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Novel Bacterial Structures in Human Blood: Cultural Isolation

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Evidence for the existence of a novel bacteriological system has been obtained from osmotically lysed and filtered human blood (membrane filters with a pore size of 0.22 μm) placed in special culture media. These blood filtrates gave rise to ordinary bacteria for 71% of the blood specimens processed from diseased humans and for 7% of those from supposedly normal humans. Morphologically, the bacteria resembled streptococcal, staphylococcal, and gram-positive filamentous (cocco-bacillary) forms. Prior to the appearance of bacteria in the media, large and small "dense bodies" were microscopically observed but disappeared when ordinary bacteria were apparent. Cultures of unlysed blood as conventionally performed were negative. These organisms may represent an adaptation of certain bacteria to life in the blood.

Recent studies (5, 6) in our laboratory demonstrated by electron microscopy morphological variations of *Streptococcus faecalis* L-phase variants in pure culture and while growing in human embryonic kidney fibroblasts. Among these variants were ultramicroscopic filamentous aggregates that resembled the nests of tubular structures reported in the glomerular endothelium of patients with lupus erythematosus and nephritis (3, 7). Another morphological variant seen in pure L-phase and in L-phase infected fibroblast cultures—a dense body (a structure ranging in size from 0.05 to 0.30 μm and postulated as a stage in the evolutionary cycle of L-phase organisms) (6)—bears a remarkable resemblance to membrane-bound, dense core particles of unknown origin described in renal biopsies of patients with membranous and sclerosing glomerulonephropathies (1). The possibility that the cause of many idiopathic renal diseases might be some form of variant bacterial parasitism led to a study of the blood of such patients and apparently normal subjects (2). This report describes the isolation of bacteria from lysed filtered blood that appear to be present in a variant bacterial phase prior to becoming ordinary bacteria.

MATERIALS AND METHODS

Media. Variant bacterial medium (VB agar) is a modification of the medium employed by Roberts and Wittler (8) and prepared as follows: 37 g of brain heart infusion (Difco Laboratories), 100 g of sucrose (Fisher Scientific Co.), 5 g of yeast extract (Difco), and 12 g of agar (Difco) dissolved in 1 liter of distilled water. The medium is dispensed in 10-ml amounts in the form of agar deeps in screw-cap tubes and autoclaved. The VB broth is the same medium as above but without agar. The media are completely devoid of antibiotics, and no additional source of serum was added other than the plasma from the specimen that was inoculated into the media.

Culture procedure. A schematic of the methodology employed is given in Fig. 1.

Blood filtrates. Filtration of the lysed blood was accomplished by placing the hypotonic saline-blood mixture in a sterile filter unit (150 ml, 0.22 μm) without grid (Falcon Div., Becton-Dickinson & Co.) with the application of 20 to 24 lb (ca. 9.07 to 10.89 kg) of negative pressure to the filter unit, using a vacuum pump.

Controls. In addition to the controls that are shown in Fig. 1, all reagents and materials utilized were cultured for elimination of contaminants. Samples from each lot of heparin (an amount equivalent to that represented in the blood samples) were added to the hypotonic saline (filtered and unfiltered) and inoculated into variant and conventional media. Also, the hypotonic saline only was processed as above. Quality control tests for sterility were routinely performed on pipettes, filter units, and all culture media employed.

Preliminary characterization of revertant bacteria. Preliminary characterization of revertant bacteria was done by Gram staining, catalase production, optochin susceptibility, growth in *S. faecalis* broth (SF medium, Difco), bacitracin susceptibility, coagulase production and testing their reactivity in 20 biochemical substrates: beta-galactosidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, citrate, H_2S, urea, tryptophan deaminase, indole, acetoin, gelatin, glucose, nitrate,

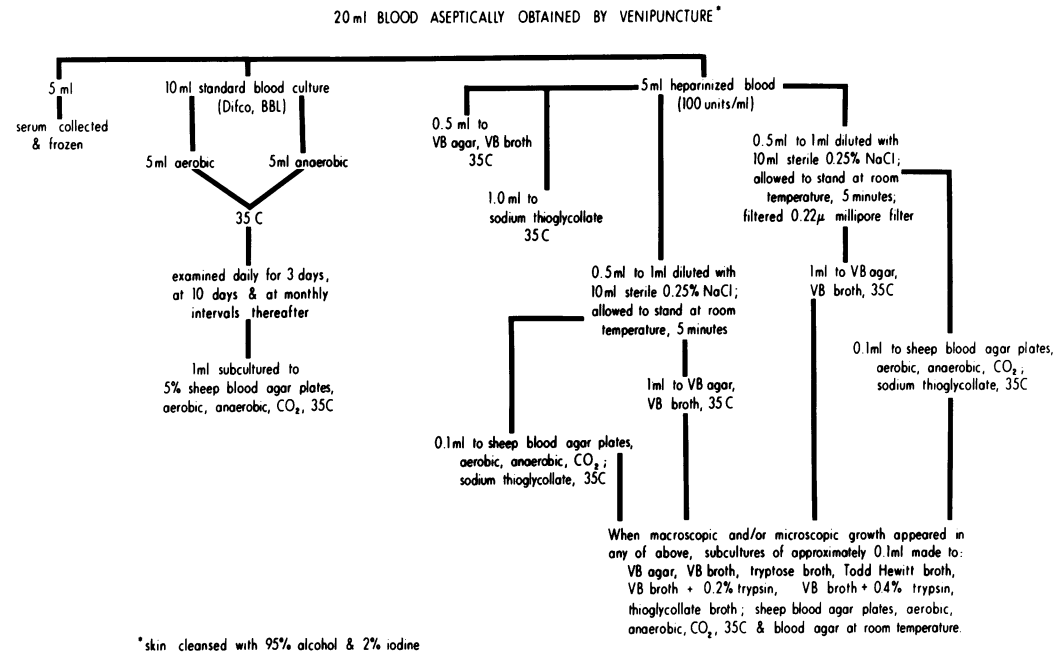


FIG. 1. *Schematic of methodology for processing blood cultures.*

mannitol, inositol, sorbitol, rhamnose, sucrose, melibiose, amygdalin, and arabinose (Analytab Products, Inc., Carle Place, N.Y.). Utilizing the Bauer-Kirby technique, antimicrobial susceptibility biograms were determined on the revertant bacteria for the following antimicrobials: ampicillin, penicillin, cephalosporins, chloramphenicol, colimycin, erythromycin, kanamycin, streptomycin, tetracycline, gentamicin, carbenicillin, nitrofurantoin, nalidixic acid, trimethoprim sulfamethoxazole, and sulfamethizole.

RESULTS

Osmotically lysed filtered blood inoculated into VB agar. As early as 2 to 3 days postinoculation, phase microscopic examination of the VB agar deeps has revealed the presence of small, dense elementary bodies (Fig. 2A) often surrounded by what appears to be a capsular-like layer; bodies stain gram negatively. As the culture ages, the center of the body may stain gram positively while the capsular-like layer is gram negative. The dense bodies may increase in numbers and appear to be of various sizes. In some instances the organisms remain in this phase, whereas those with a potential for reversion to ordinary bacteria were arranged in chain-like formation and ultimately converted to gram-positive coccal forms mainly resembling streptococci (Fig. 2D, E, and F).

Osmotically lysed filtered blood inoculated into VB broth. At 2 to 3 days after inoculation of VB broth with the lysed blood filtrate, aggregates of tubular-like structures with bulging edges can be seen microscopically; further incubation of the broth reveals bodies which appear to be "pinching-off" of the aggregated masses. They have a dense center that is surrounded by a capsular-like layer. Often the dense center appears to be diplococcal-like. Structures in the broth culture are much larger in appearance than in agar deeps (Fig. 2C). Reversion does not occur in the initially inoculated VB broth.

Subcultures from VB broth to VB agar. Subculture from the VB broth 48 to 72 h after initial inoculation to VB agar causes fragmentation of the large bodies in broth to numerous smaller dense forms that are similar to those obtained from the initially inoculated VB agar (Fig. 2A). This subculture from broth to agar greatly enhances the rate of reversion to ordinary bacteria. Furthermore, during the process of reversion, the gram-negative, capsular-like material surrounding the gram-positive center of the body appears to peel off, leaving behind gram-positive coccal forms singly or in chains. In addition to the streptococcal-like revertants, some have converted to large and small catalase-positive, single coccal organisms and filamentous-like forms (Fig. 3A and B).

It is suspected that from a given subject various morphological forms cultured might represent different stages in the life cycle of a given

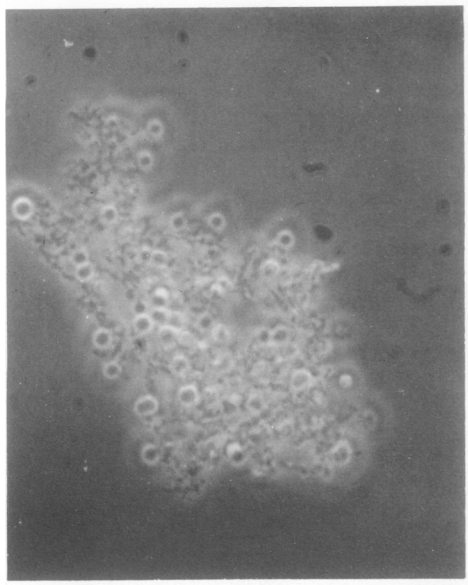
organism (which are undetected by routine blood cultures). When reversion does not occur in these initially inoculated media after 1 month of incubation, multiple subcultures from the variant agar to VB, VB-trypsin, Todd-Hewitt, tryptose, and thioglycolate broths may provoke a reversion to ordinary bacteria. If ordinary bacteria do not grow in the cultures, the structures tend to remain as dense bodies in agar deeps. Multiple attempts at getting the organisms to grow as single or L-form-like ("fried-egg") colonies on agar surfaces have failed.

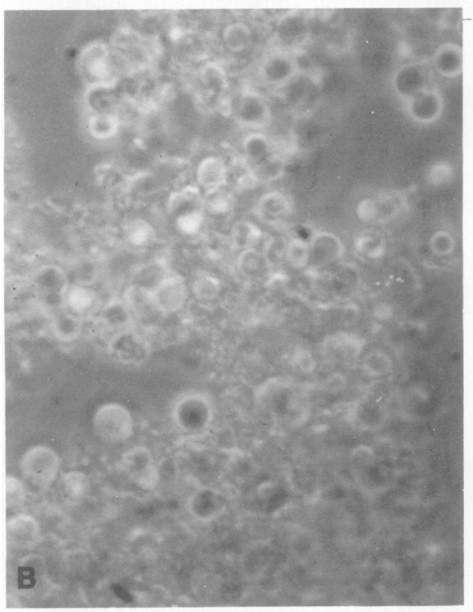
Distinguishing variants from artifacts. The variant forms observed were distinguished from artifacts (materials from lysed cells or formed elements such as platelets) by daily observation of their ability to grow and develop into dense round bodies of varying sizes, filamentous, transitional, and eventually classical organisms. Appearance of ordinary bacteria was always accompanied by disappearance in the same culture of the dense bodies. Also, only filtered specimens were considered in this paper. A further distinction was obtained by the ability of some variant forms in agar to subculture to fresh variant media without reversion to ordinary bacteria, but their growth rate on subculture appears to have been greatly curtailed. In broth it has not been possible to serially subculture. All routine bacteriological cultures of blood from subjects reported in this study were negative for ordinary bacteria after incubating for 1 month, except from one patient who had streptococci growing in the thioglycolate medium inoculated with whole blood after only 5.5 weeks of incubation. It is of interest that certain routine blood cultures incubatged for prolonged periods (>3 months) produced ordinary bacteria that appeared to be identical to the revertants obtained from a blood specimen that was osmotically lysed and filtered from the patient. This suggests that bacteria may grow in culture upon lysis of blood cells with aging, yet are not apparent during the usual time period for blood culturing.

To completely exclude the possibility of heparin as a potential source of the bacterial forms recovered, whole blood (without anticoagulant) was placed in hypotonic saline for lysis immediately after venipuncture, and the organisms were recovered from such specimens. Furthermore, the organisms were isolated from urine specimens containing blood processed identically as a blood specimen but devoid of anticoagulant.

Isolation from human blood. Table 1 shows that of the 95 diseased patients studied, 67 patients had lysed filtered blood samples producing ordinary bacteria. Also, the majority of the cultures from diseased subjects produced ordinary bacteria within 1 week of initial culture of the lysed filtrate. All cultures underwent a "dense body" phase prior to becoming ordinary bacteria. A total of 21 of the patients had idiopathic hematuria. Blood samples from 96% of these patients produced bacteria within 48 h to 2 weeks of culturing. It is of interest that of 60 normal subjects studied, only 2 produced ordinary bacteria within 1 week of culturing and 2 within 4 weeks. Organisms from normal subjects also underwent the dense body phase. Furthermore, the revertants from normal subjects were gram-positive filaments (cocco-bacillary) and cocci, which were catalase positive, and usually not streptococcal-like organisms. Diseased humans who are highly symptomatic and not receiving antimicrobials appear to have large numbers of dense bodies (particularly in broth) with a tendency towards rapid conversion to ordinary bacteria, whereas normal subjects have very few round bodies in broth culture with rare conversion to ordinary bacteria upon subculture to agar. Quantitative methodology will have to be established to definitively determine these differences.

Preliminary laboratory findings on revertants. Basically, three morphological types capable of growing on 5% sheep blood agar, thioglycolate broth, and tryptose broth and agar have arisen from the lysed, filtered blood, as discussed below. (i) The first type is streptococcal-like organisms (Fig. 2E), which have been either gamma- or alpha-hemolytic initially on sheep blood agar plates. In many instances, an alpha-prime hemolytic zone developed, followed later by beta-hemolysis upon incubating at room temperature (25°C). Many had a tendency to adhere firmly to the agar and were sticky when handled with a loop. (ii) Another revertant type is an irregularly shaped, large, gram-positive coccal form and is catalase positive and coagulase negative (Fig. 3A). These organisms are extremely tenacious on solid media and gray to white in appearance. They have a tendency to adhere to the sides of the tubes in broth culture. (iii) The third revertant type is a filamentous-like (cocco-bacillary), gram-positive form (Fig. 3B) that may initially require anaerobiosis for growth although it is aerotolerant. Most of these cocco-bacillary forms have an extremely foul odor. On sheep blood agar plates, the colonies are very small and white to gray and tend to run together as a thin film. In some instances, these morphological forms later converted into the streptococcal-like organisms already mentioned. Many of the various revertant types produced gelatinous substances

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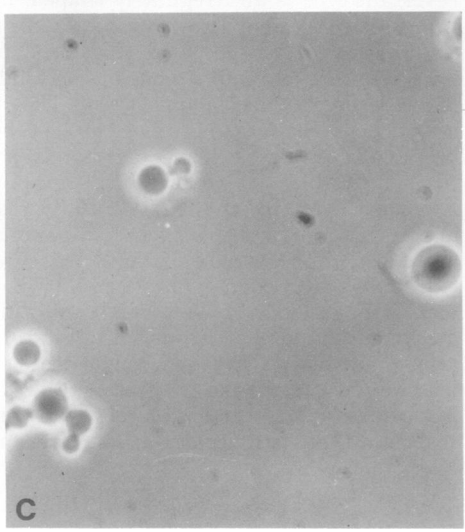
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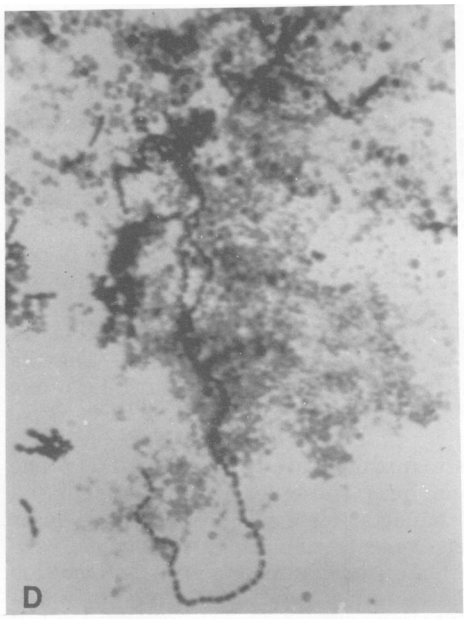
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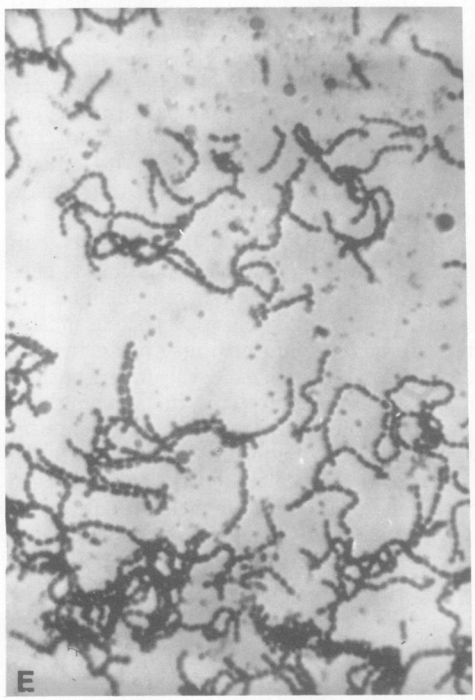
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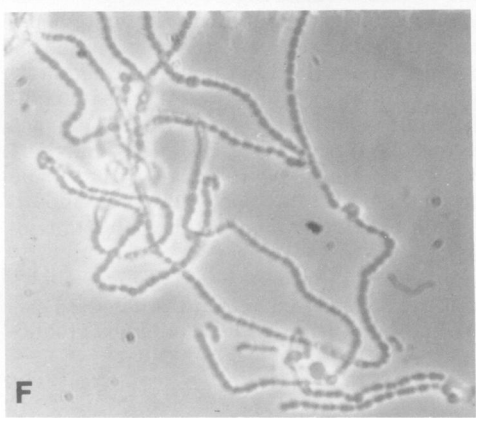
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in broth culture. We have only preliminary data on biochemical reactivities of these isolates, and no definitive conclusions can be drawn since there are great variations in biochemical patterns of reactivity, even when presumably identical colonies from a plate are tested in a given substrate. The most consistently positive reactions for the streptococcal-like revertant types are beta-galactosidase, acetoin, glucose, and sucrose, with variable reactions for urea, nitrate, amygdalin, and arabinose. Revertant streptococcal organisms from two patients were sent to the Center for Disease Control, Atlanta, Ga., for identification. The reports indicate that these organisms were alpha-hemolytic streptococci, but not enterococci. One revertant was further classified as a *S. sanguis* biotype II. The large gram-positive, catalase-positive, coagulase-negative coccal forms are most consistently beta-galactosidase, acetoin, glucose, and sucrose positive; variable reactions have been obtained with arginine and nitrate. A few of these isolates sent to the Center for Disease Control have been identified as *Staphylococcus epidermidis*. The filamentous (cocco-bacillary) revertant type was most consistently positive in arginine and glucose. It is also of interest that the filamentous type appears to be anaerobic. The filamentous form can also be biphasic, i.e., filamentous in tryptose broth and coccal-like on media containing agar. Detailed biochemical patterns of reactivity for all revertants will be the subject of another communication. The antimicrobial susceptibility biograms were particularly interesting. The streptococcal-like revertant appears to be susceptible to most antimicrobials, including those that are protein synthesis inhibitors, as well as those that interfere with the synthesis of the cell wall of bacteria. The gram-positive, large coccal bodies that are catalase positive are usually susceptible to the protein synthesis inhibitors, but many are resistant to penicillin and ampicillin and yet susceptible to cephalosporins. The filamentous type can be totally resistant to all antimicrobials tested (protein and cell wall synthesis inhibitors), but this was not consistent; in some instances these forms from certain subjects were susceptible to those antimicrobials.

DISCUSSION

The isolation of these bacteria from osmotically lysed, filtered blood is a novel finding and may open new doors to a number of mysterious host-parasite interactions, particularly those pertaining to nephropathies and certain chronic inflammatory diseases of humans suspected of being bacterial in origin. Aside from the possible significance of these studies to disease, the findings deserve complete elucidation since they modify the currently held view that the blood of humans is sterile for bacteria under conditions of health. The methodology employed revealed the presence of these bacteria, whereas conventional methods of blood culturing were negative. Unlysed blood with anticoagulant inoculated into conventional and variant media was negative, further indicating the need for disrupting blood cells prior to placing in culture media. This approach may be particularly pertinent for a bacteriological diagnosis when conventional methods yield negative findings. Furthermore, what we have much evidence for is a novel bacteriological system in which extremely small variants obtained from cultures of 0.22 μm membrane filter filtrates develop into normal size, revertant-type bacteria, as well as into very large vesiculated cells that are able to fragment to the smallest common revertible variants. Because of their small size, the variants must have dispensed with some of the classical cellular machinery of the parent form, but as we hope to prove in ongoing biochemical studies, it should still have all or at least most of the parent's genome. Although dense bodies in varying sizes and numbers were observed in culture from lysed and filtered blood of all normal subjects, few produced ordinary bacteria. Multiple subcultures to various media (Fig. 1) from the initially inoculated variant media incubated for greater than 4 weeks provoked reversion to ordinary bacteria from 18 additional normal subjects and required for 1

FIG. 2. *(A) Small dense bodies (encapsulated-like) and granular forms in VB agar developing within 3 days after culture of lysed blood. Phase photomicrograph unstained. ×2,500. (B) Subculture of VB broth to VB agar at 24 h. Fragmentation into smaller bodies as in A has already begun. Phase photomicrograph unstained. ×2,500. (C) Large round bodies in VB broth. Phase photomicrograph unstained. ×2,500. (D) Gram stain of dense bodies and streptococcal-like forms in VB agar that are unable to grow at this stage as classical bacteria on subculture to ordinary media; cocci in chains are gram positive, and small bodies are gram negative. ×2,500. (E) Gram stain of D in VB agar 2 days later. Predominant organisms are gram-positive streptococcal-like chains, with few gram-negative single bodies remaining. ×2,500. (F) Subculture E in VB agar to Todd-Hewitt broth produced a pure culture of streptococcal-like organisms in long chains. Note bulging bodies particularly at tip of chain. Organisms subcultured to 5% sheep blood agar grew as small colonies with alpha-prime hemolytic zones. Phase photomicrograph unstained. ×2,500.*

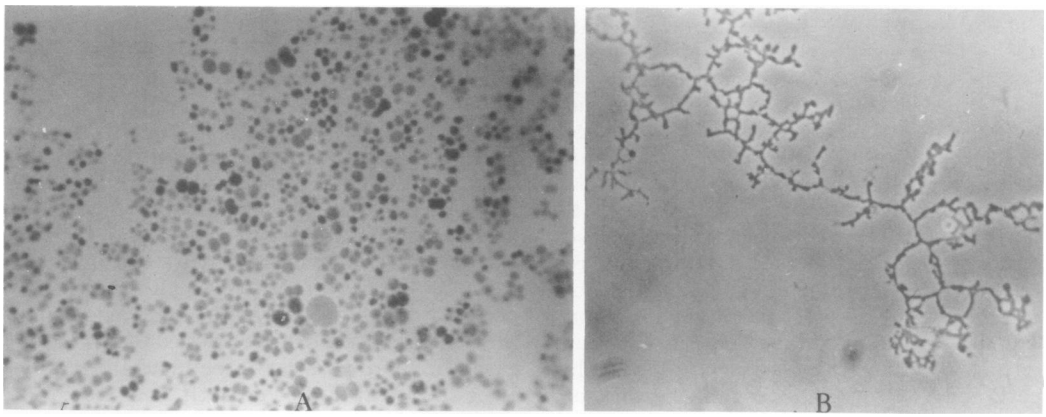

FIG. 3. *(A) Gram stain of large and small coccal revertant forms that are catalase positive. Dark cells are gram positive, and light ones are gram negative. ×2,500. (B) Filamentous form. Unstained phase photomicrograph. ×2,500.*

TABLE 1. *Isolation of variant bacteria from osmotically lysed, filtered blood (297 specimens)*

Clinical condition	No. of patients	No. with dense bodies	No. reverting to gram-positive coccus, cocco-bacillus, filamentous organisms	No. reverting within (weeks): 1	2-4	4-6	7-20	No. non-reverting
Diagnosed renal disease	64	64	49	34	15	0	0	15
Other diseases of the genitourinary tract	31	31	18	18	0	0	0	13
Total diseased	95	95	67	52	15	0	0	28
Normal	60	60	22	2	2	11	7	38

subject 20 weeks of in vitro culturing, for the appearance of ordinary bacteria, suggesting that the dense bodies from normal subjects are more stabilized. Also rapid conversion to ordinary bacteria in culture in diseased versus normal subjects may be due to the greater number of potentially revertible forms present in diseased humans when compared with normals.

An intriguing point to be raised is why are only gram-positive coccal and filamentous forms (cocco-bacillary) growing from these lysed blood filtrates? It is likely that they are hardier organisms that can survive in deleterious environments more readily than other microbes, or possibly they represent just one more phase in the life cycle of a particular group of organisms capable of existing in more than one form. In the latter case, the dense body may represent another type of differentiation in a unicellular organism. It may be that we find only gram-positive organisms because only these cells can form dense bodies, in analogy to the fact that only gram-positive bacilli can sporulate. Further research is necessary to determine whether they represent sophisticated forms of bacteria that may have adapted to life in animal tissue or whether their presence is a result of continual bombardment of tissues by ordinary organisms entering the blood from the mouth or other routes. Upon entering a deleterious environment such as blood, further development of units of novel structure and composition (dense bodies) may serve as a mechanism of survival in hostile environments. If these organisms enter the blood via the oral cavity, it is possible that some of these microbes may be the same as, or similar biochemically and physiologically to, the viridans streptococci that have been reported to have cariogenic potential (4). Of particular interest is colonization of in vivo sites by microorganisms that have unusual abilities of adherence to host tissues and cells. Many of the revertant organisms obtained from lysed blood filtrates formed gelatinous substances in broth culture and were adherent to agar surfaces. It is possible that ad-

herence of the variants in vivo may also be associated with their virulence and possibly serves as a prerequisite for pathogenicity. Furthermore, the variants might synthesize substances which, while also enabling them to attach to epithelial surfaces, could lead to mechanical entrapment for colonization of in vivo environments. The potential for these organisms to initiate a disease state may be conditioned in occurrence by some existing or developing immunological, biochemical, or physiological abnormality of the host. Because the organisms appear to be multipotential in their abilities to develop into diverse morphological forms, antigenic and biochemical modulation in vivo may well lead to immunopathological and/or toxic consequences.

We are presently looking for biochemical evidence to support the morphological observation of variant forms reverting to classical bacteria. We are studying the relationship between the base sequences of the genomes of variants and revertant-type forms. We are also trying to assess the relatedness of different isolates (morphologically diverse or similar) and to partially classify them by deoxyribonucleic acid-deoxyribonucleic acid hybridization analysis. From these studies, we should be able to conclude how diverse the variants in the blood of normal and diseased patients are and to determine their bacterial derivation.

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