosides and the ribosome, preceded nucleotides and DNA in the evolutionary development of prokaryotes. Thus, there is not just a duality of genotype and phenotype, but a trinity of genotype, ribotype, and phenotype.[85] Or, as stated by Stanier,[86] "at the level of gene products, information is accumulating which shows that the gene composition of phenotypically similar strains is closely related to ribosomal protein composition." Also, see Stanier[87] and Darnell.[97]

One of the common techniques for ascertaining differences in ribosomal structure and function is to examine ribosomes extracted from disrupted cells or to ascertain the responses of intact cells and mutants thereof with antibiotics known to affect the ribosome, i.e., erythromycin and/or chloramphenicol. This approach has been fruitful in determining discrete ribosomal differences in such organisms as *E. coli*[89,90] and *B. subtilis.*[91-93]

In the brucellosis laboratory at the University of California, Davis, we have examined all the species and biotypes for their sensitivity to erythromycin and chloramphenicol, using both a low and high concentration via antibiotic disks and measuring zones of inhibition in millimeters with calipers. All strains of *B. abortus,* type 2 were sensitive to erythromycin, and all other *B. abortus* biotypes were resistant; all biotypes of *B. suis* were susceptible, as was *B. ovis. B. melitensis, B. canis,* and *B. neotomae* were sensitive only to the higher concentrations. All species and biotypes were equally sensitive to chloramphenicol.

These results indicate that there may be marked ribosomal differences between *B. abortus,* type 2 and all its derivative biotypes. Additionally, there must be subtle but discrete

and measurable differences in ribosomal structure and function that ebb and flow through this genus (possibly switched on and off?). Be that as it may, it is known that the differences between sensitivity and resistance to erythromycin are due to changes in the ribosomal structure resulting in a lower binding affinity to this antibiotic.[93] It would appear, therefore, that one of the critical points of inquiry to help account for and to recapitulate the lineage of the species and biotypes of *Brucella* is to further ascertain the importance and occurrence of these ribosomal differences.

V. CURRENT TAXONOMIC STATUS OF THE GENUS *BRUCELLA*

There should be an orderly sequence of processes in establishing the systematics of genus, i.e., strain descriptions, identification key, and a hierarchical ordering of the organisms into a classification scheme based on known and observed evolutionary relationships. Clearly, taxonomy is a dynamic and ongoing rather than a static process.

The present classification scheme that sequesters this genus is actually a determinative identification key, and, as such, it obscures rather than reveals the evolutionary relationships that must exist among the member organisms. Additionally, the species and biotypes have been intercalated into the scheme in the chronological order in which they were isolated and described. Further disorder has been introduced into the scheme by virtue of the fact that there is no consensus on what constitutes a biotype. There is also now the recommendation that the nomenclature be changed to reflect the closeness or DNA-DNA homology[30] and that the genus be considered as one species.

A. WHAT COURSE OF ACTION?

The bottom line is that no one is certain about what constitutes a bacterial species. It is generally and somewhat loosely defined as a group of organisms sharing a mutuality of characteristics as measured phenotypically and that phenotypic discontinuities separates them into species.

In a hierarchical ordering of members of this genus, the great genetic plasticity of the species *B. abortus,* lesser plasticity of *B. suis,* and almost lack of plasticity in *B. melitensis* should be accounted for, as should the phenotypic discontinuities between each species. The working model and submodel include these differences and account for the origin of *B. ovis* and possibly of *B. canis.* Should the genus be considered a single species based on DNA-DNA homology?

DNA-DNA homology of polynucleotide sequences may give the illusion of being more genetically precise than it actually is. For example, human beings and chimpanzees have 98% homology among their nucleotide sequences,[85] and 99% of the amino acids in the proteins are the same.[94] At the bacterial level, among the serovars of *Listeria monocytogenes,* DNA relatedness has been found to be heterogeneous,[95] and the serotypes have been divided into "genomic groups". Intragroup relatedness varies from 90 to 100% homology, while intergroup relatedness falls to 25%.

In view of these disconcerting and somewhat confounding facts and before the species concept in this genus is altered, we should perhaps do what other phylogeneticists and molecular biologists are doing.

First, though overall polynucleotide homology does not separate humans from other primates, a fastidious examination of the DNA base sequences per se revealed altered positions of the protein (amino acid sequences), and these alterations, though very minimal, do separate man from African apes, orangutans and gibbons.[96]

Second is the use of recombinant DNA techniques. As stated by Slatkin,[94] "Even if we were presented with a complete DNA sequence of every individual of every species, we would still not be able to discern what makes species differences. We need to be able to

modify genes to see if we can produce new traits and even new species, and to see whether the species act as if they possess some inertia, resisting modification. The tools for this exist in using recombinant DNA."

B. BRINGING ORDER INTO THE TAXONOMY OF THE GENUS *BRUCELLA*

To do this will require consensus and consistency among members of the International Committee on Systematic Bacteriology, Subcommittee on Taxonomy of *Brucella*. Thus far, there has been little consistency and no consensus. In its 1970 meeting, even though it defined *B. suis* by both the conventional determinative methods and metabolic patterns, it assigned *B. canis* as an additional species and simultaneously accepted an organism as a biotype of *B. suis* (type 5) that had not been adequately shown even to be a *Brucella* organism.[98] This organism later was decisively excluded from the genus.[99,100] While there is no uniformity on the definition of a species, Pinigin et al.[101] are still making a plea to have *Brucella suis* type 4 considered a separate species.[101,102]

Further, indecision has been caused by Tolari et al.[103] who suggested that *B. abortus*, biotypes 3 and 6 be considered a single biotype because the only significant difference between them is a sensitivity to thionin. However, in 1984 they suggested strains of *B. suis* with slightly altered resistance to basic fuchsin be considered an additional biotype of this species.[42]

There has also been a suggestion that the species *B. melitensis* can be subdivided into five biovars on minute differences in the oxidative rates of five amino acids.[104] Since it has already been established[105] and observed many times over that age, i.e., number of previous subculturings, and lyophilization tend to cause an increase in utilization rates (but do not alter the basic metabolic pattern), it is really not possible to distinguish metabolic biovars of *B. melitensis* by their oxidative metabolism.

C. CHANGE IN NOMENCLATURE

Verger has proposed that since he considers the genus *Brucella* to be a single species, *B. melitensis*, the names of the constituent organisms reflect this monospecies concept.[30] Thus, *B. abortus* would become *B. melitensis* biovar *abortus*. In view of what we know about the evolutionary paths in this genus, this nomenclature is terribly misleading.

Until more is elucidated about the species and biotype derivations and lineages, it seems to be a good idea to adhere to the advice given in *Bergey's Manual*,[36] i.e., "the advantage of adopting a restrictive species definition must be weighed against its potential impact on well-established and accepted bacteria groups." Thus, it seems the time to consider changing the structure of the genus is when we have more finite and discrete genetic and evolutionary information, especially as to what accounts for the substantial differences among these organisms.

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Chapter 2

ANTIGENS OF *BRUCELLA*

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* Sections I and IV were the work of John W. Cherwonogrodzky, who served as general editor for the Chapter; Section II was the work of Gevard Dubray; and Section III was contributed by Edgardo Moreno and Hubert Mayer.

I. POLYSACCHARIDES OF *BRUCELLA*

A. INTRODUCTION

The species of *Brucella* have but a few known serotypes. Despite early reports of extensive cross-reaction between the species of *Brucella,*[1-3] distinct antigens between the *abortus* and *melitensis* strains were recognized.[4,5] In 1932, Wilson and Miles designated the A antigen as that which predominates on strains of *B. abortus,* and the M antigen as that which predominates on strains of *B. melitensis.*[6] Two years later, Topping reported that *B. abortus* and *B. melitensis* extracts, treated with heat and dilute acetic acid, yielded polysaccharides which were antigenic.[7] However, she was unable to show any chemical difference between the isolated components of these bacteria. Miles and Pirie[8] later reported that the antigen of *B. melitensis* was a formyl derivative of an amino-polyhydroxy compound (AP) with a specific optical rotation of $[\alpha]_D^{16} + 43°$. This material was released from a larger complex, which contained phospholipids and proteins, by a treatment with acetic acid. Although AP was stable in dilute acetic acid, it lost its antigenicity when treated with 0.1 *M* HCl (100°C/5 min).[9] More recently, Redfearn[10] demonstrated that the phenol-extracted smooth-lipopolysaccharide (SLPS) of *B. abortus* biotype 1 contained the A antigen, whereas Diaz et al.[11] suggested that both the A and M antigens were contained in lipopolysaccharide-protein complexes. Rabbit monospecific anti-A antiserum, but not anti-M nor anti-R antiserum, agglutinated both *B. abortus* and *Yersinia enterocolitica* O:9,[12,13] the cross-reactivity of these bacteria being attributed to O-polysaccharide similarities.[14]

B. COMPOSITION OF THE O-POLYSACCHARIDE OF *BRUCELLA*

It is now well recognized that SLPS, from *Brucella* species as well as from many other organisms of the *Enterobacteriaceae*, consists of an O-polysaccharide attached via the core oligosaccharide to the lipid A which anchors the molecule to the outer membrane. The major antigenic epitopes of SLPS and, indeed, of the cell reside in this O-polysaccharide. Within the core region, one of the 3-deoxy-2-octulosonate (KDO) residues has a ketosidic linkage that is acid labile, and under conditions of mild acid hydrolysis (e.g., 2% acetic acid, 100°C), it is cleaved to release the O-polysaccharide along with attached core sugars. Lipid A, which is insoluble under these conditions, precipitates.[15]

The antigenic O-polysaccharide of *B. abortus* 1119-3 SLPS was subsequently identified as a linear homopolymer of 1,2-linked 4,6-dideoxy-4-formamido-α-D-mannopyranosyl units by Caroff et al.[17] Hot 2 *M* HCl and 1 *M* H_2SO_4 destroyed this polysaccharide but not D-mannose, D-glucose, and quinovosamine, which were obtained in low yields (3.56%) and were probably derived from the terminal core oligosaccharide. The O-polysaccharide was essentially identical in structure to that of the O-polysaccharide of *Y. enterocolitica* O:9.[17] Serological cross-reactivity was confirmed by the use of murine monoclonal antibodies specific for both these antigens.[18] Murine monoclonal antibodies have also been used to show that antibodies specific for the O-polysaccharide are protective in passive immunity to *B. abortus* infection.[19]

The O-polysaccharide structure of *B. melitensis* was found to be related to that of *B. abortus* in that both were homopolymers of 4,6-dideoxy-4-formamido-α-D-mannopyranosyl units.[20,21] Although this similarity in structure provided a rationale for the cross-reaction between these organisms, it did not account for the fact that the A antigen is serologically distinct from the M antigen. Also, the optical rotations of the purified A and M O-polysaccharides differed ($[\alpha]_D + 38°$ [water] for *B. abortus* 1119-3 O-polysaccharide; $[\alpha]_D + 49°$ [water] for *B. melitensis* 16M O-polysaccharide), as did the ^1H- and ^{13}C-NMR spectra (*B. melitensis* O-polysaccharide was complex and required extensive analysis).[18,26-28] In combination with classical methods, these showed that while the A antigen is essentially a homopolymer of 1,2-linked 4,6-dideoxy-4-formamido-α-D-mannopyranosyl residues, the M antigen is a linear polymer of a repeating pentasaccharide unit comprising one 1,3-linked and four 1,2-linked 4,6-dideoxy-4-formamido-α-D-mannopyranosyl residues.[25-27]

These similarities and differences may account for the cross-reactivity and different antigenicity of the A and M antigens. That the O-polysaccharides of *B. abortus* (e.g., strain 1119-3) and *B. melitensis* (e.g., strain 16M) are the classic A and M antigens, respectively, is based on the following:

1. Rabbit cross-absorbed serotype-specific antisera for either A or M *Brucella* antigens (from G. M. Brown, National Veterinary Services, Ames, IA; or L. B. Forbes, Health of Animals Laboratory, Saskatoon, Saskatchewan) will selectively precipitate purified *B. abortus* or *B. melitensis* SLPS and will selectively bind to the homologous SLPS coated on polystyrene plates. The latter reaction is inhibited by preincubating the antisera with purified homologous O-polysaccharide.[28]
2. Murine monoclonal antibodies, with specificities for *B. abortus* or *B. melitensis* O-polysaccharides, show similar selectivity to that noted for rabbit antisera.[29] Indeed, monoclonal antibodies can substitute for rabbit cross-absorbed serotype-specific antisera when testing biovars of *Brucella spp.* for the A or M antigen.[261]

Heterologous O-polysaccharide inhibits rabbit anti-A and anti-M antisera from binding to *B. abortus* and *B. melitensis* SLPS coated onto polystyrene plates.[28] Some murine monoclonal antibodies can precipitate the O-polysaccharides and SLPS of *B. abortus* 1119-3, *Y. enterocolitica* O:9, and *B. melitensis* 16M, probably because these antibodies have affinity for common structural entities within the O-polysaccharides.[23]

With regard to the production and use of synthetic antigens, Bundle et al.[30] have linked the aliphatic chain of a fatty acid ester to the O-polysaccharide of either *B. abortus* of *Y. enterocolitica* O:9. This synthetic antigen is comparable to the SLPS of either organism in serodiagnostic tests and has the advantage of giving fewer nonspecific reactions with cattle sera tested in an enzyme-linked immunosorbent assay (ELISA).[31] Peters and Bundle also reported the synthesis of oligosaccharides of 1,2-linked 4,6-dideoxy-4-formamido-α-D-mannopyranosyl residues (i.e., the A antigen) as well as structures containing the α-1,3 linkages (i.e., the M antigen)[32] which should now provide specific diagnostic reagents.

C. CROSS-REACTIONS BETWEEN *B. ABORTUS* AND OTHER ORGANISMS

1. With Other Species of *Brucella*

As evidenced by agglutinations with rabbit cross-absorbed serotype-specific antisera, *B. abortus* cross-reacts with *B. melitensis*. The A and M O-polysaccharides consist of the same carbohydrate with most of the units linked in the same manner (i.e., α-1,2-linkage), and murine monoclonal antibodies confirm that these have common epitopes. However, cross-absorbance should have removed nonspecific antibodies. In 1932, Wilson and Miles[6] suggested that each bacterium has both antigens although in different amounts, *B. abortus* having predominantly the A antigen and *B. melitensis* having predominantly the M antigen. Agglutination of these bacteria with murine monoclonal antibodies specific for the A and M O-polysaccharides and extensive NMR spectra analysis support this conclusion.[262] Also, the species of *Brucella* consist of different biovars, strains which differ in metabolism, sensitivity to dyes, lysis by bacteriophages, and agglutination with serotype-specific antisera.[33] *B. melitensis*, for example, has biovar 1 which expresses the M antigen, biovar 2 which expresses the A antigen, and biovar 3 which expresses both the M and A antigens. It appears that biovar 3 may, indeed, have both antigens on its cell surface, while biovars 1 and 2 may have predominantly the M and A antigens, respectively, and trace amounts of the other.[262]

Despite considerable cross-reaction between *B. abortus* and *B. melitensis*, one antigen should not be used in serodiagnostic tests to detect infections due to more than one *Brucella* species. Rojas et al.[34] reported that approximately 80% of *B. melitensis*-infected human sera could be identified with either *B. abortus* or *B. melitensis* SLPS on a nitrocellulose-ELISA but that the remainder could only be identified with *B. melitensis* SLPS. For *B. ovis* infections in rams, its rough lipoproteins (RLPS)[35] and outer membrane proteins[36] are useful antigens for the detection of serum antibodies.

In considering *B. abortus* 45/20, it has been observed that cattle vaccinated with this bacterium will raise antibodies to the A and M antigens of *B. abortus* and *B. melitensis*.[37] Rabbits immunized with *B. abortus* 45/20 RLPS have antibodies that bind to the SLPS of *B. abortus* 1119-3 or *Y. enterocolitica* O:9 coated onto polystyrene plates.[261] NMR spectra of phenolic extracts of *B. abortus* 45/20 indicate that trace amounts of the O-chain polysaccharide are present on this bacterium and may account for the noted cross-reactions.[262]

2. With Bacteria of Other Genera

Brucella abortus cross-reacts with other bacterial species (for an excellent review, refer to Reference 13), the most widely known being *Y. enterocolitica* O:9. The discovery of this cross-reaction was made in Scandinavia during the 1940s. There, in spite of a successful eradication program against *B. abortus*,[38] human patients still exhibited high antibody titers. *Brucella abortus* could not be isolated from these people, and rather than having "undulant fever", their distress was gastroenteritis caused by *Y. enterocolitica* O:9.[39,40] It was subsequently found that these two organisms cross-reacted[41] because of shared antigenic determinants on the O-polysaccharides of their SLPS-protein complexes,[14,42,43] both antigens being identified as linear homopolymers of 1,2-linked 4,6-dideoxy-4-formamido-α-D-mannopyranosyl units.[16,17]

TABLE 1
Comparison of O-Polysaccharides of Cross-Reactive Bacteria

Bacterium	Component glycoside of the SLPS O-polysaccharides	Ref.
Brucella abortus 1119-3	4-Formamido-4,6-dideoxy-D-mannose	16
B. melitensis 16M	4-Formamido-4,6-dideoxy-D-mannose	27
Yersinia enterocolitica O:9	4-Formamido-4,6-dideoxy-D-mannose	17
Vibrio cholerae	*N*-Acylated 4-amino-4,6-dideoxy-D-mannose (where the acylating acid is 3-deoxy-L-glycero-tetronic acid)	44,45
Salmonella landau and *godesburg*	4-Acetamido-4,6-dideoxy-D-mannose (1 part) D-glucose (2 parts) L-fucose (1 part) 2-acetamido-2-deoxy-D-galactose (1 part)	46,47
Escherichia coli O:157H:7	4-Acetamido-4,6-dideoxy-D-mannose (1 part) D-glucose (1 part) L-fucose (1 part) 2-acetamido-2-deoxy-D-galactose (1 part)	48
Pseudomonas maltophilia 555	4-Acetamido-4,6-dideoxy-D-mannose (3 parts) D-rhamnose (1 part) 3-acetamido-3,6-dideoxy-D-galactose (1 part)	49

Other bacteria show serological cross-reactivity with *B. abortus*.[16,17,27,44-49] Structural analysis of the SLPS O-polysaccharides of these cross-reacting Gram-negative bacteria (Table 1) shows that these all contain units of N-acyl derivatives of 4-amino-4,6-α-D-mannopyranose, and it is probable that epitopes involving this aminoglycoside are responsible for the observed cross-reactivity. Some monoclonal antibodies have been raised which agglutinate one bacterium and not other cross-reactive species.[18,29] The potential exists, therefore, for differentiating even cross-reactive bacteria using a panel of monoclonal antibodies of known specificities.

D. OTHER POLYSACCHARIDES OF *BRUCELLA*

1. Polysaccharide B

Polysaccharide B (poly-B, also referred to in the literature as component 1, second component, or PB) is a low molecular weight carbohydrate released from strains of *B. melitensis* or *B. abortus* with 0.2 *M* trichloroacetic acid.[11,50] Interest was given to poly-B since *B. abortus* infected animals were reported to produce antibodies which precipitated poly-B, while vaccinated animals did not.[51,52] The composition of poly-B was controversial, some publications citing its chemical and immunologic difference from both acid hapten (AH) (the O-polysaccharide cleaved from SLPS by mild acid and heat) and native hapten (NH) (a component present in endotoxin preparations from smooth strains of *Brucella* which may be a polysaccharide-protein complex).[53-55] It was shown to have a high glucose content (89%),[54] and its reported isolation from rough, rather than smooth, cells[52] differed from traditional extractions of *Brucella* O-chain polysaccharides. Furthermore, poly-B could be isolated from disrupted cells rather than cell membranes, suggesting that poly-B resides within the cell instead of at the cell surface as does O-polysaccharide.[54]

Bundle et al.[26,56] purified poly-B from an extract of *B. melitensis* 16M. Structural analysis identified the major component as a cyclic polymer of between 17 and 24 1,2-linked β-D-glucopyranosyl residues, being essentially identical to cyclic D-glucans produced by other bacteria.[57] Poly-B appears to be present in all species of *Brucella*.[56] Purified cyclic β-D-glucan does not appear to be antigenic, and the previously described serological reactions attributed to poly-B were therefore probably due to the presence of *Brucella* O-polysaccharide in the preparations.[56,58] This is reinforced by the observation that L'vov et al.[59] noted that

the β-D-glucan will bind and form a complex with the SLPS of *B. melitensis* 16M. Therefore, the antigenicity attributed to crude poly-B preparations is probably due to its O-polysaccharide content.

2. Native Hapten

About 50 years ago, Miles and Pirie identified a water-soluble "native antigen" which upon drying or freezing would dissociate into a free lipoid and a formyl derivative of an amino-polyhydroxy compound.[8] The presence of a *Brucella* antigen that is partly O-polysaccharide and yet is distinct from either SLPS or O-polysaccharide remains controversial. Native hapten can be extracted from *Brucella* endotoxin by mild methods unlikely to break covalent bonds in the SLPS,[54] and NH has been characterized as being a large polysaccharide which lacks KDO, does not bind to erythrocytes, and may be associated with protein.[55,60-62] Others have purified NH, found it identical to *Brucella* O-polysaccharide isolated from acid treated SLPS, and have detected core glycoses and a KDO residue, suggesting that NH originates from SLPS.[63]

Moreno et al.[21,62] have identified a *Brucella* polysaccharide which differs from NH by having a small amount of lipid associated with the polysaccharide. Other Gram-negative bacteria have capsules also consisting of polysaccharide associated with lipid,[64,65] and, indeed, an external layer suggesting the presence of a capsule has been reported for *Brucella* species.[66]

E. *BRUCELLA* POLYSACCHARIDE USED TO DISCRIMINATE INFECTED FROM VACCINATED CATTLE

Diaz et al.[11,50,51] reported that animals exposed to *B. abortus* have sera which in an immunodiffusion assay (10% NaCl in the agar) show two precipitin patterns: *B. abortus* field-strain infected cattle sera precipitate both SLPS-protein complex and poly-B, while *B. abortus* S-19 vaccinated cattle sera precipitate only the SLPS-protein complex. Murine monoclonal antibodies (raised to *B. abortus* and *Y. enterocolitica* O:9 and selected for reactivity to both O-polysaccharides) when tested in an immunodiffusion assay (1% NaCl in the agar) show two precipitin patterns: type 1 monoclonal antibodies precipitate both SLPS and O-polysaccharide, while type 2 monoclonal antibodies precipitate only SLPS.[18] Approximately half of the monoclonal antibodies were of each type, and the immunoglobulin class did not appear to be a factor. The similarities between the above precipitation patterns for cattle sera and murine monoclonal antibodies was apparent, and it was found that both *B. abortus* field-strain infected cattle and *B. abortus* S-19 vaccinated cattle sera precipitate *Brucella* SLPS, while only the former cattle sera precipitate *Brucella* O-polysaccharide.[58,67] It appears likely that past reports on the discrimination between infected and vaccinated cattle sera by precipitation with poly-B was due to the *Brucella* O-polysaccharide content.

It may be speculated that the mechanism for this difference in precipitation patterns is due to epitope specificity,[68] type 1 antibodies binding to "length" epitopes along the O-chain and type 2 antibodies binding to "tip" epitopes. Figures 1 and 2 suggest that although type 1 antibodies can form a precipitate with either SLPS or O-polysaccharide, type 2 can form a precipitate only with SLPS, and then because of hydrophobic micelle formation by lipid components. *Brucella abortus* field-strain infected cattle sera may have both types of antibodies, while *B. abortus* S-19 vaccinated cattle sera may have antibodies with the activity of type 2 murine monoclonal antibodies. As the immunodiffusion assay lacks sensitivity and uses relatively large amounts of sera and antigen, an ELISA has been developed which overcomes these limitations.[69]

F. CONCLUSIONS

The composition of *B. abortus* 1119-3 and *B. melitensis* 16M O-polysaccharide has been described as having the O-polysaccharides of bacteria that cross-react with *Brucella*.

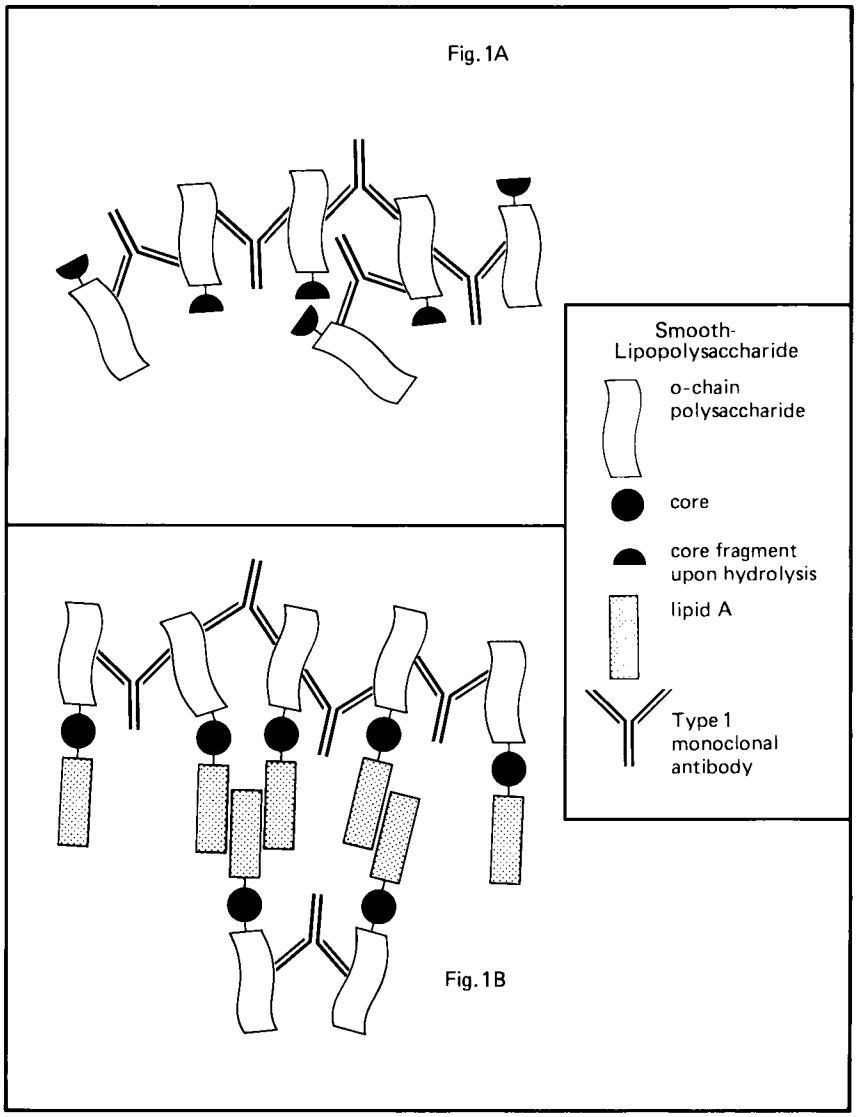

FIGURE 1. (1A) Precipitation of O-chain polysaccharide by type 1 monoclonal antibody. (1B) Precipitation of smooth-lipopolysaccharide by type 1 monoclonal antibody.

These O-polysaccharides have N-acylated derivatives of 4-amino-4,6-dideoxy-α-D-manno-pyranosyl residues in common, and it is likely that this common factor accounts for cross-reaction in serodiagnosis. Monoclonal antibodies have been raised to the A and M antigens, and some can substitute for rabbit cross-absorbed serotype-specific sera, while others can be used in a panel to differentiate *B. abortus* from other cross-reacting bacteria. Murine monoclonal antibodies have given insight into the precipitation patterns of *B. abortus* field-strain infected and *B. abortus* S-19 vaccinated cattle sera against SLPS and O-polysaccharide (crude preparations of poly-B contain *Brucella* O-polysaccharide). Schurig et al.[70] have suggested that immunogens located on the surface of invading microorganisms may be more important than internal immunogens in modulating the host's immune response. This is indirectly supported by the observation that in the production of murine monoclonal antibodies against *B. abortus* and *Y. enterocolitica* O:9 cells between 15 to 18% of the putative hybrids were LPS-specific, and of these, 60% were O-polysaccharide specific[18] (the SLPS of a bacterium is only 1 to 4% of its dry weight).[15]

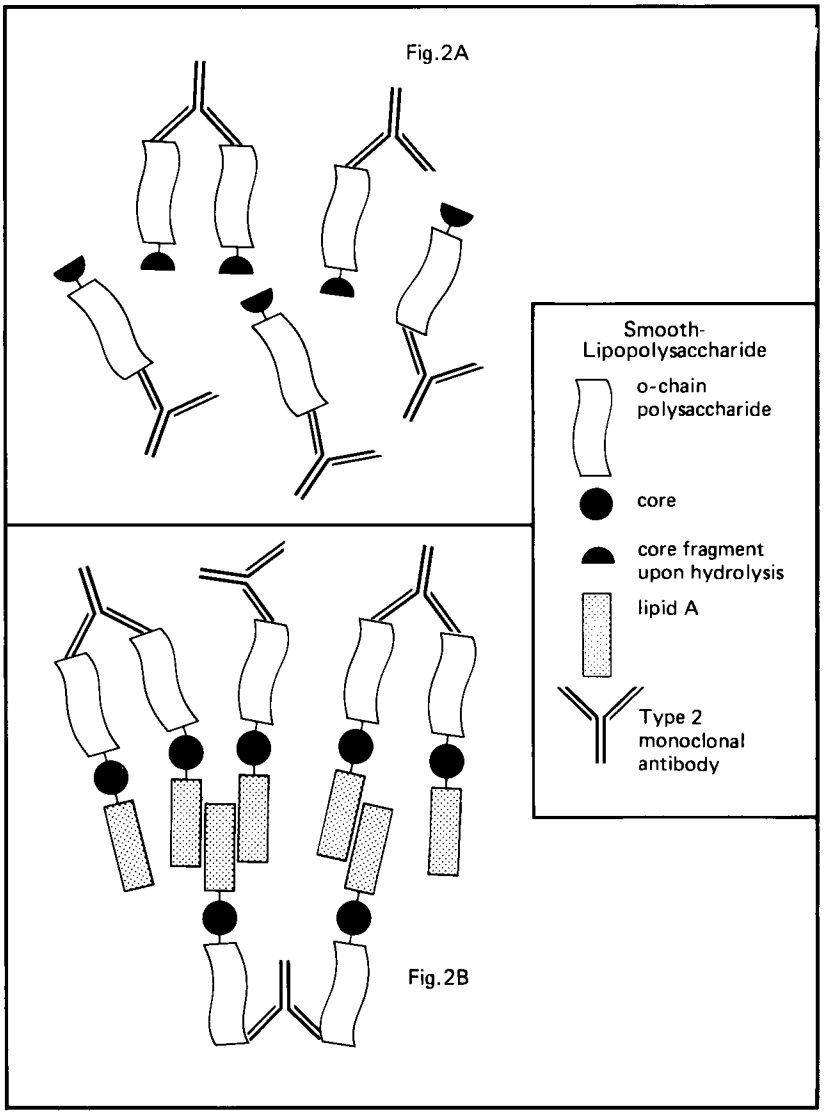

FIGURE 2. (2A) Absence of precipitation of O-chain polysaccharide by type 2 monoclonal antibody. (2B) Precipitation of smooth lipopolysaccharide by type 2 monoclonal antibody.

With the current understanding of the nature of the O-polysaccharide of *Brucella* spp. some questions remain to be answered:

1. If *Brucella* spp. are but biovars of *B. melitensis*,[71] how is the A and/or M antigen genetically regulated, and are these influenced by plasmid or bacteriophage nucleic acid?[72]
2. Although the O-polysaccharide may be immunodominant when compared to other antigens on the bacterium, are there regions of differing immunodominance on the O-polysaccharide (i.e., tip vs. length epitopes)?
3. Can either purified or conjugated antigens be used as protective vaccines, and can the immune status of the inoculated animal be differentiated (i.e., vaccinated vs. infected)?
4. Can screening serodiagnostic tests be improved by having a mixture of *B. abortus* and *B. melitensis* (A + M) antigens?

Although some of these questions are currently being investigated in our laboratories, possibly different ones are of interest to the reader. These are, indeed, interesting times, for with the advances in knowledge of antigen structure and in the development of novel reagents and technologies, the means exist to answer these questions.

II. PROTEIN ANTIGENS OF *BRUCELLA*

A. INTRODUCTION

Protein extracts of *Brucella* cells have been used as antigens, mainly in immunodiffusion and immunoelectrophoretic methods, to study either the antigenic structure of *Brucella* cells with hyperimmune sera or the immune response of experimentally and naturally infected animals and humans.[73] By an anatomic approach (e.g., isolation of cell wall) and by absorption studies of immune sera by whole cells, it has been possible to distinguish between surface (i.e., outer membrane) and internal (i.e., cytoplasmic) protein antigens. This distinction is not definitive because, as yet, little is known about the localization of *Brucella* protein antigens. There is also a need to distinguish antigens revealed by hyperimmune sera, raised by multiple injections of extracts with adjuvant, and antigens revealed by either sera or sensitized immune cells of naturally and experimentally infected hosts, raised by inoculation with live bacteria.

The major advances in the knowledge of *Brucella* protein antigens will be highlighted by a review of the distinction between cell wall and internal antigens in relation to their structure and function. Within this text, further information will be given on the method of obtaining sera from the hyperimmunized and infected host as well as the humoral and cellular immune response of the host.

B. CELL WALL PROTEIN ANTIGENS

The species of *Brucella* are members of the group of Gram-negative bacteria, and as such the cytosol of the cell is surrounded by a complex cell envelope consisting of a cytoplasmic membrane and a cell wall, the latter having a layer of peptidoglycan and an outer membrane.[74] The outer membrane, which forms the physical and functional barrier between the inside of the bacterial cell and its environment, is the first structure in contact with the host's immune cells during the early stages of disease.

1. Structure and Composition of the Cell Wall of *Brucella*

The outer membrane contains lipopolysaccharide (see Section I), proteins, and phospholipids.[75-77] Knowledge about the surface protein molecules (e.g., outer membrane proteins [Omp]) was limited for a long time because the *Brucella* cell wall had structural and biochemical differences with enterobacterial cell walls, notably:

1. The peptidoglycan layer of *Brucella* is strongly associated with the outer membrane and contains far more 1-2 glycols, as revealed by cytochemical reactions, than does the cell wall of *E. coli*.[74]
2. *Brucella* cells do not form spheroplasts under standard conditions.[78]
3. Nonionic detergents are less efficient in extracting Omp of *Brucella* cell wall as compared with *E. coli* Omp.[77,78]
4. Omp can be extracted from *Brucella* by Zwittergent® or sodium dodecyl sulfate (SDS) either without using lysozyme, provided the bacteria are not inactivated before extraction,[78] or by using lysozyme, provided the cells are inactivated by formalin or heat.[77,79]

The major Omp of *Brucella* are porins of group 2 proteins (molecular mass 35 to 40

kDa), group 3 proteins (25 to 30 kDa),[75,76,80] and a lipoprotein (8 kDa) covalently linked to peptidoglycan.[81] Group 1 proteins (88 to 94 kDa) are also found.[77,80] A covalent linkage between these proteins and SLPS has been hypothesized for *B. abortus*.[55,82] This hypothesis has not been confirmed by any structural studies.

The cell wall contains the *Brucella* protective antigens (Bpa).[73,83,84] Bpa are found in either the soluble or the insoluble fractions of extracted cells or cell walls. The soluble fractions, or the supernatants of extracts following centrifugation or ultracentrifugation, contain mainly SLPS and proteins. The insoluble fractions are found in the residues of phenol extraction, done on either cell walls (i.e., the Rh2 fraction)[85] or whole cells (i.e., the phenol insoluble [PI] fraction),[86,87] or in the residues of SDS-boiled cell walls (i.e., the SDS-I or PG fraction).[79]

2. Outer Membrane Proteins

a. Immune Response after Injection of Fractions or Purified Proteins with Adjuvant

i. Humoral Immune Response

Purified group 2 and 3 proteins in Freund's complete adjuvant (FCA) elicited antibodies against Omp and LPS when injected intradermally into cattle.[80] Immunodiffusion and immunoelectrophoretic methods showed two lines of precipitation between LPS absorbed sera and the group 2 and 3 proteins, indicating that these components were immunologically unrelated. Verstreate and Winter showed that porin preparations contained two antigens, "a" and "b", among *B. abortus* strains.[80] Antigen "b" was unique to porins, antigen "a" was common to most group 2 proteins. The common "a" antigenicity could be due to the presence of a murein determinant on both porins and group 3 proteins. These antigens were also present in most strains of *B. melitensis, B. ovis,* and *B. canis*.[80,89]

Porins, derived from *B. abortus* and in oil with immunomodulators such as muramyl dipeptide and trehalose dimycolate, when injected into cattle elicited antibodies that could be detected by ELISA.[88] Protective activity of Zwittergent® purified Omp in laboratory and domestic animals has yet to be reported.

The peptidoglycan-linked lipoprotein cross-reacts with rabbit hyperimmune sera against *E. coli* lipoprotein.[90]

Of the cell wall fractions containing Bpa (pa = protective antigens), only the SDS-I fraction has been characterized.[79] The SDS-I fraction contained two major protein and glycoprotein bands with an apparent molecular mass of 36 to 38 kDa and 25 to 27 kDa, respectively, which are Omp. Minor bands with a molecular mass of 70 and 31 kDa were present. The major isolated bands were not as protective as the entire fraction.[79] The correct identification of Bpa has been impeded by the presence of SLPS. When SLPS-free SDS-I fractions prepared from rough cells were used, these showed varying degrees of protection, but were less effective than the SDS-I fractions prepared from smooth cells. These results favor the hypothesis that probably both SLPS and proteins are involved in protection in the mouse model.

The PI fraction obtained from the phenol extraction of delipidated cells has been used as a vaccine in humans.[91] This fraction contained SLPS as shown by the serological response to SLPS dependent tests in people.[91] Further treatment of the PI fraction with deoxyribonuclease, ribonuclease, pepsin, papain, and pronase hydrolysis led to formation of fraction 4A, which contained peptidoglycan and proteins, plus a small quantity of lipids and carbohydrates. This fraction was about 5% of the original weight of PI and was still highly immunogenic in mice.[86] SDS solubilized about 55% of this material, and the soluble fraction (SF) was slightly protective in mice.[86] This fraction was not characterized in SDS-PAGE, and so it is difficult to determine whether or not it contained the 36 to 38-kDa and 25 to 27-kDa bands.

The identification of Bpa can be done indirectly by passive immunity or by assessing the protection given by the transfer of antibodies or lymphatic cells raised to reagents of

rigorously established specificity. Passively transferred polyclonal antibodies with undetermined specificities (mainly against SLPS) were shown to confer protective immunity to *B. abortus* in mice.[92] To define unique specificities, monoclonal antibody technology has been used. Monoclonal antibodies specific to the O-polysaccharide of *B. abortus,* when injected into mice prior to challenge infection, afforded significant protection.[19,93] In contrast, two IgM monoclonal antibodies specific for the porins of *B. abortus* failed to confer protection.[19] Other isotypes of monoclonal antibodies against major protein (36 to 38 kDa) and glycoprotein bands (25 to 27 kDa) have been produced, and their activity will be tested in the protective mouse model.

ii. Cellular Immune Response

Brucella-sensitized immune cells are able to transfer protective immunity in mice.[92,94] Antigens of the SDS-I fraction are involved in this protection, and the two major proteins are good candidates for inducing cell-mediated immunity. Porins, derived from *B. abortus* 45/20 and suspended in oil with immunomodulators (muramyl dipeptide and trehalose dimycolate), elicited blastogenesis and delayed type hypersensitivity when injected into cattle.[88]

b. Immune Response to Antigens during Experimental and Natural Infections

i. Humoral Immune Response

Antibodies against Omp were not detected by immunodiffusion using O-antibody absorbed sera from 11 cattle infected with field strains.[77] The antigenic reactivity of *B. ovis* Omp was further studied by immunoblotting. Antibodies of sera from *B. ovis*-vaccinated rams bound to all Zwittergent®-extracted Omp, while sera from naturally infected rams reacted only with the group 3 proteins.[36] These results have still to be confirmed because the electrophoretic separation of the proteins was poor and the group 3 proteins were located too near the RLPS bands for an accurate assessment.

ii. Cellular Immune Response

Porins were used to probe the cell-mediated immune response of cattle experimentally infected with virulent *B. abortus* field-strains and *B. abortus* strain 19.[95,96] The blastogenic response of peripheral blood lymphocytes of cattle was not indicative of the infection status, as nonpregnant heifers infected with the virulent strain 2308 reacted inconsistently while false-positive responses occurred. As a result of these difficulties, Baldwin et al. "do not recommend the lymphocyte blastogenesis test as a sole method for diagnosis of bovine brucellosis".[96]

The intradermal allergic skin test (AST) and the lymphocyte blastogenesis test (LBT) with cell wall antigens were used to identify infected humans, but no protein antigen was identified.[91] Fraction PI was also used to detect human brucellosis with LBT, but this test does not seem to be any better than the AST when melitine, the PI fraction, is used.[97]

c. Other Cell Surface Protein Antigens

Non-LPS surface antigens were detected with sera of infected and vaccinated animals by the following:

1. The A5 antigen, one of the seven distinct antigens of a soluble extract as revealed by sera from three infected cows, was shown to be present at the surface of *B. abortus* 45/20 cells.[70,98]
2. Unidentified surface antigens, prepared by a hot saline extraction of *B. abortus* 45/20 cells, were precipitated by the sera of *B. abortus* 45/20 vaccinated cattle.[99]
3. Sodium dodecylsulfate extracts of *B. abortus* 544/W were shown to contain three surface antigens: a, b, and X.[100,101] The X antigen, described by Raybould and Chantler, differentiated infected from vaccinated animals by passive hemagglutination,[100] but this assessment was not confirmed by other workers.

4. Salt-extractable protein antigens from *B. abortus* strains developed 1 to 4 immunoprecipitates when analyzed by cross-immunoelectrophoresis using infected cattle sera in the gel. In contrast, 20 to 25 immunoprecipitates developed with hyperimmune rabbit antisera.[102,103] Absorption of infected sera by whole cells did not remove immunoprecipitation against proteins, while the analogous absorption of rabbit hyperimmune sera removed 14 of the 19 immunoprecipitates. These results suggest that protein epitopes, recognized by the immune system of the rabbit after repeated injections in adjuvant, are different from those recognized by the bovine immune system.
5. Nonagglutinating antibodies, obtained by immunizing calves with *B. abortus* S-19 and then cross-absorbing the sera with *B. abortus* 45/20 cells, were able to label two (10 and 12 kDa) or three (10, 12, and 14.3 kDa) bands of a *Brucella* saline extract following SDS-PAGE and electroblotting.[104]

The results of immunoabsorption and surface localization of antigens on rough *B. abortus* 45/20 or other R cells must be interpreted with caution because of the reported nonspecific binding of IgG molecules to their surface.[70]

C. INTERNAL PROTEIN ANTIGENS

Many of the fractions obtained by cell disruption contain internal protein antigens with a variable quantity of cell wall antigens. The numeration and identification of *Brucella* antigens which are able to diffuse through agar and give a precipitate are dependent on:

1. The physical structure of the antigen, whether it is aggregated in vesicles or lamellae
2. The method of extraction of these antigens. Several of the fractions referred to as "soluble antigens" in the literature were crude and poorly characterized, either for physical or chemical properties. These protein extracts have been prepared by numerous methods, from simple extraction with water or saline buffers to cellular disruption by sonication, glass beads, or pressure. These extracts contained nucleic acids, cytoplasmic proteins, lipids, and variable amounts of SLPS or RLPS.
3. Whether the serum is obtained from infected or hyperimmunized animals (the latter may use multiple injections of live, killed, or extracted cells with or without adjuvant) and at what time it is taken
4. The concentration of antigen and antibody which must be optimal to form immunoprecipitates

1. Immune Response after Injection of Cells or Cell Fractions with Adjuvant

The number of immunoprecipitates revealed by hyperimmune sera may vary from 5 to 24.[73,102,103,105-108] A tentative identification of precipitable antigens has been done,[109] but definite relationships between the antigens described can only be established by purification and molecular characterization of the antigens and by exchanges of reagents between research groups for standardization.

2. Enumeration and Identification of Antigens during Experimental and Natural Infections

a. Humoral Immune Response

Numerous complex antigenic fractions have been analyzed by precipitation in gels (e.g., immunodiffusion, immunoelectrophoresis) against infected host sera to study the humoral immune response to *Brucella* (Table 2). As a result, there has also been considerable variation in the enumeration and identification of immunoprecipitates. The number has ranged from 0 to 9 precipitates, depending on the many experimental conditions discussed in the introduction. Only the A2 antigen has been partially characterized. Antibodies against the A2

TABLE 2
Enumeration and Identification of Antigens during Humoral Immune Response of Infected or Experimentally Infected Hosts

Species[a]	Extraction[b] method	Host[c] sera	Test[d]	Number of precipitates	Identified antigens[e]	Ref.
B. m(S)	TCA	r,g,c	ID	1—3		143
	GB	r,h		0—3 or >		144
	Phenol	c		3	SLPS, poly B	113
	Ether					113
(R)	NaCl	r		4		126
(R)		h		1—7		114
(R)		h	CIE	1—3		115
(R)		s		⩾1		116
B. m(S,R)	US	r	ID	2—6		113
B. ovis	US	r				113
B. a(S)	Acetone	c	ID	3—6		145
	Ether	c		1—5		146
	US	c	IE	9	SLPS	147
		c	—	11	SLPS	148
		c	ID	5	SLPS	149
		c	IE	3	A2	110
		c		6	A2	98, 117
		c,h	ID	0—3	SLPS?	150
	Saline, GB	c	TDI	3—4		102, 103
B. a(S)	Auto-claved	c	IE	2	SLPS	134
	Heat	c	ID	4		99
	SDS	c	IE	5		109

[a] m = *melitensis*; a = *abortus*; S = smooth; R = rough.
[b] TCA = trichloracetic acid; GB = glass beads; US = ultrasonication; SDS = sodium dodecylsulfate.
[c] c = cattle; g = goat; h = human; r = rabbit; s = sheep.
[d] ID = immunodiffusion; IE = immunoelectrophoresis; CIE = counterimmunoelectrophoresis; TDI = two-dimensional immunoelectrophoresis.
[e] SLPS = lipopolysaccharide from smooth cells; Poly B = polysaccharide B;[113] A2 = A2 antigen.[98]

antigen were found in the sera of infected cows and goats.[98,110,111,117] The A2 antigen is a heat-resistant glycoprotein of high molecular weight which has been partially purified.[112] It detected infected cattle with a moderate sensitivity (55%) and a good specificity (99%), while no A2 precipitins were detected in the sera of calves infected with *Y. enterocolitica* serotype O:9.[112]

One kinetic analysis has shown that for infected rabbits, antibodies arise first against SLPS, then against "polysaccharide B" (which has been characterized as containing a glucan and a polysaccharide related to the O-chain polysaccharide in antigenicity), and last against the surface and inner cell proteins.[113]

A saline extract (refer to the above paragraph on AST) called Brucellin-INRA, which contains at least 20 protein antigens, precipitated with the antibodies in human sera from patients with acute or chronic brucellosis when used in counterimmunoelectrophoresis (CIE).[114,115] The *Brucella* specificity of this antigen mixture eliminates serological cross-reactions due to *Y. enterocolitica* O:9 and *Vibrio cholerae* vaccination.[115] A Brucellin preparation was used in CIE to detect infected sheep and gave a high sensitivity of 82% and a high specificity of 78%.[116]

In conclusion, the host humoral response to *Brucella* is directed against SLPS and other surface or inner cell antigens. One of these antigens, the A2 antigen, was later proposed for diagnosis in immunodiffusion tests with the possibility of differentiating between infected and vaccinated animals. Nevertheless, vaccination of cattle by *B. abortus* strain B19 and of goats by *B. melitensis* strain Rev1 induces anti-A2 antibodies which may persist for 7 months after inoculation.[111,117]

b. Cellular Immune Response

The AST and the LBT have been the main tests used to probe cell-mediated immunity for identifying *Brucella*-infected humans or animals.[118] The following is a review of these assays.

i. Allergic Skin Test

The AST is now used as a diagnostic test in many countries (e.g., U.S.S.R., Italy, Greece, France, and China), and the various allergens have been well described.[119-121] The preparations of allergens are usually mixtures of cell wall and internal antigens and are not physicochemically characterized. Some saline soluble allergenic protein extracts were prepared from smooth strains of *Brucella* with the consequence of being contaminated with SLPS.[122,123] To avoid this problem, saline-soluble allergenic protein extracts (Brucellin) have been prepared for the last 15 years from rough strains of *Brucella* which are free of SLPS.[124-130] The allergenic activity of Brucellin is destroyed by pronase, suggesting that the protein molecules have the biological activity.[122,126] Of these preparations, Brucellin-INRA was the best studied and has the following criteria for being a suitable allergen for detecting delayed hypersensitivity:

1. Its physicochemical and molecular characterizations have been done.
2. It neither sensitizes nor desensitizes the host when several skin tests are made.
3. After infection a true delayed-type hypersensitivity is revealed.
4. It does not induce antibodies reactive in standard diagnostic tests (i.e., this suggests that the preparations are SLPS free).
5. The delayed hypersensitivity is specific for *Brucella*.
6. A titration assay is available, as are reference standards.
7. The allergen is stable for considerable lengths of time (at least 10 years).[125]

Brucellin-INRA contains at least 20 molecular species with no detectable LPS. A commercial preparation for sheep and goats is now available.[127] In cattle and goat herds or sheep flocks, Brucellin-INRA detected more positive infected animals than did standard tests.[131] Usually about 50% of the infected animals react in both serological and allergic tests, while only about 25% are serologically positive, and the remaining 25% are only positive by the skin test. The use of Brucellin to reveal delayed-type hypersensitivity and its use for identifying infected animals has been shown by its high specificity (greater than 95%), though it does have a relatively low sensitivity (about 60%), and by the AST which can detect a significant percentage of infected animals that test serologically as negative. These conclusions show that an AST using Brucellin would be suitable for routine surveillance to identify infected flocks and herds (e.g., beef herds[128] because for dairy herds the milk ring test can be used) and for accelerating an eradication program by using this test in addition to classical serology.

ii. Lymphocyte Blastogenesis Test

The LBT was first developed for chronic human cases of brucellosis often found to be serologically negative[132] and for serologically negative infected cattle.[133] Numerous partially

characterized antigens were used with partially purified blood lymphocytes, and several procedures were designed which have been previously discussed.[118] *Brucella abortus* soluble antigen (BASA) containing considerable amounts of SLPS, soluble proteins, and brucellin preparations were tested as antigens.[134,135] BASA was claimed to be superior to brucellin in detecting infected animals and for its ability to discriminate between infected and B19-vaccinated animals.[136] Nevertheless, the validity of using the LBT with SLPS containing antigens, such as BASA, has been seriously questioned because of observations of false-positive reactions and of problems with interpretation.[95,96,137] Also, the lymphocytes used may consist of B- and T-sensitized or immature lymphocytes which may undergo a specific or nonspecific blastogenesis in the presence of a great number of molecules (e.g., LPS and proteins) in the various extracts.[91,138] Additional research is needed on the standardization of LBT procedures as well as the preparation of purified antigens for the correct evaluation of this test.

Nevertheless, Brucellin, although contaminated with small amounts of LPS, as an antigen in LBT was able to differentiate bovine brucellosis from yersiniosis.[139] This result suggests that the *Brucella* protein in the reagent does not cross-react with the protein antigens of *Yersinia*.

D. CONCLUSIONS

Little is yet known about the protein antigens of *Brucella*. Outer membrane proteins which are potentially protective or suitable as diagnostic antigens are the best known. The use of one or a mixture of physicochemically characterized protein antigens in humoral and cellular tests can improve the diagnosis by preventing cross-reaction, since internal proteins differ antigenically from the SLPS of cross-reacting bacteria[139,140] and by detecting hosts infected by R species of *Brucella* (e.g., *B. canis* and *B. ovis*).

Progress in the knowledge of *Brucella* protective antigens, protective immune mechanisms, and diagnostic antigens will depend on the quality of the antigens (i.e., purification and characterization),[141,142] the standardization of operating procedures, and the exchange of reagents between the research groups to define the relationship between the prepared material and the reproducibility of results. The identification of protective antigens, whether SLPS or proteins, will greatly assist the production of an ideal vaccine containing only protective antigens, other than diagnostic antigens, used to differentiate infected and vaccinated hosts. Through the use of technologies such as hybridoma production and gene cloning, purified protein antigens should be available to probe unambiguously the immune response of the host.

III. *BRUCELLA* LIPIDS

A. INTRODUCTION

Members of the genus *Brucella* possess similarities with other Gram-negative bacteria, although they have properties of their own which clearly distinguish them from this group. The cell envelope, morphologically similar to that of other Gram-negative bacteria, consists of a cytoplasmic membrane, the periplasmic space, a peptidoglycan layer, and an outer membrane.[151] However, the chemical composition and physical properties make the cell envelope of *Brucella* unique among the Gram-negatives[152,153] This becomes apparent when the membrane fractions of *Brucella* are isolated and the components analyzed[21,59,62,76-78,82,154]

The role of surface macromolecules in determining the virulence of extracellular parasites has been well established.[155] However, it is not clear which function(s) the surface components play in the virulence of facultative intracellular pathogens such as *Brucella*. It is known that the rough *Brucella* types are more readily killed by polymorphonuclear leukocytes than the smooth counterparts.[154,156] It is likely that the chemistry and macromolecular or-

ganization of the surface components are important factors in the virulence of these microorganisms. Therefore, the ability of *Brucella* cells to replicate in phagocytes may be due, at least in part, to the particularities of their outer membrane.[154,157]

The group 1, 2, and 3 proteins of *Brucella* and a lipoprotein covalently linked to the peptidoglycan sacculus seem to be very similar to those present in *Enterobacteriaceae*.[75-77,81,89,152,153,158-160] Thus, it is likely that the major difference in the properties of the outer membrane of *Brucella* is in the proportion, species, and distribution of lipids (free and bound) present in this layer, as well as in their interactions with other molecules of the outer membrane.[21,81,152,161-168] The few reports on the precise composition of the *Brucella* peptidoglycan[169-171] and the cytoplasmic membrane[169-171] suggest that these two layers do not differ significantly from those of *Enterobacteriaceae*.

Brucella cell envelopes are relatively more resistant to the disrupting action of detergents and ethylene diaminetetraacetate (EDTA).[78] In addition, these bacteria are more resistant to the action of polymyxin B than other Gram-negatives,[152,172] polymyxin B is an antibiotic which interacts on membranes by its cationic detergent structure. These properties are probably due to various facts:

1. The *Brucella* LPS present in the outer membrane does not require stabilization by interactions with divalent cations.[78]
2. The properties and quantity of the 2-keto-3-deoxyoctonate (KDO) molecule(s) and the content in phosphate groups linked to the lipid A differ from other Gram-negative bacteria.[82,173]
3. The ionic interactions of some proteins associated to the peptidoglycan layer are weaker than in *Enterobacteriaceae*.[159]
4. The distribution, different proportions, and class of phospholipids in the outer membrane are different from most of the Gram-negatives studied.[152,163]
5. The overall hydrophobic forces operating in *Brucella* envelopes, in comparison with those operating in members of the *Enterobacteriaceae*, seem to be stronger.[78,152,163]

In addition, the cell walls from smooth strains are more resistant to the proteolytic action of enzymes and digestion with lysozyme than strains of rough *Brucella*.[154]

The *Brucella* outer membrane components are the principal molecules interacting with the immune system of the host.[11,54,62,75,79,80] We will devote most of this review to the structure of the "free" and "bound" lipids present in this layer, their implications on pathogenesis, their function in antibiotic susceptibility, the biological activities induced by lipid-bound macromolecules, and their significance for phylogenetic and taxonomic studies.

B. FREE LIPIDS

Free lipids are those which are readily extractable from the bacterial mass with a mixture of ethanol and diethyl ether and which are then separated by gel filtration and repurified by extracting them with chloroform/methanol. The free lipids of *Brucella* (4.5% of bacterial dry weight) include phospholipids (2% of bacterial dry weight) and the neutral lipids (2.5% of bacterial dry weight) which are separated and analyzed by different chromatographic procedures. Thiele and Schwinn[168] found that the amount of total free lipids of *Brucella* is strongly dependent on the culture media. The same seems to be true for the neutral lipid proportions of the total free lipids. Some authors have questioned the idea that the content of neutral lipids and phospholipids vary inversely from *B. abortus* through *B. suis* to *B. melitensis*, i.e., the last species containing the highest proportion of neutral lipids and the lowest of phospholipids.[168]

1. Phospholipids

From the lipid composition of *Brucella* presented in Table 3, several exceptional characteristics can be observed. Phosphatidylcholine (PC) has been found to be the major phospholipid of *Brucella*,[163,166-168] in contrast to *Enterobacteriaceae*, where phosphatidylethanolamine (PE) constitutes the major phospholipid.[174] It is striking that while PC is generally absent in most bacterial species, it is commonly abundant in plant and animal cells.[174] Recently, Moriyon et al.[152] and Gamazo and Moriyon[163] have described the proportion and distribution of phospholipids in the outer membrane (Table 4), taking advantage of the observation that *Brucella* cells release outer membrane fragments devoid of inner membrane and cytoplasmic markers but containing LPS and Omp. Phosphatidylcholine as a membrane constituent has different properties than PE.[175] In most eukaryotic membranes PE is distributed in the inner leaflet of the bilayer, while PC is mostly present in the outer leaflet. It has been established that the polar group of PE is able to form hydrogen bonds with adjacent phosphate groups.[176] Since all other characteristics of PE are very similar to those of PC, the higher viscosity given by PE in the membranes is very likely due to this property. This conclusion has been corroborated by the presence of two enzymes that sequentially methylate PE, transforming it to PC.[177] In eukaryotes, PC moves to the outer leaflet of the bilayer increasing the membrane fluidity. In Gram-negative bacteria containing PC, it is not known whether PC is also present in the outer leaflet of the outer membrane. Thiele and Schwinn[168] detected methylated PE in *Brucella* species, suggesting a similar biosynthetic pathway for PC as has been described for *Agrobacterium tumefaciens*.[178]

The outer membrane of enteric bacteria is highly asymmetric with respect to the distribution of LPS and phospholipids.[179-181] The outer leaflet is devoid of phospholipids and is almost entirely occupied by LPS, glycolipids and the external moiety of proteins. For enteric bacteria, this distribution might be important since they live in the presence of high concentration of detergent cholates in the intestinal tract.[180] In *Brucella* a respective asymmetric distribution in the outer membrane might make the cell less susceptible to the action of phospholipases, proteolytic enzymes, and lysozyme.[154] However, the possibility of having a different distribution of macromolecules in the bilayer of *Brucella*, with significant quantities of phospholipids in the outer leaflet of the outer membrane, cannot be ruled out at the moment.

As proposed by Gamazo and Moriyon,[163] it is likely that the outer membrane fragments released by *Brucella* represent more the average lipid than the average protein composition of the outer membrane. In comparison with the cell envelopes, the outer membrane fragments are enriched in PC (Table 4). Since the total PC content of *Brucella* is similar to the amount of PC found in the respective outer membrane,[163,168] it is feasible to suggest that most, if not all, of the PC is present in this layer.

Phosphatidylglycerol (PG) and diphosphatidylglycerol (DG), also present in *Brucella*, are probably the most commonly occurring bacterial phospholipids. Diphosphatidylglycerol usually occurs together with PG due to their biosynthetic relationship.[182] Diphosphatidylglycerol is the most apolar of the phospholipids, a property resulting from the presence of four acyl residues in the molecule instead of only two.[183] Phosphatidylserine, present in small amounts in *Brucella* as well as in other Gram-negative bacteria, is recognized as an intermediate in the biosynthesis of PE.[183] Phosphatidylinositol, characteristic of the membranes of eukaryotic cells and of some mycobacteria, has not been detected in *Brucella*.[168]

It is known that cyclopropane fatty acids occur frequently in bacterial lipids. The C17:0 cyclic seems to be more frequent in Gram-negative bacteria and the C19:0 cyclic (lactobacillic

TABLE 3
Lipid Composition of *Brucella*[a]

	Free lipids (4.5% of bacterial dry weight)															
	Bound lipids				Phospholipids (2% of bacterial dry weight)[b]								Neutral lipids (2.5% of the bacterial dry weight)[c]			
	LPS (2.5%)[d]		Lipoprotein		DG (20.1%)[e]		PG (9.4%)[e]		PE (32.9%)[e]		PC (37.6%)[e]		Wax-like esters (6%)[f]	1,2- and 1,3-diglycerides, diol monoesters, α-glycol esters, and other acyl esters (28%)[f]	Ornithine-containing lipids (32%)[f]	
Fatty acid	A	E[g]	A	E	1	2[h]	1	2	1	2	1	2			A	E
12:0	—	—	—	—	t[i]	t	1.1[j]	t	1.5	t	t	t	6.4	2.6	t(?)[k]	
14:0	—	—	6.8	12.0	1.1	t	1.6	t	4.1	t	t	t	12.8	3.2	t(?)	
15:0	—	—	—	—	1.3	t	t	t	t	t	t	t	5.4	0.7	—	—
16:0	—	37.0	28.3	20.7	48.9	16.0	72.6	40.4	53.9	10.7	52.7	7.6	34.5	27.3	30.4	—
17:0	—	1.8	—	—	2.5	t	t	t	t	0.2	1.4	0.3	2.0	1.5	0.3(?)	
18:0		4.5	22.6	24.1	6.4	5.2	4.5	6.2	8.4	2.7	4.0	3.6	8.9	9.0	12.2	—
20:0	—	—	4.9	9.9	—	—	—	—	—	—	—	—	—	—	—	—
16:1	—	—	2.9	1.1	39.0	1.9	9.7	4.3	11.1	1.8	31.2	0.7	8.8	4.1	—	1.9
17:1	—	—	—	—	—	—	—	—	—	—	—	—	t	1.3	—	—
18:1	—	—	12.1	3.2	0.9	16.4	t	9.5	10.9	13.9	3.6	8.3	9.8	17.8	—	18.4
3-OH-12:0	7.8	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
3-OH-13:0	t	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
3-OH-14:0	15.4	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
3-OH-15:0	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
3-OH-16:0	15.1	12.4	—	—	—	—	—	—	—	—	—	—	—	—	—	—
3-OH-18:0	—	3.5	—	—	—	—	—	—	—	—	—	—	—	—	—	—
17:Ocyclic	—	—	1.8	3.5	—	—	—	—	—	—	—	—	—	—	—	—
19:Ocyclic	—	—	1.7	0.8	t	57.2	6.8	39.8	7.4	69.6	7.1	79.5	—	31.3	—	29.3
Other	1.3(?)		18.9	23.7	t	3.3	3.7	t	2.7	1.1	—	t	11.4	1.2	7.5(?)	

[a] LPS data are unpublished results obtained with *B. melitensis* by E. Moreno and H. Mayer. Similar results were obtained with *B. abortus*. Fatty acid composition of lipoprotein was obtained from Gomez-Miguel and Moriyon[81] with *B. abortus*. The phospholipid and neutral lipid compositions were taken from Thiele and Schwinn[168] with *B. melitensis*.

[b] Diphosphatidylglycerol (DG), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), phosphatidylcholine (PC). Phosphatidylserine is present in trace quantities in *B. melitensis* and 5.3% in *B. abortus*.

[c] Ubiquinone Q_{10} is the predominant component of neutral lipids, comprising 30% on a dry weight basis.

[d] Expressed as % of the bacterial dry weight.[90]

[e] Expressed as % of dry weight of the total phospholipids.

[f] Expressed as % per dry weight of the total neutral lipids.[154]

[g] Amide- (A) and ester (E)-linked fatty acids. The hydroxylated fatty acids exist as acyl-oxyacyl residues: 3-O-(16:0)12:0, 3-O-(16:0)13:0, 3-O-(16:0)14:0, and 3-O-(18:0)14:0.

[h] Position of the fatty acid in the individual phospholipid.

[i] Trace (t).

[j] In LPS and neutral lipids, numbers express % fatty acid per dry weight of total (A + E) fatty acids. In the lipoprotein and phospholipids, numbers represent % of the corresponding species (A or E linked; 1 or 2 position) of fatty acid.

[k] Linkage is not known (?).