IMMUNOLOGICAL STUDIES ON RHINOSPORIDIAL ENDOSPORES

A THESIS SUBMITTED TO THE

UNIVERSITY OF PERADENIYA

SRI LANKA

FOR THE DEGREE OF

MASTER OF PHILOSOPHY

BY

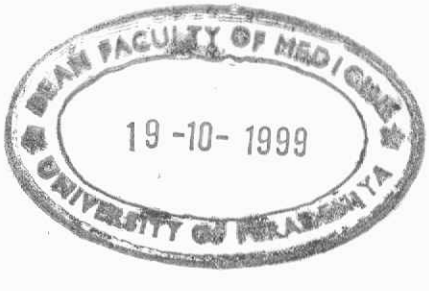
DHAMMIKA NANDA ATAPATTU

BSc. Honours (Sri Lanka)

MB.BS. (Sri Lanka)

Diploma in Medical Microbiology (Sri Lanka)

October 1999



ABSTRACT

Review of our current knowledge of *R.seeberi* to identify areas of obscurity.

The disease rhinosporidiosis, caused by *Rhinosporidium seeberi*, was first described by Seeber in 1900 who regarded the pathogen as a coccidian parasite. In 1922 Ashworth concluded that the organism belonged to the Phycomecetes, sub-order Chtridineae. Minchin and Fantham (1905) studied the disease and named the pathogen *Rhinosporidium kinealyi*. Zschokke (1913) described a similar disease in horses and named the pathogen *Rhinosporidium equi*. The horse pathogen is now regarded as being identical with *R.seeberi*. The current name *Rhinospidium seeberi* was given by Wernicke in 1903 to this anomalous fungal-like organism, but the nature and taxonomy of *R.seeberi* remain obscure.

Rhinosporidiosis has been described as a sporadic disease in about 68 countries but it is endemic in tropical regions, especially in India and Sri Lanka, and to a lesser extent in Uganda, Brazil and Argentina. The only minor human epidemic occurred in Yugoslavia, while swans showed the disease in epidemic form in the United States of America. Human patients have been of a wide age group, from 4 months to 94 years, although young adults are predominantly affected. Disease in the respiratory tract has

been described (Karunaratne, 1964) as occurring predominantly in males, while ocular disease has shown a preponderant female incidence. Polypoidal growths in humans have occurred most commonly in nasal, naso-pharyngeal sites, while sites of lesser incidence have included the urethra, vagina, rectum, subcutaneous tissue and the oral cavity. Disseminated disease is rare and had involved the viscera and the bones. In some animals - dogs, cattle, horses, mules, swans and ducks - the disease has been detected. The epidemiology of rhinosporidiosis is not completely known in respect of the habitat of the pathogen, host predisposition, and the mode of infection. It is not known whether non-diseased carriers exist.

Diagnosis of rhinospioridiosis is primarily by histology of biopsied or resected tissue, and by cytodiagnosis on smears of material from the surface of polyps or from needle aspirates.

There are a very few reports on the immunology of *R.seeberi* and on immune responses in diseased hosts. Circulating rhinosporidial antigen has been reported in only one report on human cases; the absence of detectable anti-rhinosporidial antibody in human patients was also reported in that paper. Only one report exists on cell mediated immunity in rhinosporidiosis; the results were inconclusive.

Histologically rhinosporidial tissue shows a variable degree of infiltration with inflammatory cells, which include mononuclear lymphocytes, macrophages and giant cells, which might suggest at least the operation of non-specific defences.

Treatment is primarily surgical with excision of the growths and cauterisation of the base. Drugs used have included antimony and iodine compounds. Dapsone remains the only drug with documented value, in combination with surgery. Since Dapsone is also used for leprosy, it is desirable that an alternative drug against *R.seeberi* be found to lessen the use of Dapsone and reduce the development of resistance of *Mycobacterium leprae*.

R.seeberi forms various morphological stages, the nomenclature of which was revised by Kennedy et al. (1995). Endospores develop into juvenile, immature and mature sporangia; when mature, the sporangia contain several thousand endospores which are released through a pore in the sporangium, into the tissues, to continue the cycle of development. Electron dense bodies within the endospores have been regarded as the ultimate generative unit of *R.seeberi*, although the nature and significance of the electron dense bodies have long been in dispute. Ultra-structural studies are needed to provide more information on structure in relation to pathogenicity and immunology. Due

to the absence of a method for the purification and separation of endospores and sporangia, studies on these aspects have not hitherto been possible.

The work reported in this thesis.

In this study, a simple, reliable, reproducible and inexpensive method for the purification and separation of the various developmental stages of *R.seeberi*, has been developed. The method involved the centrifugation of homogenates of rhinosporidial tissue on density gradients of Percoll. By altering the density of Percoll in layered columns or in graded density columns, separation of endospores from sporangia was achieved. Since Percoll is a biologically non-toxic medium, the separated fractions could be used for immunological, pathogenicity and drug sensitivity studies.

The isolated bodies were examined by different staining procedures, Leishmann's and the Periodic acid Schiff stains, and with the nucleic acid staining agent Acridine Orange. Indirect immunofluorescence (IF) with anti-rhinosporidial antiserum prepared in rabbits and with serum from human rhinosporidiosis patients was also used to identify and characterize the isolated bodies. The IF technique showed that rhinosporidial extracts with Freund's Incomplete Adjuvant are immunogenic and that rhinospoiridial patients have anti-rhinosporidial antibody. The endospores and sporangia showed

individual patterns of labelling with the IF reagents and their significance in respect of the location of the antigens and their nature are discussed. The matrix of the sporangia in which is embedded the endospores appeared to show the most intense fluorescence and perhaps, therefore, the greatest antigenicity.

Electron microscopic studies were done on purified endospores and sporangia. The presence of multi-layered walls, micro-fibrillae on the endospores and lamellated walls on the endospores were identified. The electron dense bodies were visualized.

Analysis of the antigens of the endospores and sporangia was done by SDS-PAG electrophoresis with staining of the isolated protein bands with Coomassie Blue. Western blotting of these bands and labelling with anti-rhinosporidial antibody (from experimental rabbits or human patients) and tracing with anti-human or anti-rabbit peroxidase linked antibody, indicated the presence of multiple antigens on *R.seeberi*. Novel antigens were seen to appear on the mature sporangial walls. Evidence indicated that extracts from purified endospores/sporangia could show the presence of human proteins, probably immunoglobulin G, modified immunoglobulin G or "immunoglobulin G like" molecules. The possibility that this indicates that *R.seeberi*'s antigens mimic human proteins or are able to bind human proteins on to the surface of the developmental stages, is discussed. The need to identify this and other human proteins on the rhinosporidial endospore or

sporangial surface is pointed out. These findings could indicate mechanisms through which *R.seeberi* evades the host's immune defences, which could account for the chronicity of the disease and dissemination.

In vitro studies in different liquid media at different temperatures demonstrated development from stage to stage, rather than growth. While the endospores and sporangia enlarged in size, the sporangia seemed to liberate their endospores. Only a small proportion of the endospores was seen to change in morphology. More accurate and sensitive methods for demonstrating growth and increase in numbers of endospores and sporangia with their contained endospores would be needed for confirmation of these preliminary observations; these methods would include the quantitative determination of DNA content.

Examination of the endospores/sporangia revealed the presence of high quantities of D-galactose, which indicated affinities of *R.seeberi* with the Chtridiomycetaceae. Chitids are considered to be a primitive taxon amongst the prokaryotes. While the sharing of biochemical characters does not provide a definite indication of taxonomic affinities between microorganisms, definitive characterisation with nucleic acid sequence studies will be needed to determine the taxonomic position of *R.seeberi.*

The areas which require further work are the status of cell mediated immunity and capacity of rhinosporidial antigens to induce CMI responses, the unequivocal establishment of growth in addition to development in vitro, and based on the latter, the determination of drug sensitivity of *R. seeberi* to drugs as alternatives to Dapsone in view of the latter's use in leprosy in the same population groups as rhinosporidial patients, in India and Sri Lanka.