

PATHOGENESIS OF CANDIDA ALBICANS INFECTION FOLLOWING ANTIBIOTIC THERAPY

I. THE EFFECT OF ANTIBIOTICS ON THE GROWTH OF CANDIDA ALBICANS

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In reporting the work of a penicillin therapeutic research unit, Christie and Garrod (1944) cited a case of actinomycosis treated with penicillin by the intravenous drip method. The patient succumbed during therapy to a *Pseudomonas aeruginosa* septicemia with the primary focus at the site of the infusion. Although these authors did not ascribe any particular influence of the therapy on the rapid development of the fatal septicemia, nevertheless, in later reports, they were impressed by the number of secondary infections with gram negative organisms which followed upon the continued use of penicillin. Since that time there have been many such reports and these have been the subject of a number of recent review articles (Editorial, 1952; Finland, 1951; Garrod, 1951; Keefer, 1951; Miller, 1951). Many microorganisms have been incriminated now as opportunists, the most frequent offenders being gram negative bacteria and the yeast-like fungus *Candida albicans*.

A number of theories have been proposed to explain the increased incidence of candidiasis as a complication of antibiotic therapy. One is the "suppression with substitution" thesis (Miller, 1951). This maintains that the administration of antibiotics upsets the equilibrium in which the normal flora exists, resulting in the virtual elimination of the susceptible microorganisms and, as a consequence of reducing the numbers competing for the environmental food supply, permitting the resistant species to increase vastly their population and thereby to overwhelm the host resistance. A second theory (Harris, 1950) claims that the normal flora supplies certain nutritional requirements to the host. This author postulates that the disturbance in the normal flora, as a consequence of antibiotic therapy, results in a nutritional disturbance which affects the integrity of the mucous membranes of the host, opening a portal of entry for microorganisms which

normally are unable to penetrate the intact healthy mucosa. A third explanation suggests that some of the antibiotics directly stimulate the growth and/or virulence of *C. albicans* (Foley and Winter, 1949; Pappenfort and Schnall, 1951; Moore, 1951).

It occurred to the present authors that an approach to an understanding of this problem would best be made through quantitative *in vitro* studies of the effect of antibiotics on *C. albicans* in isolated systems. The results of such investigations then could be applied to *in vivo* environments. The work reported here represents the results obtained from the *in vitro* studies.

EXPERIMENTAL METHODS

Stock cultures of four strains of *C. albicans* were maintained on Sabouraud's glucose agar incubated continuously at 37 C and transferred to fresh media at weekly intervals. For all other purposes the medium used was a semisynthetic broth (Keeney and Eriksen, 1949) with the following composition: K₂HPO₄, 0.75 g; NaCl, 1.00 g; MgSO₄·7H₂O, 1.50 g; ferric citrate, 0.02 g; glucose, 10.00 g; casamino acids (Difco), 5.00 g; distilled H₂O to make 1 L. The pH of the broth was adjusted to 6.0, and the medium was dispensed in 50 ml volumes in 250 ml Erlenmeyer flasks followed by sterilization in the autoclave at 15 lb for 15 minutes. In some cases the antibiotics were dissolved in the broth and the mixture sterilized by filtration through a sintered glass disc.

For making cell counts, turbidity measurements, and nitrogen determinations the fungus growth was separated from the broth by centrifugation and washed three times with 0.9 per cent aqueous NaCl adjusted to pH 6.0 with N HCl. The saline solution was made acid because a precipitate formed in the broth under neutral and alkaline conditions. This precipitate dis-

solved in the acid saline solution. Cell counts were made with a Levy counting chamber with the Neubauer ruling. Turbidity measurements were done with a Klett-Summerson photoelectric colorimeter. The micro-Kjeldahl method as described by Kabat and Mayer (1948) was used in making nitrogen determinations.

In preliminary experiments a comparison was made between direct cell counts and nitrogen determinations as methods for standardizing the initial inoculum. For the former method the cellular suspension in the final washing was centrifuged at high speed for 15 minutes in a calibrated tube. The volume of cells was read directly and then diluted quantitatively to a volume convenient for making counts in the Levy chamber. An analysis of repeated counts revealed that a single determination might deviate by 10 per cent or more from the average of a series of such counts.

The micro-Kjeldahl determination of nitrogen proved to be a much more reliable index of cellular growth. Several flasks of the broth medium were inoculated from stock cultures, and the growth after 48 hours' incubation was separated and washed repeatedly by centrifugation. Quantitative dilutions of the sediment were made and samples from each dilution were measured for turbidity and for total cell nitrogen. Statistical analysis of these results indicated that the chances against an individual nitrogen determination exceeding the average by 5 per cent or more were 100:1. This method was used exclusively for standardization of inoculum and for determination of the results of growing the *C. albicans* in the presence of the various antibiotics.

Several flasks of the broth medium were inoculated from stock cultures and incubated for 48 hours, at which time the cells were separated and washed repeatedly. Quantitative dilutions were made from an initial suspension of these washed cells and the turbidity of the suspensions measured. From a previously calibrated curve for the turbidity-total cell nitrogen relationship the concentration of cell nitrogen in the suspension was interpolated, and the original dilution was re-adjusted with sterile saline solution to contain 0.1 mg nitrogen per milliliter (mg N/ml). One-half milliliter of this final suspension, or 0.05 mg N/ml, was used for inoculating each flask of broth in the experiment. The antibiotics were

dissolved in the broth to make a series of tenfold dilutions. The highest concentration was selected at approximately 100 times the maximum blood level commonly attained. For orally administered antibiotics this approaches the level attained in the stool. An exception had to be made with aureomycin, however, since at this concentration all of the aureomycin preparation did not go into solution. Nevertheless, the highest concentration of aureomycin used is considerably greater than the common therapeutic levels in serum and only slightly less than that commonly attained in stool (Welch, 1950). In the streptomycin series the pH of the broth was adjusted to 7.5 since this antibiotic is most active under alkaline conditions. Control flasks were run with each series of experiments, and every experiment was run in duplicate. All the cultures were incubated at 37 C for 48 hours. At the termination of the incubation period the flasks were placed in a boiling water bath for 15 minutes, the cells were separated and washed repeatedly, and then transferred quantitatively to a 50 ml volumetric flask and made up to volume. Aliquots were removed for nitrogen determinations.

RESULTS

The results presented in figure 1 are typical. A series of 21 controls, in which *C. albicans* was grown in the broth and harvested in the same manner as for the experiments with the antibiotics, yielded an average of 1.01 mg N/ml with a standard deviation of 0.07. The value $\pm 3\sigma$ has been plotted to indicate the limits of possible random variations in the procedure. It is obvious that, whereas penicillin, chloramphenicol, terramycin, and streptomycin had no effect—either stimulatory or inhibitory—on the growth of *C. albicans*, aureomycin very definitely increased the total amount of growth as compared to the controls. Statistical analysis of the results with aureomycin would indicate that the chances against this effect being caused by random variation are of the order of 1,000,000:1. All four strains of *C. albicans* yielded the same results in slightly varying degree.

The stimulation of *C. albicans* by aureomycin was not confined only to one type of preparation. The same procedure was repeated with three different lots of aureomycin, and the results are shown in figure 2. The lot of aureomycin with

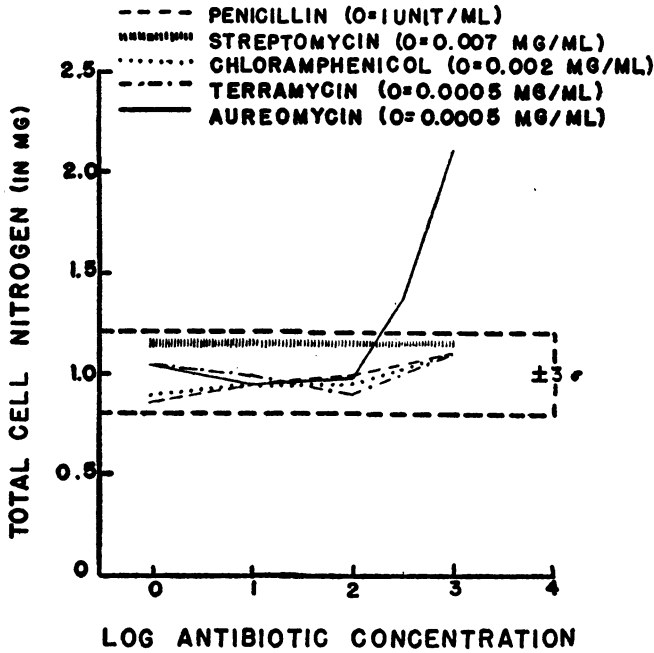


Figure 1. The effect of various antibiotics on the growth of *Candida albicans*, strain 412. The $\pm 3\sigma$ limits were calculated from a series of 21 control flasks. The slightly higher values for streptomycin result from growth in broth with an initial pH of 7.5.

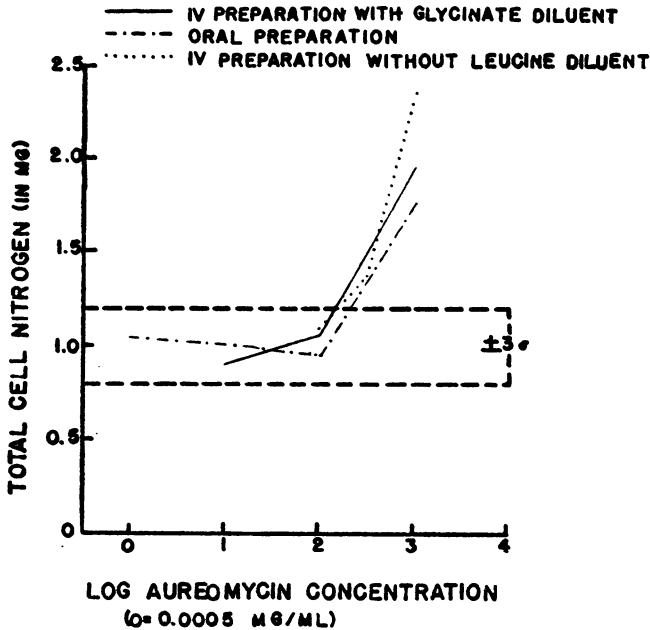


Figure 2. The effect on the growth of *Candida albicans*, strain 412, of three different lots of commercially available aureomycin preparations. One intravenous preparation was supplied with leucine diluent in separate vials but only the vial containing aureomycin alone was used.

leucine diluent for intravenous administration was used without the diluent to test the effect of the antibiotic separately from any commercially added substances. This vial contained pure aureomycin hydrochloride made by lyophilizing a sterile solution of the antibiotic. The greatest amount of stimulation recorded in these experiments was obtained with this preparation.

It was found that, under the conditions of the experiment, a precipitate appeared in the higher concentrations of aureomycin flasks which had not been inoculated with the fungus. The total nitrogen in this precipitate proved to be negligible, however, and did not affect the results sufficiently to warrant subtracting this value from the total nitrogen obtained for the flasks which had been inoculated.

DISCUSSION

From the literature of the last few years it would appear that an increased incidence of candidiasis is a definite complication of antibiotic therapy. McVay and Sprunt (1951) in culture studies from oral, vaginal, and rectal specimens on a total of 186 patients found that, when aureomycin was administered, 63 per cent of their patients yielded positive stool cultures for *C. albicans* where none had been positive before therapy. This has been observed with most of the antibiotics administered orally or parenterally. Adequate proof of the biological mechanisms involved is still lacking although several explanations have been offered.

Harris (1949, 1950) felt that the mucous membrane complications following aureomycin therapy were attributable to vitamin B complex deficiency resulting from the elimination of the intestinal bacteria necessary for the synthesis or utilization of these compounds. When a potent vitamin B complex preparation was administered parenterally to patients receiving aureomycin, he found the incidence and severity of mucous membrane lesions reduced. As Harris points out, however, this combined treatment was studied in only 11 patients, and, furthermore, the fact that the lesions develop rapidly would argue against the etiology of avitaminosis. In spite of these arguments, he felt that the reduction in the numbers of the intestinal bacteria would allow the overgrowth of *C. albicans* which then could invade tissues whose resistance had been lowered by a vitamin B complex deficiency.

Woods, Manning, and Patterson (1951) reported *in vitro* experiments which gave no indication that *C. albicans* was stimulated by aureomycin, penicillin, or chloramphenicol. They stated that, in their opinion, ". . . the most important single factor in the overgrowth of *C. albicans* following the use of antibiotics . . ." is the elimination of bacteria competing with this fungus for the environmental food supply. The highest concentration of aureomycin used in their studies was 0.1 mg/ml, and it is only at higher levels of antibiotic concentration that the stimulatory effect of aureomycin becomes apparent. Furthermore, the role played by mere competition by bacteria for nutritive substrate cannot be of major importance since a number of studies of the effect of antibiotics on the intestinal flora indicate that the change is qualitative rather than quantitative; in fact the total bacterial count after continued antibiotic therapy frequently exceeds that existing before the administration of the drugs (Bierman and Jawetz, 1951; Dearing and Heilman, 1950; Lipman *et al.*, 1946; Marshall *et al.*, 1950; Metzger and Shapse, 1950; Spaulding *et al.*, 1949).

The studies of both Moore (1951) and Pappenfort and Schnall (1951) are in conflict with the *in vitro* results reported by Woods, Manning, and Patterson (1951). Moore grew *C. albicans* in Sabouraud's glucose broth containing crystalline aureomycin hydrochloride in a concentration of 0.2 mg per ml and noted that in these flasks the yeast growth appeared to be approximately twice as great as in flasks inoculated similarly but without the antibiotic. In addition, he reported that the cells on the bottom of the flasks in close proximity to undissolved crystals of the antibiotic were considerably larger than normal cells, and that the yeast cells in the veil of growth on the surface of the broth were markedly smaller than normal. Both of these observations were interpreted as evidence of stimulation of the growth of *C. albicans* in the presence of aureomycin. It may be noted here that the concentration of aureomycin used by Moore falls in the range of concentrations resulting in evidence of increased growth under the conditions of the experiments reported by the present authors. Pappenfort and Schnall measured the effect of aureomycin on *C. albicans* using the diffusion plate method. The organisms were suspended in Sabouraud's glucose agar in a petri dish, and a

solution of aureomycin was placed in cups in the agar. A zone of increased growth was noted in 4 days in the area of diffusion around the cups. The same effect was obtained with 6 different lots of aureomycin as prepared for oral administration, but no stimulation appeared with either of 2 lots of aureomycin as prepared for parenteral administration. In both of these reports, all observations were made macroscopically or microscopically, and no attempt was made to obtain results on a quantitative basis. In the experiments reported by the present authors the same macroscopic observations were noted, and they were corroborated by the quantitative determination of increased cellular metabolism in terms of cellular nitrogen. The quantitative methods revealed that not only oral aureomycin preparations but also parenteral preparations were capable of stimulating the growth of *C. albicans*.

It would appear that direct stimulation of *C. albicans* by aureomycin should receive consideration in any explanation of this problem. While this would account for the greater frequency with which aureomycin is implicated as compared to the other antibiotics, it would not explain why the same complication arises after therapy with the other drugs for which no similar stimulatory effect has been demonstrable. It has been suggested that changes in the pH of the intestinal menstrium and also that an enriched medium resulting from the destruction of intestinal bacteria both play a part (Foley and Winter, 1949; Woods *et al.*, 1951). Since *C. albicans* grows equally well over a wide range of pH, it would seem doubtful that such a change could play a significant role in the pathogenic biology of this microorganism in this instance. Furthermore, *C. albicans* has only very minimal essential growth factor requirements, and it would seem questionable whether the death of even large numbers of bacteria would add to the environment any materials which were required by this yeast and which had not been available previously. It might very well be that some of the normal bacterial inhabitants of the intestinal tract exert a specific antibiosis effect on *C. albicans*, and that the elimination of this agent permits the overgrowth of the fungus and allows it to exert its full pathogenic potentialities. If this should be so, the presence or absence of this antibiosis and its elimination following antibiotic therapy could explain the presence of *C.*

albicans in some apparently normal intestinal tracts and also its rapid appearance subsequent to therapy. The direct stimulatory effect of aureomycin would account for the fact that this antibiotic is involved more frequently than the others since the enhancement of the *C. albicans* growth might be sufficient to overcome the specific antibiosis. Further studies along these lines are now in progress.

SUMMARY

Determination of the total cell nitrogen with the micro-Kjeldahl method is a reliable measure of the amount of growth of *Candida albicans*.

Three different lots of aureomycin hydrochloride when present in a broth culture medium in concentrations greater than 0.1 mg per ml stimulate the growth of *C. albicans* significantly while penicillin, chloramphenicol, streptomycin, and terramycin do not show a similar effect.

Several explanations for the increased incidence of candidiasis following continued antibiotic therapy are discussed.

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