PUBLICATIONS OF THE CAGLIARI INSTITUTE OF HYGIENE

RESEARCH ON A NEW ANTIBIOTIC

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CAGLIARI - Tip. C. E. L. 1948

Research on a new antibiotic

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Studies on a new fungus

In the course of studies aimed at searching for germs endowed with antibiotic potency I focused my attention on an examination of the microbial flora of sea water in the vicinity of a sewage outlet, taking as my starting point the assumption that the self-purification processes of the water itself were to some extent also the result of bacterial antagonism and that there was good reason to expect that the water contained germs (mycetes and schizomycetes) whose study would yield interesting results from this point of view.

The germ we have studied is, in fact, one of the numerous bacterial and fungal isolates whose antibiotic potency was assayed.

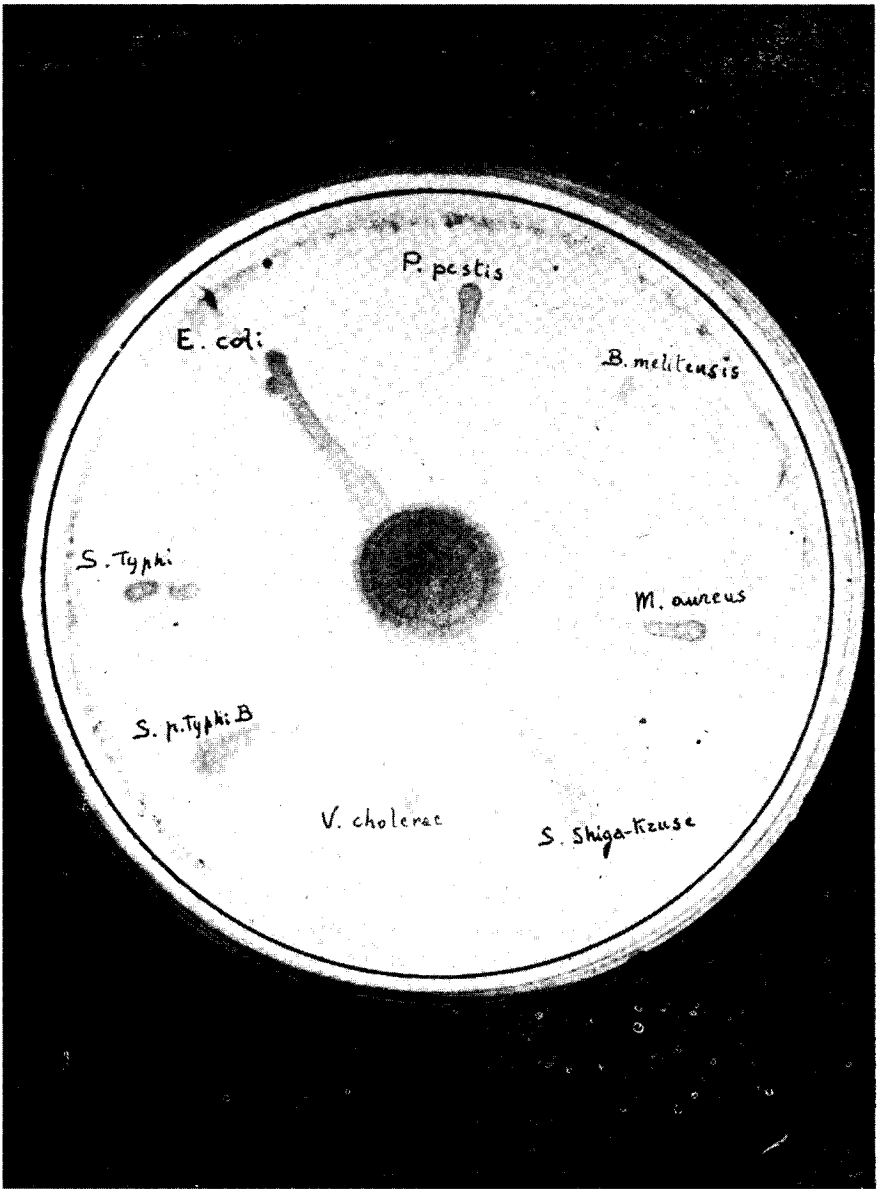
It was isolated in July 1945 from a sample of water taken from the above-mentioned location, seeded on common agar and incubated at room temperature. On completion of development, colonies of a very large number of germs were isolated, and for each of these we assayed the antagonistic potency against Staphylococcus aureus, Eberthella typhi, V.cholerae, B.anthracis and Br.melitensis.

For this purpose, a smear was produced on agar plates and, after 3 to 4 days' development, a loopful of culture broth of the above-mentioned germs was deposited in the vicinity of the development area. It thus proved possible to identify in preliminary fashion the existence, if any, of an antibiotic principle in the agar in the zone bordering on the development area.

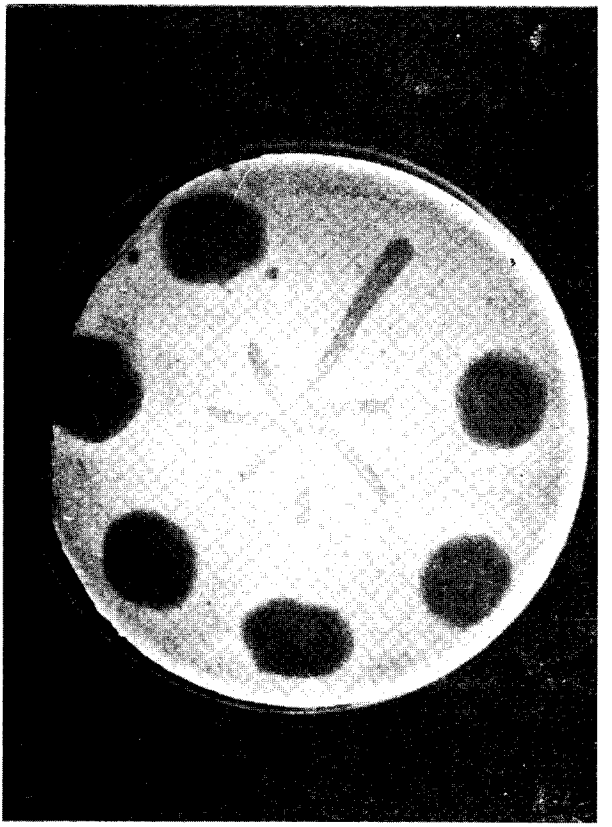
With this very simple technique, we were able to study hundreds of germs and to choose, from among them, our particular fungus, which right from the very first few isolations was found to possess pronounced inhibitory activity.

Study of the germ

The colonies in common agar or on Sabouraud Agar at three daysí development have diameters ranging from 0.7 to 0.8 cm. They are white in colour and are composed of a more compact central zone consisting of a tangle of hyphae surrounded by a halo of radial filaments.



Three-day colonies. The various bacterial species are seeded with loops up to the margin of the fungal colony. The undeveloped area indicates the potency of the antibiotic on the individual species.



Three-day fungal colonies. Eberthella typhi was loop-seeded up to the margins of the individual colonies and, for control purposes, in the space between two colonies.

Characteristics of the antibiotic principle

At the present time we are unable to give any information regarding the chemical composition of the principle. All we can do is indicate a number of its characteristics.

Heat resistance. In solution in extracts or culture the germ presents a certain degree of resistance to temperature. Even temperatures of 100°C, albeit only for a few minutes, fail to destroy it.

Time resistance. Assays on materials in the dry state could not be performed. In sodium phosphate solution the bactericidal titre at temperatures of 4 to 5°C decreases slowly but progressively. At a temperature of 37°C disappearance of the antibiotic potency is distinctly more rapid.

Relationships between antibiotic principle and pH of the medium. Production of the antibiotic principle is greater around days 7 to 8 on media containing starch or carbohydrates. This production coincides with changes in the pH of the culture medium, an initial decrease in pH being observed followed by a slow increase.

Initial pH	7.2
after 1 day	6.8
after 2 days	6.6
after 3 days	6.6
after 4 days	6.6
after 5 days	6.7
after 6 days	6.8
after 7 days	7.0
after 8 days	7.2
after 9 days	7.4
after 10 days	7.6
after 11 days	7.8

Production of the antibiotic principle, which appears to be very substantial in agar, is subject to limitations in cultures in liquid media. The first few tests conducted on Czapek and other synthetic media yielded thoroughly unsatisfactory results. The concentration of the antibiotic principle was so low, in fact, that the dilution of the culture liquid allowing inhibition of development generally speaking did not exceed 1 to 10.

A better response was obtained with common broth containing 1% glucose and starch. In this medium, titres of 1 : 15 were achieved around days 4 to 8, whereas in common broth such titres were obtained around days 10 to 13. The antibiotic principle content in cultures maintained at a temperature of 30°C declines rapidly almost to the point of complete disappearance.

Numerous attempts were made to improve the production of antibiotic principle on liquid media.

The actions of various amino acids such as histidine, leucine, glycolic acid and valine, as well as of uric, glutamic and tartaric acid were studied.

Broths with extracts of germinated barley, beer yeast, wheat germs and peas were tested, but the results were not very satisfactory. A slight advantage was achieved only with the addition of hypoxanthine, valine, cystine and glutamic acid. It is not hard to perceive a link between the formulae of the latter three substances.

Progressive selection of the fungus with agar cultures. By means of patient consecutive isolations of colonies obtained on plates we were able, after several hundred cultures in series, to achieve strains which yielded production of antibiotic principle active up to the 1:100 dilution in glucose-starch broth around day 10.

Particularly worthy of note is the fact that these characteristics can easily be lost when the strains are maintained in liquid medium at ambient temperature and even at a temperature of 5°C.

Extraction of the antibiotic principle. The antibiotic principle was extracted from agar cultures and from liquid cultures.

The antibiotic principle can be extracted from agar cultures. As we have already said, when a fragment of agar from a point adjacent to a colony of Cephalosporium is placed in broth, the Cephalosporium prevents further development of Eberthella typhi. This indicates that the principle evidently spreads in the aqueous medium of the culture. The principle, however, can be extracted not only with water, but also with alcohol. Cephalosporium culture agar is ground down in a flask and suspended in alcohol at 95°C. The alcohol, which turns more or less bright yellow in colour, extracts the principle. Cultures are particularly active around days 5 to 6, whereas the younger and older (beyond days 8-10) do not contain detectable amounts of the principle. The active principle can be concentrated by evaporating the alcohol at low temperature in a vacuum.

Extraction from liquid cultures has been the subject of lengthy investigations aimed at achieving purification of the principle and concentration in a small volume. Owing to the lack of adequate equipment, the difficulties encountered in this connection have been enormous.

The methods used for the preparation of penicillin were tried but the results were not very satisfactory.

Thus, in the end, we prepared a concentrate by rapidly evaporating the culture liquid under air flow at 25°C after filtering through cotton to a concentration equal to 1:10 and precipitating the protein substances with ethyl alcohol at 98°C in the proportion of 1.5 parts to 1 of concentrate.

From the cotton-filtered liquid thus obtained the alcohol is distilled by vacuum aspiration and heating to 37-40°C.

The liquid obtained is yellow-brown in colour and slightly fluorescent. There can be no doubt that it contains many other substances in addition to the active principle.

Clinical trials with the culture liquid

Trials have been conducted directly with the culture liquid on both staphylococcal and streptococcal surgical clinical conditions, particularly carbuncles, phlegmons and abscesses, especially by direct inoculation into the focus for inflammation.

a) In all the cases treated in which an inflammatory process was in progress, during the infiltration stage or with an abscess collection already formed, at the very first inoculation of the inflamed tissue in the former case and on injection into the abscess cavity in the latter, a disagreeable local burning sensation lasting a few hours and gradually diminishing in intensity was followed by disappearance of the tensive, throbbing pain typical of such foci of inflammation.

The pain, when it recurred, which in no case happened within 12-24 h if there was no second administration, never reached the same degree of severity as the patient experienced prior to treatment.

b) Disappearance of the pressive, throbbing pain in the site of inflammation was invariably accompanied by a sense of general well-being.

c) Except for a few cases presenting a particular pattern of behaviour, fever yielded to the first administration of the treatment, sometimes even in cases of critically high temperatures.

d) Particularly worthy of note were the findings relating to the inflammation foci, which provided unequivocal evidence of the efficacy of the study preparation.

The very first inoculation of abscess collections was followed by distension: heat, redness and swelling disappeared within 24 h, paralleling the above-mentioned disappearance of pain and functional impairment. Relief of subjective pain was also accompanied by cessation of tenderness on palpation of the affected area.

The regression and even the arrest of the inflammatory process could be clearly observed during the infiltration phase; in these cases, infiltration of the drug into the inflammatory tissues was followed by healing.

e) The clinical findings are confirmed by the results of bacteriological examinations.

In all the cases studied, with only a few exceptions, the samples of material taken from the infection sites proved bacteriologically sterile.

A particular application was used in a case of tinea and in a kerion by means of packs with mycelial pads which formed in the fungal culture. Healing of the ringworm was obtained in 40 days, while the kerion took only 20 days to heal.

Clinical trials with culture liquid extracts

The culture liquid, in the 10% concentrate form produced according to the technique described above, was prepared for general rather than local use and was inoculated intravenously, intramuscularly and rectally.

The liquid, especially when inoculated intravenously, produces a substantial feverish reaction with the result that it cannot be used regularly by this route. The febrile reaction is less when the administration is intramuscular, but such injections prove painful for most (though not all) patients and are poorly tolerated. Rectal administration is generally well tolerated, though the absorption is probably limited in adults.

This extract has been used, obviously with a certain amount of difficulty due to the above-mentioned drawbacks, in cases of typhoid fever, paratyphoid fever A, paratyphoid fever B and brucellosis.

The results can be summarised as follows:

1) The patient's general condition generally shows a marked improvement, particularly in cases of Eberthella infection.

2) The fever resulting from the pyrogenic effect of the product - due, in all probability, not to the antibiotic content, but to the substantial impurities it undoubtedly contains (so much so that there are differences between preparations despite their having the same content of antibiotic principle) - increases over the first 3 to 4 days, then fluctuates for a period and eventually falls after discontinuing the drug around days 4 to 6.

Sometimes the drop in temperature occurs in 24 h, while in serious cases of typhoid fever with various complications the reduction in temperature may take as long as 7 to 8 days.

3) In Brucella infections, a recurrence of fever has sometimes been noted on discontinuing the treatment too soon.

4) No other adverse events worthy of note have been observed above and beyond those already mentioned.

CONCLUSIONS

The results of the present studies appear to suggest that this antibiotic principle produced from Cephalosporium acremonium may have a very extensive range of applications.

Its in-vitro activity against staphylococci, streptococci, B.anthracis, Eberthella typhi, V.cholerae, B.pestis and B.melitensis, and its efficacy in staphylococcal and streptococcal infections, typhoid fever and brucellosis, as well as in-vivo trials in human subjects, despite the limitations due to difficulties in extracting the antibiotic principle, suggest that this antibiotic may have a distinctly promising therapeutic potential.

These findings have been reported here in the hope that other better equipped institutes may be able to make greater progress in the selection of the fungus and in the culture preparation and extraction of the antibiotic.