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Treponematoses Research

Report of a WHO Scientific Group

This report contains the collective views of an international group of experts and does not necessarily represent the decisions or the stated policy of the World Health Organization.

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Geneva, 18-25 November 1969

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TREPONEMATOSES RESEARCH

Report of a WHO Scientific Group

A WHO Scientific Group on Treponematoses Research met in Geneva from 18 to 25 November 1969. The meeting was opened by Dr A. M.-M. Payne, Assistant Director-General, who welcomed the participants on behalf of the Director-General.

1. TREPONEMES

The Group recognized that knowledge of many aspects of the biology of treponemes is limited and that it is not possible at present to make pathogenic organisms grow *in vitro*. This constitutes a major obstacle in most treponemal research. Consequently, recent information on the characteristics, structure, metabolism, survival factors, etc. of pathogenic and other treponemes is dealt with in some detail in this report.

WHO has published extensive bibliographical reviews of the morphology, culture and survival of *Treponema pallidum* and associated organisms (Willcox & Guthe, 1966) and of the Reiter treponeme (Wallace & Harris, 1967). These reviews provided useful reference material for research workers in treponematoses.

1.1 Characteristics

The anatomy of treponemes was shrewdly interpreted by Noguchi (1928), but confirmation of his description has only recently been provided by electron microscopic studies. Today many aspects of treponemal structure are quite clear, but others remain obscure. Deficiencies in knowledge are largely due to the fragility of treponemes, especially their susceptibility to osmotic changes, and to the resulting problem of distinguishing facts from artefacts.

Pathogenic treponemes, which are among the thinnest members of the genus *Treponema*, have a transverse diameter seldom exceeding 0.15 μ and a length of 5–15 μ. For this reason the mass of a single treponeme is much less than that of bacterial cells, and possibly the immunogenic stimulus it can provoke is also less.

Pathogenic treponemes possess remarkable plasticity, as revealed by their movement in the cellular portion of lesions. In such a situation, and especially when the extracellular fluid is viscous, treponemes become more

serpentine than spiral as they migrate through the confined and tortuous intercellular spaces.

The basic structure of the treponeme is a procaryotic [1] cellular mass surrounded by 2 trilaminar membranous structures, each ranging from 7.5 to 15 nm in thickness, with the organs of motility situated between the two membranes. Ultra-thin sections of treponemes have revealed several subcellular structures within the cytoplasm ; these include ribosomes, mesosomes, and filamentous nuclear areas, as seen in other bacterial cells (Jepsen et al., 1968 ; Ovčinnikov & Delektorskij, 1966, 1969 ; Pillot et al., 1964). The limiting membranes that surround the treponemal cell have been called by a variety of terms, but they are being referred to with increasing frequency as the cell wall and cell membrane, thereby bringing the terminology into line with that employed for the eubacteria. Undoubtedly they function in these capacities, but perhaps not in the same sequence as in most bacterial cells. The outer membrane of treponemes, unlike the cell wall of true bacteria, is very responsive to changes in environmental osmotic pressure. Cultivable treponemes will expand like a balloon when subjected to osmotic shock (Hardy & Nell, 1961). It is the outer membrane only that expands while the remainder of the cell coils inside. If such spherical organisms are additionally subjected to physical or chemical trauma sufficient to rupture the outer membrane, they will immediately snap back to their original spiral shape, proving that the basic thread-like shape of treponemes is not due to the integrity of the outer membrane. Visible evidence in support of this has been provided by many of the early electron microscopy studies of treponemes, where the organisms retained their basic morphology despite intentional (Mölbert, 1956 ; Swain, 1955) or inadvertent (Morton et al., 1951) destruction of the outer membrane.

The outer membrane of pathogenic treponemes appears to be less sensitive to osmotic changes than that of non-pathogenic, cultivable treponemes. Nevertheless, these organisms also balloon and form spherical bodies in the presence of an adverse environment.

Although the outer membrane does not give the treponeme rigidity, it possesses the selective permeability properties associated with the cell membrane of eubacteria and thus serves as the osmotic barrier of the treponemal cell (Hardy & Nell, 1961). Its chemical composition has not been fully established, but its integrity can be destroyed by lipolytic agents, glucosidases (Pillot, 1965), and by at least one proteolytic enzyme, pepsin (Swain, 1955).

If the outer coating of the treponeme behaves as a " cell membrane ", the inner membranous structure functions like a cell wall. In addition to providing such rigidity as the treponemal cell possesses, it also contains the mucopeptide layer characteristic of bacterial cell walls. Visual con-

[1] A procaryotic cell is one that has no nuclear membrane.

firmation of this has been provided in electron micrographs of the Reiter treponeme grown in the presence of sublethal concentrations of penicillin (Pillot, 1965).

For a number of years, various investigators have suggested that some spirochaetes possess a " protective " slime layer or capsule on their surface (Christiansen, 1963 ; Jones et al., 1968). The evidence for this is entirely circumstantial, e.g., the mucoid substance in syphilitic lesions (D'Alessandro & Zaffiro, 1961 ; Listgarten et al., 1963). Direct visualization of a capsule in electron micrographs has been claimed by some investigators (e.g., Ovčinnikov & Delektorskij, 1969), but others disagree with this interpretation of the structures observed.

Motility apparatus

The motility apparatus of treponemes is in many ways analogous to that of bacterial flagella, but its location is unique. It consists of one or more fibrils, approximately 17 nm in diameter, which wind around the cell body between the inner and outer cell membranes. This location, first revealed in partially digested cells (Swain, 1955), has since been thoroughly established by many other studies utilizing ultra-thin sections (Jepsen et al., 1968 ; Ovčinnikov & Delektorskij, 1966, 1969 ; Pillot et al., 1964). The fibrils arise from basal granules located subterminally in the cytoplasm at both ends of the cell. From this point they penetrate the inner membrane to the intra-membranous space, from where they may extend to, and even beyond, the distal end of the cell. Fibrils arising from opposite ends of the organism overlap for part or all of the cell length. In most instances the distal ends of fibrils are unattached, but remain inside the outer membranous coat even when they extend beyond the limits of the cell body. However, some investigators are of the opinion that fibrils may sometimes re-enter the cytoplasm and become re-attached in the mid-portion of the cell (Ovčinnikov & Delektorskij, 1969).

In *T. pallidum* it is usually possible to observe 3 fibrils arising at each end of the organism, so that as many as 6 fibrils can be observed at various points along the cell length. Some investigators who have studied oral spirochaetes (Listgarten et al., 1963) are of the opinion that fibril numbers have taxonomic significance and may be valuable in establishing treponemal species. Up to now, too few pathogenic treponeme strains have been studied to permit the constancy of such features to be established. However, limited studies indicate that *T. cuniculi* and *T. pertenue* have the same number of spirals as *T. pallidum*, so this feature appears to be of no value in differentiating these species (Ovčinnikov & Delektorskij, 1970[1]). This finding supports earlier observations that there are no differences in this respect between *T. pallidum* and *T. pertenue* (Mölbert, 1956).

[1] Pre-publication data made available to the Scientific Group.

Fission

Cell division in treponemes occurs most often, if not always, by transverse fission. Incompletely divided cells can be seen frequently by darkfield microscopy of early lesions or young cultures. Electron micrographs reveal that such cells are held together by a common, or incompletely divided, outer membrane. There may also be overlapping of fibrils originating from the 2 daughter cells, but the cell bodies are totally separate (Jepsen et al., 1968).

Separation of dividing cells may result from the gradual pulling apart of the daughter cells, with concomitant stretching of the terminal portion of the outer membrane. Such a mechanism would readily explain the differences in appearance of the 2 ends of *T. pallidum* (Ovčinnikov & Delektorskij, 1969). Moreover, the subterminal " sausage-shaped " structures found in one end of treponemes could be the mesosomes that are usually present at the fission site of bacteria (Ryter, 1968).

In preparations of vigorously motile treponemes, 2 intertwined spirochaetes can occasionally be found. This could be the result of longitudinal division, but there is no evidence to support this view.

Structure under altered environmental conditions

It has long been known that spherical or granular forms of treponemes can be found both in old lesions of syphilis (Wartin & Olsen, 1930) and in aging cultures of non-pathogenic treponemes (DeLamater et al., 1951b). These structures, which are usually free-standing but may be attached to spiral forms, have been called cysts, spherical bodies, or granules. Recently it has also been suggested that they may be protoplasts or L-forms of treponemes. Regardless of their nomenclature, many investigators have looked upon them as altered, but viable, treponemal forms (Bladen & Hampp, 1964 ; Listgarten et al., 1963 ; Ovčinnikov & Delektorskij, 1969 ; Ryter, 1968).

Whether the smaller bodies, granules, are another stage of the cysts or spherical bodies, or whether they arise under totally different conditions, is still to be determined. Moreover, there is no evidence that either type of body is viable. It has been clearly proved, indeed, that spherical bodies identical to those arising spontaneously can be produced by osmotic shock, and spheres so formed are non-viable. It can be reasoned from this that all atypical forms of treponemes probably arise from poor environmental conditions, but it has not yet been established whether any are viable and, if some are, how long they may remain so.

Sublethal concentrations of penicillin lead to the production of atypical forms of the Reiter treponeme. These probably arise from herniations in the inner limiting membrane (the cell wall) and are probably analogous to partial protoplast formation. In the early stages these cells are viable,

but it is not yet known how long they remain so, and whether they can progress to completely spherical forms. It is likely, but as yet unproved, that other environmental conditions yielding unbalanced growth of the cell wall produce similar atypical but viable treponemes.

1.2 Physiology

Metabolism

What little is known about the metabolism of treponemes has been gleaned from two sources: (*a*) attempts to achieve *in vitro* survival of pathogenic treponemes, and (*b*) the conditions required for cultivation of the indigenous and similar spirochaetes. A few findings that seem most relevant to the pathogenic species are commented on below.

In thorough studies of the *in vitro* survival of *T. pallidum* (Nelson, 1948), a medium was devised that sustained viable treponemes for more than 15 days. The necessity of a lower-end environmental eH for treponemal survival was demonstrated. A recent study has suggested a lower as well as an upper limit of eH for *T. pallidum* survival (Metzger & Smogor, 1966); similar conditions have been reported as necessary for the growth of an oral treponeme (Socransky & Hubersak, 1967). It can be assumed from these studies that the various metabolic pathways in *T. pallidum* function only at a suitably lowered oxidation-reduction potential. A requirement by *T. pallidum* for pyruvate has also been demonstrated (Nelson, 1948), but this simply implies that treponemes utilize one of various possible fermentation pathways as an energy source. The demonstration that glucose enhances *T. pallidum* survival (Kimm et al., 1960) further supports this assumption. However, it is pointed out that the Reiter treponeme has recently been found to possess cytochromes, suggesting that it may be able to use molecular O_2 under certain conditions (Kawata, 1967).

Some investigators maintain that pathogenic treponemes are anaerobic *in vitro* only, and are aerobic in the tissues. The presence of many treponemes near the surface of open lesions is offered as evidence to support this belief. At the same time, it must be remembered that open lesions contain a mixture of bacteria, some of which could maintain the eH at a level sufficiently low for *T. pallidum* survival. In this context it may be noted that *T. carateum*, the causal agent of pinta, exists nearly exclusively in the lower Malpighian layers of the epidermis.

Many of the cultivable treponemes have a requirement for some factor present in serum. The serum factor required by the Reiter treponeme was identified as albumin by Little & Subbarow (1945). Subsequent studies demonstrated that this protein served a dual role as a "detoxifying" agent and as the source for an essential long-chain fatty acid carried in bound form by albumin (Oyama et al., 1953). Not all investigators have obtained growth of Reiter treponemes in media supplemented with albumin, with

serum fraction V, or even with the serum albumin fraction obtained by "salting out" the globulins. Growth approaching that of whole serum was achieved when the globulin fraction of serum was used.

All the nutritional requirements of the Reiter treponeme have been identified (Steinman et al., 1952) but their relevance to the pathogenic treponemes is still unknown.

Whether or not treponemes have a complex life cycle has been debated for many years. Among proponents of the complex life-cycle theory have been Levaditi and his associates (Levaditi & Li Yuan Po, 1930; Levaditi et al., 1927) and DeLamater et al. (1950, 1951a). Other studies, notably those of Pillot (1965), have not confirmed this view.

1.3 Survival and culture

Artificial media

During the past 50 years the *in vitro* cultivation of *T. pallidum* has been claimed a number of times, but only twice since 1930. In one instance, a medium for the isolation of *T. pallidum* was patented (Ichelson, 1950). but its effectiveness has never been proved. In addition, partial success was once reported (Boak et al, 1949) but again this work could not be reproduced.[1]

Of the numerous strains reputedly isolated prior to 1930 only a few are available today, and each has a dubious pedigree. All are morphologically larger than *T. pallidum* recovered from tissues, and none can be propagated *in vivo*. The two remaining American isolates (the non-pathogenic Nichols strain and one Noguchi strain), supposedly recovered from rabbit lesions, are antigenically, morphologically, and culturally very similar, but differ in all these respects from the European isolates. The latter also form a closely related group and were probably recovered by direct cultivation from human beings. What these micro-organisms were originally will probably never be known, but they are now almost indistinguishable from some indigenous treponemes.

In view of the foregoing, any discussion of *in vitro* cultivation must be limited to treponemes in the normal microbial flora of man (or mammals) and to the few free-living forms, such as *T. zuelzeri*, that have recently been recognized (Veldkamp, 1960). As regards indigenous treponemes, it should be emphasized that only a fraction of the observed varieties have so far been cultured and the methods employed for their isolation have been very diverse.

The cultivable treponemes represent a very heterogeneous group both morphologically and antigenically, and their growth requirements vary

[1] A news report that appeared in *Med. Wld News* (14 October, 1966) was not followed by the publication of scientific evidence.

widely. With the exception of one or two free-living forms, all are obligate anaerobes requiring an unusually low environmental eH. This can be most satisfactorily achieved by adding a salt of either cysteine or thioglycolic acid to the growth medium. For some treponemes the concentration of such reducing agents is critical, and growth inhibition may result if the level is too high or too low.

Depression of eH is not the only function of sulfydryl compounds; some are essential for maintaining viability. The Nelson survival medium used in the TPI test contains 3 such compounds balanced so as to reduce eH adequately while retaining a non-toxic environment.

For most if not all treponemes, sugar fermentation represents the primary source of energy; there is no evidence that amino acids can be used for this purpose. Some treponemes ferment a variety of sugars, but no systematic study of these has been made. The end-products of fermentation that have been identified are of the mixed acid type (Moureau, 1955), but this may not be true for all species. In some of the cultivable spirochaetes, growth is so slight that fermentation studies are impracticable.

All cultivable treponemes grow best in rich media such as meat infusions or enzymatic digests of proteins. However, the nitrogen needs of some treponemes can be satisfied by acid hydrolysates; mixtures of individual amino acids are also effective, but virtually all naturally-occurring amino acids are required and the respective concentrations can be quite critical.

Most cultivable treponemes also require some form of " native " protein for *in vitro* growth. This need can usually be met by supplementing the growth medium with serum or ascitic fluid (bile-free). Most treponemes can utilize such supplements from a variety of mammalian species, but a few will tolerate only a quite small number of sera. For the Reiter treponeme, albumin was found to be the serum fraction required for growth (Little & Subbarow, 1945), and later studies demonstrated a long-chain fatty acid to be the actual growth factor in the albumin preparation (Oyama et al., 1953). More recently, studies on an oral treponeme demonstrated that the serum supplement could be replaced by a euglobulin, but whether or not this protein also acted as a lipid carrier was not determined.

A number of cultivable spirochaetes have specific requirements for short-chain rather than long-chain fatty acids. This is the case with some of the very small oral treponemes and many rumen organisms. The compounds involved are principally those with branched chains of 5–7 carbon atoms. Most of these spirochaetes live in association with other microorganisms, upon which they depend for a supply of these acids in nature. Since *T. pallidum* does not normally exist in a mixed microbial flora, requirements such as these are probably irrelevant to growth studies of pathogenic treponemes.

Unsuccessful attempts have been made to cultivate *T. pallidum* in the presence of killed Kazan and Reiter treponemes, a source of preformed

growth factors. The reason for this approach was the growth of *Mycobacterium lepraemurium* in a medium containing saprophytic mycobacteria.

The attempt to explain why *T. pallidum* cannot be cultivated *in vitro* has received discouragingly little help from studies of the cultivable indigenous spirochaetes. The only definite results of these studies have been to reaffirm the anaerobic nature of most, if not all, treponemes and to reveal the generalized requirement for a preformed fatty acid of one type or another. On the other hand, the studies provide an indication that a proper balance of various nutrients may be a key to the *in vitro* cultivation of the pathogenic treponemes. Although a great number of elements have been tried and it is unlikely that an essential growth factor has been overlooked, they may not have been presented to the organisms in proper balance with other nutrients.

Tissue culture

A number of investigators have attempted to grow treponemes in tissue culture, but successful results have been achieved only with non-pathogenic organisms, and even in these the contribution made by the tissue was not convincing. Failures may be explained by the fact that tissue cultures cannot be maintained under anaerobic conditions long enough for marked growth of an organism with a division time of approximately 30 hours. Success in the future may depend on (*a*) techniques that will permit tissue cultures to be kept anaerobic for longer periods of time, (*b*) newer knowledge of growth factors, or (*c*) organ culture.

Growth in vivo

In considering the growth of treponemes within the vertebrate host, it should be borne in mind that this genus of micro-organism is ubiquitous in nature and can be found as part of the mixed microbial flora in various anatomical sites of birds and many mammals. The indigenous treponemes live primarily in body cavities in close proximity to tissues, but some at least are opportunist pathogens, and can invade tissues if they receive the proper stimulus. Is it not known what the necessary stimuli are, but they seem to be dependent on the presence of a mixture of other bacteria.

Spirochaetes resembling treponemes have been found in several genera of insects, as well as in vertebrate hosts. Those associated with several species of *Drosophila* are of particular interest because of their apparent sex-linked influence upon their host, i.e., infected female fruitflies fail to produce viable male offspring (Poulson & Sakaguchi, 1961).

The so-called pathogenic treponemes naturally inhabit primates only, with the exception of *T. cuniculi* which is a pathogen of low virulence for rabbits. Further information concerning the range of experimental hosts in which disease has been produced by these organisms is given in section 2.

Specific knowledge of the requirements for *in vivo* growth is quite limited. Studies in rabbits have revealed that *T. pallidum* prefers a temperature slightly below 37°C for optimum growth, and this may also be true for *T. pertenue*. Syphilitic lesions show a predilection for appearing at sites of injured tissue, but the reasons are unknown. The role of immune phenomena in inhibiting *in vivo* growth is obvious, but it should be emphasized that this effect is limited to immunity produced by identical or closely related pathogens. The fact that *T. pallidum* and similar organisms produce demonstrable lesions in only a limited number of tissues in the rabbit indicates that local environmental conditions play a major role in growth *in vivo*. Such conditions may be physical, nutritional, or immunological, and may be primarily responsible for " natural " host resistance to treponemal infection.

In judging claims of propagation of pathogenic treponemes, the most convincing criterion is the retention of pathogenicity as evidenced by production of a characteristic lesion and demonstration of the presence of treponemes. Another, but less convincing, criterion is a rising titre of some antitreponemal antibody.

1.4 Classification

A wide variety of non-pathogenic treponemes exist in nature as part of the mixed microbial flora indigenous to many vertebrates, including man. In addition, several insect species have been shown to harbour treponeme-like micro-organisms (Poulson & Sakaguchi, 1961) and several free-living forms have also been described. At present there is no adequate means of identifying and classifying these micro-organisms except by size, shape, and normal habitat.

Current knowledge of the biological and immunological properties of this group of micro-organisms is insufficient for a detailed taxonomic ordering of the genus *Treponema*. There is evidence suggesting that host range susceptibility may be of value in differentiating between species of pathogenic treponemes, but too few strains have been studied for this method to be recommended at present.

Until further information becomes available, it seems advisable to continue identifying species of treponemes pathogenic for vertebrates according to the disease condition with which they are normally associated. In two species, sub-species should probably be recognized as shown below :

Species	*Sub-species*
T. pallidum	*T. pallidum*, variant : endemic non-venereal
T. pertenue	*T. pertenue*, variant : cynocephalus
T. carateum	
T. cuniculi	