RECENT ADVANCES IN RHINOSPORIDIOSIS AND RHINOSPORIDIUM SEEBERI

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Abstract

Rhinosporidiosis and its causative pathogen Rhinosporidium seeberi have been known for over a hundred years. Yet unresolved enigmas in rhinosporidiosis include the mode of infection, mechanisms of spread, mechanisms of immunity, some aspects of histopathology e.g. the significance of transepidermal elimination of sporangia, the cause of the variation in cell infiltration patterns in rhinosporidial tissues and their correlations with immune status, and the absence of the Splendore-Hoeppli reaction which is well-marked in invasive, classical mycoses.

This review describes the main features of rhinosporidiosis and discusses recent work which clarifies some of these enigmas. Recent work included in this review are molecular biological classification of R.seeberi among the hydrophilic organisms of the former DRIP clade, establishment of a method for the purification of the developmental stages, and some aspects of the immunology of R.seeberi with reference to mechanisms of immune evasion - antigenic variation, host immunoglobulin binding, immune deviation in relation to the chronicity, recurrence and dissemination seen in rhinosporidiosis. The mechanism of endospore release from the sporangium has been described. Some problems involved in the resolution of enigmas that persist are briefly discussed.

Key words: Rhinosporidiosis, R.seeberi, immune mechanisms

Rhinosporidiosis, reviewed in 1998, has been known for over a hundred years since its first description in Argentina. It is a chronic disease, with frequent recurrence after surgery, and occasional dissemination from the initial focus which is most commonly seen in upper respiratory sites. It occurs universally, although it is endemic in south Asia, notably southern India and in Sri Lanka. Increased migration to the west of persons who acquired rhinosporidiosis in their native Asian countries has resulted in the increasing occurrence of this disease in the West. Contrasting with other infective diseases which are of more recent identification and characterisation, there are still many basic issues, concerning the pathogen Rhinosporidium seeberi and the disease, which have not been resolved. Indeed it has proved, in our experience, to be a difficult experimental problem both methodologically as well as theoretically.

The literature on rhinosporidiosis and on the different morphological stages of R.seeberi contains many synonymous, overlapping terms; for example, the morphological element for which the proposed term is “Electron Dense Body”, was earlier termed spherule, electron-dense circular structure, protrusion of cell wall, electron-dense inclusion, germinative body, sporozoite, spore, sporule and spherical body. This review uses the terminology proposed by Kennedy et al.

Rhinosporidiosis

Epidemiology

Rhinosporidiosis has been reported from about 70 countries with diverse geographical features although the highest incidence has been from India and Sri Lanka. Though most cases of human rhinosporidiosis in western temperate and middle eastern countries occurred in expatriate Indians who probably acquired the disease in their native lands, a few cases have been reported in persons, living in the west, who have never travelled to endemic areas.

Rhinosporidiosis is an infective disease in the sense that the tissue lesions are always associated with the presence of the pathogen. No evidence has been adduced that it is also an infectious disease, as no transmission has ever been documented of cross-infection between members of the same family or between animals and humans. Incidentally, it is pertinent to point out the differentiation between these two terms - infective and infectious - as they appear to be often misused in articles as well as in texts. A clear illustration of this difference.
is in respect of tuberculosis and disease caused by mycobacteria other than *M. tuberculosis* (MOTT). The lesions in tuberculosis are associated with the presence of *M. tuberculosis* at the diseased sites and tuberculosis is hence an infective disease. It can be spread from patient to susceptible persons and is therefore, also infectious. On the other hand disease caused by opportunistic MOTT, while being infective in the sense defined above, is not infectious in not being spread from the patient to other persons.

In addition to the more numerous cases in humans, rhinosporidiosis has also been documented as having occurred in several species of farm, domestic and wild animals - cattle, buffaloes, dogs, cats, goats, horses, mules, several species of ducks, swans, geese and water fowl.

The great majority of cases are sporadic. In the 1990s, outbreaks in humans and swans were reported; the single outbreak of ocular and nasal rhinosporidiosis in humans was in Serbia,1 and a lake in which all the patients had bathed was incriminated as the source of the pathogen. The outbreak of ocular and cutaneous rhinosporidiosis in swans was reported from Florida, USA.2

A curious feature in the incidence of the disease is that while several hundreds of persons bathe in the stagnant waters, as in Sri Lanka, only a few develop progressive disease; this might indicate the existence of predisposing, though obscure, factors in the host. The only patient factor on which there is some data is blood groups. In India the highest incidence of rhinosporidiosis was in Group O (70%) though in the population Groups A, B, and O are distributed “fairly equally”; the next highest incidence was in Group AB though in the general population Group AB is rare.4 Jain5 in India reported that the blood group distribution was too variable to draw any conclusion. In a small series of 18 Sri Lankan patients, the distribution was different; Groups A Rh+ and O Rh+ having 33% each, Group B Rh+ with 22%, and Group AB Rh+ with the lowest of 11%, with a population distribution of 21, 43, 26 and 5% respectively (Arseculeratne et al, 2001 unpublished data). Larger series are necessary for meaningful analyses. Other genotypic features such as HLA types also need to be investigated. The possibility that non-specific immune reactivity in the host, especially related to macrophages, might be important in the failure of *R. seeberi* to establish an initial focus of infection, is suggested by investigations discussed later in this review.

Reasons for endemicity

It has also to be explained why the disease is of high endemicity in certain regions of southern India and in the dry zone of Sri Lanka. If indeed stagnant ground waters are the natural habitat, then the chemical and physical characteristics of these waters need definition. In addition, other aquatic micro-organisms might also be relevant to a possible synergistic action in the establishment of natural rhinosporidiosis. There are examples of such synergism of bacteria, with parasites - lactobacilli with *Trichomonas*, and *Wolbachia* with filarial nematodes.

Mode of infection

The presumed mode of infection from the natural aquatic habitat of *R. seeberi*, is through the traumatised epithelium (“transepithelial infection”) most commonly in nasal sites. The occurrence of rhinosporidiosis in river-sand workers, in India and in Sri Lanka, is particularly relevant to such a mode of infection, through abrasions caused by sand particles with the pathogen in the putative habitat - ground water. Trauma from *R. seeberi* contaminated stones used for mopping-up residual drops of urine is claimed to be responsible for anterior urethral rhinosporidiosis in the male.

Mode of spread

‘Auto-inoculation’

‘Auto-inoculation’ was considered by Karunaratne7 in his classical monograph on rhinosporidiosis, to be the explanation for the occurrence of satellite lesions adjacent to granulomas especially in the upper respiratory sites and for local spread. Spillage of endospores from polyps after trauma or surgery is thought to be followed by ‘auto-inoculation’ through the adjacent epithelium.

Haematogenous spread

There is evidence for haematogenous spread of rhinosporidiosis to anatomically distant sites.8 The development of subcutaneous granulomata in the limbs, without breach of the overlying skin, could be attributed to such haematogenous dissemination, from a subclinical, upper respiratory focus of infection.

Lymphatic spread

The mode of regional spread, however, is controversial. Ashworth9 suggested the possibility of lymphatic spread but none of the numerous reports on the histopathology of localised or disseminated rhinosporidiosis has described the occurrence of
lymphadenitis. One reason could be that histological examination of the lymph nodes, especially in disseminated cases, has apparently not been done. The first report on the occurrence of inguinal lymphadenitis in a case of disseminated rhinosporidiosis which involved the lower limbs was made in 2002, describing the criteria on which the identification of the lymph node tissue was made; the site of the biopsied lump, the presence of a fibrous capsule, subcapsular sinus, fibrous trabeculae from the capsule (Fig. 1), and residual collections of lymphocytes probably representing the follicular B-cell zones despite the extensive presence of sporangia in this region of the node. The apparent rarity of lymphadenitis in rhinosporidiosis, contrasting with its frequency in systemic mycotic disease, is remarkable; this rarity might be related to the diverse mechanisms of immune evasion by R.seeberi discussed later in this review.

Figure 1: Inguinal lymphadenitis in rhinosporidiosis involving the lower limbs; showing capsule and trabeculae from capsule into parenchyma of lymph node. (H&E. Original magnification x 400).

**Clinical features**

The great majority of cases occur in upper respiratory sites, notably the anterior nares, the nasal cavity - the inferior turbinates, septum and floor. Posteriorly, rhinosporidial polyps occur in the nasopharynx, larynx, and soft palate; the buccal cavity is only rarely affected. The disease, while being of special interest to oto-rhinolaryngologists, is of interest to dermatologists and ophthalmologists as well, through the occurrence of granulomas in the skin, subcutaneous tissues and eye. About 15% of cases of rhinosporidiosis are ocular in location, in the bulbar and palpebral conjunctiva. Rhinosporidiosis of the lacrimal sac and naso-lacrimal duct has been documented and it has been suggested that the primary site of rhinosporidiosis is the lacrimal sac with downward spread to the nasal passages through the naso-lacrimal duct; this view is untenable since nasal rhinosporidiosis has occurred as the primary lesion while in other cases a concomitant infection of the lacrimal apparatus has been on the contralateral side.

Other sites of solitary polyps include the external urethral meatus especially in males. The absence of rhinosporidiosis in the sexual partners of these patients is cogent evidence that the disease is neither infectious nor contagious. Dissemination to the limbs, trunk and viscera has been described in a few cases, with a rare fatality especially when the brain was involved. In rhinosporidial lesions in the limbs, a notable feature has been the destruction of underlying bone. Rare cases of spontaneous regression of nasal polyps have been recorded.

**Pathology**

Characteristically, rhinosporidial lesions in the nasal passages are polypoidal, granular, red in colour due to pronounced vascularity, with a surface containing yellowish pin head-sized spots which represent underlying mature sporangia. A covering of mucoid secretions is not uncommon. Naso-pharyngeal polyps are often multi-lobed with a variegated appearance, with typical strawberry like regions and other areas which have relatively less vascular lobes with smooth surfaces. Polyps on the face and trunk could simulate verrucous warts, and are either pedunculated, or sessile on broad bases.

Rhinosporidial granulomas in disseminated cases occur as subcutaneous lumps with unbroken skin. These sometimes present clinically as ulcerated growths which could mimic malignant lesions such as sarcomas and carcinomas.

Rhinosporidial histopathology was described in detail by Beattie,\textsuperscript{11} Tirumurti,\textsuperscript{12} Ashworth,\textsuperscript{9} and by Karunaratne.\textsuperscript{6, 7} These classical descriptions have not been advanced upon in the prolific literature since these descriptions were written. In most histological sections, the organism is present in all stages of its development and these appearances have led to the tentative identification of its 'life cycle' described below.

The stroma which is either fibro-myxomatous or fibrous contains chronic inflammatory cells which include macrophages and lymphocytes, while neutrophils are numerous around free endospores. In granulomatous tissue, giant cells occur often within sporangia and in the stroma. Fibrosis is prominent, notably, in non-respiratory sites. A noteworthy feature is the variability of stromal and cellular reactions even within tissues from a single patient. These variations are further discussed under immunity.
Diagnosis

Histopathology

The definitive diagnosis of rhinosporidiosis is by histopathology on biopsied or resected tissues, with the identification of the pathogen in its diverse stages, rather than the stromal and cellular responses of the host.

Although the developmental stages of the pathogen have definitive characteristics which allow of a histopathological diagnosis, some notable features include the wide variation within a single specimen, between lesions in different sites of the same and in different hosts, in respect of both numbers and identity of developmental stages of the endospores and sporangia, as well as in the stromal and cellular response of the host. These variations are further considered below under immunity.

The histopathology of animal rhinosporidiosis is essentially similar to that in human disease. The variations found in the stromal and cell infiltration patterns in human rhinosporidiosis were also described in rhinosporidial tissue in swans.

Pitfalls in the histopathological diagnosis of rhinosporidiosis.

There are, however, instances when confusion, misdiagnosis or a false negative diagnosis can be made on histopathology. Such instances were reviewed elsewhere and included: (a) rhinosporidial tissue, especially in naso-pharyngeal rhinosporidiosis, in which rhinosporidial bodies were absent despite the presence of marked cell infiltrates; other portions of the polyp however had rhinosporidial bodies. Inappropriate selection of portions of such polyps for histology might result in a false-negative diagnosis. (b) sporangia with atypical walls in which the well-marked, bilamellar thick wall was absent. (c) only fragments of the sporangial wall, with or without endospores, were occasionally visible. (d) in some sections no typical rhinosporidial bodies were visible although a well-marked cell infiltrate was present, with the presence of occasional PAS positive bodies which were similar to the rhinosporidial endospores; these might indicate incipient or slow-growing rhinosporidiosis or conceivably rhinosporidial bodies altered by immune reactions. These instances were discussed with reference to anti-rhinosporidial immunological responses in the host, especially the possibility of free rhinosporidial antigen in the polyps causing immune distraction and consequent immune escape, the occurrence of suppressor reactions of T-lymphocytes, and the role of anti-rhinosporidial antibody in cell-mediated cytotoxicity.

As noteworthy in rhinosporidiosis, is the absence of the Splendore-Hoeppli (antibody-mediated) eosinophilic deposit around rhinosporidial bodies, though this reaction is well described as occurring in (mycelial) mycotic infections as well as with a wide range of infecting bacteria. This absence is all the more surprising because rhinosporidial patients show high titres of anti-rhinosporidial antibody.

A problem, though uncommon, in differential histopathological diagnosis is that of subcutaneous Spherulo-cystic disease (Myospherulosis) which has been described as “reminiscent of an endosporulating fungus such as rhinosporidiosis” and as a “distant cousin of rhinosporidiosis”. This entity was recently ascribed to erythrocytes, altered by lipids; the altered erythrocytes do not stain with Gomori methamine-silver which readily stains R. seeberi.

In adiaspiromycosis the sporangial walls of the causative Chrysosporium parvum var. crescens are much thicker with large adiaconidia (200-400 mm). Differentiation from Coccidioides immitis could be done on morphological grounds; there are no EDBs in C. immitis, the intra-sporangial endospores of R. seeberi are larger and more numerous, the endospores of C. immitis are much smaller (2-5m) while arthroconidia and hyphae may be found in the coccidiomycotic lesions. Only the empty sporangia of R. seeberi might be confused for those of C. immitis.

Cytodiagnosis

Cytodiagnosis on aspirates from rhinosporidial lumps or on smears of secretions from the surfaces of accessible polyps and fine-needle aspirates from lumps provide, with suitable stains, distinctive diagnostic features; caution was however recommended with smears of material from respiratory sites, in the identification of endospores; which could be confused with epithelial cells, especially from the naso-pharynx, in which the residual cytoplasm and large nuclei might simulate the residual mucoid sporangial material around the endospores (referred to as ‘comet’ forms by Beattie), and the endospores themselves, respectively. The Periodic acid-Schiff stain will discriminate between these, as the endospores stain markedly magenta while the epithelial cells are PAS-negative. The presence of Electron Dense Bodies in the endospores is useful in confirmation of rhinosporidial identity.

Transepidermal elimination of rhinosporidial sporangia

Reference to this phenomenon (Fig. 2) in rhinosporidiosis has been made in only two reports. It was assumed that this phenomenon represented a non-
specific defence mechanism of the host for the expulsion of rhinosporidial sporangia. Deducing from some histopathological features, especially the orientation of the pore of the mature sporangium towards the epithelium, and on a parallel with similar histological appearances of the amphibian skin pathogen Batrachochytrium, it was postulated that this phenomenon could, on the other hand, be the pathogen’s dispersal mechanism for rhinosporidial endospores.

Figure 2: Trans-epidermal elimination of the sporangium at the surface of the epithelium with extrusion of the rhinosporidial endospores; bulbar conjunctival rhinosporidiosis. (H&E. Original magnification x 400).

Mechanisms of immunity

In the absence of pure preparations of the developmental stages of R.seeberi, uncontaminated by host tissue and other micro-organisms, and consequently the absence of specific tests for immune responses in diseased hosts, it is not surprising that there was very little data, till recently, on the immune responses in rhinosporidiosis. Crude preparations of endospores and sporangia from homogenised and filtered human rhinosporidial tissue, which have hitherto been used, would have contained human material and even contaminating micro-organisms. It is possible that the lines of precipitation seen in double diffusion tests with crude extracts of R.seeberi might have been due to human proteins, which were either contaminants in these crude preparations, or bound to rhinosporidial antigens, and which would have induced anti-human antibodies in the experimental sera used by Chitravel et al. Suspensions of endospores only used in tests for immune responses, might also have had the disadvantage of excluding the sporangial stages and hence antigens which are not present in endospores.

Cell-mediated immune responses (CMIR)

The only definitive report on Cell-Mediated Immune (CMI) responses in human patients was that of Chitravel et al which claimed that Leucocyte Migration Inhibition (LMI) was detected in patients with disease of less than 9 years duration, but that the LMI decreased when the disease was of longer duration. No explanation was given for that observation but these authors suggested the possibility that immune suppression might occur.

CMI responses (CMIR) in human rhinosporidiosis were re-examined recently by (a) immunohistology with monoclonal antibodies to specific cell markers on cells in rhinosporidial tissues, (b) in vitro lymphoproliferative responses (LPR) of peripheral blood lymphocytes from rhinosporidial patients to Concanavalin A (Con A) and to extracts from purified rhinosporidial endospores and sporangia.

Immunohistology showed that the cell infiltrate in human rhinosporidial polyps was mixed, having consisted of many plasma cells, fewer CD 68+ macrophages, CD 3+ and CD 56/57+ NK lymphocytes which were positive for CD 3 as well; CD 4+ lymphocytes were present though scarce. CD 8+ suppressor/cytotoxic lymphocytes were numerous and most of the CD 8+ cells were TIA-1+ and therefore of the cytotoxic sub-type.

In LPR assays in vitro, rhinosporidial lymphocytes showed stimulatory responses to Con A, but some of these samples showed significantly diminished responses to rhinosporidial antigens, indicating suppressor responses in human rhinosporidiosis. The intensity of the depression however bore no relation to the site, duration, the number of lesions, or to the occurrence of dissemination. These results demonstrate that while a CMIR does develop in human rhinosporidiosis, suppressor responses also occur.

There are, hitherto, no reports of studies on anti-rhinosporidial CMI and humoral responses in experimental animals. CMIR to R.seeberi in experimental mice were recently examined by the quantitative foot-pad response of the Delayed Type Hypersensitivity (DTH), with histopathology of the treated foot-pads for confirmation of the DTH response. It was demonstrated that sonicated suspensions of rhinosporidial endospores and sporangia, used for sensitisation and challenge, evoked well-marked DTH foot-pad responses of a magnitude similar to that evoked by T-dependent antigens such as sheep red blood cells. The foot-pads challenged with rhinosporidial extracts showed histopathology which was typical of DTH reactions in the mouse.

A noteworthy finding in these mouse experiments was that with repeated sensitisation with rhinosporidial
extracts, the DTH foot-pad response decreased significantly in intensity while the humoral immune response (HIR) was significantly elevated, in comparison with the CMIR and HIR to a single sensitising dose of rhinosporidial antigen. This phenomenon is thus one of Immune Deviation which has been ascribed to the switch, on repeated administration of antigens, from activation of CD4+ Th-0 cells to the production of CD4+ Th-2 cells, probably mediated by cytokines, after an initial production of CD4+ Th-1 cells. Th-1 cells induce delayed-type hypersensitivity while Th-2 cells encourage antibody production. The ensuing decrease of DTH reactivity with the switch from Th-1 to Th-2 might contribute to decreased anti-rhinosporidial cell-mediated immunity and might be an explanation for the chronicity, recurrence and dissemination in clinical rhinosporidiosis.

Humoral immune responses (HIR)

The only report on tests for anti-rhinosporidial antibody in human patients was by Chitravel et al where no antibody was detected in double diffusion or CIE tests. Anti-rhinosporidial HIR in human patients as well as in experimental mice and rabbits after immunisation with rhinosporidial extracts, were demonstrated by indirect immunofluorescence tests which used Percoll-purified, sonically disrupted endospores and sporangia as antigen. Rhinosporidial patients had appreciable titres (over 1/240) while only 2/30 of clinically non-rhinosporidial persons showed low (1/30) titres; these two persons were from an area, endemic for rhinosporidiosis and might have been subclinically exposed to the organism with consequent immunisation (Arseculeratne et al 2001, unpublished data). Precipitin lines were also detected on CIE gels, with these rhinosporidial patients’ sera. CD20 + B-lymphocytes were detected in rhinosporidial polyps especially around sporangia.

Immunological correlates of histopathology

A histological feature in both human and animal rhinosporidiosis is the variation of the stromal and cell infiltration patterns. Intact sporangia containing endospores or degenerate, empty sporangia are found either surrounded by a well-marked cell infiltrate of especially mononuclear cells or in a stroma devoid of such cells. On the other hand, well-marked cell infiltration exists without rhinosporidial bodies. These variations could possibly be correlated with both the specific immune responses or the mechanisms of immune evasion shown by the pathogen.

Treatment

Although cases of spontaneous regression have been recorded, they are rare, and the mode of treatment remains surgical. Total excision of the polyp, preferably by electro-cautery, is recommended. Pedunculated polyps permit of radical removal while excision of sessile polyps with broad bases of attachment to the underlying tissues are sometimes followed by recurrence due to spillage of endospores on the adjacent mucosa. Extensive growths, as on the penis, might require amputation of the affected site.

The failure to propagate Rhinosporidium seeberi in vitro, with the inability to establish experimental rhinosporidiosis, have prevented the determination of the sensitivity of Rhinosporidium seeberi to drugs that might have clinical application. While several anti-bacterial and anti-fungal drugs have been tested clinically, but unsuccessfully, the only drug which was found to have some anti-rhinosporidial effect is dapsone (4,4-diaminodiphenyl sulphone) which appears to arrest the maturation of the sporangia and to promote fibrosis in the stroma, when used as an adjunct to surgery. A special reason for the need to develop drug therapies as alternatives to dapsone is the precipitation by dapsone of haemolytic reactions in patients with G6PD deficiency which co-exists in the same geographical regions in Sri Lanka which also have a high endemicity of rhinosporidiosis.

There have been no innovations in treatment since surgery and dapsone were introduced.

Rhinosporidium seeberi

The identification of the organism in rhinosporidiosis in horses and in buffaloes led to it being named Rhinosporidium equi and R.ayyari respectively. R. kinealyi was the name given by Minchin and Fantham to the organism detected in rhinosporidial tissue studied by O’Kinealyi in 1903. Vanbreuseghem also referred to R.amazonicum. These four names however are now replaced by R.seeberi, as they are considered to represent a single species.

As in 1998, several enigmas exist even today regarding R.seeberi - taxonomy, the question of strain variation, habitat, purification of the stages in its life cycle, antigenic structure, the nature and significance of the intra-endosporial Electron Dense Body (EDB) and the ‘laminated (multilamellar) body’, the failure of attempts at sustained in vitro culture, and the failure to establish rhinosporidiosis in experimental animals.
Taxonomy

*R.seeberi* had first been regarded as a sporozoan by Malbran, its discoverer, in 1892, as a protozoan by Seeber who first published a description of the pathogen and then, as a phycomycete by Ashworth in 1923. Through molecular biological analysis of the organism’s ribosomal DNA, Herr *et al* classified the organism in a new clade which was named the *Mesomycetozoa* (Fig. 3), which includes fish and amphibian pathogens in the former DRIP clade (*Dermocystidium*, the rosette agent, *Ichthyophonus* and *Psorospermium*). It is of interest that the histopathology of these fish and amphibian diseases closely resembles that of rhinosporidiosis. In addition, morphological similarities were noted between *R.seeberi* and these pathogens. Indeed it was speculated by Herr *et al* that some of these pathogens could be classified in the genus *Rhinosporidium* with the suggestion that *Rhinosporidium* is a monotypic genus. An independent group of workers supported this conclusion concerning taxonomy, in that their analysis of *R.seeberi* 18S rRNA from infected tissue showed that this organism is a protist “from a novel clade of parasites that infect fish and amphibians”. These studies finally resolve the debate on the taxonomy of *R.seeberi*, particularly that it is not a classic fungus “but rather the first known human pathogen from the DRIPs clade, a novel clade of aquatic protistan parasites.

Figure 3: Phylogenetic comparison from analysis of ribosomal DNA of *R.seeberi* showing affinities of *R.seeberi* to members of the Mesomycetozoa clade.

Mention must be made of two earlier reports which had radically different conclusions on the identity of the aetiological agent of rhinosporidiosis, if only to highlight pitfalls in the study of the aetiology of this disease and because these radically different views are still being quoted by authors on rhinosporidiosis.

Ahuwalia *et al* on ultrastructural and biochemical grounds concluded that the bodies seen in rhinosporidial tissues are not micro-organisms, but are carbohydrate-containing bodies derived from cellular lysosomes. Later Ahluwalia* decided that the cause of rhinosporidiosis was rather the ubiquitous, aquatic cyanobacterium *Microcystis aeruginosa*. Apart from the bizarre carbohydrate theory, *M. aeruginosa*, a common cyanobacterium in ground waters, proved to be an effective red-herring on account of (a) its presence in the same type of waters which are the putative habitat of *R.seeberi*, (b) its possession of ‘round bodies’, like
R. seeberi, (c) the fact that it was isolated from rhinosporidial polyps. No genetic or immunological homologies were reported. Refutations of Ahluwalia’s views have been made on the basis of (1) non-contamination of the organism’s DNA with human DNA, (2) absence of sampling from rhinosporidial tissues uncontaminated with ground water and absence of controls in Ahluwalia’s work, from normal people inhabiting the same areas from where her water samples were collected, (3) ultrastructural studies, (4) inability of *M. aeruginosa* to cause rhinosporidiosis in experimental animals, (5) the absence of reactivity of this cyanobacterium with human and rabbit anti-rhinosporidial antibody in immunofluorescence tests, (6) absence of reactions with anti-rhinosporidial sera in Western Blots and finally (7) the absence of amplification in PCR tests with primers based on the sequence data published by Herr et al. The pitfalls arising from sampling of rhinosporidial polyps which were exposed to water containing *M. aeruginosa* were also pointed out. A previous report on the isolation of the causative agent of rhinosporidiosis, a mycelial fungus, was also flawed for some of these reasons.

**Life cycle and Morphology**

In the absence of sustained *in vitro* culture, the development of *R. seeberi* (Fig. 4) has been deduced from its appearances in rhinosporidial tissue. Current knowledge is based on the classical descriptions of Beattie, Ashworth, Tirumurti and of Karunaratne, and no new information has been added since then.

**Figure 4:** ‘Life cycle’ of *R. seeberi* as deduced from histopathological appearances. 1 = trophocyte (juvenile sporangium); 2, 3 = immature, bilamellar sporangia; 4a and 4b intermediate sporangia with centrifugal and centripetal maturation of endospores, respectively; 5 = mature sporangium with pore through which mature endospores in mucoid matrix, are exiting; 6 = free endospores with residual mucoid material giving the ‘comet’ appearance of Beattie; 7a = free Electron Dense Bodies which are the ultimate infective unit; 7b = free Electron Dense Body surrounded by other Electron Dense Bodies which are nutritive granules. Not to scale.
The least controversial stage is the juvenile sporangium (trophocyte, 6-100μm) which is thin-walled enclosing a single nucleus in a granular cytoplasm. Further development through stages of immature, intermediate and mature sporangia (50-400 μm) is accompanied by changes in the thickness and lamination of the walls, and appearance of nuclei around which the cytoplasm condenses to form the endospores. These are, when mature, thick-walled spherical bodies (approximately 10-12 μm in diameter). The maturation of the endospores in the intermediate sporangia has been thought to be either centripetal with their origin in the inner wall of the sporangium, or centrifugal with the most mature endospores along the sporangium’s inner wall. Characteristically, the mature endospores contain up to about twenty, spherical, 1-3 μm Electron Dense Bodies (EDB). Ashworth\(^7\) considered them to be proteinaceous nutritive reserves, although Kannan-Kutty and Gomes\(^{41}\) and Kannan-Kutty and Teh\(^{42}\) considered them to be the precursors of the trophocytes.

The thin-walled juvenile sporangia (15-40 μm, trophocytes), develop into immature, intermediate, and mature sporangia which contain several hundreds of mature endospores embedded in a mucoid matrix. Each mature sporangium contains an operculum or pore through which the endospores are extruded; the recently-matured sporangium is surrounded by a thickened ring of the sporangial wall, or centrifugal with the most mature endospores along the sporangium’s inner wall. Characteristically, the mature endospores contain about twenty, spherical, 1-3 μm Electron Dense Bodies (EDB). Ashworth\(^7\) considered them to be proteinaceous nutritive reserves, although Kannan-Kutty and Gomes\(^{41}\) and Kannan-Kutty and Teh\(^{42}\) considered them to be the precursors of the trophocytes.

The developmental stages can be readily identified by special fungus stains such as the Gomori methenamine silver, Gridley’s and the periodic acid-Schiff stains, although the identification of the stages can also be made with the routine haematoxylin and eosin stain.

Ultrastructurally, endospores were described by Kannan-Kutty and Teh\(^{42}\) as possessing a golgi apparatus, mitochondria and an endoplasmic reticulum. There is more or less consensus on the appearances of the sporangia at all stages of development. Juvenile sporangia were found to have unilamellar walls with a ‘capsule’ of fibrils and hazy, electron-dense material. More mature sporangia have bilamellar walls - a thinner electron-dense outer and a thicker, translucent inner wall - surrounded by the ‘capsule’. A nucleus with a nucleolus and nuclear membrane was described by Kannan-Kutty and Teh\(^{42}\) in the developing endospore. ‘Laminated (multilamellar) Bodies’, approximately 2 μm in diameter, composed of granular material surrounded by two or more electron-dense rings have been described by many authors in juvenile and intermediate sporangia. Kannan-Kutty and Gomez\(^{41}\) regarded these laminated bodies as precursors of the endospores. Apple\(^{41}\) described such bodies in endospores which he regarded as precursors of juvenile sporangia.

### Endospore release by rhinosporidial sporangia

Sporangia are well known to possess a pore, surrounded by a thickened ring of the sporangial wall, through which their endospores are extruded into the surrounding tissues to perpetuate the ‘life cycle’. It had been assumed that the extrusion resulted from tissue pressure from outside the sporangia. Evidence, also documented on a video recording, was produced for the operation of an active, vital mechanism, probably osmotic, for the extrusion of the endospores.\(^{44}\) The apparently high antigenicity of the intra-sporangial material, as revealed by the high intensity of fluorescence on indirect immunofluorescence tests with anti-rhinosporidial antibody,\(^{29}\) might be expected to generate antibody with the formation of immune complexes which, on expansion in volume as in the Quellung reaction with pneumococcal capsular polysaccharide, could conceivably aid in the expulsion of the endospores; indeed the need for water in this process of endospore release was noted by Mendoza \textit{et al.}\(^{44}\)

### Purification of developmental stages

Several gaps in our knowledge on \textit{R.seebesi} arose from the difficulty of obtaining pure preparations of the developmental stages of \textit{R.seebesi}, uncontaminated with host material and micro-organisms. This failure prevented unequivocal observations and conclusions on the biology and immunology of the pathogen, as well as on immunity mechanisms in diseased hosts. For instance, the possible contamination of crude preparations of rhinosporidial endospores by human material when the preparations were made from human rhinosporidial polyps, could have led to the identification of ‘rhinosporidial antigenaemia’ on immunoprecipitation in gel-diffusion tests;\(^{22}\) it is possible that the reactions observed were between the contaminating human antigens and the corresponding antibodies from the animal sera which were obtained by immunisation against these crude rhinosporidial preparations.

A method for the isolation of pure preparations of endospores and sporangia in all stages of development (juvenile, immature and mature) based on centrifugation in density gradients of Percoll, of homogenised suspensions of rhinosporidial polyps, was described in 1999.\(^{29}\) The EDBs may also be obtained by sonic disintegration of the purified endospores. When the Percoll purification is done under aseptic conditions, the isolated rhinosporidial bodies are free even of bacterial contamination and could be used in tests which require bacteria-free preparations.
In vitro Culture

Numerous attempts have been made for many decades to propagate R. seeberi, from either rhinosporidial tissue or from saprophytic sources, on inanimate as well as cell-containing media in vitro. Various types of culture media with nutritional additives have been used. There has been no report of successful in vitro culture and propagation in subculture. Grover reported a rise in the number of endospores and formation of sporangia in suspensions of crushed rhinosporidial tissue in vitro at low temperatures. We have made similar observations (Arseculeratne 1997, unpublished data) that suspensions of endospores in repeatedly frozen and thawed suspensions when incubated at room temperature, show the formation of sporangia although we have not seen mature sporangia extruding endospores formed in repetition of the life cycle. These observations indicate the viability and development of the endospores but sustained propagation with repetition of the ‘life cycle’ described above has apparently not been achieved on any culture system hitherto used.

The report of Levy et al. that the organism from canine nasal rhinosporidiosis grew in cultures of human rectal epithelioid cells with the formation of polyps, has had no independent confirmation.

Immunology

Studies on the immunology of the developmental stages of R. seeberi are scarce. One reason is undoubtedly the absence of an in vitro culture method for obtaining pure cultures of the organism. The recent development of a method quoted above, based on gradient centrifugation in Percoll columns, has made available pure preparations of endospores and various stages of sporangial development. Studies on the immunology of R. seeberi with these preparations are in progress in our laboratory.

Immuno-electron microscopy was used to demonstrate an antigen which first appears immediately under the mature sporangial wall; this antigen was apparently absent in sporangia of earlier stages of maturation and in endospores. As the authors stated, this was “the first report in which an antigenic material with a potential role in the immunology of rhinosporidiosis has been detected”. This finding raises the possibility that the antigenic variation might contribute to immune evasion.

A finding of special interest to studies on the clinical characteristics of rhinosporidiosis - chronicity, recurrence and dissemination - is that sonic extracts of rhinosporidial endospores and sporangia on Western Blots show evidence of the presence of human immunoglobulins on the rhinosporidial antigen bands, suggesting either bound immunoglobulins or antigens with epitopes, cross reacting with those on human immunoglobulins. This might indicate immunoglobulin-binding proteins in R. seeberi, a phenomenon also described in bacteria, which might contribute to immune evasion by this pathogen.

Though anti-rhinosporidial humoral and cell-mediated immune responses do occur in human rhinosporidiosis, there are indications that R. seeberi evades specific adaptive immunity through several mechanisms which might explain some hitherto enigmatic aspects of rhinosporidiosis viz., chronicity, recurrence and dissemination:

(a) antigen sequestration. The thick wall of the endospores, which contains chitin and cellulose, is impervious to the exit of intra-endosporial antigens and impermeable to immune destruction. Phagocytosis and ensuing destruction of endospores might however result in antigen release.

(b) antigenic variation, based on the findings of Herr et al. that a new antigen appears for the first time in the wall of maturing sporangia.

(c) immune suppression affecting the cell-mediated immune responses.

(d) Immune distraction. The cell-infiltration patterns in rhinosporidial tissues in some cases indicates that infiltrates occur in some areas of the tissue in which rhinosporidial bodies are absent, suggesting that the infiltrates were in response to free rhinosporidial antigen. If this were the case, the possibility of immune distraction by free antigen might conceivably contribute to immune evasion by R. seeberi.

(e) Immune deviation. Experimental studies on CMIR in mice to R. seeberi demonstrated the occurrence of immune deviation which might further contribute to immune evasion through decreasing CMI reactivity.

(f) Binding of host immunoglobulins. Antigens of R. seeberi, examined by SDS-PAGE and Western Blotting were found to have bound human immunoglobulin. Such binding might mask the pathogen’s antigens, interfering with anti-rhinosporidial immune responses.
Problems yet unsolved

Habitat

There is much circumstantial evidence for the view that the natural habitat of *R. seeberi* is ground water. Yet the unequivocal demonstration of the pathogen in these waters is yet to be made. One difficulty with methods which rely on morphology of the developmental stages of *R. seeberi*, especially the endospores, is the unwarranted assumption that the stages seen in diseased tissues have the same morphology as that of these bodies in the natural habitat. Aquatic animals and water plants have been examined by microscopy and animal inoculation with negative results. Molecular biological investigation of this problem is in progress in our laboratory.

Morphology

The significance of the EDB needs clarification, though the evidence points to their possessing nucleic acids as suggested by positive Feulgen staining, and being DNAase sensitive, and hence being infective units; the EDB, when released by sonic disintegration of endospores, stained yellow-orange with Acridine Orange (Arseculeratne 1996, unpublished data). Investigation of the infective nature of these bodies awaits the establishment of an experimental model for rhinosporidiosis. Ultramicroscopic studies to elucidate the structure of the EDB are also needed. The nature of the Laminated (multilamellar) Bodies also needs clarification.

Biology

The absence of a method for the *in vitro* propagation of *R. seeberi* undoubtedly accounts for the paucity of data on the biology of *R. seeberi*. Vanbreuseghem reported that African rhinosporidial endospores are smaller than Indian endospores; we have no evidence that endospores from rhinosporidial tissues from different clinical presentations in Sri Lanka show differences in size.

There is a recent report of strain variation in the genotype of *R. seeberi*, as revealed by amplification by randomly selected primers (RAPD) of DNA extracted from purified endospores and sporangia from Sri Lankan cases. Variations, especially in virulence properties, if they exist, might be relevant to the spectrum in clinical disease as well as to the variations seen in histopathology of rhinosporidial granulomata. Comparison of strains of *R. seeberi* from different countries would be of interest.

Experimental pathogenicity

A major obstacle in experimental investigations on rhinosporidiosis is the absence of an animal model for the disease. Animals that have shown natural disease, such as horses, cattle, dogs, frogs and fish, are among the animals, apart from laboratory animals, and snails, that have been tested for experimental pathogenicity of *R. seeberi*, with negative results. The armadillo which well supports the growth of the *in vitro* uncultivable *Mycobacterium leprae*, was also found to be incapable of sustaining the growth of *R. seeberi* (L. Ajello, 2002, personal communication). Reports exist on the failure to establish rhinosporidiosis in iatrogenically (steroids, cyclophosphamide) immunsuppressed animals. Congenitally immunodeficient SCID and NUDE mice too were recently shown to be insusceptible. A uniform finding in all these negative reports was that the injected rhinosporidial bodies had disappeared from the site within a few weeks of inoculation. Non-specific immune mechanisms, such as through phagocytes, notably macrophages, might be involved in this process. An experimental animal model of rhinosporidiosis will be of use in drug-response trials, investigation of immunity mechanisms, and further definition of the ‘life cycle’ of *R. seeberi* in natural disease. A basic question which also needs resolution through an experimental model of pathogenicity is, what is the ultimate generative, infective unit of *R. seeberi*? Is it the endospore, as most authors have assumed, or is it its Electron Dense Body from which arises the laminated body and then the trophocyte as suggested by Kannan-Kutty and Gomez?

References


