FURTHER TESTS IN MAMMALS, REPTILES AND AN AMPHIBIAN TO DELINEATE THE HOST RANGE OF THE MOSQUITO FUNGUS *CULICINOMYCES* SP.

R. C. MULLEY, J. R. EGERTON, A. W. SWEENEY[1] AND W. J. HARTLEY

Department of Veterinary Clinical Studies, The University of Sydney, Camden,
New South Wales, 2570, Australia

ABSTRACT. Normal and immunosuppressed mice and a horse were inoculated intradermally and subcutaneously with suspensions of conidia and hyphae of *Culicinomyces* sp. A transient local inflammatory reaction developed at the site of inoculation but attempts to reisolate the fungus were unsuccessful.

Tortoises, toads and 2 species of lizard were each dosed orally and subcutaneously with a suspension of conidia and hyphae of *Culicinomyces* sp. Macroscopic and histological examination of selected tissues showed no apparent lesions as a result of oral dosing. Fungal hyphae and conidia were demonstrated at subcutaneous inoculation sites for up to 3 weeks in lizards, 18 days in toads and 10 weeks in tortoises. The fungus was in circumscribed lesions and remained viable for a limited period despite a chronic inflammatory response. Tortoises inoculated orally and subcutaneously with fungal suspension remained healthy for up to 12 months afterwards. At autopsy there was no evidence of fungus at the inoculation site.

INTRODUCTION

An Australian isolate of the fungal genus *Culicinomyces* Couch, Romney and Rao, is being investigated as a potential biocontrol agent of mosquito larvae (Sweeney et al. 1973). Its effects on nontarget organisms in the aquatic environment and its safety for vertebrate animals need to be established. Laboratory observations to define the host range suggest that susceptible invertebrate hosts of this fungus may be restricted to a limited range of aquatic dipterous larvae (Sweeney 1975, 1979, Knight 1980). Earlier experiments on vertebrate safety showed no clinical signs or lesions in test animals following oral administration of a large daily dose of infective spores (10^6 conidia/ml) for several weeks in laboratory animals (mice, rats and guinea pigs), farm animals (sheep and cattle) and 2 species of wild ducks (Egerton et al. 1978). However, there was still a possibility that the fungus could invade skin wounds or induce allergenic responses in animals chronically exposed to it.

This paper reports the results of experiments in which the fungus was administered dermally and subdermally to normal and immunosuppressed mice and to a horse. The fungus was also administered orally and subcutaneously to two species of native Australian lizards, a species of freshwater tortoise and a species of toad to determine whether poikilotherms were more suitable hosts than mammals.

MATERIALS AND METHODS

Cultures containing conidia and hyphae of *Culicinomyces* were prepared as previously described (Egerton et al. 1978). The infectivity of each batch of fungal culture for mosquito larvae was confirmed before use. The procedures used for the test animals are as follows:—

HORSE—Fungal cultures containing 2.8×10^7 conidia/ml and hyphae were inoculated intradermally into a series of sites on the abaxial metacarpal and anterior pectoral regions of a pony mare. In addition, 1 ml of culture was swabbed onto scarified areas of the mucocutaneous junction in the nostril. Contralateral control sites were inoculated with the same volume of sterile culture

[1] Malaria Research Unit, Australian Army, Ingleburn, 2174, Australia.

medium. Biopsies were taken from the sites of inoculation on days 1, 2, 3, 7, 14, and 21 post-inoculation and examined histologically for evidence of fungal growth. Smears of swabs taken from the nostrils were stained and examined for fungus. Blood samples were collected on days 0, 1, 2, 3, 7, 14, and 21 post-inoculation and values for Hb, RBC, WBC, PCV, MCV, MCHC, differential WBC count and total protein were determined.

MICE—Twenty normal mice and 20 mice treated with 20 mg/kg cyclophosphamide were injected subcutaneously and intradermally with 0.5 ml of culture containing 2.8×10^7 conidia/ml and hyphae. Contralateral control sites were inoculated with sterile growth medium. Mice were killed at 0, 6, 12, 24, 48, 72 and 96 hr and 7, 14, and 21 days after inoculation and selected tissues collected and examined histologically for evidence of *Culicinomyces*. Attempts were also made to reisolate the fungus from the injection sites.

REPTILES—Five common blue tongue lizards (*Tiliqua scincoides scincoides*) and 3 bearded dragons (*Amphibolurus barbatus minor*) were each given a 2 ml oral dose daily for 7 days and a 1 ml subcutaneous inoculation of fungal culture containing 4.2×10^7 conidia/ml and hyphae. Four blue tongue lizards and one bearded dragon were treated with control sterile growth media. Treated and control blue tongues were killed 8, 29, and 72 days after dosing and treated bearded dragons were killed 8, 21, and 24 days after dosing. The control bearded dragon was killed 24 days after treatment. All animals were autopsied and portions of small intestine, large intestine, liver, kidney, lung, heart and portions of the subcutaneous injectioin sites were collected and preserved in formalin. Attempts were made to reisolate the fungus from the injection sites.

In addition 16 common long necked tortoises (*Chelodina longicollis*) were each dosed orally for 7 days with 2 ml of fungal culture containing 4.2×10^7 conidia/ml and hyphae. These tortoises were also inoculated subcutaneously with 1 ml of the same culture. Eight control tortoises were dosed and inoculated with sterile growth medium. One control and one treated tortoise were killed at 0, 3, 7, 10, 17, and 28 days, and 6 wk. and 10 wk. after inoculation. All test and control animals were autopsied and specimens of tissue from organs and injection sites collected for culture and histology. A further 8 treated tortoises were kept for 52 wk, killed, autopsied, and specimens collected as above. All tortoises were housed in a suitably constructed enclosure containing pond and dry ground for the duration of the experiment.

AMPHIBIANS—Six toads (*Bufo marinus*) were treated orally and subcutaneously using the same fungal culture and dosing regime as the tortoises. Six control toads were treated with sterile growth medium. Two control and 2 treated toads were killed at 0, 7, and 18 days after inoculation, autopsied, and tissue specimens collected for culture and histology. All toads were kept in a similar enclosure to the tortoises during the experiment.

HISTOLOGY. All tissues were processed by standrd techniques, stained with either haematoxylin and eosin (H & E) or Grocott-Gomori methenamine silvernitrate, and examined microscopically.

RESULTS

HORSE—No evidence of tissue damage or growth of the organism was observed. There were no changes in the general health of the test animal and no alterations in haematological values were detected. Scarified sites healed within 3 days. There were no detectable differences between infected and control sites.

MICE—Marked inflammatory responses developed at sites inoculated with fungi in both normal and immunosuppressed mice. Palpable swellings formed at these sites and elements of fungal

hyphae and spores were observed in them up to 14 days after inoculation. However, the lesions were clearly circumscribed and there was no evidence of tissue invasion. Attempts to culture fungi from sites of inoculation in mice were unsuccessful. There was no tissue response to inoculation of sterile growth medium at control sites.

REPTILES—The fungus was recultured from subcutaneous inoculation sites at 8 days post-inoculation in blue tongue lizards and at 8 and 21 days post-inoculation in bearded dragon lizards. The fungus was also recultured from subcutaneous inoculation sites in tortoises up to 10 wk post-inoculation. Fungi were visible in sections cut and stained from the inoculation sites but were again contained in circumscribed lesions with no evidence of tissue invasion. The organism remained viable for a limited period in these sites despite a chronic inflammatory response. Histology of selected body tissues showed no evidence of tissue invasion as a result of oral dosing in any of the treated reptiles.

AMPHIBIANS—The fungus was recultured from subcutaneous inoculation sites at 7 and 18 days post-inoculation in toads but again these injection sites were clearly circumscribed with no evidence of tissue invasion. Histology of selected body tissues also proved negative for fungus.

DISCUSSION

The results support the view (Egerton et al. 1978) that *Culicinomyces* sp. is unlikely to succeed as a pathogen of mammals because of the inability of the organism to grow above 30°C. Attempts to reisolate *Culicinomyces* from inoculation sites on the nasal mucosa, trunk and extremities of the experimental horse failed. Tests were done in the horse because another entomogenous fungus *Conidiobolus coronatus* has been isolated from skin lesions in both man (Emmons et al. 1970) and horses (Hutchins and Johnston 1972).

Immunosuppression of mice prior to exposure to the fungus did not aid its establishment and attempts to reisolate the fungus from subcutaneous inoculation sites of normal and immunosuppressed mice also failed.

The persistence of the organism in poikilotherms for a short period was perhaps predictable. Fungal hyphae and conidia were demonstrated in subcutaneous inoculation sites for up to 3 wk in lizards, 18 days in toads and 10 wk in tortoises. However, the lesions were all circumscribed by a local inflammatory reaction and there was no evidence of tissue invasion. Neither illness nor pathological changes resulted from oral dosing of reptiles and amphibians, indicating that the fungus was either destroyed in the gastro-intestinal tract or voided in faeces.

From observations made on reptiles and amphibians in these experiments it seems unlikely that the fungus *Culicinomyces* could invade skin wounds of poikilotherms no matter how suitable the dermal and sub-dermal environment. The doses of fungi used in these experiments were many times greater than would ever be contacted by these animals in the wild and still there was no evidence that the organism by itself caused disease in any of the reptiles tested or that it persisted for more than a short time at the site of inoculation.

Culicinomyces is a naturally occurring fungus in the aquatic environment and no reports exist in the literature of its isolation from any pathological process in wild or domesticated animals.

ACKNOWLEDGMENT

References Cited

Egerton, J. R., W. J. Hartley, R. C. Mulley and A. W. Sweeney. 1978. Susceptibility of labo-

ratory and farm animals and two species of duck to the mosquito fungus *Culicinomyces* sp. Mosq. News 38:260–263.

Emmons, C. W., C. H. Binford and J. P. Utz. 1970. Medical Mycology, 2nd ed. Lea and Febiger, Philadelphia. 508 pp.

Hutchins, D. R. and K. G. Johnston. 1972. Phycomycosis in the horse. Aust. Vet. J. 48:269–278.

Knight, A. L. 1980. Host range and temperature requirements of *Culicinomyces clavosporus*. J. Invertebr. Pathol. 36:423–425.

Sweeney, A. W. 1975. The insect pathogenic fungus *Culicinomyces* in mosquitoes and other hosts. Aust. J. Zool. 23:59–64.

Sweeney, A. W. 1979. Further observations on the host range of the mosquito fungus *Culicinomyces*. Mosq. News 39:140–142.

Sweeney, A. W., D. J. Lee, C. Panter and L. W. Burgess. 1973. A fungal pathogen for mosquito larvae with potential as a microbial insecticide. Search (Syd.) 4:344.