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Lesions experimentally produced by fungi implicated in extrinsic allergic alveolitis

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SUMMARY

The ability of *Aureobasidium pullulans*, *Cladosporium herbarum* and *Cryptostroma corticale* to produce local lesions in the rabbit was examined. Both *C. corticale* and *A. pullulans* can survive *in vivo* and produce the inflammatory response typical of mycetoma. *C. herbarum* failed to grow or survive *in vivo*.

INTRODUCTION

The disease condition of extrinsic allergic alveolitis can be caused by several organisms, including both fungi and actinomycetes (Salvaggio & Buechner, 1971; Pepys, 1969). The actinomycetes which have been implicated, namely, *Thermoactinomyces vulgaris* and *Thermoactinomyces sacchari* have been shown to be capable of causing local mycetoma (Stretton & Bulman, 1974).

Cladosporium herbarum and *Aureobasidium* (*Pullularia*) *pullulans* are common airborne fungi which occur frequently on plants and other objects (Bernstein & Feinberg, 1942; Gregory & Lacey, 1963). Though *Aureobasidium pullulans* has been implicated by Cohen, Merigan, Kosek & Eldridge (1967) in sequoiosis, and *C. herbarum* has been associated by Bernton & Thorn (1937) and Tomsikova, Dura & Novackova (1973) with causing allergic syndromes, neither organism has been considered to be capable of causing local infection. Wynne & Gott (1956) have reported the isolation of *A. pullulans* from granulomas in patients with Hodgkin's disease and it has also been isolated from patients with rheumatoid arthritis (Arthritis & Rheumatism Council, 1966). However, it has been suggested by Emmons, Binford & Utz (1970) that *A. pullulans* can be equated with *Cladosporium werneckii*, an epidermal pathogen, although Cooke (1959) accepted them as different species.

Cryptostroma corticale is the causal agent of maple-bark disease (Towey, Sweany & Huron, 1932) and the organism was obtained by Emanuel, Wenzel & Lawton (1966) from the granulomatous nodules by lung biopsy.

This study examines the ability of these organisms as hyphae or spores to survive and produce local lesions, apart from the granulomatous nodules which occur in the lung.

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MATERIALS AND METHODS

Organisms

Cryptostroma corticale was isolated from infected maple bark, kindly provided by F. J. Wenzel, Marshefield Clinic, U.S.A. *Aureobasidium pullulans* CMI2456 (isolated originally from lymph node mycetoma) and *Cladosporium herbarum* CMI131128 were used throughout.

Media

The organisms were maintained on a medium containing (g./l.) mycological peptone, 10; and dextrose, 40. Solidified media were prepared by the addition of Oxoid Agar no. 3, 1·5 %. The ability to grow on this medium was examined at 28° and 37° C.

Morphological characteristics

The organisms were examined for purity, including the presence of contaminants, before injection and after isolation from experimentally infected animals. *Cryptostroma corticale* was examined according to the description of Emanuel, Wenzel & Lawton (1966), *A. pullulans* according to the description of Wynne & Gott (1956) and *C. herbarum* by its morphological character. They were also examined for haemolytic activity on the nutrient medium with an added 5 % v/v defibrinated horse blood.

Preparation of suspensions

Two ml. of a spore suspension, containing 10^6 spores/ml., were inoculated into 200 ml. of liquid medium for the production of the hyphal form and all organisms were incubated for 48 hr. at 28° C. The hyphae were removed by centrifugation at 4,000 ***g*** and washed 4 times with sterile normal saline (0·9 % w/v) solution. On examination by phase-contrast microscopy these suspensions were free from spores.

Spore suspensions were prepared after inoculation on the surface of 200 ml. of solidified medium in Roux flasks and incubating for 6 days at 28° C. The spores were removed from the surface of the agar by washing with sterile normal saline and then depositing the spores by centrifugation at 4,000 ***g*** and washing 4 times with sterile normal saline.

The deposited cells were resuspended in sterile normal saline using a glass tissue homogenizer (Jencons (Scientific) Ltd., Herts) and the cell density adjusted to give *ca.* 10^2 spores/ml., or 10^2 hyphal fragments/ml. using a Thoma counting chamber. The cell density was also checked to be of the same order in each case by measuring the optical density at 420 nm. (SP500, Pye Unicam, Cambridge). The number of viable units was also checked by carrying out pour-plate counts before injection; in all cases this was *ca.* 10^2/ml.

The suspensions were also plated out on the solidified medium and were found to be free from organisms other than the required fungi.

Suspensions of killed cells were prepared by dividing the above suspensions and heating in an autoclave at 120° C. for 20 min. The absence of viable organisms

from these suspensions was checked by streaking out and by the inoculation of 1 ml. of the suspension into nutrient medium and incubating at 28° C.

Experimental animals and injection procedure

Male Dutch rabbits weighing $2{\cdot}0 \pm 0{\cdot}4$ kg. were used throughout. When the intramuscular (i.m.) route was employed 0·3 ml. of the required suspension was injected into the flank and for the subcutaneous (s.c.) route, 0·2 ml. The rectal temperature was measured daily after injection. Rabbits were killed at intervals of 7 and 14 days after injection.

After necropsy samples of kidney, liver, spleen and any obviously infected tissue were inoculated on nutrient medium and incubated aerobically. Also, representative samples of tissue, including obvious lesions, were excised, fixed immediately in mercuric chloride-formalin solution, dehydrated and embedded in paraffin. Sections of 5 μm. thickness were cut and stained with haematoxylin and eosin, Gram stain, periodic acid Schiff (PAS) and for calcification by the alizarin red S method (Pearse, 1972). Smears prepared from the pus were also examined immediately after necropsy using the PAS stain.

Samples of the pus were checked, by streaking out on the maintenance medium and nutrient agar, for the presence of organisms other than the appropriate organism.

RESULTS

All the organisms grew well on the medium described at 28° C. *C. corticale* grew slowly at 37°. *Aureobasidium pullulans* gave very scanty growth at 37° and *C. herbarum* failed to grow at all at 37°. There was no detectable haemolysis, before or after isolation, of any organism from the rabbit.

There was no significant alteration in the body temperature following injection of any of the organisms.

In the case of *C. corticale* and *A. pullulans* nodules were detected on necropsy when either hyphae or spores had been injected. There were no nodules present when *C. herbarum* was implicated.

The nodules produced by *A. pullulans* were always small (*ca.* 0·5 cm.) and located at the site of injection, typically in the m. semimembranosus (Pl. 1, fig. 1). The periphery of the nodule was avascular.

C. corticale produced larger nodules, *ca.* 3 cm. diameter, located typically on the m. semimembranosus (fig. 2). The periphery of the nodule was marked by an increased vascularity. The pus obtained from the nodule was pale yellow in colour, of a cream-like texture, and contained hyphae. Nodules were produced when either hyphae or spores were injected in 80% of the animals, by the s.c. or i.m. routes.

In no case were the lesions seen to be spreading, and the nodules were all located at or near the site of initial infection. The *post mortem* examination showed no nodule present on the liver, spleen or kidney, nor were any of the organisms recovered from these organs.

In all cases where the killed cells were injected there was no reaction at the site

of injection nor was there any development of nodules, even at the site of the injection.

There were no organisms present in the samples of pus removed from the nodules other than those initially injected, as judged by their morphology and colonial appearance. Viable organisms were recovered from the nodules produced by *A. pullulans* and *C. corticale;* this included those occurring just at the site of injection. The pus from *C. corticale* contained *ca.* 100 viable units/ml. and *A. pullulans* 50/ml.

The histopathological examination revealed the presence of abscesses showing a typical inflammatory response. The type of abscess produced was similar when either *A. pullulans* or *C. corticale* was the cause (Pl. 1, fig. 3 and Pl. 2, fig. 4). There was evidence of necrosis of muscle fibres, particularly in the case of *C. corticale*, and fibrosis was noted around the periphery of the nodule (figs. 3 and 4). The organisms which produced the limited infections were present predominantly as hyphae, even after the injection of spore suspensions. In the nodules of *C. corticale* some spores remained ungerminated (fig. 5) and normal hyphae could be detected. There were fewer and shorter hyphae present when *A. pullulans* was seen in nodules (fig. 6). There was no evidence of calcification in any of the nodules, even after 14 days.

No organisms were detected in any of the organs examined histopathologically, only in the nodules themselves. There were no histological changes at the site of injection when killed cells had been injected.

DISCUSSION

The ability of *C. corticale* and *A. pullulans* to produce local infections was somewhat unexpected in view of their apparent difficulty in growing at 37° C. *in vitro*. In fact Emanuel *et al.* (1966) stated that *C. corticale* did not grow at 37° C. We found that, although growth was slow, it did occur and subsequently F. J. Wenzel (personal communication) has confirmed this observation. The observation that *A. pullulans* and the plant pathogen *C. corticale* can produce local infection supports the evidence of Lie-Kian-Joe, Tjoei Eng, Kertopati & Emmons (1957) that a plant pathogen *Cercospora apii* can also cause mycetoma of the thigh and thorax as well as being the causal agent of leaf-spot disease.

The lack of growth of *C. herbarum*, *in vivo*, was not surprising in view of its lack of growth at 37° C. *in vitro*, and in this work it served as a suitable control representing a commonly saprophytic group with species which are occasionally pathogenic (Emmons *et al.* 1970).

Both *C. corticale* and *A. pullulans* can survive *in vivo*. The presence of hyphae in the nodules following injection of spore suspensions suggests that limited growth and some differentiation had taken place. Nodules similar to those produced in this instance can be produced by the injection of foreign material which gives a non-specific inflammatory response. However, this is unlikely to have happened, as the injection of heat-killed cells failed to produce nodules or elicit any response, as seen by histological examination.

The organisms did not appear to produce any tissue-destroying enzymes, as judged from the histopathological examination. They were certainly deficient in haemolysin. The tissue destruction which would be necessary to allow even limited spreading could come from a release of proteolytic enzymes from the degranulating leucocytes (Cline, 1970) which are present around the periphery of the nodule.

The spread of infection is limited by fibrosis around the nodule, and similar fibrosis is common in cases of deposition of agricultural dusts in the lung (Rankin, Kobayashi, Barbee & Dickie, 1965). A feature of the nodules produced by these organisms is the absence of calcification, which in other infections may help to limit the spread of the infecting organisms.

The low numbers of organisms present in the nodule are not unexpected in view of the difficulty frequently encountered in obtaining the causal organism from such a lesion (Emmons *et al.* 1970). The pleomorphism exhibited in the case of *A. pullulans* is typical of several fungi where the form seen *in vivo* can differ significantly from that seen *in vitro*. This is seen in sporotrichosis (Howard, 1961) or in actinomycotic mycetoma (Georg *et al.* 1972; Stretton & Bulman, 1974).

With the organisms being present in large numbers in certain environments (Gregory & Lacey, 1963) and *A. pullulans*, in particular, being ubiquitous, the role of these fungi as possible opportunistic invaders may need reappraisal. Emmons (1962) considered the situation of several pathogenic fungi and concluded that so long as the environmental conditions were favourable they were vigorous and self-sufficient saprophytes and were parasites by accident. This is probably true of saprophytic fungi which are opportunistic invaders.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Nodules produced after 7 days following i.m. infection with *Aureobasidium pullulans* as a spore suspension located on the m. semimembranosus. × 1.

Fig. 2. Nodule produced after 7 days following i.m. infection with *Cryptostroma corticale* as a spore suspension, located on the m. semimembranosus. × 0·5.

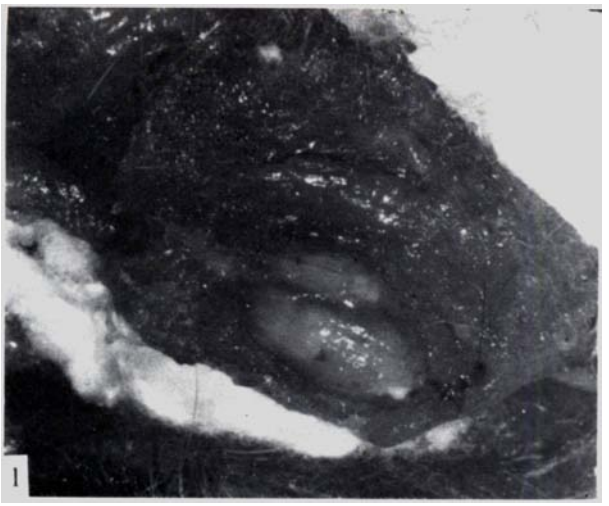
Fig. 3. Nodule produced by *A. pullulans* after 7 days in the m. semimembranosus showing necrosis of muscle fibres and limited fibrosis. H. & E. × 400.

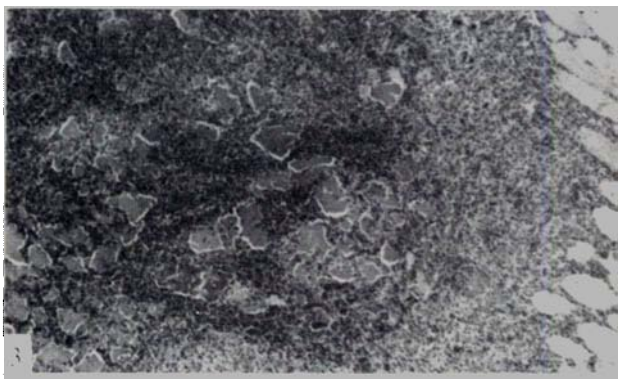
PLATE 2

Fig. 4. Nodule produced by *C. corticale* after 7 days in the m. semimembranosus showing necrotic muscle fibres, fibrosis and inflammatory response. H. & E. × 160.

Fig. 5. Hyphae and spores present in nodule produced by *C. corticale*. PAS, × 1000.

Fig. 6. Short hyphae present in nodule produced by *A. pullulans*. Gram × 1000.





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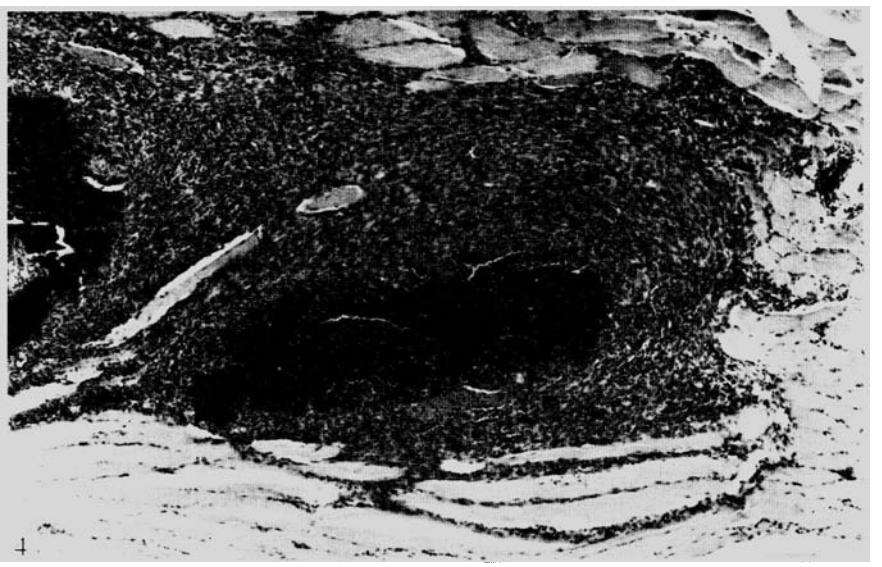

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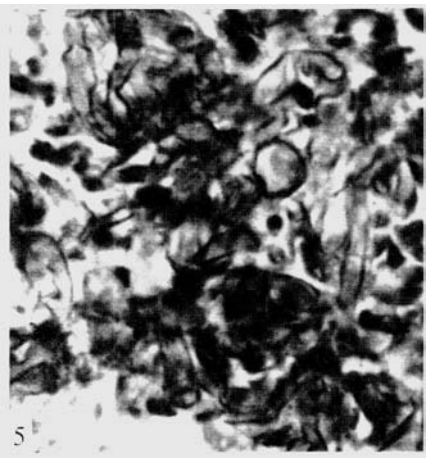

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