Pathogenicity and immunobiology of *Treponema pallidum*

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Introduction

*Treponema pallidum* occupies an important niche in the historical development of our knowledge of infectious disease, but, unlike most bacterial pathogens, it has defied most of our attempts to unravel its biochemistry and to learn about its structure and function at the molecular level. Much of the difficulty in studying this organism stems from our inability to culture it in vitro; despite some recent advances in growth of the organism in cell culture systems in vitro (Fieldsteel et al., 1981; Norris, 1982), serial subculture has still not been achieved and yields remain limited. It has not been possible to study the biochemistry and physiology of growth processes in any detail in vitro. Reliance on the relatively small numbers of organisms which can be obtained from infected animals has effectively precluded fractionation, purification and analysis of almost all the significant antigenic and structural components of the organism. An additional deficiency, and perhaps the most significant in view of current advances in the application of molecular genetics to manipulation of the genetic constitution of micro-organisms, is our inability to isolate clonal populations of treponemes (conventionally achieved with bacteria by colony selection from growth on solid media). This ability is a fundamental requirement for the assessment of genetic variability and its exploitation for the identification of markers of biological function.

Despite these technical problems, recent advances in methodology have made possible more meaningful investigation of molecular structure and function of *T. pallidum*. Knowledge of the pathogenicity (i.e., attributes that confer pathogenic properties) of many bacterial genera has advanced rapidly recently. In the context of such work, comparative studies of disease processes in syphilitic infection, and of biological properties of *T. pallidum*, have made possible some deductions about the nature of the treponemal surface and its function in interaction with the host. A very significant recent advance has been in the technology available for immunochemical analysis of infectious organisms, and the application of modern methods to *T. pallidum* has led to an explosion of knowledge of its antigenic constituent molecules in the past 5 years. Furthermore, the exploitation of monoclonal antibodies and recombinant DNA technology now enables us to identify individual antigens of the organism which are significant, and offers the potential for their manufacture and application in immunodiagnosis and, perhaps, eventually in vaccination.

Aspects of the disease process

Syphilis is not an acute disease and does not involve immediate, severe localised or systemic damage to the infected host. In primary lesions, significant numbers of treponemes accumulate in the tissues during the incubation period of days or weeks without inducing overt pathological changes. The eventual trigger of damage in primary lesions remains unclear, but several lines of evidence suggest that it is not simply excitation of an acute inflammatory response by toxic or inflammatory substances released from or displayed by treponemes in the tissues. First, polymorphonuclear leukocytes of the acute inflammatory response are not prominent in the cellular infiltrate that eventually accompanies formation of the primary lesion, and comprises predominantly mononuclear cells (Walter and Israel, 1979). Ultrastructural examination of infected tissues at the primary stage, both in human lesions (Drusin et al., 1969) and in infected rabbits (Penn, 1981) shows large numbers of treponemes to be present, in extracellular locations and often adjacent to blood vessels. Despite this accessibility to cells of the host defence system, the classical acute inflammatory processes of margination and extravasation of neutrophils have not been observed in infected animals (Penn, 1981; Penn and Clay, 1982) (fig. 1). A lack of
inflammatory potential of the organisms has been shown by their failure to stimulate recruitment of leukocytes into subcutaneously implanted, perforated plastic chambers in guinea pigs and rabbits. In contrast, gonococci (representative of pyogenic, gram-negative bacteria) stimulated a massive influx of polymorphonuclear leukocytes into these sites (Penn, 1981). Furthermore, direct inoculation of large numbers of viable treponemes (up to \(1 \times 10^8\) per site) intradermally in rabbits treated with penicillin stimulated little response—a transient (6–12 h) erythema, almost undetectable macroscopically. Similar numbers of gonococci inoculated similarly induced marked erythema persisting 24–48 h, and oedematous swelling of the skin (unpublished observations).

In contrast with the above observations, Mush et al. (1983) did report infiltration of leukocytes including polymorphs into dermal tissues inoculated with viable T. pallidum, but the numbers of infiltrating cells were small. I should emphasise that my interpretation of the observations of lack of acute inflammatory processes is that in some circumstances (the early stages of the disease, in “typical” primary lesions or the particular experimental conditions described), it is possible for large numbers of organisms to be present in the tissues without exciting acute host reactions. This does not preclude such responses occurring in other situations: the protean pathological manifestations of syphilis are well known, and in some circumstances acute inflammation may be prominent. In the resolution of primary lesions, cellular infiltration is also significant but is more of the character of a chronic inflammatory process.

The second aspect of the disease process which I wish to highlight is the absence of fever as a prominent feature of syphilis. In primary syphilis, fever is virtually absent, and in secondary syphilis it is mild. Only in the Jarisch-Herxheimer reaction has an appreciable element of fever been widely reported (reviewed by Young et al., 1982), and even then temperature rises are often not great. The occurrence of fever in syphilis has been investigated in the rabbit. Acutely infected rabbits showed a variety of rectal temperatures; although temperatures were somewhat raised in some cases, others were normal or even depressed, and the mean temperature of a group of infected rabbits was not significantly higher than that of normal rabbits (Penn et al., 1985b). Further investigation of the pyrogenicity of T. pallidum showed that intravenous inoculation into rabbits of ultrasonically disintegrated organisms stimulated a negligible febrile response compared with similar numbers of the classically inflammatory organism Neisseria gonorrhoeae (Penn et al., 1985b). Rabbits inoculated by this route are extremely sensitive to bacterial pyrogens, and this lack of response is strongly indicative of the absence of such materials (see below). It is worth noting that T. pallidum is extremely sensitive to temperatures slightly higher than the normal body temperature of man—a property of the organism which was exploited in the development of fever therapy for syphilis early this century. It is thus perhaps necessary for the survival of the organism that it should not induce strong fever responses.

In addition to the aspects of the disease process in vivo outlined above, I would like to emphasise an unusual property of T. pallidum in vitro which also bears on the ability of the organism to exist within the host without stimulating defensive responses. Although T. pallidum cells contain potent antigens, shown by the strong reactivity of treponemes subjected to disruptive fixation procedures with syphilitic sera in fluorescent antibody tests, such sera do not react with intact, living treponemes in their native state (Penn, 1981). Again, this is a significant difference from many other bacterial pathogens which are known to have potent antigens exposed on their surfaces in infection, free to combine with specific antibody. Examples are the fimbrae and outer-membrane proteins of gonococci (Heckels, 1984) and many other gram-negative bacteria. We are now learning rapidly the detailed molecular characteristics of potent treponemal antigens (see below), and the surface architecture of the organism which makes possible the concealment of such antigenic species is an enigma which I would like to examine in more detail.

**Properties of the surface of T. pallidum**

Ultrastructural examination is the obvious starting point in an investigation of the nature of the cell surface. Treponemes of several species, including T. pallidum, have been examined ultrastructurally in some detail (reviewed by Hovind-Hougen, 1983), and a consensus view of the general structure of T. pallidum has emerged (Sell and Norris, 1983). The most important implication from a number of past studies in relation to the nature of the exposed treponemal surface is that there is a distinct outer membrane of typical lipid bilayer structure. Although such a structure has been demonstrated unequivocally by some workers (e.g., Johnson et al., 1973), it has not always been observed (Penn and Lichfield, 1982; Sell and Norris, 1983). In our hands, postfixation in uranyl acetate after conven-
Fig. 1. Electronmicrograph of rabbit testis infected 14 days earlier with *T. pallidum* (Birmingham strain). Note the absence of margination or extravasation of leukocytes, despite the presence of numerous treponemes in the perivascular tissues. Ultrathin section, × 10 000. (Penn and Clay, 1982).
tional fixation procedures with glutaraldehyde and osmium tetroxide was necessary to ensure the preservation of the outer membrane during dehydration of fixed specimens before embedding. This may give some clues to the nature of the outer membrane of *T. pallidum*. A high protein content in the membrane would be expected to result in efficient fixation by osmium tetroxide, but this was not achieved. Uranyl acetate is reputed to combine effectively with phospholipids (Glauert, 1975). Thus, this outer membrane may have a relatively high content of phospholipid and a low protein content, compared with those of other gram-negative bacteria which are effectively fixed by glutaraldehyde and osmium tetroxide alone (Penn and Lichfield, 1982).

Meaningful investigations of the surface structures of bacteria have, in the past, relied heavily on the preparation of subcellular fractions, particularly of cell envelope and membrane components. In this as in the investigation of many aspects of the biology of *T. pallidum*, inability to culture the organisms to obtain sufficient material for fractionation procedures has proved a severe handicap. Nevertheless, modern techniques can provide useful information on the composition of the small amounts of material available from organisms obtained from infected tissues, and for this purpose, as well as to obtain information on the solubility of the treponemal surface several simple extraction procedures applicable to small volumes of treponemal suspensions were attempted. Immunoelectrophoretic analysis in agarose gels with polyvalent rabbit sera from convalescent and subsequently hyperimmunised rabbits to detect extracted antigenic material did not demonstrate significant breakdown of the treponemal surface after vigorous agitation in chaotropic salt solutions or chelating agents. The treponemal surface was, however, very susceptible to attack by various detergents (Penn and Rhodes, 1982).

The non-ionic detergent Triton X-100 was highly effective in dilute solution (0.1–0.2% v/v in 0.05 M Tris-HCl buffer, pH 8.0–8.5) when treponemes were suspended at concentrations in the range (1 x 10⁹)–(1 x 10¹⁰)/ml and incubated at 37°C for 30 min. This treatment resulted in extraction of a major antigenic species detectable by immunoelectrophoresis (Penn and Rhodes, 1982), and in significant ultrastructural changes in the treated treponemes. In thin section, after post-fixation with uranyl acetate to preserve any outer-membrane structure present, treponemes were shown to have lost their outer membranes, resulting in detachment of endoflagella (axial filaments) from their normal location adjacent to the cell wall. By negative staining, the distinct sharp outline of the treponemal surface was lost, the release of endoflagella from their axial location along the cell body was clear, and intracytoplasmic detail, particularly of the cytoplasmic filaments, became visible. The latter indicates that stain had penetrated the cell wall after detergent treatment, which does not occur with native treponemes. However, this penetration of stain should be interpreted cautiously in relation to outer-membrane integrity and the effects of detergent treatment. The very clear definition of the endoflagella by negative staining even in the most gently handled intact treponemes suggests that phosphotungstate ions can penetrate the intact outer membrane and contrast with the endoflagella. The critical effect of detergent in allowing penetration of stain to the cytoplasmic filaments may, therefore, be to render the cytoplasmic membrane permeable, and extraction of proteins in solution by detergents (see below) should be interpreted in this light.

The susceptibility of the treponemal outer membrane to non-ionic detergents such as Triton X-100 has implications for our understanding of the structure of the membrane. The outer membranes of several gram-negative bacteria are quite resistant to such treatment, unless their structural integrity is weakened, e.g., by removal of divalent cations, which are believed to cross-link lipopolysaccharide (LPS) residues by ionic interactions, with chelating agents (Schnaitman, 1971). The detergent susceptibility of the treponemal outer membrane, even in the presence of excess divalent Mg²⁺ (Penn et al., 1985b) suggests, therefore, that structural elements equivalent to the LPS of many other gram-negative bacteria are absent.

An alternative approach to the study of membrane susceptibility to solvents or disruptive agents is to monitor the biological effects of exposure of membranes to such agents. With most pathogenic bacteria this can be done easily, e.g., by monitoring lethality in terms of viable counts. *T. pallidum* cannot be studied in this way because viability can be assessed only by infectivity tests that are laborious, expensive and poorly quantitative. It is, however, possible to monitor motility of fresh treponemal suspensions relatively simply, and although not all motile treponemes are fully viable in terms of infectivity, viable treponemes are undoubtedly motile. Using motility as an indicator of treponemal survival, it was found that *T. pallidum* was extremely susceptible to the anionic detergent sodium dodecyl sulphate; concentrations as low as 0.01% caused immediate loss of motility (Penn et
This contrasts with the relative resistance of many intestinal gram-negative bacteria, which may withstand concentrations at least 100-fold higher. Such resistance is believed to be due to the protective effect of LPS in the enterobacterial outer membrane (Wright and Tipper, 1979). The same technique was used to assess the effect on treponemal viability of the antibiotic polymyxin B, which is known to have an affinity for LPS molecules in the outer membrane of gram-negative bacteria (Perkins, 1983). The motility of *T. pallidum* was unaffected by concentrations of polymyxin B more than ten times higher than are lethal for "smooth" strains of *Salmonella typhimurium* (Teuber, 1974). Moreover, the effects of polymyxin B include induction of morphological changes in the outer membrane (Wiegand and Quandt, 1982). Comparison of the ultrastructure of polymyxin-treated *T. pallidum* and *S. typhimurium* showed that while the latter was severely affected, the outer membrane of the former remained unchanged. Taken together, these observations suggest the absence of molecules with structural attributes of LPS from the treponemal outer membrane.

LPS, particularly the "O-antigen" polysaccharide side chain, are also important antigens of gram-negative pathogens. Conventionally, LPS can be extracted from bacterial cultures by methods such as phenol-water or petroleum-ether extraction. Again, this is not possible with *T. pallidum* because of the lack of large quantities of cultured bacteria. Tests for chemical markers of the presence of LPS such as keto-deoxy-octulosonate proved impracticable for the same reason. Recent developments in separation methods for complex antigenic mixtures have, however, included the application of immunoblotting for the detection of LPS species by their antigenic activity, and often very characteristic immunoblot patterns of protease-stable antigens are obtained. Application of this technique for the detection of LPS-like antigenic molecular species in *T. pallidum* failed to detect any such molecules; indeed, no protease-stable antigens were observed other than some activity at the leading front of the polycrystalside electrophoresis gel which may have been attributable to low-mol.-wt lipids. In contrast the cultivable, non-pathogenic and antigenically related Reiter strain of *T. phagedenis* (the Reiter treponeme) contained a characteristic series of protease-stable antigens of discretely stepped mol. wts, typical of the ladder pattern seen with "smooth" LPS (Bailey et al., 1985).

We have thus derived several lines of evidence to suggest that *T. pallidum* does not elaborate an LPS analogous to those of many other gram-negative bacterial pathogens. Furthermore, Hardy and Levin (1983) found that the *Limulus* amoebocyte lysate test for LPS gave negative results with this organism. I believe this is likely to be highly significant in the pathogenesis of syphilis, in which, as we have seen, there appears to be very limited host responses to the organism at critical stages in the infectious process. It remains possible, and perhaps probable, that the outer membrane of *T. pallidum* may contain some other glycolipid that might fulfil some of the structural roles of LPS without conferring the biological effects of the latter upon the organism.

### Antigenic structure of *T. pallidum*

Knowledge of the constituent antigens of *T. pallidum*, their disposition in or on the surface of the organism, and their biological functions and biochemical properties, is important for several reasons. First, a full understanding of the host-parasite relationship and the mechanisms of induction of disease cannot be gained without defining the individual antigens that interact with the immune system. Second, any attempt to induce artificial immunity with the newer approaches to vaccination which are currently being developed depends upon the correct choice of antigen for biotechnological manipulation. Third, the immunodiagnosis of syphilis, which relies heavily on detection of specific antibody in the serum, can be performed most effectively if the important antigens are defined, and if the individual antibody responses to such antigens in different clinical circumstances are understood.

We have already seen that treatment of *T. pallidum* cells with Triton X-100 or other detergents effectively extracts outer-membrane components. Examination by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of soluble fractions separated by centrifugation from the residual cells after detergent treatment showed that only a very small subset of the treponemal cell polypeptide complement was dissolved by this procedure (Penn et al., 1985b). Notably, an abundant protein of apparent mol. wt 47 000 was almost quantitatively removed from the treponemes, suggesting that this may be an outer-membrane component, although a periplasmic, cell wall or even cytoplasmic membrane location for this antigen cannot be ruled out (see above). Monoclonal antibodies directed against this protein do not label the surface of intact, viable treponemes as shown by immunogold electronmicroscopy (A. Cockayne and M. J. Bailey, unpublished observation). It
should be acknowledged, however, that there have been contradictory reports about ability to surface-label treponemes with either monoclonal or polyclonal antibodies (e.g., Jones et al., 1984). In view of the difficulty of establishing the integrity of treponemes subjected to such procedures, and the rather sparse nature of antibody coating that has been reported, such reports must be interpreted with caution. Thus, we have no firm evidence for significant surface exposure of this antigen. It is, however, very clear that the 47 000-mol. wt Triton-soluble antigen is dominant in inducing the antibody response in early infection in rabbits, and antibody to it remains prominent in convalescent sera, as shown by immunoblot analysis (M. J. Bailey, unpublished observation; reviewed by Penn et al., 1986). The prominence of such antibodies in the early immune response is also seen in human infection (Penn et al., 1986), suggesting that this may be a useful antigen for immunodiagnostic use.

Another prominent antigenic constituent of T. pallidum is the axial filament or endoflagellum. Although located within or beneath the outer membrane (hence endo-, a nomenclature proposed at the WHO-UCLA sponsored discussion meeting on pathogenic treponemes, Los Angeles, December 1985), these appear to be structurally and functionally analogous to other bacterial flagella. Simple immuno-electronmicroscopy of treponemes treated with detergent to remove the overlying outer membrane and exposed to polyclonal convalescent or hyperimmune rabbit antisera, revealed prominent coating of endoflagella by antibody molecules (fig. 2; Penn and Rhodes, 1982). Identification of major constituent polypeptides of this antigen was achieved by partial purification of endoflagella by modifications of the methods developed by Bharier and Allis (1974) and Hardy et al. (1975). This entailed detergent extraction of the outer membrane, detachment of endoflagella by shearing by high speed homogenisation, and partial purification of detached fragments by differential centrifugation (Penn et al., 1985a). SDS-PAGE analysis of preparations that appeared to be free of other particulate matter by electronmicroscopy revealed a distinctive pattern of one prominent band and a doublet of less prominent bands of lower mol. wt. A similar pattern was obtained with identical preparations from the cultivable, non-pathogenic and antigenically-related Reiter treponeme; mol. wts of the most prominent bands were 37 000 for T. pallidum and 38 500 for the Reiter treponeme (Penn et al., 1985a).

Immunoblot analysis of antibody responses to T. pallidum polypeptides in sera from infected rabbits, obtained at frequent intervals after immunisation, showed that antibody to the major endoflagellar polypeptide (mol. wt 37 000) was prominent in the earliest antibody detectable, 7–10 days after intra-testicular infection (M. J. Bailey, unpublished observation; Hanff et al., 1983; Lukehart et al., 1986; Penn et al., 1986). Antibody against this polypeptide was also prominent in sera from patients with primary and secondary syphilis (M. J. Bailey, unpublished observation). These observations suggest that detection of antibodies to the 37 000-mol. wt polypeptide may be a useful diagnostic test. Several monoclonal antibodies reactive with this polypeptide by immunoblot have been produced (M. J. Bailey, unpublished observations), and immunogold electronmicroscopy has confirmed that some of these bind to native endoflagella. Comparison of reactivities of monoclonal antibodies by immunoblot with the major endoflagellar polypeptides of T. pallidum and of the Reiter treponeme showed that, while some antibodies reacted with antigens of both species, others were specific for T. pallidum. It appears, therefore, that there may be endoflagellar epitopes specific for T. pallidum which could be exploited most usefully for diagnostic purposes.

Monoclonal antibodies have proved to be useful reagents in defining the core and sheath structure of T. pallidum endoflagella (A. Cockayne, unpublished observations). Ultrastructural observations have indicated for some time that endoflagellar structure is heterogeneous (Hovind-Hougen, 1983), but no analytical evidence has been available to support the idea that endoflagella may comprise a core structure with a sheath which, under some circumstances, may be removed leading to a change in ultrastructural appearance. Recently, a monoclonal antibody reactive with intact endoflagella, as revealed by immuno-electronmicroscopy, was shown not to react with thinner, core-like structures which were sometimes seen in treponemal suspensions. Furthermore, treatment with 6M urea produced core-like structures unreactive with the monoclonal antibody, and dissolved the 37 000-mol. wt polypeptide permitting its separation from treponemal suspensions (with attached endoflagellar sheaths) when the latter were collected by centrifugation. Thus the 37 000-mol. wt polypeptide appeared to represent an endoflagellar sheath structure.

Identification and characterisation of prominent polypeptide antigens of T. pallidum is a necessary step towards understanding and exploiting for practical purposes individual antigens involved in the immune response in infection. Unusually
among prokaryotic cells, *T. pallidum* and certain other treponemes including the Reiter treponeme contain prominent filamentous structures within the cytoplasm (Hovind-Hougen, 1983). These filaments are apparently connected with the endoflagellar basal bodies and probably take part in the generation of motility. Because of their prominence as structural components of the organism, and their presumed functional analogy between species, these filaments may comprise major polypeptides of similar mol. wts present in both species. Preparations enriched for filamentous structures—endoflagella and cytoplasmic filaments—contain one of the prominent treponemal polypeptides with a mol. wt c. 80 000 in *T. pallidum*. A monoclonal antibody has again proved useful in indicating analogy between this polypeptide and a similar component of the Reiter treponeme which share antigenicity in immunoblot tests (A. Cockayne, unpublished observations). This evidence strongly points to the 80 000-mol. wt polypeptide as a major component of the cytoplasmic filaments. Disappointingly, the monoclonal antibody failed to label cytoplasmic filaments in a way that could be detected by immuno-electron-microscopy.

**Future approaches**

Despite the recent progress I have charted in our knowledge of aspects of the pathogenicity and
immunogenicity of *T. pallidum*, our understanding remains rudimentary. Technical problems arising from the lack of cultured organisms for observation and analysis still render impossible many potentially valuable experimental approaches. Furthermore, the whole area of biological manipulation which would follow from the availability of clonal mutants with specific alterations in properties for more, the whole area of biological manipulation and analysis still render impossible many potentially valuable experimental approaches. Further-
comparative studies of their biological behaviour, detailed characterisation of the organism than has hitherto been possible. Pioneering work by Walfield et al. (1982), Stamm et al. (1982) and Norgard and Miller (1983) established the feasibility of cloning and expression in *Escherichia coli* of *T. pallidum* genes encoding antigenic polypeptides recognisable by antibodies in syphilitic sera. We have adopted this approach and identified six different phenotypes of recombinant *E. coli* expressing *T. pallidum* antigens encoded by plasmids derived from pAT153 (M. J. Bailey, unpublished observations). Some of these antigens are associated with anti-treponemal (immobilising) activity of corresponding polyclonal or monoclonal antisera, and their study may well illuminate aspects of the biological interaction between bacterium and host. This technology also paves the way for exploitation of recombinant treponemal antigens as immunodiagnostic or even immunoprophylactic agents.

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