Interactions between Some Aural Aspergillus Species and Bacteria

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SUMMARY

Some interactions of Aspergillus species with Staphylococcus aureus and Pseudomonas aeruginosa, all of which are concerned in otitis externa, are described. The pigments pyocyanine, 1-hydroxyphenazine and the fluorescent green pigments produced by P. aeruginosa inhibited the germination of spores of Aspergillus terreus; the most toxic of these pigments was 1-hydroxyphenazine. P. aeruginosa did not produce fluorescent pigments in Czapek-Dox medium unless the ferrous sulphate was omitted and asparagine added. Following incubation of the Aspergillus species in this medium, however, P. aeruginosa grew and produced fluorescent pigment therein. S. aureus produced antifungal toxic material which was capable of diffusing through cellophan and causing distortion of A. terreus hyphae.

INTRODUCTION

Yeasts and filamentous fungi, mainly Aspergillus species, have often been recorded as the cause of otitis externa and fungal infections are often associated with bacterial infection especially after mastoidectomy or fenestration operations. Attempts to elucidate the relationship of bacteria and fungi in mixed infections have been unsuccessful (Haley, 1950a, b; Singer et al. 1952; Leshin, 1953; Stuart & Blank, 1955; Powell, English & Duncan, 1962). Whether a fungus can be the primary cause of otitis externa remains undecided. Smyth (1962) and others have suggested that the secretions of the apocrine and sebaceous glands protect the healthy ear from invading organisms and that fungal infection occurs only after these glands have been damaged by bacteria or some other agency or when the host resistance is otherwise diminished; but English (1963) found that both Aspergillus fumigatus and A. terreus grew on the cerumen produced by these glands, as well as on keratin. English & Stanley (1966) made preliminary observations on the interactions of four Aspergillus species with four common aural bacteria. The present paper reports an examination of some of these interactions.

METHODS

Organisms and general methods. Cultures of Aspergillus flavus, A. fumigatus, A. niger, A. terreus, Staphylococcus aureus and Pseudomonas aeruginosa were obtained from cases of otitis externa. The strain of S. aureus could not be typed but gave positive

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results in egg yolk and lipase tests. The fungi were maintained on glucose peptone agar and the bacteria on nutrient agar.

All incubations were at 37°. The media used were nutrient broth, nutrient agar, glucose peptone broth, glucose peptone agar (4% glucose, 1% peptone), Czapek-Dox liquid and Czapek-Dox agar (Thom & Church, 1926). Filtrates of fungal cultures were obtained by means of a membrane filter of pore size 0.45 μ.

Interaction between bacteria and fungi. An aqueous suspension of the spores (3 x 10^6 spores/ml.) of one of the species of Aspergillus was prepared from 3 to 5 day agar cultures; 0.1 ml. samples of suspension were added to each of eight 50 ml. conical flasks containing 10 ml. nutrient broth. These were incubated in a shaken water bath. Flasks 1 to 7 were further inoculated, respectively, at 0, 4, 8, 12, 16, 20 and 24 hr after the addition of the fungus spores, with 0.1 ml. of a 1/100 dilution in nutrient broth of a 16 hr nutrient broth culture of the bacterium to be tested. After incubation for 48 hr, fungal inhibition was estimated macroscopically and microscopically. To determine the viability of fungus, drops of the mixed culture were placed on Czapek-Dox agar, a medium which allowed growth of Aspergillus species even in the presence of the bacteria.

Antifungal toxic material of Staphylococcus aureus. Two layers of agar medium separated by sterile cellophane were poured into 9 cm. Petri dishes. The lower layer contained 5 ml. nutrient agar and 0.1 ml. bacterial inoculum; the upper layer contained 5 ml. glucose peptone agar and 0.1 ml. of Aspergillus terreus inoculum. The inocula of both organisms were prepared as described above. A bacterial culture filtrate was obtained by means of a membrane filter of pore size 0.45 μ. The bacterial filtrate was tested for fungal toxicity by adding 10 ml. of a 24 hr nutrient broth culture filtrate of S. aureus to 10 ml. of nutrient agar seeded with 0.1 ml. of fungal spore suspension. In the control cultures 10 ml. of nutrient broth replaced the bacterial filtrate.

Preparation of bacterial pigment solutions. Solutions of pyocyanine, 1-hydroxyphenazine (both chloroform soluble) and the fluorescent green pigments (water soluble) were prepared from Pseudomonas aeruginosa cultures by modifications of the methods of Schoental (1941), Young (1947) and Liu, Abe & Bates (1961). The pigments were extracted from cultures incubated in 10 ml. amounts of nutrient broth + 1% glycerol for 7 days. Pyocyanine was extracted from 50 ml. of broth culture with successive 10 ml. amount of chloroform; this pigment was then transferred to dilute HCl in which it was stored until required. After neutralization, re-extraction into chloroform and evaporation, the pigment was used in a phosphate buffer solution at pH 7.3.

1-Hydroxyphenazine was present in such small quantities in the cultures that it was prepared from the stock acidic pyocyanine solution (Rodd, 1959), extracted in chloroform, evaporated and dissolved in phosphate buffer solution (pH 7.3). Fluorescent green pigments were obtained by dialysis and concentration of cultures from which the above two pigments and the bacteria had already been removed. Two ml. of each of the pyocyanine and fluorescent solutions thus prepared was equivalent to the amount of these pigments contained in 10 ml. of culture, while 2 ml. of 1-hydroxyphenazine solution was the quantity obtained by converting the pyocyanine present in 10 ml. of culture.

Toxicity of the pigments of Pseudomas aeruginosa to fungi. Two ml. bacterial filtrate
were added to 10 ml. molten nutrient agar containing 0.5 ml. fungal inoculum prepared as before. Microscopic examination for spore germination was made hourly from 8 to 14 hr in lactophenol cotton blue mounts. Spores were considered to have germinated when the germ tube length was equal to the diameter of the spore. Percentage germination was based on counts of 400 spores. Control counts were made on nutrient agar plates without added bacterial filtrate.

RESULTS

Pseudomonas aeruginosa inhibited the growth of both Aspergillus terreus and A. fumigatus in broth cultures when added within 20 hr but was never fungicidal. In contrast, when Staphylococcus aureus was grown in mixed culture with A. fumigatus or A. terreus the bacterium inhibited growth of the fungi when added within 16 hr; it both decreased their growth rate and induced repeated dichotomy of the hyphal tips, the latter effect being more pronounced with A. terreus than with A. fumigatus. The growth of A. fumigatus and A. terreus was compared in pure culture and in the presence of S. aureus added after 8 hr growth of the fungus in: (a) nutrient broth; (b) nutrient broth + 1% glucose or glycerol; (c) glucose peptone broth. The toxic effect of S. aureus was greater in media (b) and (c) than in (a). In pure culture no abnormalities of the fungi were observed in any of these media. In the presence of S. aureus, A. fumigatus exhibited repeated apical dichotomy of the hyphae while A. terreus showed also subapical swellings in glucose or glycerol containing media (Pl. I a, b, c, d).

I-hydroxyphenazine was much more toxic to spore germination of Aspergillus terreus than either of the other two substances (Fig. 1). Abnormal hyphal development was not observed in the presence of any of the three pigments. Spore germination of A. fumigatus, A. flavus and A. niger was also inhibited by I-hydroxyphenazine.
In mixed cultures *Pseudomonas aeruginosa* grew and produced its fluorescent green pigments on Czapek-Dox agar only when colonies of the *Aspergillus* species were present. The fungi were only slightly inhibited by *P. aeruginosa* on this medium. *P. aeruginosa* also grew and produced these pigments in filtrates of 24 hr Czapek-Dox liquid cultures of *Aspergillus terreus* and *A. fumigatus*, suggesting that in this medium the fungi caused a change necessary for bacterial growth and pigment production. No pH change took place in the 24 hr broth culture. The addition to Czapek-Dox liquid medium of glucose, glycerol or asparagine (1 %), peptone or casamino acids (0.3 %), nicotinic acid, pyrodoxin, thiamine or riboflavin (10 µg./l.), did not induce pigment production, although glucose permitted good bacterial growth without pigment production. Investigation showed that ferrous sulphate was inhibitory at the normal concentration of 10 mg./l and also at 1 mg/l. and that a medium without added ferrous sulphate still did not support pigment production unless 0.025 % asparagine was also added; then strong pigmentation resulted. Replacement of the asparagine with 1 % peptone allowed only weak pigment production. This suggested that the fungi decreased the iron content of Czapek-Dox agar to a value suitable for bacterial pigment formation and also supplied asparagine or some related substance required by *P. aeruginosa*. However, paper chromatography of the fungal filtrate did not show asparagine, aspartic acid or any other amino acid.

Layered plates of *Aspergillus terreus* and *Staphylococcus aureus* showed that *S. aureus* produced material capable of diffusing through agar and cellophan and causing the same hyphal distortion as in growth of mixed cultures. The addition of a bacterial culture filtrate to nutrient agar seeded with the *A. terreus* resulted in decreased fungal growth rate but no hyphal abnormalities. Investigation of the properties of this filterable antifungal material showed that: (i) incubation for 24 hr was optimum for production of the toxic material; (ii) the amount of toxic material produced by *S. aureus* in nutrient broth was not increased by adding 1 % glucose or glycerol; (iii) the toxic material withstood autoclaving at 116° for 10 min., was adsorbed on activated charcoal by boiling, was not extracted by chloroform or ether and was not precipitated by ethanol or acetone from aqueous solution. Since a concentrated solution of the toxic material was not prepared it was impossible to ascertain whether it was identical with that bacterial product which diffused through cellophan and induced hyphal tip branching in mixed fungal and bacterial culture.

**DISCUSSION**

Stokes, Pick & Woodward (1942) found that 1-hydroxyphenazine was more active against dermatophytes and *Candida albicans* than pyocyanine while the reverse was true for bacteria. My results show that *Aspergillus terreus* behaved like dermatophytes. Very little is known of the identity or quantity of substances available within the human ear for growth of micro-organisms. If conditions in the ear resemble those in nutrient agar, the toxic material of *Pseudomonas aeruginosa* might be expected to prevent fungal infection, but Singer et al. (1952) found *P. aeruginosa* in 50 % of ears infected with Aspergillus species. On the other hand, the interaction might resemble more closely that observed on Czapek-Dox agar where one organism benefited from the presence of the other on the particular substrate. The production of toxins by *Shigella dysenteriae* (Dubos & Geiger, 1946) and *Corynebacterium diphtheriae* (Pappenheimer,
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1947) is related to the concentration of iron in the medium and perhaps the inhibitory effect of iron observed in the present work was a complex reaction such as that seen with C. diphtheriae.

The filterable antifungal toxic material produced by Staphylococcus aureus is not identical with any of the well-known toxins such as the haemolysins, dermonecrotins, hyaluronidase and leucocidins produced by this organism (Elek, 1959), since it withstands autoclaving. The addition of glucose to nutrient broth did not increase the amount of filterable antifungal toxic material by S. aureus but increased the sensitivity of the fungus to the bacterium in mixed culture. The production of dichotomously branched hyphal tips in the presence of S. aureus is similar to the response which may occur in otomycosis and in pulmonary aspergillosis. It is suggested that the hyphal swellings seen in Aspergillus terreus are severe expressions of the swelling noted by Robertson (1958) to occur before division of the hyphal tip following any check in growth which lasts 60 seconds or more. Only a small proportion of the A. terreus hyphal tips produced these swellings. A. fumigatus, which is a faster growing organism than A. terreus, showed less sensitivity to S. aureus both in nutrient broth and in the glucose or glycerol containing media.

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REFERENCES


EXPLANATION OF PLATE

(a) Aspergillus terreus 20 hr culture in nutrient broth with Staphylococcus aureus added after 8 hr. (b) A. terreus+S. aureus as (a) in glucose peptone broth. (c) A. fumigatus+S. aureus as (a) in glucose peptone broth. (d) A. terreus 20 hr culture in nutrient broth. Magnification × 800.