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Bacterial Persistence and Expression of Disease

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INTRODUCTION

A review concerned with the pathology generated by the persistence of difficult-to-culture or nonculturable bacteria in human infections must necessarily take into account not only their morphologic and metabolic diversity but also the consequences of their interaction with other microorganisms and their host (121, 122). When bacteria were first recognized as causing disease, they were seen to be relatively stable cell wall-containing forms that grew and expressed their toxic metabolites in vitro. These forms tended to be quite virulent, so connecting them to particular diseases was not difficult. Since then, technologic developments have revealed a continuum of microbial agents, viruses (and smaller) to protozoa, each diverse and continually changing. The interaction of these organisms within the host can lead to the enhancement or depression of their individual properties. Clinical expression of

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their presence in the host depends on the genetic vulnerability of the host, the particular environmental stresses, and the number and location of such consortia. The clinician who faces this tangled scenario must quantitate and define the dynamic that has led to the patient's illness.

Survival of a species requires that a reasonable identity be maintained. Over time, mechanisms to maintain "self" have evolved. Because life must be nourished by life, it is inevitable that subtle parasitism should also evolve. Many such relationships have been so successful that both host and invading organism benefit. Such a process, which transiently reduces immune competence, can occur episodically in healthy subjects in association with various stresses or in particular diseases, such as rubeola. As one ages, there tends to be an insidious accumulation of intracellular microbial forms. Such quiescent organisms tend to be activated to a more toxic form when homeostatic disturbances threaten their cellular loci. The numbers and locations of cells involved with one or more types of organisms determine the clinical reaction. It can be very difficult to decide whether a new illness is due to a new organism or to an interaction with one or more cryptic organisms. These interactions can be as complex as the well-known increase in toxicity of *Corynebacterium diphtheriae* when this bacterium is infected by bacteriophage. The distinction between phage genes and bacterial genes is blurred with respect to both function and reality. It is conceivable that much of the heredity of bacteria is of viral origin, because many unknown defective proviruses may exist in nature; on the other hand, phages may be fragments of bacterial DNA that have acquired the capacity for independent reproduction. Indeed, with a history of mutual interaction of viruses and bacteria over the course of evolution, the question of sharply distinguishing their genes must be meaningless. These philosophic concepts are implicit in any discussion of the role of dormant, persistent, difficult-to-culture, and impossible-to-culture bacteria in disease.

Many bacteria can be visualized by electron microscopy (EM) but cannot be grown in culture. Nucleic acid analyses can approximate the locations of these bacteria on the phylogenetic tree. Unfortunately, none of these sophisticated laboratory procedures are consistently successful in identifying such organisms, nor are they guides to optimal therapy. Cryptic organisms, whether intra- or extracellular, are ubiquitous; their role in disease requires more than the demonstration of their presence. Quantifying and identifying the cells most parasitized are impractical clinical approaches. Koch's postulates cannot be fulfilled, because it is impossible to precisely duplicate all the variables that are involved in disease expression. In such a situation, technology plays a lesser role, and the art of medicine prevails. This art involves time-consuming questions and observations directed toward identifying the patient's susceptibility to stress and the frequency and character of that stress. Certainly, any patient with a history of recurrent infections and persistent disability is sending signals that this phenomenon is occurring. The so-called autoimmune disorders, in which no organisms can be identified by routine techniques, are suspect in this regard.

The selection of antimicrobial agents for patients with cryptic infections can be quite frustrating. Even if an organism grows in vitro, it may not represent the primary pathogen. In addition, drug susceptibility testing fails to reveal the action of the agent on the infecting organism's toxicity and capacity to adhere to cell membranes in vivo.

For good reason, physicians have been discouraged from the indiscriminate use of antimicrobial agents without strong cultural evidence that the therapy is appropriate and that the severity of the illness justifies the risk of side effects. However, in recent years, many once idiopathic, chronic, debilitating disorders have been discovered to be caused by microbial agents. Cultural techniques available to most physicians fail to reveal the pathogen. In such situations, when there is microscopic and/or molecular evidence for bacterial presence in clinical specimens, the cautious use of antimicrobial agents can be justified. Case presentations at the end of this review tend to support this philosophy.

CWDB AS PERSISTERS: SUMMARY OF BASIC BIOLOGY OF CWDB L-FORMS

A considerable body of experimental and clinical evidence supports the concept that cell wall-deficient/defective bacteria (CWDB) may be agents of disease. Although the pathogenic potential of CWDB for humans and laboratory animals has been the subject of many journal reports (26, 27, 50, 61, 64, 66–68, 83, 88, 96, 137, 139, 145, 181, 182) and several books (51, 84, 129, 136), the results of these studies have often been inconclusive, sometimes contradictory, and always clouded with controversy. Frequently, CWDB have been regarded as laboratory curiosities of little or no clinical significance. Contrary to this viewpoint, the central thesis of our review is to demonstrate that dormant bacteria in the form of CWDB, nutritionally deficient organisms, or difficult-to-culture bacteria in general may serve as cryptic agents of disease in a variety of human infections. We believe that "CWDB" is the term most broadly inclusive and descriptive of aberrant bacterial forms present in clinical specimens. The term L-form has been retained for historical perspective and for accuracy in reviewing the literature. Since there is no uniform terminology, we suggest that the term CWDB best reflects the anatomy of bacteria in clinical specimens. These organisms have been shown by EM to be partially or completely cell wall deficient, and they may or may not revert to walled forms in vitro (52, 53, 88). Although the usage is controversial, most microbiologists would agree that the term L-form describes an organism that is derived or induced from a bacterium and has no rigid cell wall. When L-forms are grown on solid media, they produce colonies that resemble those of mycoplasmas. However, few of the CWDB from clinical specimens grow as the classic "fried egg" colonies in vitro. In the presence of an inducing agent such as penicillin, L-form colonies can be propagated indefinitely in appropriate media. When the inducing agent is removed, the L-form may revert (unstable L-form) or may be stabilized (stable L-form) and be no longer able to revert (129). CWDB from clinical specimens can assume all of these forms in vivo and in vitro (51).

It is generally agreed among scientists that CWDB are extraordinarily intriguing, interesting tools for biological study, yet the most neglected area of research has been on the role of these organisms in disease, particularly in host-pathogen interactions. We believe that before we explore the clinical relevance and pathogenic potentialities of dormant bacteria in specific diseases, a brief summary of the basic biology of L-forms will help clarify the role of aberrant bacteria in latency and chronicity of certain infectious diseases.

History

In the early literature, most of the studies of bacterial L-forms were closely linked to the pleuropneumonia-like organisms (PPLO) that later came to be known as mycoplasmas. Klieneberger (109) is credited with having described the first L-forms (named in honor of the Lister Institute, where she worked). Although Dienes (35) demonstrated that the or-

ganism described by Klieneberger was, in fact, a bacterium, *Streptobacillus moniliformis*, Klieneberger initially described the colonies in culture as resembling pleuropneumonia-like organisms. She thought that these organisms were symbionts with *S. moniliformis* in culture. Dienes challenged Klieneberger's concept of symbiosis and proved that the L-form colonies observed by Klieneberger could return to a bacillary state. Later, Dienes was successful in isolating L-form colonies from several other bacterial species showing spontaneous transformation and established that bacteria could multiply in the absence of a rigid cell wall (34, 36, 37, 47, 48, 51, 84, 129, 136). Since that time, a voluminous literature on the morphological, serological, and biochemical properties of L-forms has been produced (4, 5, 23, 24, 27, 28, 30, 38–46, 51, 72, 74, 76–78, 80, 81, 84, 89–92, 94, 95, 100, 103–106, 110–112, 114–116, 118, 120, 123–130, 132, 133, 142, 144, 154–157, 160, 172, 174, 175, 179, 183–185).

Induction of a Wall-Deficient/Defective State and Spontaneous Change to L-Forms

Probably all known bacterial species can be converted to CWDB by a variety of inducing agents. Among the best known of such agents are cell wall-inhibiting antibiotics, high concentrations of amino acids (notably glycine and phenylalanine agents such as lysostaphin), and peptidases and lytic enzymes that digest the murein of the cell wall. It has been demonstrated that L-form colonies can be propagated indefinitely in the presence of inducing agents in suitable media (128, 131). Removal of the inducing agent may result in reversion to the parent bacterial form, or the organisms may remain stabilized as L-forms that are incapable of reversion to a wall-containing form. Host-induced cell wall modifications of bacteria have been demonstrated. For example, in an ultrastructural analysis of *Nocardia asteroides* during experimental infections in mice, Beaman (6) demonstrated that significant structural modification of the cell envelope occurred as the organisms adapted and grew within the host. The least-virulent strains underwent the greatest alteration, and the more-virulent organisms were affected the least. In addition, there were concomitant tinctorial changes (Gram reaction and acid fastness) that suggested that chemical modification had occurred in the cell wall as the result of growth of *N. asteroides* within the host.

General Characteristics

The nutritional requirements of in vitro-induced L-forms are usually similar to those of the parent bacterium from which they were derived (129). Enriched media are most often necessary to grow the organisms in vitro from clinical specimens. Media supplemented with animal sera or lysates of blood cells and yeast extract are most useful (51). Some L-forms may require osmotically stabilized media for continued propagation. However, L-forms induced on hyperosmotic media have been adapted to grow on media of normal osmolarity (144). CWDB can survive in environments with osmolalities lower than their intracellular osmotic pressure. The intracellular osmotic pressure for gram-negative bacteria is 300 to 400 mosmol/kg of water, and that for gram-positive bacteria is $\geq$900 mosmol/kg. Organisms confronted with an environment of lower osmolality, such as human serum or ordinary bacteriologic media (270 to 300 mosmol/kg), must intrinsically stabilize their cytoplasmic membranes to survive (144). Divalent cations or polyamines or an adaptive change to a higher ratio of saturated to unsaturated fatty acids in the membrane may satisfy this requirement for preserving the integrity of the cytoplasmic membrane (56, 116). All morphological variations, including binary fission, budding, and filamentous growth, have been observed as means of replication for CWDB. The consensus is that most CWDB usually divide by binary fission.

It is no longer controversial that many bacteria, such as *Neisseria gonorrhoeae*, *Bacteroides fragilis*, *Haemophilis influenzae*, *N. asteroides*, and various species of *Bacillus*, can undergo spontaneous change to the L-form (129). Organisms undergoing this transformation can replicate serially as nonrigid cells that are spherical and highly pleomorphic; many are larger than some human cells and are the source of seed-like elementary bodies as small as some viruses. It has been reported that L-forms retain, in general, the antigenic, biochemical, and metabolic characteristics of the parent wall-containing form. In our experience, aberrant bacterial forms isolated from clinical specimens do not always retain the identical characteristics of the parent bacterium, because a variety of biotypes that were presumably derived from a single genus and species in a given patient can be demonstrated. These biotype differences are particularly demonstrable in patients with long histories of chronic diseases suspected of being bacterial in origin. It is not uncommon to demonstrate in the initial revertant culture from clinical specimens variations in biochemical patterns of reactivity of individual, morphologically identical colonies. When these properties are stable, it is possible that these changes represent mutational events that may have been favored by prolonged in vivo maintenance and selection of CWDB, such as in a chronic infectious disease (52).

Landman et al. (110–113) performed some of the more definitive studies on L-forms as a genetic system. These investigators and others (184) have provided answers to questions on the nature of the heritable mechanism accounting for the stability of L-forms. The gene complement of L-forms is probably the same as that of the parent bacterium from which the L-form was derived. There does not seem to be a gain or loss of nucleic acid when bacterial cells convert to L-forms or when they revert to the wall-containing bacterium. The early work of Landman showed that every cell of *Bacillus subtilis* could convert to an L-form and that every L-form could regain the cell wall. Landman's conclusions focused on two key events: commitment (loss of ability to form a wall-containing bacillus) and reversion (ability of the cell to regain a cell wall). For *B. subtilis*, commitment occurred as the last traces of cell wall were removed by lysozyme. For gram-negative bacteria, such as *Escherichia* and *Salmonella* spp., it was postulated that commitment occurs as the last vestige of the lipoprotein-lipopolysaccharide (cell wall) is lost. Formation of stable L-forms as a result of mutations were excluded from the generalization that loss of the cell wall triggers commitment.

Reversion to a wall-containing form differs greatly among various bacterial genera. For *B. subtilis*, Landman reported that an early step in the process is the formation of a wall layer outside the cell membrane; formation of the septum and mesosomes follows this initial step. We emphasize that the clinical significance of persistence of L-forms and reverting parent bacterial cells in vivo may well depend on those host factors and/or inducing agents that regulate the ability of the organism to transform. It is possible that suboptimal dosages of wall-inhibiting antimicrobial agents or other chemotherapeutic agents will create wall-deficient variants in vivo and will over time encourage their persistence. Time-lapse photography of growing cultures has shown many alterations in morphology that lead to extreme pleomorphism upon exposure of the organisms to a variety of inducing agents. Because CWDB may be a part of every growing bacterial population, inducing agents might also play a role in allowing the selective growth of the aberrant forms. The in vitro observations of CWDB grown

on penicillin gradient plates might well be extended to analogous situations in patients treated suboptimally with a variety of agents capable of inducing the formation of CWDB in vivo.

L-forms have been shown to be immunogenic for a variety of hosts (120). Many of the bacterial membrane components have common antigenic determinants with mammalian tissue. Both humoral and cell-mediated immunity to L-forms have been demonstrated. Inflammatory reactions and autoallergic types of reaction have been shown in hosts immunized with L-forms. The persistence of the L-form might therefore lead to continuous low-level immunogenic stimulation in the host and trigger immunopathologic events. It is possible that many of the so-called autoimmune diseases represent immune reactions initiated by persisting CWDB that cross-react with host tissue antigens. Immunological disease initiated by CWDB has been grossly overlooked and deserves investigation.

RELATIONSHIP OF THE L-FORMS TO MYCOPLASMAS

Although L-forms resemble mycoplasmas in individual and colonial morphology, the evolutionary relationship of L-forms to mycoplasmas remains controversial. Neimark (147) proposed an interesting hypothesis to explain the genetic events leading to the formation of mycoplasmas. He hypothesized that unequal crossing-over between homologous rRNA genes could have resulted in losses of rRNA genes, and rearrangements could have occurred as well. This process may have been repeated several times. It has been reported that *Clostridium bifermentans* is related to lactobacilli, and Dams et al. (31) indicated that mycoplasmas evolved from lactic acid bacteria. In studies of 5S RNA sequences from *Cloridium innocuum* and eight mycoplasmas, Rogers et al. (164) concluded that the mycoplasmas evolved sequentially from clostridia. They also concluded that as a result of repeated events, *Mycoplasma* and *Ureaplasma* spp. arose from *Spiroplasma* spp. From the evidence available in the literature, it seems that mycoplasmas are a diverse group of wall-less prokaryotes derived from various bacteria. It has been convincingly demonstrated by immunologic methodology that acholeplasmas are descended from streptococci, specifically from groups N and D (148). It therefore seems logical to conclude from molecular and immunologic data that mycoplasmas are not a true phylogenetic class and that they are not descended from one single common ancestor. If we are to extend these findings to clinical relevance, it is tempting to speculate that in vivo genetic events may lead to development of bacteria with aberrant cell wall morphology and physiology and may involve complex interactions among a variety of bacteria and host cells. Such interactions might lead to persistence of a dormant bacterial phase in patients with infectious diseases. This may be a continual biologic process in all living hosts, with the host environment serving as the determinant for evolution, persistence, and survival of morphologically altered microbes.

A teleologic approach to the evolutionary relationship between mycoplasmas and CWDB should consider the survival advantage of an organism with a cell wall in a hostile primordial environment. Only after the appearance of higher life forms was there a protective niche for mutant microbia.

EXPERIMENTAL STUDIES WITH LABORATORY-INDUCED CWDB: HOST-PATHOGEN INTERACTIONS

Demonstration of the Phenomena of Bacterial Persistence and Reversion in Cell Culture

It has been known for a very long time that many life forms, such as the malaria parasite, trypanosomes, and chlamydiae, have complex intracellular/extracellular life cycles with remarkable pleomorphism of the phases. The truism that nature's successful survival tricks tend to be repeated was confirmed by Green et al. in 1974 (79), when they were able to demonstrate that this strategy is also used by bacteria. In a series of experiments, human embryonic kidney fibroblasts (HEK) were infected with *Enterococcus* (formerly *Streptococcus*) *faecalis* CWDB (a relatively stable L-form capable of reverting to a wall-containing form, i.e., an unstable L-form) under defined growth conditions. For the first few days after the inoculum was introduced into the cell culture system, the cells appeared normal by EM, even though they contained phagocytized vesicular L-forms. However, a series of subsequent EMs revealed gradual autolysis of these bodies and the generation of many elementary bodies that were diverse in size and morphology but still no evidence of injury to the HEK. With the appearance of transitional forms containing some cell wall fragments, injury and death of the HEK cells occurred along with complete reversion of many of the transitional variants to bacteria with complete cell walls. Reversions were sporadic, occurring in various experiments on days 4, 5, 14, 25, 29, 30, 56, and 63 postinfection. These findings documented for the first time that an unstable L-form was linked with the phenomena of persistence and reversion in a cell culture system. Survival of the unstable L-form over long periods of time in this system was particularly fascinating in view of the fact that the unstable L-form survived only 14 to 48 h in nutrient media in the absence of HEK cells (based on EM visualization of dead forms and inability to culture any organisms). It therefore appeared that the HEK cells were providing some mode of protection.

On the other hand, a stable L-form (incapable of reverting to a parent form) derived from another *E. faecalis* strain inoculated into HEK cells resulted in positive cultural findings for up to 73 days postinfection. The specific fluorescence of persisting stable L-form dense bodies was confirmed by an indirect immunofluorescence test that utilized specific antibodies to the stable L-form. Differences in the culturabilities of the stable and unstable L-forms in infected HEK cells at various time intervals postinfection may have reflected their basic morphological differences: namely, the prevalence of dense opaque forms and numerous free-floating dense bodies in the stable L-form cultures in contrast to a preponderance of the vesiculated forms containing dense bodies within the vesicles of the unstable L-form.

These data clearly indicated that the stable L-forms could persist for prolonged periods in cells, remained viable, and were culturable from these cells as L-forms per se. Because the stable L-form appeared to coexist with human cells, causing no classical pathology, its fate (assuming that it could never regain the ability to revert in this HEK cell system) remained unclear. It would therefore be worthwhile to prolong the experimentation beyond the points reported by these investigators to determine whether organisms survive and whether prolonged survival causes pathology to host cells. These findings with reverting and stable L-forms in this cell culture system have broad implications for the entire field of bacterially caused persisting and relapsing infectious diseases.

Could the phenomena of persistence and reversion observed with the group D relatively stable enterococcal L-form in a cell culture system be extrapolated to in vivo situations involving other kinds of bacteria? Fundamentally, could the occurrence of viable persisting bodies be a universal feature of bacterial L-forms, and if it is, what is the chemical and immunologic nature of these bodies? These questions are pertinent because a theme of persistence and relapse runs throughout the wealth

of literature on the otherwise diverse diseases with which L-forms have been associated (51, 84, 129, 136). In an effort to provide possible answers to these questions, a most expedient approach was an attempt to understand the reproductive cycle of the L-form in order to gain insight into clinical management of patients suspected of harboring CWDB.

Dense Elementary Bodies Derived from Enterococcal L-Forms in Cell Culture

Green et al. (80) attempted to organize into a logical sequence the observations made on growth characteristics, morphology, and ultrastructure of the unstable L-form of *E. faecalis* (the strain used in the HEK system described above). They hypothesized a reproductive cycle in which small, dense, nonvesiculated L-forms are the central (core) element. These forms divide and bud rapidly. In addition, the dense forms appear to be capable of growth and development within vesicles of mature mother forms. When these forms are released from the vesicles into the surrounding fluid medium, further growth occurs, resulting in the development of immature and ultimately mature mother forms. Under conditions unfavorable for L-form growth, these dense forms develop first into transitional forms and then into wall-containing organisms. These investigators reasoned that these dense forms might be considered undifferentiated "stem cells" with the capacity to develop along several different routes, depending upon the stimulus received. The pathology observed in the HEK cell system inoculated with this relatively stable L-form was triggered when the L-form was reverting to the wall-containing bacterial form. These authors assumed from their morphological data that the accumulation of electron-opaque material and the formation of mesosome-like structures were synonymous with aging and death as well as being prerequisites for reversion to the bacterial form. Therefore, reversion to the wall-containing form as well as aging, with subsequent death, of the L-form may be the effect of common causes, namely, the depletion of available nutrients and the accumulation of toxic products in the growth medium.

Reversion of the L-form may aptly illustrate one of the immutable laws of nature: when faced with unfavorable environmental conditions, an organism must adapt or die. This may raise the question of how a compromised CWDB mobilizes the energy necessary for reversion to the bacterial walled phase. One can speculate that these forms are genetically programmed to develop a cell wall when nutrients and energy sources are wanting. Certainly there is an evolutionary advantage to being an independent, free-living form able to forage for the best nutrients. The unstable L-form has probably taken up its parasitic residence within the cell only because there are impossible hazards in the extracellular milieu.

Proposed Developmental Stages in a Pathogenic Bacterium

The hypothesized reproductive cycle for a relatively stable L-phase variant of *E. faecalis* (80) suggested that tiny dense bodies are capable of developing into undifferentiated stem cells. Domingue hypothesized (57) that one large vesiculated parent (the mature form) may develop into many elementary bodies that become undifferentiated dense forms that may then be extruded from the parent as propagating organisms. Such forms might retain the ability to mature within the vesicle of the parent or even outside of it after rupture as long as they remain attached to it. Such extruded bodies contain a bacterial genome and minimal metabolic capability (i.e., enzymes and cofactors) sufficient to initiate energy production and biosynthesis. They may reproduce as dormant forms without cell walls and may revert to cell wall-containing bacteria, or they may do both. Therefore, production of the dense bodies within the vesiculated parent would represent still another type of bacterial differentiation.

Interestingly, Margulis et al. (134) reported previously unknown comparable metamorphic phenomena in free-living spirochetes. Margulis in collaboration with Guerrero et al. (82) demonstrated that large spirochetal cells (100 μm long, between 0.4 and 3.0 μm wide) contain smaller intracytoplasmic spirochetes. These organisms, which were grown in mixed culture and studied live and by transmission EM, form membrane-bound dense bodies. The authors demonstrated that the protoplasmic cylinder of the spirochete was replete with spherical granules that were 20 to 32 nm in diameter. These cylinders also had three to six periplasmic flagella (26 nm) that were inserted subterminally in the cell. There were comparably granulated and flagellated small spirochetes located inside the protoplasmic cylinder and in the periplasm of the larger cells. It was observed that when the spirochete was exposed to air, movement became erratic, and the protoplasmic cylinders refracted and lay folded inside the outer membrane, forming retractile membranous structures. From one to four structures per still-moving spirochete were seen. The investigators believe that the refractile, membranous bodies may provide a morphological basis for possible oxygen and desiccation resistance of the organisms. They conclude that these transformations may relate to (i) the enrichability of spirochetes from desiccating microbial mats, (ii) the formation of spirochete round bodies, and (iii) the unpredictable appearance of spirochetes in tissues of patients with syphilis and Lyme disease.

The observations by Green et al. (80) of electron-dense cytoplasmic bodies within parent cells of wall-defective enterococci and other gram-positive bacteria (51) and the observations by Margulis et al. (82, 134), who described 2 to 12 protoplasmic cylinders inside a single common membrane, might suggest that symptom reappearance in certain chronic bacterial infectious diseases, including spirochetosis, is related to bacterial differentiation into resistant forms (elementary dense cytoplasmic bodies) that persist in tissue. It has been reported that elementary bodies derived from L-forms of at least 0.24 μm may, in fact, grow into undifferentiated forms (80). Smaller dense bodies within vesicles are assumed not to play a major role in reproduction—they are claimed to be either deficient or devoid of DNA. Domingue believes that these dense bodies might be capable of maturation within the parental vesicle or even after its rupture. In any case, the role of dense bodies as resistant forms of pathogenic bacteria deserves further investigation (57). Regarding reversion to a wall-containing bacterium, Green et al. (80) hypothesized that the L-form does not separate completely from the newly formed classical bacterium but that instead, components of the L-form are incorporated into the walled forms. The failure to observe transformation of autonomous dense bodies into bacterial forms with cell walls supports this interpretation.

Formation of Urinary Bladder Calculi in Experimental Animals by *Proteus mirabilis* L-Forms

Braude and Sieminski (22) demonstrated the capacity of cell wall-deficient variants to produce urinary bladder calculi. Stable *P. mirabilis* L-forms having strong urease activity were inoculated into the renal medullas of rats. L-forms were recovered from the kidneys or urine of the animals for 11 weeks but not later. The reverted parent bacillary form was never cultured. At 2 weeks, bladder calculi appeared in 2 of 12 rats; by 11 weeks, they had appeared in 8 of 15 rats; and from 2 to

11 weeks, they appeared in 35 of 85 rats. After 8 weeks, there were no calculi after intrarenal broth injections in 24 control rats. Sixty percent of the calculi tested were composed of magnesium ammonium phosphate, and the remainder contained chiefly hydroxy apatite. Growth-inhibiting circulating antibody to the L-form developed in all rats. Progressive stone development after L-form cultures became negative may suggest that antibody prevented cultivation of persisting L-forms. It was concluded that urease of L-forms apparently produced the bladder stones by creating alkalinity that precipitated apatite and magnesium ammonium phosphate. The data clearly demonstrated that L-forms can contribute to progressive disease despite negative cultures. In animals given chloramphenicol, no stones occurred in 19 rats given the antibiotic immediately; when chloramphenicol was started at 1 week after injection of the L-forms, stone formation occurred in only one animal. Further evidence suggesting that the living L-form per se was responsible for stone formation was obtained by demonstrating that no stones were formed in 35 of 35 animals receiving acetone-killed L-forms. This stable L-form was originally isolated by exposure to penicillin and never reverted to bacillary forms in vitro or in vivo.

Role of In Vitro-Induced L-Forms in Pyelonephritis

Ponig et al. (161) demonstrated that in vitro-stabilized L-forms derived from *Escherichia coli* O111 may, when injected intravenously into rats, revert to the classical parent walled form within 1 day. It was shown that in animals sacrificed at 4 h postinoculation, 10 of 10 animals had L-forms in the kidneys without evidence of the reverted parent organism. However, at 1 day postinoculation, 9 of 10 animals showed the parent form; 1 of 10 had L-forms only. At 1 and 2 weeks postinoculation, CWDB could not be cultured from the kidneys, yet three animals were positive for the parent bacterial form at 1 week, and nine animals were positive at 2 weeks. At 3 weeks, CWDB grew from one animal, and one had the parent form. At 4 weeks, 1 of 10 animals had CWDB only, and 0 of 10 had the parent bacterial form. At 1, 2, and 4 weeks, 3 of 10 animals in each group showed chronic infiltrates with tissue destruction. Although at 4 weeks only one animal had positive cultural findings of CWDB, three had a chronic infiltrate with tissue destruction, and one had an infiltrate with pyelitis. CWDB were grown from the urine of only one animal, and this was at 1 day postinoculation. Reversion to the parent form occurred as early as 4 h, since the urine of one animal was positive for the parent wall-containing organism at that time; two positives occurred at 1 day postinoculation, and two occurred at 3 weeks postinoculation. Although the damage to the kidneys in the animals inoculated with CWDB (compared with those groups injected with the parent bacterial form in the same experiments) was not as extensive, it is significant that three of the animals had chronic infiltrates with tissue destruction at 4 weeks in the absence of the parent bacterial form.

Persistence of Experimentally Induced L-Forms in Antibiotic-Treated and Untreated Animals

In a study by Domingue et al. (70), it was demonstrated that antibiotic treatment of animals infected with unstable *E. coli* O4 CWDB (in vitro induced with penicillin, reverting to the parent form upon removal of the inducer) led to the persistence of these aberrant bacteria in a viable state. Over time, penicillin treatment of the animals converted the unstable CWDB to stable nonreverting L-forms in vivo. The persistence of L-forms in kidney tissue for 171 days and the presence of antigenic material for 237 days were indicative of the cryptic parasitism of these organisms. By utilizing indirect immunofluorescence with antibodies to the L-form, numerous fluorescent bodies were revealed in the kidney tissue, yet histologic studies did not show evidence of classic histopathology compatible with pyelonephritis in those animals kept on penicillin therapy. Untreated animals from which reverted bacterial cells could be cultured were clearly diseased. These data further substantiated the hypothesis for the persistence of CWDB in chronic renal disease and the role of the L-form in sustaining bacterial presence in the tissue.

Phagocytosis of CWDB

Evidence exists suggesting that the uptake and intracellular persistence of viable CWDB may affect phagocytic action against these forms. Such a change could well interfere with the treatment of bacterial infections. The association of group A streptococcal L-forms (induced in vitro with penicillin) with human polymorphonuclear leukocytes (PMNs) and mouse peritoneal macrophages was intensively studied by Schmitt-Slomska and colleagues (170, 171). The L-forms were readily associated with the cells and often appeared to have been phagocytized. The phagocytic cells were not affected if the inoculum was within defined limits. There was no evidence of lysosome activation. The survival of these L-forms was also studied in the presence of human and animal sera and of PMNs and mouse peritoneal macrophages. It has been confirmed that bactericidal action against ordinary bacteria with cell walls is related to the antibody complement system, whereas the killing effect of phagocytic cells on L-forms is less marked. Thus, phagocytosis may protect some L-forms from the action of serum, allowing these forms to be phagocytized and to survive within these cells. This may explain the difficulty in demonstrating L-forms from human blood cultures. Measures to disrupt host cells followed by inoculation into specialized osmotically stabilized media may improve the yield of aberrant organisms. These studies have shown that the pathogenic potential of CWDB is related to their prevalence during antibiotic therapy (170, 171). The increased susceptibility of bacteria (pretreated with certain antibiotics) to leukocytic action may be diminished for CWDB (143).

Autoradiographic localization of tritiated thymidine labeled-*E. coli* O4 CWDB has been investigated in an in vitro phagocytic system containing PMNs in an effort to determine whether L-forms were capable of surviving phagocytosis (63). This study revealed a significantly lower phagocytic index (percent PMNs containing ingested organisms) for the CWDB than for the parent bacteria. The results of multiple experiments indicated that the phagocytic indices for CWDB were depressed at all times. A representative experiment showed that after the phagocytic mixture had stood for 30 min, CWDB had a phagocytic index of 5.3 compared with 44 for the parent bacteria when both were compared in the presence of specific antisera for the CWDB. Exposure of the phagocytic system to the parent *E. coli* O4 antiserum indicated that CWDB phagocytosis had an index of 10.6 compared to 45 for the parent bacterium.

The authors reasoned that it was preferable to perform the phagocytic experiments with unstable (reverting) rather than with stable (nonreverting) CWDB, because unstable CWDB are the usual isolates from clinical specimens. Under host conditions allowing for formation and survival of CWDB in vivo, these unstable forms would probably be the earliest subjected to host defense mechanisms. In these experiments, labeling with tritiated thymidine proved to be a useful method for localizing the organisms. Although there may have been

attachment of the CWDB to the PMNs, it did not appear that the CWDB underwent extensive ingestion by the phagocytes. Much remains to be learned about which molecules on the surfaces of CWDB aid in resisting and surviving phagocytosis. Because there is convincing evidence for the persistence of CWDB in vivo, the mechanisms by which CWDB avoid the bactericidal action of host cells deserve extensive exploration. The data from this study clearly suggest that CWDB resist phagocytic activity in vitro.

Host-Pathogen Interactions with CWDB in Germfree Animals

It was demonstrated (60) that CWDB (unstable, reverting L-forms) derived from *P. mirabilis* persisted for up to 12 weeks in penicillin-treated germfree rats (Fisher strain 344), eliciting an antibody response to the CWDB that cross-reacted with the parent classical bacteria from which they were derived. A high percentage of positive cultures for CWDB were demonstrable (brain, liver, spleen, and kidneys) in the earliest period after inoculation of the CWDB (<1 to 24 h), but the number of positive cultures markedly decreased in later periods (72 h to 6 weeks). The cultures grew almost equal numbers of bacteria from the various organs, with the liver and spleen most frequently positive. The stool cultures became positive at 72 h and were positive in all subsequent cultures (except for one) for up to 12 weeks. These investigators found that the axenic rat is an excellent host for the study of CWDB because it has no preexisting histological abnormalities or antibodies and no microbial contaminants. In this study, the CWDB did not revert to the parent bacterial form in vivo, or if reversion occurred, CWDB were not observed on multiple sections that were stained for bacteria. There is the possibility that transitional, pleomorphic forms with only vestiges of a cell wall may have resisted staining. These investigators concluded that this *P. mirabilis* L-form could persist for prolonged periods without evoking discernible histologic reactions in the tissues, further substantiating the role of the L-form in persistent infection.

A stable L-form derived from *E. faecalis* that had been continually passaged and propagated in vitro as an L-form without reversion (probably a permanent stable mutant without a cell wall) was injected into Fisher strain 344 axenic rats (59). The L-forms were recovered for up to 3 days following injection into the rats. An inducing agent was not used to stabilize the L-form in vivo. There appeared to be an increase in the numbers of organisms per gram of tissue in animals sacrificed at 3 days (for given doses, i.e., 10^8 and 10^{10}) compared to those sacrificed at 2 h or dying within 12 h. These results suggested that multiplication of the organisms occurred in vivo. Failure to culture the organisms in vitro as colonies beyond 3 days may have indicated that the organisms were nonviable after this time. However, the persistence of antigens derived from the L-form were shown in the tissues at 12 weeks postinjection, and the continual rise in antibody titer suggested that the organisms remained in the tissues but could not be cultured in vitro. This same strain was used in a human embryonic cell culture system and could be cultured as an L-form for up to 73 days postinfection (79). As with the stable L-form in cell culture, there was minimal evidence of histopathologic damage caused by this organism. It would be of interest to study prolonged persistence of various L-forms in gnotobiotic animals to determine whether compromising the animals immunologically and/or physiologically affects the pathogenesis of a CWDB infection.

An overall conclusion to be drawn from all animal studies employing unstable and stable L-forms as infecting agents is that L-forms are most pathogenic when reverting to the parent bacteria as transitional forms with part of the cell wall or as fully reverted organisms. The exception is the finding of Kagan et al., who demonstrated that group A streptococcal L-forms per se were pathogenic in their models (97, 98). The overall theme of persistence in the wall-deficient/defective phase is supported by most of the studies and provides a basis for reevaluating the mechanisms of persistence of bacteria in relapsing and mysterious diseases suspected of being bacterial in origin.

SPONTANEOUS CONVERSION OF BACTERIA INTO CWDB

Transformation of Bacteria into CWDB In Vivo

It has been reported in experimental studies of pyelonephritis that certain antibiotics can transform bacteria into CWDB in vivo (1, 177). Development of CWDB in untreated animals has also been reported in an experimental *E. coli* pyelonephritis study by Demonty (33). Unfortunately, the role of CWDB in experimental diseases has produced conflicting findings. At the center of the controversy is the effect of antibiotics on the in vivo conversion and persistence of CWDB. Research is needed to determine whether the concentration of wall-inhibiting antibiotics in tissues and body fluids contributes to the maintenance of these forms in vivo. The studies on cell cultures and in certain animal models strongly suggest that these phenomena occur, either spontaneously or during treatment with antibiotics. Some investigators have concluded that L-forms might account for the persistence of bacteria but not for inflammation. Others have implicated revertants of bacteria with aberrant walls in experimentally produced pyelonephritis. Guze et al. (85), in a study on *Klebsiella pneumoniae* L-forms and the effect of growth as an L-form on the virulence of reverted *K. pneumoniae*, reported that there were no signs of disease in mice experimentally infected with the L-forms. These findings conflict with those of Kagan et al. (97, 100), who, as described above, have reported that group A streptococcal L-forms per se are pathogenic.

The mechanism of action of penicillin on the production of CWDB in vivo is also unclear. Schmitt-Slomska et al. converted group A streptococci into CWDB (unstable, reverting L-forms) in experimentally infected, penicillin-treated mice (171) and monkeys (98, 99). One parent strain inoculated into animals, as well as the isolated CWDB and their in vivo revertants, was resistant to tetracycline (MIC, 50 μg/ml). The CWDB produced in vivo by penicillin remained resistant to tetracycline and developed insensitivity to penicillin. The L-forms of this strain that were stabilized (nonreverting) in vitro were susceptible to tetracycline.

In extensive studies by Kagan and Schmitt-Slomska (98, 99), the spontaneous production in vivo of CWDB in subacute streptococcal infection of monkeys not treated with penicillin suggested to those authors that penicillin might have only a selective action. By inhibiting the multiplication of virulent organisms, penicillin might allow cellular or humoral host factors to transform the less-virulent bacteria into penicillin-resistant CWDB. In a study on experimental endocarditis produced by methicillin-resistant *Staphylococcus epidermidis*, Archer et al. (3) found that semisynthetic penicillin and cephalosporins did not protect against experimental endocarditis, whereas vancomycin, gentamicin, and rifampin were protective. The selection in vivo of bacterial subpopulations resistant to beta-lactam antibiotics might account for these results. Failures of antibiotic treatment could also be due to the localiza-

tion of CWDB and their revertants in the host. Schmitt-Slomska et al. (169) isolated *Brucella suis* CWDB (unstable CWDB, reverting forms) from the spleens of penicillin-treated mice experimentally infected with a virulent strain of *B. suis*. These CWDB were isolated simultaneously on penicillin-containing, osmotically stabilized medium and on antibiotic-free media several days after the last penicillin injection (and in the total absence of ordinary parent bacteria), confirming that these CWDB were induced in vivo during the *Brucella* infection. Their presence in spleen cells and the difficulty in isolating tissue-free L-form colonies suggested to these authors that the CWDB could persist in the mouse as intracellular parasites that were difficult to grow in vitro. Therefore, failure by routine bacteriologic culture to demonstrate CWDB in cultures from clinical specimens does not negate their in vivo presence or persistence in a suspected bacterial infection.

Transformation of Bacteria into CWDB in Cell Cultures

Cell culture cell lines can coexist for very long periods with wall-less bacteria, such as *Mycoplasma* spp., and these bacteria can be insensitive to antibiotic treatment. The intracellular localization of CWDB may explain the poor results achieved with antibiotics for decontamination of cell cultures chronically infected with *Mycoplasma* spp. and for the treatment of recurrent bacterial disease. Typical L-form colonies were isolated from hamster kidney cell cultures by Hatten and Sulkin (86, 87) after experimental infection with classic bacterial forms of *Brucella abortus*. Penicillin or streptomycin alone did not eliminate the bacterium or its L-forms from infected cells, and it was significant that the L-form cultures were positive after 7 to 14 days of treatment. It is of interest that after treatment with tetracycline, either alone or in combination with penicillin or streptomycin, the L-forms survived for longer periods than did the ordinary bacteria. Schmitt-Slomska et al. (167, 168) confirmed the possibility of spontaneous conversion of bacteria into CWDB in antibiotic-free cell cultures. Human diploid cells infected with viable group A streptococci survived and thereafter divided and prospered normally. After several divisions of the human cells, the microorganisms persisted only as large, isolated, swollen cocci and could not be grown on ordinary bacteriologic media. When cultured on L-form media, however, typical L-form colonies developed. They grew well on media containing penicillin or vancomycin. After several passages on antibiotic-free media, the organisms reverted to the group A parent streptococci. In other experiments, the authors revealed that the in vitro-produced group A streptococcal L-forms multiplied intracellularly and passed from cell to cell without affecting the cell line's morphology or the rate of cell division. Similar findings with *E. faecalis* in cell culture were noted by Green et al. (79). The ability of *Streptococcus pyogenes* L-forms to grow in human heart and kidney cells with destruction of the cell cultures was reported by Leon and Panos (116), who confirmed the earlier work of Kagan et al. (98–100) and Madoff (129) on the cytopathogenic effects of group A streptococcal L-forms. Spontaneous reversion in vivo without cultural evidence of ordinary bacteria in vitro may have accounted for the pathology shown by Leon and Kagan.

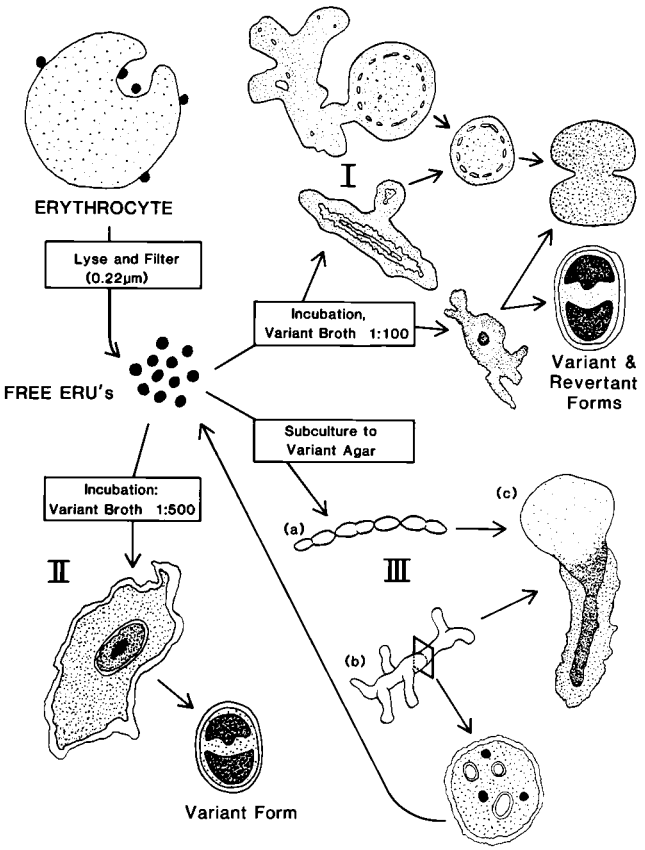

FIG. 1. Proposed interrelationships and hypothesized life cycle of isolated cell wall-deficient forms. We hypothesize from our cultural and morphological data that elementary reproductive units (ERU) adherent to erythrocytes (Fig. 2) are freed upon lysis in vitro and pass through a 0.22-μm-pore-size filter. Depending upon the conditions of culture, three morphologically distinct groups of forms are observed. Lysate cultured in variant broth at a 1:100 dilution yields a population of large spheroid and elongate forms (group I) (Fig. 3 through 5). Also observed are small round bodies, thought to represent ERUs, entrapped within an amorphous flocculent material, probably lysate debris. It is hypothesized that finely granular, L-form spheroids lacking cell walls arise from or in association with these large forms; such L-forms may also arise directly from ERUs. The granular spheroids then give rise, through a complex series of intermediate forms, to revertant bacterial organisms with thin, but distinct cell walls. Incubation of lysate in variant broth at a 1:500 dilution yields variant bacterial forms with thin cell walls in association with flocculent material (group II) (Fig. 7 and 8). Forms in group I are rarely, if ever, observed in 1:500 dilution cultures. In contrast to reversion in group I cultures (which may take many months), the appearance of revertants within group II cultures is rapid and occurs frequently after as little as 24 to 48 h of incubation. Subculture of group II forms onto variant agar yields a third morphologically distinct group of forms, called group III. These include elongate cocci (a), branching forms (b), and intermediate forms (c) (here greatly enlarged). Whereas coccal forms exhibit a thin to absent cell wall, the branching forms and the branching portions of intermediate forms have thick though electron-lucent coats. Within branching forms from older cultures, a variety of dense bodies and vesicles (see enlargement of b) are hypothesized as possessing reproductive potential as ERUs and being capable of reinitiating the cycle under appropriate culture conditions.

L-Forms of *Nocardia* spp.: Host-Induced Cell Wall Modification of *Nocardia* spp. and Its Role in Pathogenesis

The elegant experimental studies by Beaman and colleagues (6–16) have provided a solid basis for explaining the diversity and heterogeneity of *Nocardia* spp. Considerable convincing evidence from Beaman's laboratory has shown that L-forms of *Nocardia* spp. are important in the host pathogen interaction in experimental infection. He clearly demonstrated that the nocardial L-form is involved in bacterial persistence, latency, and recrudescence of disease caused by certain strains of nocardiae. The in vitro-induced L-forms of *Nocardia caviae* 112 and *N. asteroides* GUH-2 have been shown to be pathogenic for mice. Additionally, L-forms of *N. asteroides* GUH-5 were isolated from the cerebrospinal fluid of a human prior to the onset of disseminated disease. Approximately 10^4 L-form colonies per ml of cerebrospinal fluid were recovered on L-form medium from four separate samples collected several weeks

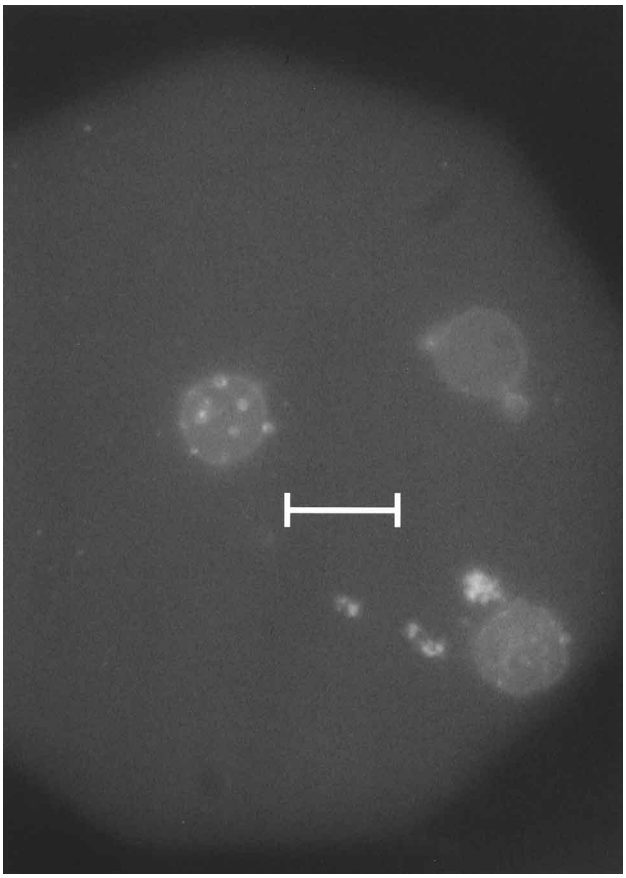

FIG. 2. Acridine orange stain of erythrocytes in the urine of a nephritic patient. There is a yellow-green fluorescence (DNA) of bodies associated with the cellular plasma membrane. Wet preparation, oil immersion. Bar, 10 μm.

apart. The identity of the organisms was established by reverting the L-forms to typical cells of *N. asteroides*. These revertants were shown to be antigenically related to the strain of *N. asteroides* isolated previously from the patient's sputum. Walled forms of *N. asteroides* were not isolated from the cerebrospinal fluid of the patient. After several months, the patient died as a result of brain abscess and extensive central nervous system infection. The author did not indicate whether nocardiae were isolated from the abscess.

Bourgeois and Beaman (20, 21) demonstrated that the passage of *N. asteroides* 10905 through an L-form state resulted in considerable alteration in the physiological, morphological, ultrastructural, and biochemical properties of the microorganism. It is of interest that the longer the organism remained as an L-form prior to reversion, the greater were the changes in these properties. Beaman concluded that these changes were stable and probably represented mutational events. Beaman reasoned that the passage of cells through the CWDB phase may have potentiated mutations or selected for multifunctional mutational events. A number of other scientists have found frequent aberrant forms of *Nocardia* spp. in clinical material and reported that these altered organisms were intimately involved in chronic, progressive nocardial infections (51, 84, 129, 136).

In his mouse studies, Beaman has essentially satisfied Koch's postulates with in vitro- and in vivo-induced L-forms of *Nocardia* spp. by clearly demonstrating that these L-forms can be induced within the host, they can survive and persist in a latent phase within the host, and they can induce a pathologic response resulting in the formation of mycetoma-like lesions after a prolonged latent period within the host. Thus, it is likely that an analogous situation occurs in naturally acquired infections. Furthermore, these findings could explain the clinical results reported by others that demonstrate nocardial disease in the absence of microscopically observable ordinary bacteria in specimens yet subsequent appearance of *Nocardia* species in culture after prolonged incubation (51, 84, 129, 136). Removal of the L-form from inhibitory host factors may have initiated reversion to the nocardial bacillary phase in vitro.

EXPERIMENTAL STUDIES WITH BACTERIAL VARIANTS DERIVED FROM HUMAN SPECIMENS

Ultrastructure of CWDB in Human Blood and Urine

Fine-structure studies (88) have documented the growth on variant media of morphologically heterogeneous bacterial forms derived from blood lysates. Heparinized human blood was lysed in 0.25% sterile hypotonic saline, filtered through a 0.22-μm-pore-size filter, and inoculated into variant solid and liquid media (2, 65). Although the precise immunologic and bacteriologic interrelationships between these heterogenous structures is not known, the morphologic interrelationships and patterns of growth permitted the formulation of a tentative hypothesis concerning the life cycles of forms in this culture system (88) (Fig. 1). It is clear that the erythrocyte is a key element in this cycle: the observations of dense bodies within

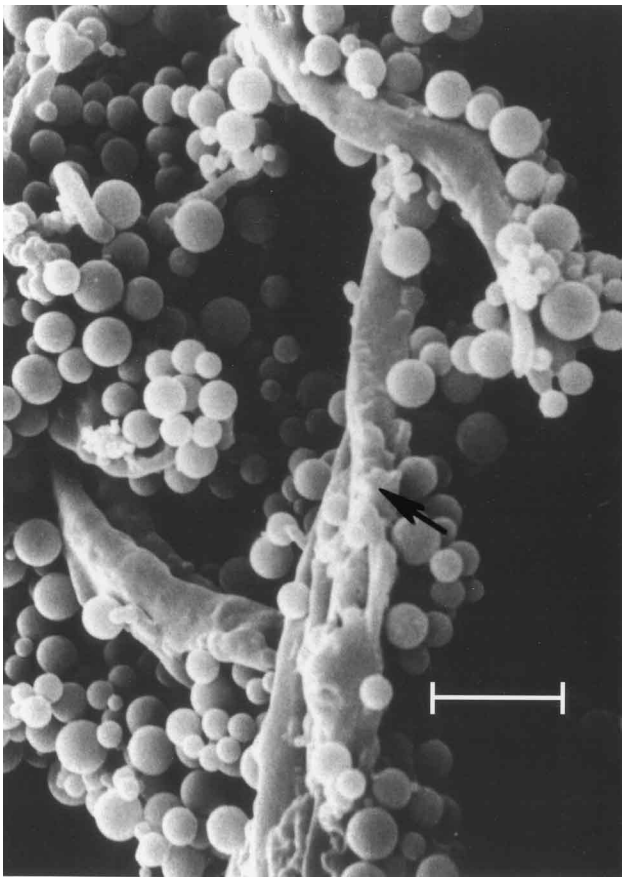

FIG. 3. Scanning EM of elongate and spheroidal bodies harvested from lysed filtered blood cultured in variant broth. Bar, 10 μm.

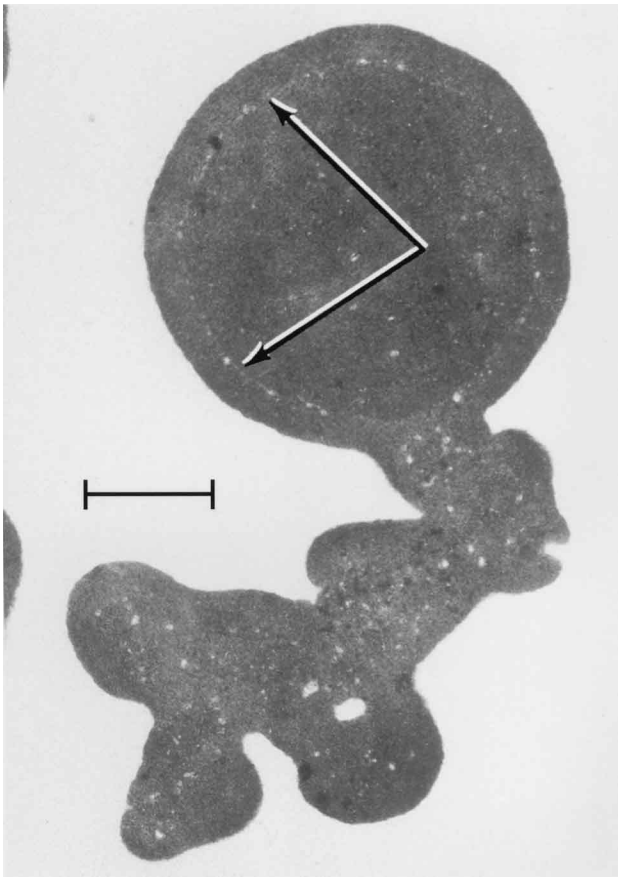

FIG. 4. Transmission EM of Fig. 3-type specimen, showing continuity of branching forms and spheroidal bodies. Arrows indicate an electron-lucent vesicular ring, which delineates an outer surface region from a more electron-opaque core. Bar, 1.2 μm.

invaginations of the erythrocyte cell surface and upon the plasmalemma suggested that these bodies may represent the minimal reproductive units that pass through the 0.22-μm-pore-size filter used in the preparation of the filtered lysate for culture (Fig. 2). Most bodies ranged in size from 0.17 to 0.20 μm in diameter and would thus pass into the lysate. Once within a culture medium of variant broth, these particles were surrounded by a thick cell coat or capsule (Fig. 3–8). Some of this material may be of lysate origin (proteins, perhaps including hemoglobin, as suggested by Nelson [149]) or may at least in part be synthesized by the developing organism. Once free of the coating material, these forms were recognizable as bacterial variants of diverse morphology. Many of the variants observed were not dissimilar to those described in studies of L-forms of bacteria (88). The routine presence of both CWDB and walled forms, together with intermediate forms, strongly suggested the origin of classical bacteria from these variant forms. The subculture of the variants arising within a variant broth to a variant agar provided an opportunity to document a cultural and morphologic lability among coccal and filamentous forms. Forms intermediate between these two morphologic types strongly suggested their origin from a common source and their interconvertibility. The production of both vesicles and small dense granules within short filamentous forms suggested that these represented, upon their release from the parent organism, a population of elementary reproductive units capable of reinitiating the life cycle.

The authors acknowledged that an artificial situation was created for rapid release of these organisms by exposing whole blood to osmotic lysis, which allowed for subsequent growth in vitro of the bacterial variants in an enriched medium (88). Nevertheless, an analogous though more subtle situation may very well exist in vivo. It is theorized that while the organisms are cell associated, there is probably no immunologic reaction to the organisms, although various host environmental factors could affect the cellular multiplication of these forms in cells. Overloading of host cells with organisms that slowly replicate would very likely lead, with time, to lysis of whole host cells and release of the elementary bacterial forms. If external conditions are then conducive for maintaining the viability of these released elementary bacterial particles, they conceivably could mobilize the necessary machinery (energy) needed to partially or completely convert to ordinary bacteria or could remain as stable CWDB. If they revert partially or completely, overt signs of disease may appear. Transformation from a minimal reproductive unit to a transitional or fully reverted bacterium tends to be associated with the synthesis of toxins, changes in antigenic structure, and formation of physiologic by-products. The CWDB might be equally detrimental to host tissues, but the effect might be more subtle histopathologically and hence not as readily recognizable. More important, the immunologic event(s) triggered by release of bacterial antigens and microbially altered host cell antigens could precipitate a variety of immunopathologic conditions. The demonstration of cryptic bacterial forms in filtered blood lysate undetected by conven-

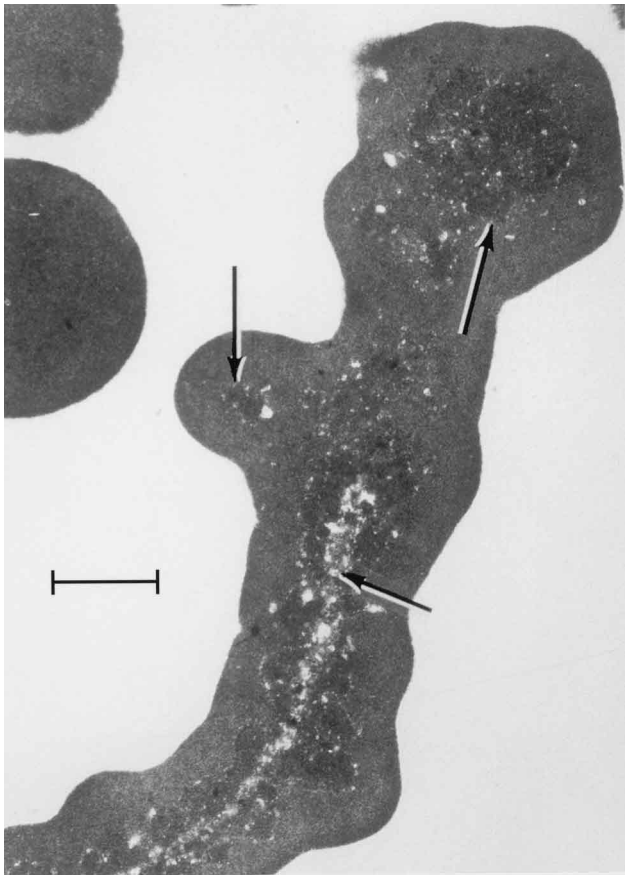

FIG. 5. Transmission EM of Fig. 3-type specimen, showing the elongate bodies with an electron-lucent, presumably nucleoid region within its central portion. Bar, 1.4 μm.

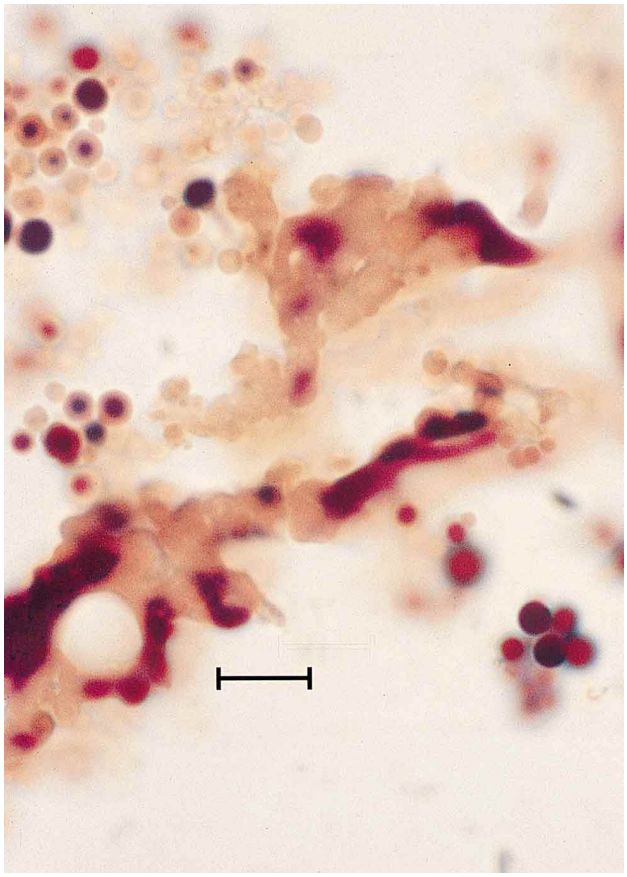

FIG. 6. Elongate bodies and spheroids derived from Fig. 3-type culture embedded, sectioned, and stained with Brown and Brenn's tissue stain. Note the diversity of staining that suggests an uneven entrapment of metabolically active material (DNA and membraneous material). Bar, 10 μm.

tional bacteriologic culture of whole blood, coupled with other circumstantial evidence for persistence of variant bacterial forms in kidney tissue (50), will, it is hoped, emphasize the need to consider seriously the possible importance of these organisms in human disease. Additional supportive evidence has been obtained in guinea pigs sensitized with variant or revertant bacterial antigens (gram-positive coccal forms). The animals were shown to develop immunopathologic effects upon intravenous challenge with variants. The effect was even more pronounced upon injection of variant culture supernatant. Furthermore, injection of large amounts of culture supernatant without prior sensitization provoked toxic reactions with death of animals suggesting that toxic substances were associated with these unusual forms (see the next section of this review).

Reports of unusual microbial forms in whole blood have appeared in the literature for many years, and these forms have been associated with a variety of diseases (51, 84, 129, 136). Convincing supportive evidence for the presence of nonconventional bacteria in human blood comes from the work of Bisset and Bartlett (19), who concluded that an organism identified as *Bacillus licheniformis* (endoparasiticus) exists as an L-form associated with the erythrocytes of a large portion of normal humans. They stated that organisms associated with the L-form cycle have in the past been described as different microorganisms, such as L-forms of various bacteria or mycoplasmas, and a diphtheroid state that was thought to belong to the genera *Corynebacterium* and *Listeria*. Therefore, the variable morphology of the reverting bacterial forms in blood has been a source of confusion. Bisset and Bartlett (19) stated that the blood isolates may pass through stages resembling organisms of the genera *Mycoplasma*, *Mycobacterium*, *Streptococcus*, *Corynebacterium*, *Listeria*, *Micrococcus*, and *Bacillus.*

In DNA relatedness studies performed in the laboratory of Domingue (52), isolates from human blood appeared to be different from those reported by Bisset and Bartlett, and more than one type of organism could be isolated from blood lysates. In contrast to Bisset, Domingue et al. observed no sporulating or diphtheroid cells. Spheroplasts of *Bacillus* spp. as described by Bisset exhibited a distinct cell coat similar to that observed in association with some forms in the Domingue studies. It is also of note that the postulated elongate "mother cell" of *Bacillus* spp. described by Bisset apparently bears at least two types of granular inclusions, the larger of which somewhat resembles granular inclusions (thought to represent elementary reproductive units) similar to those described by Domingue (52, 53). It is significant as well that many of the "granules" within erythrocytes, reported as possible progenitors of corynebacterium-like organisms, lie within the size range of similar dense bodies reported by Heidger et al. (88), i.e., 0.13 to 0.20 μm. Additional information, such as immunologic and nucleic acid characterization of the forms described by Heidger et al., may contribute to the substantiation or modification of their life cycle model constructed on the basis of morphological evidence and cultural data. Such un-

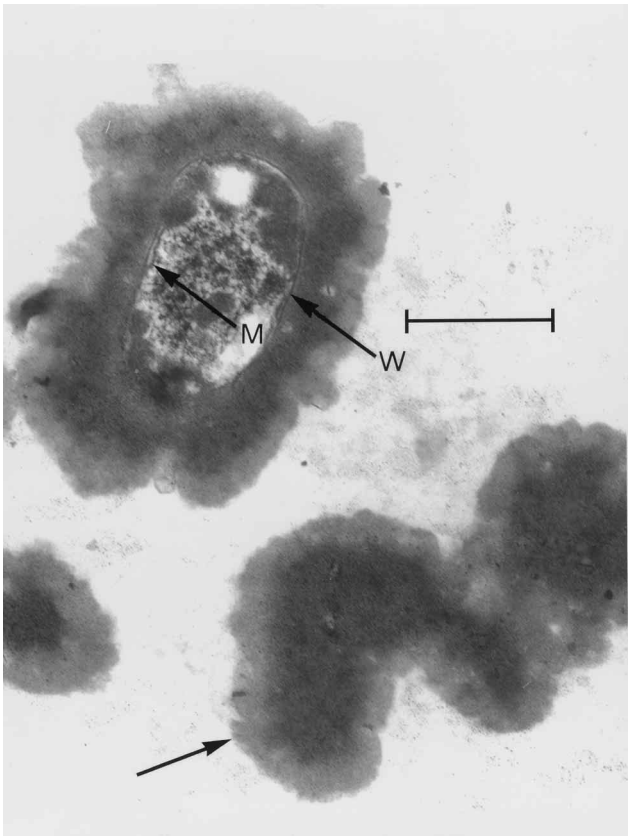

FIG. 7. Transmission EM of Fig. 3-type specimen, showing a spheroidal form in which a bacterium-like cell is evolving within the core. Note the wall (W) and internal membranes (M). Another spheroidal form (bottom arrow) contains no microbial structures. Bar, 0.51 μm.

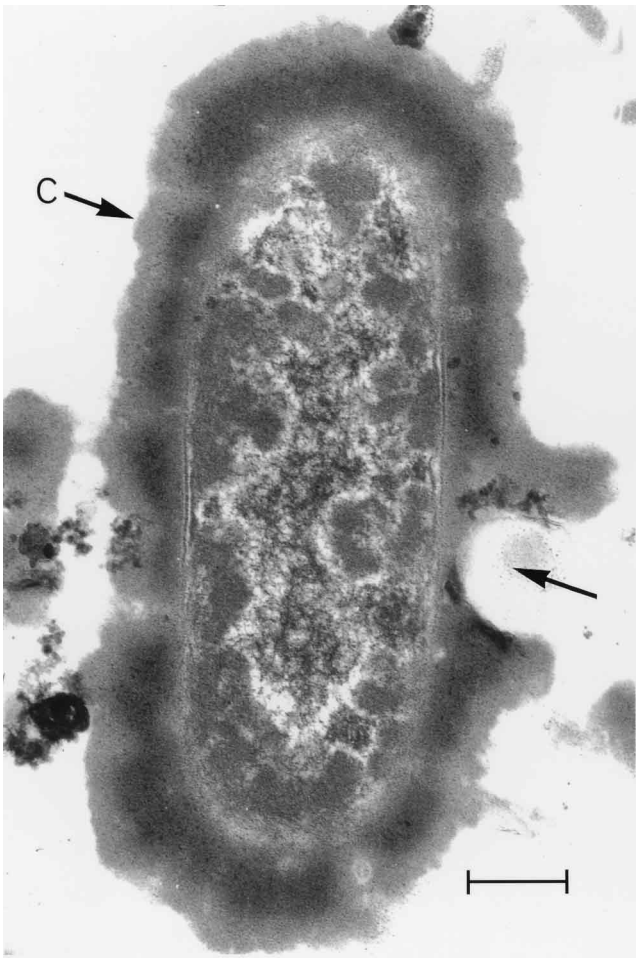

FIG. 8. Transmission EM of Fig. 3-type specimen, showing a bacillus-like form within thick capsular material (C), some of which is being lost (arrow). The cytoplasm of the cell shows scattered ribosomes and a fibrillar nucleoid-like core. Bar, 0.27 μm.

derstanding will also contribute to our knowledge of the relationship of these unusual organisms to disease.

Exaggerated Immune Responses and Toxic Responses in Guinea Pigs Sensitized with CWDB Derived from Human Blood Lysate and CWDB Culture Supernatants

An exaggerated immune response (anaphylaxis) has been demonstrated in over 300 guinea pigs sensitized and challenged with spheroid-containing CWDB or spheroid culture supernatant fluids derived from human blood (54). In experiments with spheroids alone, the spheroid-containing CWDB supernatants were more efficient in bringing about the reaction than were the spheroids. Autoclaving of the sensitizing preparation did not interfere with its sensitizing effects. The data suggested that soluble antigens are associated with the blood-lysate derived spheroid-containing CWDB and that they are potent initiators of an anaphylactic reaction in guinea pigs sensitized with either the spheroids or spheroid culture supernatants. Transfer of serum derived from sensitized guinea pigs to normal guinea pigs caused a severe reaction without death in 14 of 22 animals passively immunized and challenged with the spheroid culture supernatants, which was consonant with the anaphylactic reaction observed in the actively sensitized animals.

Further studies were performed to determine whether the culture supernatants per se were toxic. Guinea pigs that had not been previously sensitized were injected intravenously with various amounts of culture supernatant. Two milliliters of spheroid culture supernatant elicited a violent reaction not unlike that observed in sensitized guinea pigs, and nine of nine animals succumbed to the injection within 24 h. To ensure that the reaction and death were not due to the volume of culture injected into the animals, 2 ml of uninoculated broth medium was injected into eight animals; it did not elicit a reaction in any of them. These data indicated that toxic substances were associated with the culture supernatant fluid derived from spheroid-containing CWDB cultures of blood lysate from humans with chronic renal disease (idiopathic hematuria, nephrotic syndrome, systemic lupus erythematosus, and renal Fanconi syndrome). Large amounts injected into guinea pigs can produce a fatal reaction.

Attempts were made to learn more about the nature of the antigen(s) eliciting the reaction in sensitized and nonsensitized animals. Spheroid culture supernatants were dialyzed against distilled water. Upon dialysis, a precipitate formed within the dialysis membrane. Animals sensitized with the material remaining in the dialysis membrane demonstrated a severe reaction, with death, upon challenge with the spheroid culture supernatant fluid or the material remaining in the dialysis bag. Of 46 animals, 46 had a severe reaction and 25 died within 15 min of injection of the challenge dose. Forty-five normal guinea pigs that had not been previously sensitized with any antigens did not react upon challenge with spheroids, spheroid culture supernatants, autoclaved spheroids, or culture supernatants and dialyzed supernatants in amounts of less than 2 ml. The sensitizing and challenging antigens were not completely characterized; however, preliminary ultrafiltration indicated that the molecular weight of the material provoking the exaggerated immune response after challenge of sensitized animals was less than 30,000. Chemical studies indicated that the material contained 60% protein, 8% carbohydrate, and 32% lipid.

Revertant bacteria derived from spheroid-containing CWDB obtained from the lysed filtered blood of the patient were also used as sensitizing antigen(s). A severe reaction (such as that described for the spheroids containing CWDB) developed in 8 of 10 animals, with 6 succumbing to the challenge dose of spheroid culture supernatant. Controls sensitized with revertant bacteria but challenged with uninoculated broth medium were unaffected. Sensitizing the animals with uninoculated culture medium and incomplete Freund's adjuvant and then challenging them with spheroids and spheroid culture supernatants did not evoke a reaction, indicating that the sensitizing antigen(s) was derived from the spheroids containing CWDB or culture supernatants.

When the urine sediment of a patient with a chronic renal disease (devoid of ordinary culturable bacteria but containing CWDB) was mixed with incomplete Freund's adjuvant and injected as sensitizing antigen(s) into guinea pigs, five of five animals reacted violently upon injection of the spheroid culture supernatant derived from the patient's lysed blood, and four of five animals died (54). Therefore, urine sediments containing CWDB but devoid of ordinary bacteria also sensitized animals for the exaggerated response upon challenge with spheroid culture supernatants derived from the patient's blood lysate. These data suggested that there were shared CWDB antigens in the urine and blood of the same patient. The overall conclusion from these studies is that both anaphylactic- or anaphylactoid-like immune pathology and toxic re-

actions are associated with spheroids containing CWDB derived from blood lysates and urine of patients with chronic renal disease.

Groups of 50 guinea pigs were repeatedly injected with either pure culture of CWDB or reverted bacteria (gram-positive cocci) derived from human blood lysate of patients with chronic renal disease, and a third uninoculated group served as a control (55). Repeated injection of CWDB intravenously (group 1) or reverted bacteria subcutaneously (group 2) over a 2-year period led to the recovery of both CWDB and partially reverted organisms (transitional forms) from the kidneys of the animals at death. The animals were clearly diseased at necropsy. In the two groups injected with organisms, both CWDB and parent bacteria were isolated, suggesting interconversion of both types of organisms in vivo. The organisms recovered from the animals were identical to those initially injected, but certain biochemical patterns of reactivity had changed, suggesting that phenotypic alterations had occurred in those organisms. These variable reactions on biochemical substrates occurred more frequently with isolates obtained from animals inoculated with CWDB than with parent bacteria. Control animals remained healthy, and no organisms were grown from their tissues. These preliminary data indicate that CWDB derived from the blood lysates of patients with chronic renal disease survive and multiply when inoculated into experimental animals. At necropsy, the organisms can be recovered from the kidneys of the animals.

CLINICAL RELEVANCE OF BACTERIAL PERSISTERS AND EXPRESSION OF DISEASE

The following cases will illustrate and put into context some of the diverse clinical effects of difficult-to-culture, dormant CWDB and the presently nonculturable bacteria or both in pathologic tissues. The findings reported for the first patient (71) occurred because the chronicity and metabolic bankruptcy of her physical state amplified mechanisms that are less severe and more evanescent in most patients. The prepared minds and special skills of her caretakers made recognition of these phenomena possible. We believe that the clinical data described here in each case are necessary for the practicing physician, who must put the bacteriologic information into the perspective of its diagnostic and therapeutic utility. The details concerning reassembly of microbial fragments within spheroidal polymers is referenced (29, 32, 71, 119, 149, 151, 153) and deserves consideration by scientists skilled in disciplines such as cellular adhesion, transposition of genetic elements, and microbial reassembly as mechanisms for the genesis of unusual organisms. The cases subsequent to the first are more familiar and straightforward and illustrate the concept that we have barely scraped the surface in defining the presence, variety, and effects of microbes in all life forms.

Idiopathic Urinary Diseases

We monitored a series of patients with obvious renal disease whose urine samples were significantly abnormal for sustained periods (50, 52, 57, 58, 62, 64–69). Proteinuria and the presence of nucleated cells and erythrocytes indicated that glomerular capillary membranes had been breached. On occasion, inspissated mucoprotein released by renal tubular cell membranes formed casts that entrapped cellular elements that had passed through the glomeruli as well as epithelial cells shed by the renal tubules. Occasionally, recognizable bacteria were seen, but most often, no classic organisms could be identified. In the following three cases, urine samples examined by routine cultural techniques and bright-light microscopy were reported to be negative. However, phase oil immersion microscopy demonstrated the presence of morphologically aberrant bacterial forms; staining of the urine sediment with acridine orange and examination by fluorescence microscopy revealed that these forms contained nucleic acid.

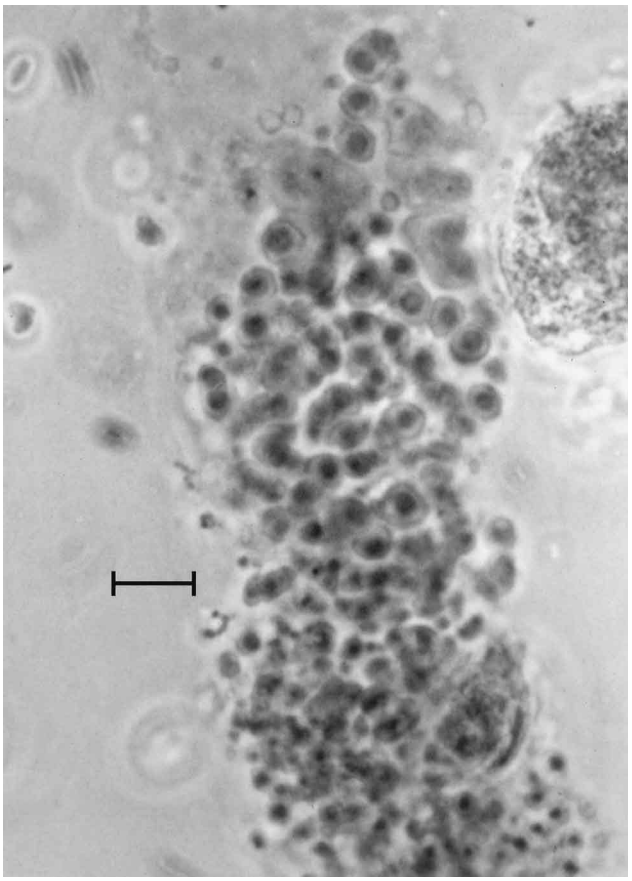

FIG. 9. Freshly passed urine from patient with renal Fanconi syndrome containing casts with very large granules with dense cores. Phase oil immersion. Bar, 10 μm. (Reprinted with permission from reference 71.)

Renal Fanconi syndrome. In 1979, we reported the case of a child who had been chronically ill since 4 months of age with recurrent infections associated with fever, diarrhea, and anemia (71). A *Salmonella* spp. was grown from her stool when she was hospitalized at 4 months and again at 5 months of age. During hospitalization at 9 months of age, it was noted that she was small but developing normally and had an appropriate weight. Laboratory studies were essentially normal except for a macrocytic anemia, which resolved with folic acid therapy. She was first seen by us when she was about 3 years of age, at which time she was hospitalized with fever, vomiting, and diarrhea. She had grown very poorly in the previous year and was markedly undernourished, with muscle wasting. Studies done at the time of her acute illness revealed a normal blood urea nitrogen and creatinine but moderate acidosis, hypokalemia, and a very low serum phosphorus. Urine clearance studies revealed an inappropriate phosphaturia, moderate glucosuria and proteinuria, and a generalized aminoaciduria, findings consistent with a diagnosis of renal Fanconi syndrome. Her renal tubular dysfunction persisted after correction of the acid-base balance and hypokalemia. A striking finding on examination of her urine were the very large casts packed with remarkably large sphe-

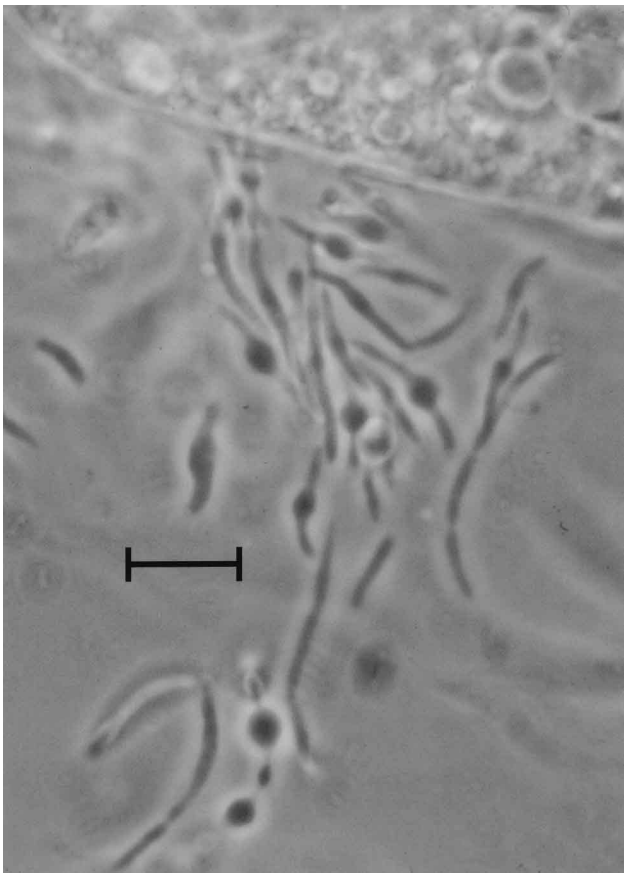

FIG. 10. Casts (see Fig. 9) incubated under a sterile coverslip for 2 days, showing granules developing filamentous extrusions which elongate, segment, and finally evolve streptococcal forms. Phase oil immersion. Bar, 10 μm. (Reprinted with permission from reference 71.)

roidal granules. Similar, even larger granules were seen free in the urine. She continued to be monitored by us from this time until her death at 6 years. In the interval, she had frequent episodes of gastroenteritis and respiratory infections that required hospitalization. She grew very little and weighed only about 9 kg at the time of her death from heart failure. She had developed increasing renal insufficiency.

In the 3 years before her death, repeated blood cultures were positive for CWDB that reverted rapidly to streptococci; conventional cultures showed no growth. Even more striking was the evolution of streptococci from the large granules in the casts when the urine sediment was incubated under sterile sealed coverslips (Fig. 9 and 10). It is known that spheroidal bodies can be generated in vitro by polymers in solution (153). It has been noted that such bodies can achieve a kind of metabolism, the sophistication of which is determined by their entrapped nucleic acid and membrane-associated enzymes. In addition, growth and division of such probionts depends on the nutrients in the solution that bathes them. All the conditions necessary to subserve a similar phenomenon seem present in this patient, i.e., partially degraded cell wall-defective streptococci in the glomerular filtrate, an abundant mucoprotein polymer generated by the renal tubular epithelial cell, and a urine rich in such nutrients as amino acids and glucose.

As previously described, blood culture for CWDB involves using whole heparinized blood or centrifuged washed erythrocytes. The blood cells (mainly erythrocytes) are lysed and filtered to remove any conventional bacteria; the filtrate is inoculated into a medium similar to that used for growing mycoplasma. This procedure is essentially the introduction of an iron-rich polymer from erythrocytes whose C3 membrane receptors carry a variety of antigens to the mononuclear phagocyte system for processing (166). We have examined spheroids generated in culture with acridine orange and noted associated nucleic acid staining of coccobacillary organisms. Spheroids from the patient's cultures were fixed, embedded, sectioned, and stained much as tissue sections. Postmortem sections of this patient's kidney and liver revealed spheroids, with the same morphology and staining properties as those obtained from culture, within casts in renal tubules and in some renal pelvic epithelial cells (Fig. 11 through 14). In addition, similar spheroids were seen in many hypertrophied hepatocytes.

Nephrotic syndrome. In the last 20 years, we have monitored a series of children with minimal-change nephrotic syndrome, a chronic, relapsing renal disease that can develop as early as 2 years of age. The condition usually presents with an insidious edema that dramatically increases when the child develops an infection. Laboratory studies reveal a heavy proteinuria, a depressed serum albumin, and an elevated cholesterol. Blood volume contraction is recognized by an elevated hematocrit and a scarce, markedly concentrated urine. A tendency to coagulopathy is usually present (17, 117).

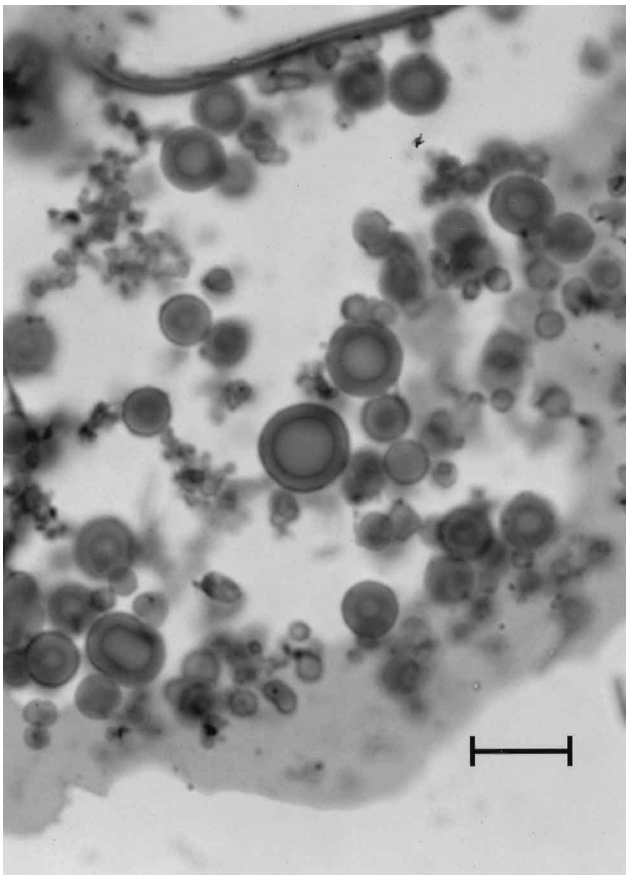

FIG. 11. Embedded, sectioned, and stained spheroidal forms harvested from a culture of lysed blood in variant broth. Further incubation of these bodies led to a positive culture for streptococci. Note the silver-staining inner lamination and outer membrane. Jones methenamine silver stain. Bar, 10 μm. (Reprinted with permission from reference 71.)

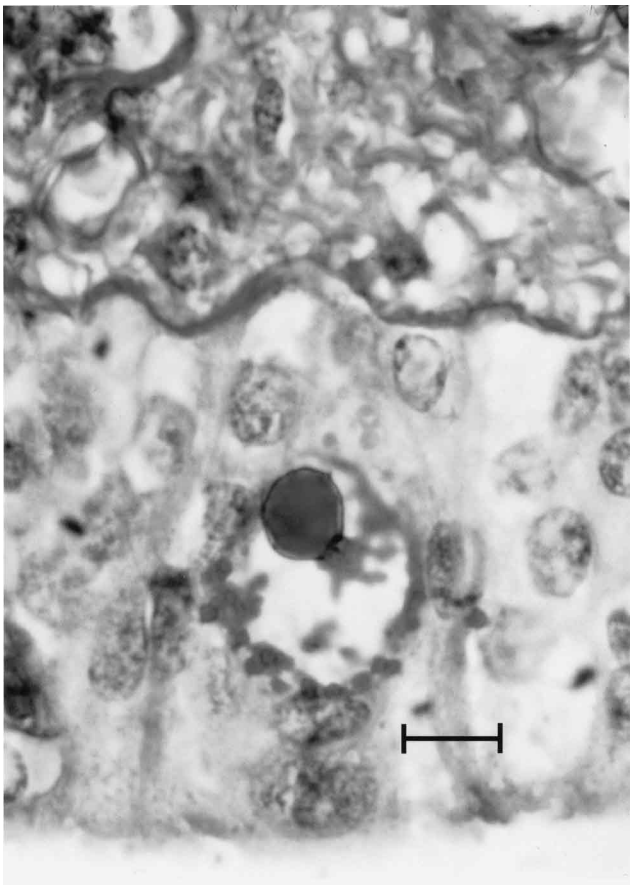

FIG. 12. Cyst within a hypertrophied renal pelvic epithelial cell from the patient with Fanconi syndrome, showing a large spheroidal body with a dense core, a slender relatively lucent cortex, and a hematoxylin-staining outer membrane. Note clusters of small spheroidal bodies associated with the cyst membrane as well as the outer membrane of the large spheroid. Periodic acid-Schiff stain. Bar, 10 μm. (Reprinted with permission from reference 71.)

When the urine samples of these children in early relapse are reviewed by oil immersion phase microscopy, one invariably sees an extraordinary number of 1- to 2-μm-diameter coccus-like CWDB embedded within a mucinous matrix (181) as well as within casts and renal tubular cells (Fig. 15 through 17). Acridine orange staining of the urine sediment confirms their nucleic acid content (Fig. 18). On occasion, there is a tiny elementary body on the plasma membrane of the organism. If incubated under a coverslip, it can be seen to be the source of a filamentous growth. After prolonged relapse, lipidemia and lipiduria occur. Since CWDB, like mycoplasma, adsorb lipid avidly, many of the organisms are obscured by this refractile fatty coating. CWDB within renal epithelial cells also adsorb lipid and have been named "oval fat bodies"; they are considered pathognomonic of this disorder. When such a cell is incubated under a sterile coverslip, the lipid can be seen to escape and lie around the cell as refractile globules. The cell can then be seen to be packed with the organisms that had adsorbed the lipid (93, 181).

EM of such urine sediment shows moderate autolysis of the larger, mature forms, but the elementary body lying within a small cyst adjacent to the plasma membrane seems quite intact and capable of the growth viewed in culture (Fig. 19). EM of a proximal renal tubular cell from a nephrotic patient reveals an undistorted organism that precisely matches the urinary form (Fig. 20).

The combination of infection and poor tissue perfusion can lead rapidly to serious morbidity; the first therapeutic priority is to control infection and blood volume. Therapy with appropriate antimicrobial agents and steroids over several weeks usually brings about clinical remission and the normalization of most chemistry levels. However, a persistent elevation in serum cholesterol and depression of serum immunoglobulin G is predictive of a continued tendency to relapse (173).

It is our belief that in these patients, an occult persisting infection becomes established at or before the onset of the acute illness and may lead to immune dysfunction produced by residence of CWDB within certain cells of the host. Many clinical aspects of the disease could be explained if macrophages were a significant locus; there are many examples of such chronic bacterial parasitism within macrophages. Tumor necrosis factor, the cytokine released largely by the macrophage, reduces the level of lipoprotein lipase so that a marked hyperlipidemia develops (18, 178). It induces coagulopathy and in conjunction with interleukin 1 and lymphotoxin serves as an osteoclast-activating factor that mobilizes calcium from bone. Numerous inflammatory disorders of diverse origin may depend on the production of tumor necrosis factor. Of particular interest is the excessive production of collagenase and prostaglandin E_2, which may lead to the loss of cartilage. Curiously, children with recurrent relapses tend to have very soft ears, even during long intervals of remission.

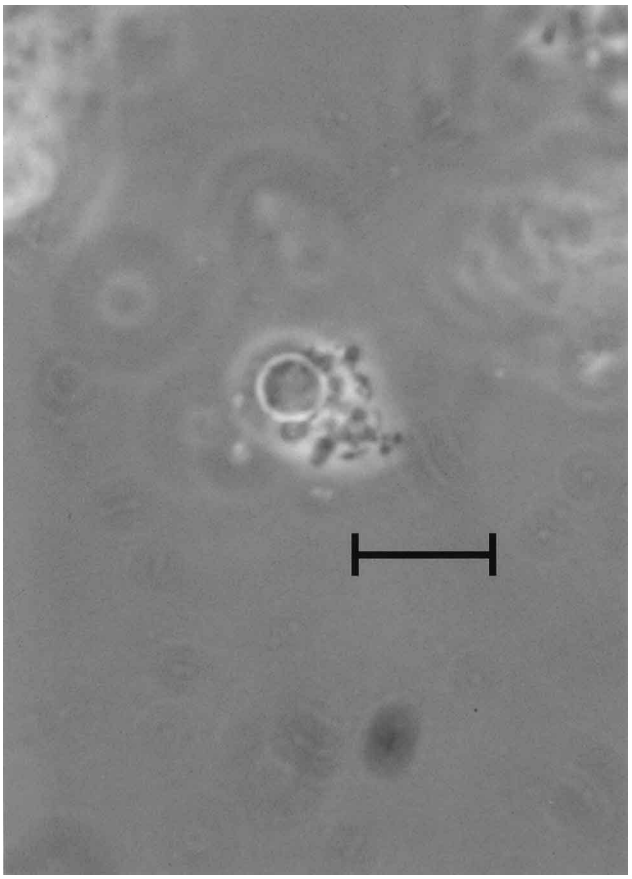

FIG. 13. A large spheroid with an associated cluster of smaller spheroids seen in the urine of the patient with Fanconi syndrome. Note its resemblance to the intracystic body in Fig. 12. Phase oil immersion. Bar, 10 μm.

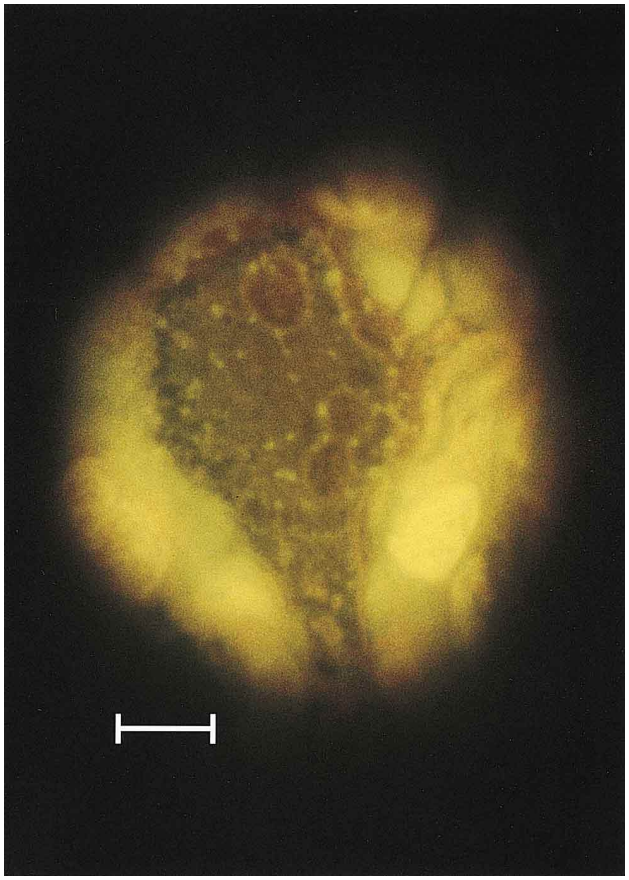

FIG. 14. Acridine orange-stained frozen section of the renal pelvic epithelium of the patient with Fanconi syndrome. Note within an epithelial cell the yellow-green fluorescence (DNA) defining the cystic wall and the membranes surrounding large intracystic spheroidal bodies. Bar, 10 μm.

Blood cultures from the patients in cases described above grew only CWDB on L-form medium and nothing at all by routine techniques. Reversion was always to gram-positive cocci; this is also true of the case that follows. The presence of L-forms of gram-positive cocci in such diseases as rheumatic fever, a variety of nephropathies, and aphthous stomatitis, to name only a few, are thought to relate to the shared biochemical properties of these organisms and host tissue. The severity of expression of pathology seems to relate less to the organism than to the vulnerability of the host.

Idiopathic hematuria. The patient was a sexually active 22-year-old white female who had no serious illness until her early twenties, when she developed recurrent urinary tract infections requiring antimicrobial therapy (69). At 21 years of age, she required surgery for an ectopic pregnancy involving the right fallopian tube. Two weeks later, she developed fever, severe headaches, and gross hematuria, which did not resolve when she was treated with cephalexin. Over the next 3 months, she had extensive urologic studies that included repeated microscopic examination and routine cultures of the urine. No leukocytes or bacteria were seen, and the cultures were negative for growth. The urologic studies were normal except for the atypical finding of a low-riding right kidney and a short right ureter. On cystoscopy, bloody urine was seen to be draining only from the right ureter. Right ureteral urine was submitted for phase microscopy, EM, and culture on L-form medium. All revealed the presence of CWDB. The revertants on culture proved to be *Streptococcus agalactiae* and *E. faecalis*; the same organisms grew with a very low colony count on routine culture after a prolonged incubation of 96 h. The revertants had in common only a susceptibility to nitrofurantoin.

Within 4 days of starting this drug, the patient's hematuria resolved. This medication was continued for 6 weeks, during which time her clinical status showed continued improvement. Two weeks after discontinuing this medication, the urine was normal by microscopy and showed no growth on L-form medium. She was monitored for 3 years, during which time she remained well. She also had a normal pregnancy.

Because this patient was well both before and after this illness, no significant genetic/metabolic derangement need be invoked. However, the atypical right kidney and ureter as well as the right ectopic pregnancy suggest that anatomic factors predisposed the patient to recurrent infections. In the year before her ectopic pregnancy, short courses of antimicrobial agents could have led to induction, selection, and persistence of CWDB resistant to many drugs.

Glomerulonephritis and Rheumatic Fever

Streptococcal infections frequently precede many chronic relapsing disorders, such as glomerulonephritis and rheumatic fever, during which no streptococci can be cultured (25). Because perturbations of immune function continue (152), there is a view that the streptococcus has altered some tissue to such

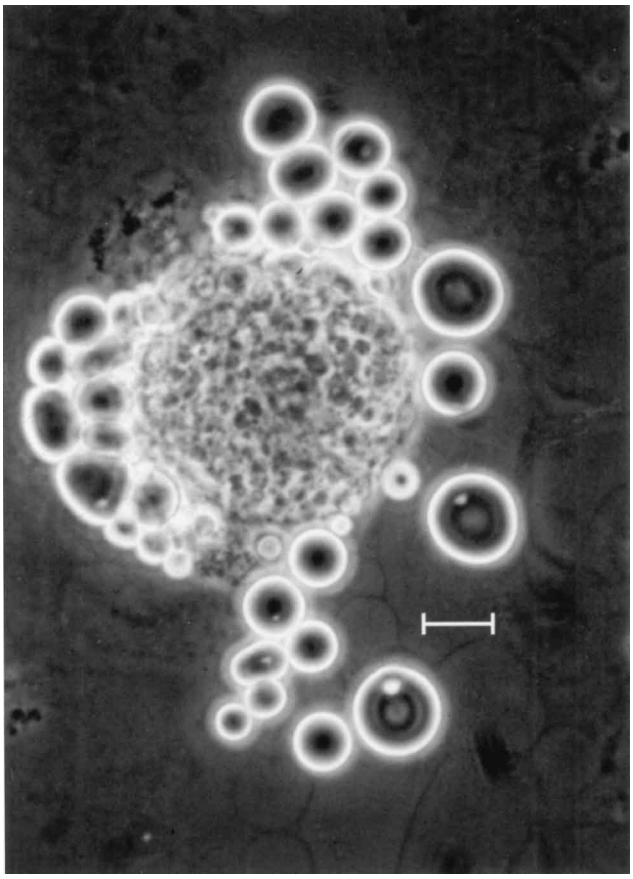

FIG. 15. Urine from a nephrotic patient containing an oval fat body (a renal tubular epithelial cell filled with lipid granules) which, when incubated under a sealed coverslip, releases globules of lipid that had been adsorbed by intracellular CWDB-like forms. Phase oil immersion. Bar, 10 μm.

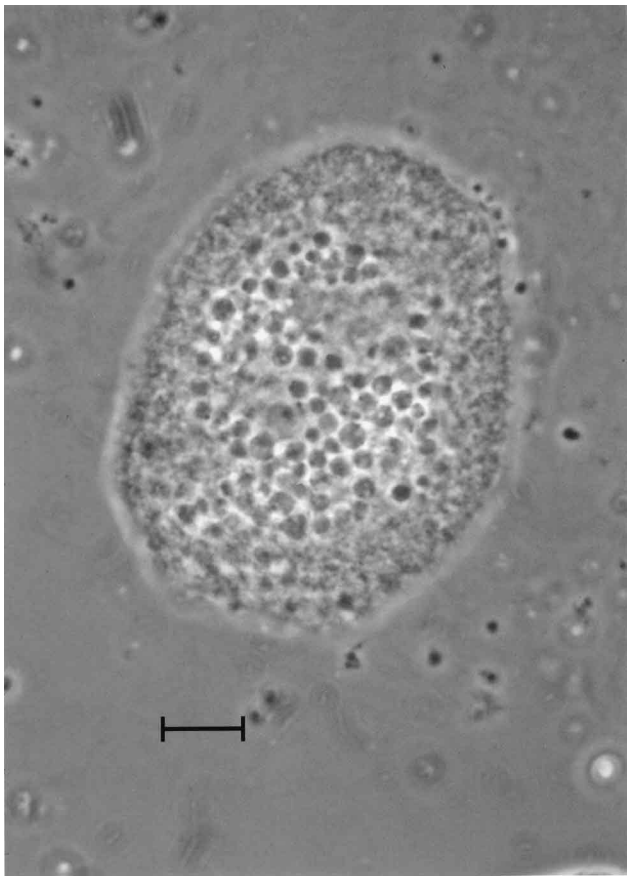

FIG. 16. A hypertrophied renal tubular epithelial cell filled with CWDB-like forms seen in the urine of a nephrotic patient. Phase oil immersion. Bar, 10 μm.

a degree that the tissue is no longer recognized as self and the mechanism of autoimmunity is invoked. Because infection of most individuals with identical strains of these streptococci fails to produce clinical pathology, the responsible dynamic is believed to be some form of immune incompetence leading to autoimmunity.

A search for a persisting endogenous antigen has been less vigorous. Largely unrecognized in clinical circles are the extensive changes in phenotypic expression of bacteria in different environments. Because immune incompetence equates with the inability to fully degrade foreign antigens, including bacteria, an assortment of bacteria which have lost either all or part of their cell walls persists in the host (152). These forms are less antigenic and have subtle differences in morphology and function and therefore various effects on reactive immune mechanisms and their consequent clinical expression (102, 137). Routinely used culture media may select out forms that fail to represent the more pathogenic members of this assorted microbial family. In contrast, culture media designed for growth of more fastidious organisms are more likely to reveal bacterial variants in blood, urine, and other body fluids. The rapidity with which CWDB revert to their parent form often correlates with the activity of the disease. EM examinations of tissues from patients with streptococcus-related collagen diseases have often identified CWDB (159).

Because the means of culturing CWDB are not presently available to many physicians, an assumption that CWD streptococci are the primary stimulus activating the disease can be justified. For many years, prophylactic penicillin has been administered to patients with rheumatic fever in order to prevent streptococcal reinfection; it seems more likely that the beneficial effect derives from its capacity to reduce the generation of the highly antigenic cell wall of endogenous organisms. A consideration should be given to the use of antimicrobial agents effective against the cell wall-defective form of streptococci.

Aphthous Stomatitis

Barile et al. (5a) in 1963 and Wray in 1982 (182) made the observation that recurrent aphthous stomatitis (RAS) was not caused by a mycoplasma, as they had suspected, but by the in situ reversion of the CWDB form of *Streptococcus sanguis* to its parent form. They further established that the responsible antigen was contained in the bacterial cell wall and that this immunologic reaction was peculiar to individuals with a genetic predisposition to RAS. The clinical expression of this painful disorder varied from an occasional mild flare-up with a minimal number of small ulcers to severe and prolonged episodes that could involve not only the buccal mucosa but the urinary tract, eye (iritis), and joints. Studies conducted at that time demonstrated that antibodies derived from these patients, in contrast to those from healthy persons, had an affinity for fetal oral mucosae.

Since then, the following information has helped define the

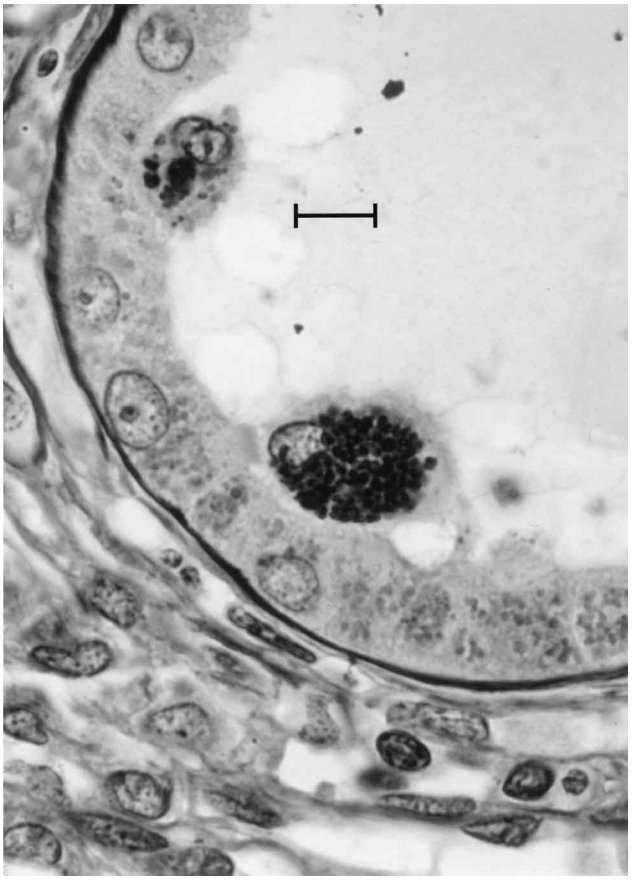

FIG. 17. Surgical specimen from a patient with nephrotic syndrome, showing a hypertrophied renal tubular epithelial cell within a renal tubule. Note the intense silver staining of the contained intracellular CWDB-like forms. Jones methenamine silver stain. Bar, 10 μm.

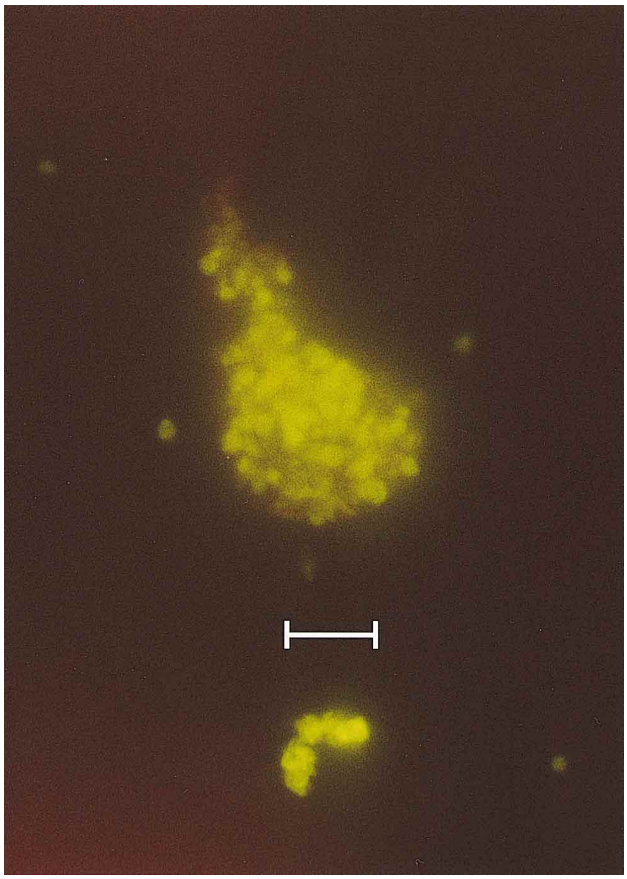

FIG. 18. Acridine orange stain of a renal tubular epithelial cell in the urine of a nephrotic patient. Note the yellow-green fluorescence (DNA) of the contained CWDB-like form. Oil immersion. Bar, 10 μm.

role of the host in the expression of this condition (182). An increased frequency of HLA types A2 and Bw44 in afflicted patients has been shown. Examination of the lesions has revealed an increased number of immunoglobulin E-bearing lymphocytes, and mast cells have been noted in increased numbers during the prodromal phases; such findings are consistent with relapse being precipitated by dietary allergens. Patients with RAS, but not control patients, have circulating complement-binding antibodies against human oral mucosal extracts which localize to the intracellular cytoplasmic areas of the prickle cell layer of the oral epithelium. Circulating immunoglobulin G antibodies against *S. sanguis* 2A are consistently increased in these patients with RAS. There was a significant reduction in these patients' lymphocyte proliferation to *S. sanguis* 2A regardless of the disease activity. Cytokine production in patients with RAS showed no significant difference from that in controls, nor was macrophage function thought to be deranged. It is interesting that a streptococcus is so often incriminated in this confused identity between microbes and host.

Whipple's Disease

In 1907, Whipple described the first case of a rare, chronic, relapsing systemic illness that typically presented with a nonspecific history of weakness, arthralgia, intermittent fever, and weight loss. It was only when intractable diarrhea and cachexia appeared and surgical procedures were indicated that the character of the pathology was defined. Before antimicrobial agents were available, most patients died of this disorder. At postmortem examination, it was noted that many organ systems were involved and showed essentially the same pathology found in the intestine, i.e., granulomatous lesions in the upper intestinal wall in which tiny silver-staining bacillary forms are seen free in the tissues as well as intracellularly (75). The organisms are strongly periodic acid-Schiff stain positive, weakly gram positive, and not acid-fast. The substance in the foamy macrophages that contain the bacilli is believed to be a polysaccharide derived from the bacterial cell wall. To date, no one has convincingly cultured an etiologic agent. Biopsies done when the patient is acutely ill do show an assortment of other bacteria associated with the granulomas; the growth of these bacteria on culture has considerably confused the nature of the pathogenic mechanisms. After 1960, EM of the intestinal lesions consistently revealed tiny bacillary bodies and the details of their cell wall, which had both an underlying and overlying trilaminar membrane (49). This unusual configuration resembled those of *Nocardia* and *Mycobacteria* spp. and of intracellular forms seen in patients with sarcoidosis (145).

After PCR was developed and an extensive library of the sequences of known bacteria was accumulated, it became possible to design a phylogenetic tree that maps genetic relatedness with a rough timing of microbial evolution. It has become obvious that no precise match can be found for many bacteria; it is estimated that fewer than 10% of all bacteria have been identified. Relman et al. (163) successfully designed a probe

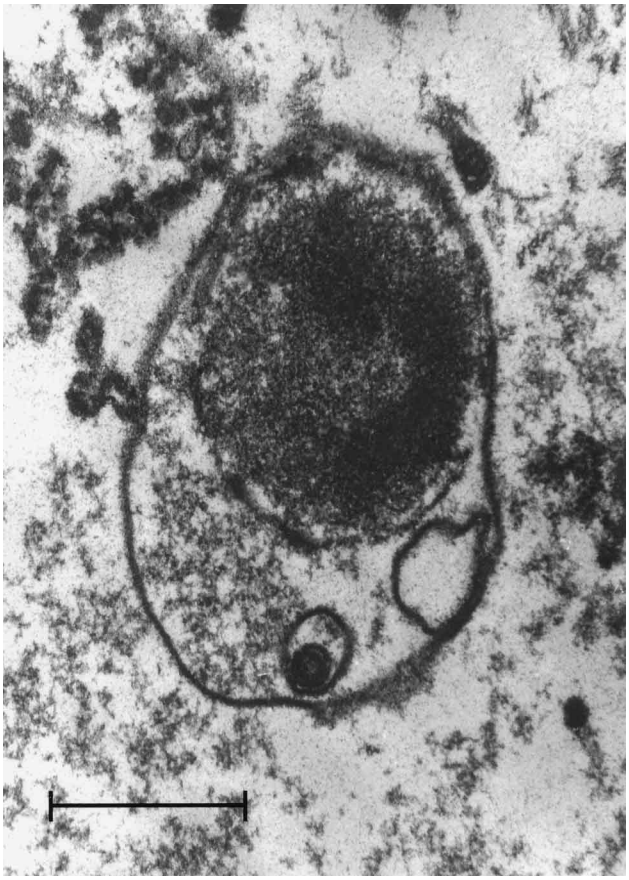

FIG. 19. Transmission EM of urine from a nephrotic patient, showing a CWDB-like form containing a large autolyzed dense body and a well-preserved elementary body within a vesicle near the cytoplasmic membrane. Bar, 0.5 μm.

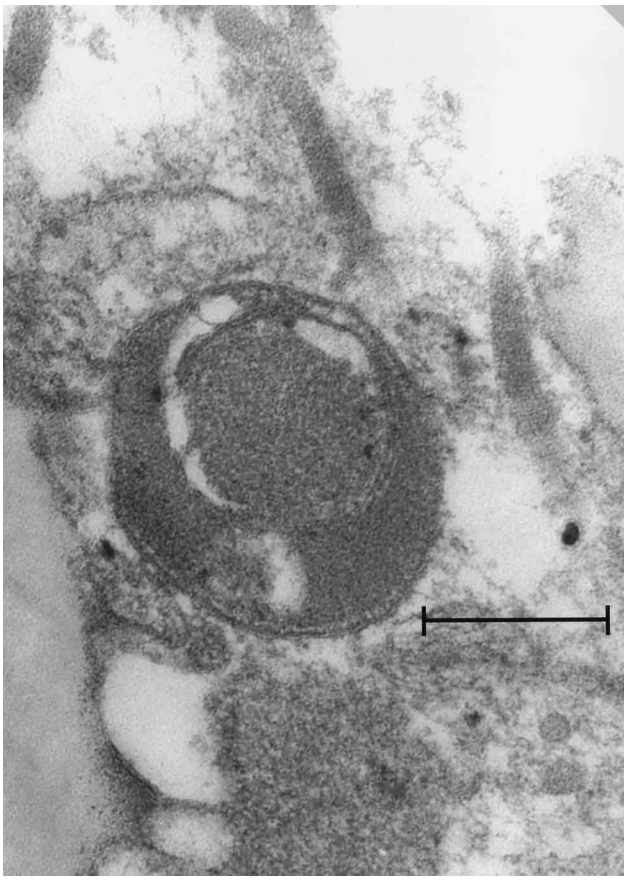

FIG. 20. Transmission EM of a CWDB-like form within a proximal renal tubular epithelial cell of a nephrotic patient containing two vesiculated forms. The large, dense body and the smaller body (lower left, near membrane) lying within membranous vesicles are well-defined and resemble the morphology of the urinary form (see Fig. 19). Bar, 0.5 μm.

based on that of organisms with the closest genetic match to the Whipple bacillus, identified it as an actinomycete, and named it *Tropheryma whippellii*.

Empiric antimicrobial therapy with a variety of drugs has brought about a dramatic resolution of this once intractable disease (101). In a matter of days, the gastrointestinal symptoms resolve, and within a few months, the patients seem fully recovered. During periods of remission, intestinal histology returns to normal and no bacteria are seen within the intestinal wall. However, relapses can occur even after years of remission. These patients are then treated with parenteral antimicrobial agents for about 2 weeks, after which trimethoprim-sulfasoxazole is given orally for 1 year.

Studies have not revealed any defects in the immune system except for a moderate decrease in number of lymphocytes and percentage of T cells even after recovery, and the lymphocytes are less responsive to mitogen. The cutaneous tuberculin reaction to purified protein derivative tends to be quite depressed.

Crohn's Disease

Crohn's disease is a chronic relapsing granulomatous disorder in which only segments of the bowel are involved; intervening portions are entirely normal. The ileocecal area is most frequently affected, but any part of the gastrointestinal tract from esophagus to rectum can become diseased. All layers of the wall are involved with a noncaseating granulomatous process in which deep ulcers can lead to perforation. The mesentery and lymph nodes draining the affected bowel share the same pathology. The onset can occur in children, in whom the disease tends to be most severe, and in the elderly but is most commonly seen between the second and fourth decades. Remissions can be quite long if the pathologic tissue is surgically removed; unfortunately, relapses almost invariably occur, so operations are performed only for such emergencies as perforation and obstruction.

Significant symptoms in the more severely affected patients are cramping abdominal pain and a painful palpable mass in the right lower quadrant when the ileocecal area is diseased. The systemic evidence of this debilitating process can be fever, anorexia, weight loss, and anemia. Additionally, there may be associated arthralgias, ankylosing spondylitis, uveitis, aphthous ulcers, and liver disease. Amyloidosis can be a complication of Crohn's disease. A variable depression of cell-mediated immunity has been found in these patients even when they are in remission. There can be some degree of anergy to tuberculin antigens, a high frequency of failure to respond to dinitrochlorobenzene, and a diminished reactivity of cultured circulating lymphocytes to phytohemagglutinin.

Of particular interest is the observation that about half of these patients have a positive Kveim skin test (which uses a sterilized suspension of human sarcoid lymph node). There has always been a strong suspicion that a mycobacterial organism (107, 108) is the transmissible agent responsible for this disease, but repeated efforts to isolate the responsible organism were unsuccessful. More recent studies have succeeded in demonstrating a cell wall-defective form of mycobacterium (141) that is believed to be responsible for the pathology. Although many free-living mycobacteria can be cultured from the human intestinal tract, no cell wall-defective mycobacteria were found in 27 controls but they were found in 12% of patients with Crohn's disease and in 16% with ulcerative colitis. Pleomorphic, variably acid-fast bacteria were found in 52% of ulcerative colitis patients and only 7% of controls. A monoclonal antibody designed to react with a glycolipid present in both *Mycobacterium avium* and mycobacteria from Crohn's disease patients showed the glycolipid to be present only in superficial intestinal tissue of controls but also in the submucosae and subserosae of patients with Crohn's disease. Antibody titers to *Mycobacterium paratuberculosis* are highly elevated in patients with Crohn's disease, so this species is a preferred candidate for its causation. Goats are known to be susceptible to a granulomatous intestinal disease caused by *M. paratuberculosis*. A revertant strain of *M. paratuberculosis* from a patient with Crohn's disease has produced ileal granulomas when fed to infant goats. Impressive studies have implicated viruses and *Pseudomonas* spp. as possibly playing a role in the expression of this disease (158). Therapy is largely supportive and empiric. Steroids and sulfasalazine have been used to control inflammation. Moss et al. (146) reported treating 44 patients for 6 months or longer with broad-spectrum antimicrobial agents. Most of them received symptomatic relief, and in many, there was radiographic evidence of significant resolution of stenosis, ulceration, and abdominal masses. The X rays of five of these patients returned to normal.

Mycobacterial Infection

Mycobacteria are distinguished by a cell wall containing 60% lipid that acts as a deterrent to the entry of injurious substances; this wall gives mycobacteria a survival advantage, but the consequence of this defense is slow growth secondary to restriction in the uptake of nutrients. In contrast to the gen-

eration time of approximately 20 min for most bacteria, mycobacteria need 12 h at best to double their numbers. Experimental animal studies have clarified some of the dynamics of the host-pathogen interaction. The initial infection is brought about by the uptake of organisms by macrophages at mucosal surfaces; various metabolites produced by the organism so modify the phagosomal membrane and the intraphagosomal milieu that it becomes a habitat for replication (176). The clinical reaction to this invasion may be imperceptible or may produce severe immune reactivity leading to the production of a wide range of autoantibodies (159). Ideally, the reactive phase is relatively benign, and defensive mechanisms supervene. Proliferation and recruitment of macrophages and other cells mediating inflammation isolate the microbe from the circulation; over time, there is caseation of the core and fibrosis of the tubercle. However, the potentially infective microbe can persist in the tubercle for decades and be activated when immune competence is compromised by such disorders as malnutrition, old age, and virus infections such as measles and AIDS.

Once heightened immunity develops, an increased level of lysozyme is generated (165) and the bacterial cell wall can be degraded. Judge and Mattman (96, 138) believe that it is the CWDB that preferentially persists in the tissues and blood of patients after allergy to the cell wall has been established. Mattman (138) has been successful in culturing such forms from blood within less than a week when classic cultures failed to grow or grew very slowly. She reported that the wall-deficient mycobacteria usually do not revert in vitro, that they are acid-fast (with an intensified Kinyoun's stain), and that their biograms and immunologic profiles are consistent with those of the parent bacterium. She has also reported that blood cultures for CWDB from patients with *Mycobacterium leprae* and sarcoidosis also produce microbial variants (96, 139, 140). It is her conclusion that these are stable L-forms that can insidiously produce significant pathology.

Miscellaneous Difficult-To-Culture Bacteria

An intact immune system can mask the potential pathogenicity of endogenous microorganisms. The clinical expression of many diseases in AIDS patients illuminates this phenomenon. *Mycobacterium*, *Cryptococcus*, and *Salmonella* spp. are among the many organisms repressed; even more alarming is the recognition that under immunocompromising circumstances, saprophytic bacteria can create serious pathology. In Third World countries, malnutrition, poor sanitation, and pervasive exposure to such parasites as *Plasmodium* and *Giardia* spp. have obviously accounted for susceptibility to lethal infections. More recently, the death of large numbers of young adults with AIDS has stimulated the development of techniques that are increasingly clarifying the dynamic of the relationship of microorganisms to the immune system. The cases that follow are briefly reported to illustrate the scope of the investigations.

***Bartonella* spp.** Before 1990, it was not possible to isolate and identify *Bartonella* spp. by available culture methods. Since that time, molecular techniques have confirmed the presence of these difficult-to-culture bacteria in clinical specimens, allowing precise and cultural identification. The slender silver-staining bacilli seen in pathologic tissues have been successfully cultured in special cell-free media after incubation periods of as long as 15 days. The nucleic acid sequence of the organism within bacillary angiomatosis lesions of patients with AIDS was first characterized by Relman et al. (162), who placed it in the order *Rickettsiales* and gave it the genus and species name of *Rochalimaea henselae*. Since then, appreciation of additional genetic and cultural properties of the order *Rickettsiales* has led to a reclassification that has moved this organism to the family *Bartonellaceae* and the genus *Bartonella*. The genus *Bartonella* had previously included *Bartonella bacilliformis*, the etiologic agent of Oroya fever, and now includes *B. vinsonii* (Canadian vole disease), *B. quintana* (trench fever, bacillary angiomatosis), *B. henselae* (cat scratch disease, bacillary angiomatosis, and bacillary peliosis), and *B. elizabethae* (endocarditis).

The diversity of clinical expression of infection by any of these organisms reflects the biochemical/immunologic vulnerability of the patient. Severely immunocompromised patients such as those with AIDS tend to have clinically expressed sepsis and a constellation of symptoms relating to the organ system most severely compromised; neovascular skin lesions and lymphadenopathy have more specific diagnostic value. Although *B. henselae* can be associated with severe morbidity in the patient with AIDS, it is usually relatively benign and self-limited in immune competent children who acquire cat scratch disease from a cat that is an asymptomatic carrier. *B. bacilliformis* is transmitted by the sand fly in areas of endemicity and may produce no symptoms or a chronic relapsing disorder associated with a severe hemolytic anemia and, in the late stages, verruga, a nodular neovascular skin lesion. All of these organisms share a common therapeutic response and are best treated with doxycycline or erythromycin. The kinship of members of this species illustrates that their genetic relationship tends to be predictive of their similar potential pathologies and antimicrobial responses.

Unidentified hemotropic bacteria. In 1979, Archer et al. (2) reported the case of a 49-year-old white male who had required surgery when he was 20 years old to remove his spleen, ruptured in an automobile accident. He had been otherwise essentially well until he was about 45 years old, after which moderately severe arthralgias developed, which were adequately controlled by nonsteroidal analgesics. In the year before his initial hospitalization, he lost weight and had chills, fever, and night sweats. On physical examination, he was found to have generalized lymphadenopathy and purpuric, tender, nodular lesions on his feet and petechiae on his legs. Extensive laboratory studies appropriate for a patient with a collagen disorder were carried out and were unremarkable. Cultures for *Mycobacterium* and *Mycoplasma* spp., CWDB, and viruses as well as animal inoculations with the patient's tissue were done and showed no growth. The intensity of this search was prompted by the extraordinary appearance of his Wright's stained blood smear, which was remarkable not only for the appearance of the expected Howell-Jolly bodies but also for the presence of very small bacilli adherent to more than 60% of the erythrocytes. Skin biopsy and bone marrow specimens showed tremendous numbers of similar organisms lying free in the interstitial tissues. A gram-stained smear was faintly positive; the EM of the bacillus seemed to suggest an ultrastructure characteristic of gram-negative bacilli.

Within a week of initiating therapy with intravenous vancomycin, the patient had become asymptomatic. At the end of 1 month's therapy, no bacilli were seen on blood smears. Remission lasted only 3 weeks after therapy was discontinued. He was again hospitalized and found to have erythrocyte-adherent bacilli. Initial treatment with tetracycline was unsuccessful and was changed to treatment with intravenous cefazolin plus intramuscular streptomycin, to which he again responded within a few days. Treatment was discontinued after 6 weeks, at which time no bacilli were seen in a blood smear. He relapsed again 2 months later with essentially the same clinical picture and

was treated successfully with cephalexin for 4 months as an outpatient.

Three months after cephalexin was discontinued, symptoms again recurred. The bone marrow was found to be heavily parasitized with bacilli and was believed to be the primary locus for initiating relapses. Because chloramphenicol is believed to be the drug of choice for bone marrow penetration, this drug was given intravenously for 10 days and then orally for 1 months. A bone marrow biopsy sample obtained 3 weeks after initiation of this therapy was free of bacilli. Cephalexin was then administered for the next 10 months, after which the patient remained well for a year.

Hemotropic bacteria are rarely reported to infect humans, although Archer et al. had previously reported three similar cases in patients with lupus erythematosus. The expression of disease by hemotropic bacteria has been demonstrated in splenectomized animals, in whom such infections are not rare (73).

Hypothesis for a Dormant Bacterial Phase of *Treponema pallidum* Resulting in Spirochetal Persistence in Syphilis: an Analog?

T. pallidum, the causative agent of syphilis, has not been successfully cultured on artificial culture media in vitro. It has, however, been maintained in animal hosts by inoculation. Noordhoek et al. (150) reported on the use of PCR in patients with neurosyphilis; they employed nested primer pairs based on the DNA sequence of the 39-kDa *bmp* gene of *T. pallidum* subsp. *pallidum*. After concentration of DNA, it was possible to detect a level of about 100 treponemes in 1 ml of cerebrospinal fluid. Cerebrospinal fluid samples from a total of 29 symptomatic and asymptomatic patients with neurosyphilis were tested for the presence of treponemal DNA before and at various intervals after intravenous treatment with penicillin. Prior to the penicillin treatment, these investigators detected *T. pallidum* DNA in 5 of 7 patients with acute symptomatic neurosyphilis, in none of the 4 patients with chronic symptomatic neurosyphilis tested before treatment, and in 2 of 16 patients with asymptomatic neurosyphilis. They unexpectedly found that *T. pallidum* DNA was also often detected in cerebrospinal fluid long after intravenous treatment with penicillin, sometimes up to 3 years after therapy. The authors concluded from the results of their study that the presence of *T. pallidum* DNA in cerebrospinal fluid does not necessarily mean that these patients were treated inadequately, because none of the patients had a clinical relapse by 2 to 9 years after treatment. It is important to determine whether treponemal DNA detected by PCR originates from treponemes that are viable at the time of sampling or from killed or lysed spirochetes. DNA appears to be a stable biopolymer, and the data of these investigators suggest that it might remain present in cerebrospinal fluid for prolonged periods after assumed effective treatment (and possible killing of *T. pallidum*) by intravenous penicillin treatment.

We speculate that the persistence of *T. pallidum* DNA (despite the absence of symptoms) may also represent nucleic acid derived from dormant, viable persistent forms of the organism (intracytoplasmic dense bodies as previously described in this review) that may or may not elicit clinical symptoms yet maintain the dominant presence of the microbe in tissues and contribute to spirochetal persistence and relapse. Microorganisms within the genera *Treponema*, *Borrelia*, and *Leptospira* are often characterized by large cyst-like bodies that are present in their developmental cycles. These cyst-like structures have been well documented at the EM level. These cyst-like bodies resemble L-form large bodies (57, 58). With free-living spirochetes, a classic spirochete may appear in the interior of the cyst. They also resemble L-forms in their erratic regeneration when transferred to fresh culture medium. The spirochetal cysts differ from L-form bodies by forming only a few spirochetes rather than the numerous bacterial forms packing a reverting L-form body. Tiny retractile dense forms and their development into spiral organisms are also well documented (136).

CONCLUSION

In the last few decades, an increasing percentage of the population has become immunocompromised. Some mechanisms for this increase are aging; autoimmunity; congenital, metabolic, and degenerative disorders; and AIDS. The life of a patient so affected is prolonged by therapy with hormones, antimicrobial agents, and immunosuppressants. It is therefore not surprising that pleomorphic, dormant, and mutant bacterial populations arise in vivo when the bacteria are exposed to agents that interfere with structural components and metabolic processes essential to survival of the microbe. These altered pleomorphic, aberrant forms may be of serious pathologic consequences to the host as a cause of persistent infection. Some possible mechanisms for dormant bacteria expressing disease are the transient immune dysfunctions that can be brought about by severe physical or emotional stress. In addition, the introduction of a coinfecting symbiotic organism can adversely alter the relationship of host and microbe. The concept of normal bacterial flora is a statistical one that derives from the immune competence of most of the population. It is unwise to dismiss the pathogenic capacities of any microbe in a patient with a mysterious illness. Bacteriologic advances, which include special culture media and stains, EM, and PCR, have revealed an increasing number of previously unidentifiable organisms in a variety of pathologic conditions. Because it is estimated that less than 10% of all bacteria have been identified, it is inevitable that the present system of microbial classification will become increasingly cumbersome and counterproductive clinically. A correlating transition to one with more potential should be explored. The present trend toward using sequence-based identification of difficult-to-culture and nonculturable organisms should successfully achieve this end. It is hoped that clinicians frustrated by negative cultures in obviously infected patients will encourage microbiology laboratories to expand their diagnostic capabilities so that the role of these more fastidious microorganisms can be appreciated.

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