Cell Wall-Deficient Bacteria

Basic Principles and Clinical Significance

Gerald J. Domingue, *Editor*

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Tulane University School of Medicine
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PREFACE

There is a considerable body of experimental and clinical evidence—much of which has never been published—supporting the concept that cell wall-deficient bacteria (CWDB) may be agents of disease. Although the pathogenic potential of CWDB for humans and laboratory animals has been the subject of journal reports, the results of many of these findings have been inconclusive, sometimes contradictory, and often clouded with controversy. Frequently, these aberrant bacteria have been regarded as laboratory curiosities of little or no clinical significance. This book dispels that myth by providing up-to-date experimental and clinical data which describe and amplify the processes of cryptic parasitization with CWDB. These data demonstrate that CWDB can be isolated from the tissues and body fluids of patients when ordinary bacterial culture of the same specimens yield negative results. Herein, the central thesis is that cryptic parasitization with CWDB is an important bacteriologic entity often overlooked in clinical medicine. Most written accounts on the subject have dealt with the fundamental microbiology of CWDB. Although the expertly edited volume by Dr. Lucien Guze in 1968, the monograph by Dr. Lida Mattman in 1974, and the proceedings of a conference in Montpellier, France in 1976 edited by Dr. Jacques Roux, called attention to the possible relevance of CWDB in medicine, there are no current books whose primary focus is on the clinical significance of these unusual bacteria.

It is generally agreed among scientists that CWDB are extraordinarily intriguing and interesting tools for biological study, yet the most neglected research area has been on the role of these organisms in disease and particularly in host-parasite interactions. This book meshes fundamental data

with clinical relevance. For some diseases described, it has been clearly established that CWDB can be induced within a suitable host; they can survive and persist in a latent state within the host; and they can induce pathologic responses compatible with disease. The observations delineated in this book regarding the roles of CWDB in experimental infections provide some understanding of the mechanisms of both latency and chronicity which typify certain infections.

This book does not purport to show that all definitive answers have been obtained to the questions about the role of CWDB in disease. Quite the contrary, the purpose here is to call timely attention to this neglected area in medicine so that others will want to explore this exciting and provocative subject. It is hoped that much of the information presented in this volume will convince the reader of the worthiness of continuing to search for a buried bacterial genome as a cause of persisting infection and disease in certain patients. The intent has been to try to make a field which has traditionally been very murky for the clinician much clearer. In this way, it is hoped that it will be possible to make more worthwhile clinical and laboratory observations and interpretations which, finally, will encourage and facilitate additional research.

I proudly salute all contributors and thank each one for their willingness to participate as authors. I am most grateful to my technical assistant, Mrs. Kamma Pontoppidan, for her competence and for her help in compiling data. To the following research technicians who have been a part of my research program, I express my sincere appreciation: Gail Fernandez, Carole Richtmyer, Sunita Prabhu, Lisa Matthews, Trudy Crow Oswald, Dwight Hardy, Debbie Duckworth, Celia Stokes, Pearl Gervais and Kathy Fabricant. To my former students, Drs. Mary Green, James McLaughlin, Reutai Sakulramrung, Rudolf Bruppacher, Ali Salhi and to post doctoral fellows, Drs. Gary Frentz, Bruce Turner, Keith Lloyd, Andy Daniels, Bertholdt Ponig, Michel Auger, Alfred Colfry, Leo Lowentritt and Karolyn Hardaway who participated as collaborators in various phases of my research activities, I express my sincere thanks. I am most grateful to the following faculty associates for their assistance and advice: Drs. Jorgen Schlegel, Hannah Woody, Norman Woody, Paul Heidger, Jr., Melanie Ehrlich, James Roberts, Blackwell Evans, Gary Frentz, Ronald Lewis and Richard Harrison. Special thanks go to my many clinical colleagues at Tulane University Medical Center, and Ochsner Foundation, Methodist, Southern Baptist and Jo Ellen Smith Hospitals in New Orleans.

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Gerald J. Domingue

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Chapter 1

BASIC BIOLOGY OF CELL WALL-DEFICIENT BACTERIA

Suzanne K. Patterson and Richard W. Gilpin

NOMENCLATURE

Historical

The origin of studies on cell wall-deficient bacteria (CWDB) is closely tied to the earlier discovery and research with *Mycoplasmas* (formerly known as pleuropneumonia-like organisms - PPLO). The first report on isolation of an L-form in 1935 was by Klieneberger-Nobel (60), who found an organism which had a colony morphology similar to PPLO bacteria. This organism was first considered to be a PPLO. Dienes (21) and Van Rooyen (131) soon found that this culture was actually derived from a Gram positive bacterium, *Streptobacillus moniliformis*. Once the bacterial parentage was established, Klieneberger-Nobel described this culture with atypical colony morphology as a stable colony form which did not revert to the usual parent bacterium colony. She named these bacteria "L-forms" after the Lister Institute where she worked.

Current

The difficulties in sorting out the terminology applied to

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L-forms and associated CWDB can be traced to their morphological similarities with Mycoplasma. As research with L-forms increased, so did the number of terms in the literature. Certain terms or designations have been applied to both L-forms and Mycoplasma. For example, "L" was originally used to designate Mycoplasma strains studied by Kleineberger-Nobel. Mycoplasma and L-forms were considered to be "filterable", meaning that they could pass through filters with pore sizes that usually retained bacteria but not viruses. Both entities often grow with a characteristic "fried-egg" colony morphology on agar medium. Light microscopy and electron microscopy show that L-forms and Mycoplasma also have similar morphologies.

The terms, "elementary bodies" (0.1-0.3 µm diameter) and "large bodies" (5-50 µm diameter) have been used to describe morphological units found in both. Some other terms used in the literature in reference to L-forms are: "L-phase","L-phase variant", and "L-variant". In addition, some authors have used the morphologically descriptive terms, "spheroplast" or "protoplast" to describe L-forms.

It is important to be aware of these terms and how they are defined. Clearly, it would be helpful to establish uniform terminology. In the monograph, Cell Wall-Deficient Forms (88), Mattman put together some definitions of the various terms used for L-forms and other morphologically related bacteria. We suggest that the terminology should be simplified to reflect the original definitions. Some of the terms put forth by investigators are listed below. Those that we feel should be retained are marked with an asterisk.

*CELL WALL-DEFICIENT BACTERIA: Bacteria which have lost all or part of their cell wall. This term is broadly inclusive (88). ELEMENTARY BODY: Very small morphological units found in L-form cultures; considered by some to be the smallest reproductive unit (22). FILTERABLE FORMS: Bacteria which can pass through a filter with a pore size of 0.20 µm or less and remain viable; may apply to L-forms and Mycoplasma (21,25,61,62,84). G-FORMS: Granular colonies associated with some antibiotic-induced cell wall-deficient bacteria. They revert to the parent bacterium colony morphology when antibiotic is removed and are thought to be an intermediate stage in the development of L-forms (145). *INDUCTION: A process by which a parent bacterium undergoes partial or complete loss of cell wall material by in vitro manipulation of the environment or

metabolism of the parent organism, resulting in an L-form (107). *L-FORM: Derived from bacteria; having no rigid cell wall; able to reproduce; not able to revert to the parent bacterium; a colony morphology on solid medium described as "fried-egg" (23,54,62,63,91,99,135,137). *L-FORM COLONY: Colony formed by an L-form on solid medium. A "fried-egg" colony with a dense center that penetrates the agar, surrounded by growth that spreads over the agar surface (21). L-PHASE: Term proposed to replace L-form (29). L-PHASE VARIANT: Term proposed to replace L-form (29,88). LARGE BODY: Large morphological unit (up to 50 μm) found in L-form cultures; considered by some to play a role in L-form reproduction (23). *PROTOPLAST: Bacteria with no demonstrable cell wall. Most authors include in this definition the inability to reproduce (7,32,59,67,70,85,90,134,137). *REVERSION: Return of a cell wall-deficient form to the morphology of the parent bacterium with formation of a cell wall (21,25). *SPHEROPLAST: Bacteria which retain some cell wall components. Usually not considered able to reproduce (7,59,70, 85,90,127). TRANSITIONAL FORM: Bacteria which have the properties of an L-form but are capable of reverting to the parent bacterial form. Unstable L-form and L-phase variant have been used to describe transitional forms (56, 57,58,135,137).

Recommendations

In light of the confusion in terminology, the use of more than one term to describe the same thing seems counter-productive. For instance, the term "L-phase variant" instead of "L-form" does not provide any additional information. A return to some of the original terms is strongly endorsed as a logical approach to keeping terminology uniform and readily understandable. This is not a new idea. In 1958 Lederberg and St. Clair (74) stated that "The time is perhaps nearly ripe for a notation that better reflects our concepts of these (L-form) structures."

We recommend that only the terms CELL WALL-DEFICIENT BACTERIA, INDUCTION, L-FORM, L-FORM COLONY, PROTOPLAST, REVERSION, and SPHEROPLAST be retained. The terms SPHEROPLAST and PROTOPLAST would be used most appropriately as descriptions of cell morphology seen by electron microscopy. No cell wall is observed in protoplasts, but some observable cell wall is present on spheroplasts.

ULTRASTRUCTURE

When considering L-form morphology it is helpful to be familiar with the morphology of classical, cell wall-containing bacteria; morphological entities such as spheroplasts and protoplasts that resemble L-forms; and members of the class Mollicutes such as Mycoplasma. Light and electron microscopy are used to observe the structure of individual L-forms.

Ultrastructure of Classical Bacteria

L-forms can be induced from both Gram negative and Gram positive bacteria. It is easier to induce them from Gram positive bacteria, however. This may be related to the structural differences between the cell walls of Gram positive (Fig. 1) and -negative bacteria (Fig. 2). Gram positive bacteria have a trilaminar (18) cell wall composed of a thick (15-50 nm) peptidoglycan matrix containing other

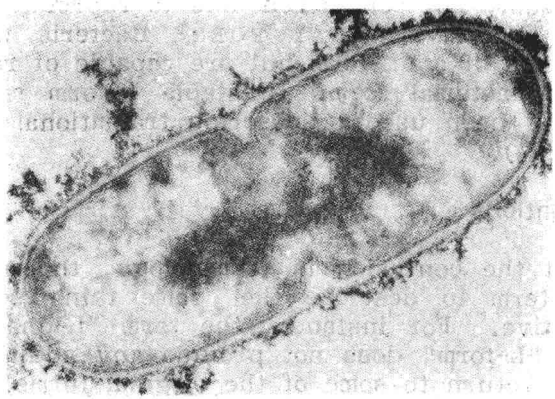

Fig. 1 Structure of a Gram positive bacterium (Streptococcus mutans) in thin section. The thick trilaminar cell wall can be seen external to the electronlucent cytoplasmic membrane. Magnification: 104,000 X. Courtesy of John J. Bozzola, Ph.D.

macromolecules such as teichoic acid and lipoteichoic acid. Gram negative bacteria have a more complex cell wall. The peptidoglycan is thinner (1-3 nm) and is not easily visualized by electron microscopy. An outer cell wall membrane containing lipopolysaccharide is anchored to the peptidoglycan by lipoprotein. Other proteins, carbohydrates and lipids are also associated with cell walls of both Gram

positive and -negative bacteria.

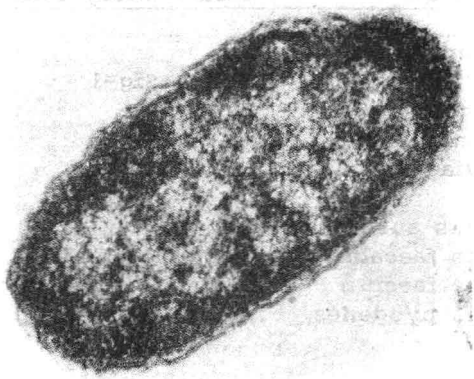

Fig. 2 Structure of a Gram negative bacterium (Legionella pneumophila) in thin section. The wavy cell wall outer membrane can be seen external to the cytoplasmic membrane. Magnification: 11,000 X. Courtesy of John J. Bozzola, Ph.D.

L-form Induction

The construction of the Gram positive cell wall facilitates its removal by in vitro techniques. This may result in the induction of L-forms under the appropriate conditions. This has been demonstrated with Bacillus species (33,71) C. botulinum (9), M. lysodeikticus (115), S. aureus (133), Streptobacillus (107), and Streptococcus species (44). The induction methods often include treatment with cell wall lytic enzymes such as lysozyme (134) or antibiotics such as penicillin (129), that affect cell wall synthesis. Usually, protoplasts produced by these procedures do not retain the ability to replicate as L-forms.

Because Gram negative bacteria have a more complex cell wall organization, subjecting them to similar enzymatic and/or antibiotic induction pressures often gives rise to spheroplasts which retain some vestages of the cell wall. N. meningitidis and N. gonorrhoeae L-forms have been produced by penicillin treatment (111). L-forms have also been induced from E. coli (125), Haemophilus (73), P. mirabilis (23), and Salmonella (66,117). Some well documented L-forms are listed in Table 1. This is not intended to be a complete tabulation.

Table 1. L-forms Reported in the Literature

Parent Bacterium	Strain Designation	Reference
Bacillus licheniformis	Slf	(33,142)
Bacillus subtilis	Sal-1, Sig-1	(39,146)
Escherichia coli	Ecl	(27)
Neisseria meningitidis		(112)
Nocardia caviae		(2)
Proteus		(93,138)
Staphylococcus aureus	209 PL	(28)
Streptococcus faecalis	(GK) T53,T531	(94,95)
Streptococcus faecium	F24	(43,59)
Streptococcus pyogenes	AED, Type 12	(99,102, 104,121, 144)

It is not easy to determine whether an L-form has been induced by these procedures. Protoplasts or spheroplasts may lyse, revert to classical bacteria, or become L-forms. Spheroplasts and/or L-forms of E. coli described by Gumpert and Taubenek (46) grew as L-forms on medium supplemented with penicillin but reverted when penicillin was removed. This is typical of many recently induced L-forms. It has been proposed that a mutational event may be necessary to produce an L-form which does not revert (69,74,86). Hoyer and King have shown an actual loss of genome in a streptococcal L-form (50), which lends further support to this theory.

L-form Ultrastructure

It is difficult to distinguish between various cell wall-deficient bacteria, including Mycoplasma, by light or electron microscopy. Typical findings are presented in order to illustrate this point.

Light Microscopy

L-forms can be observed by light microscopy of stained L-forms and colonies and by phase contrast microscopy of wet mounts. Most light microscopy of stained L- forms has been done with colonies on solid medium. Dienes (21) developed a staining technique using Methylene and Azure blue to stain L-form colonies on agar blocks after they were

placed on a glass slide and sealed under a coverslip. Colonies can also be stained in situ without cutting out agar blocks (119), as shown in Fig. 3.

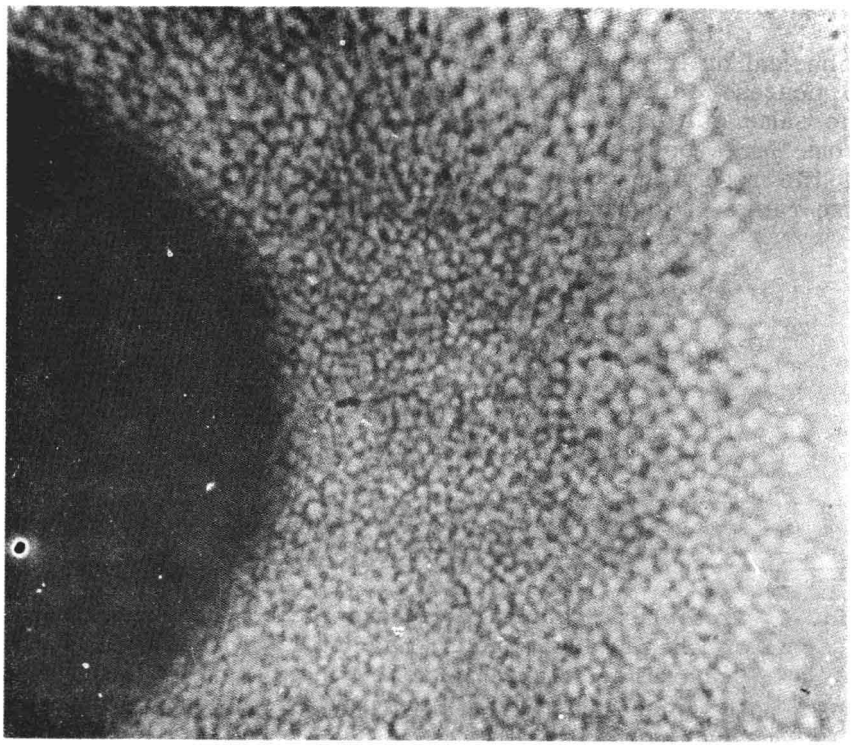

Fig. 3 Dark center and light edge of a S. aureus L-form colony on agar medium stained in situ with the Dienes stain. Approximate magnification 215 X.

An acridine orange method has also been used to stain L-form colonies and individual L-forms in broth cultures (89). A fluorescent microscope is needed for this procedure, however. Clive amd Landman (16) used methylene blue and safranin to differentiate Gram positive bacteria from L-form colonies on membrane filters. L-forms are uniformly Gram negative and have no cell wall. Therefore, Gram stained smears show a pink background of cellular debris with no discernable structures.

Oil immersion, phase contrast microscopy is the best way to observe viable L-forms. The following figures com-