SENSITIVITY CHANGES OF ACTINOMYCES BOVIS TO PENICILLIN AND STREPTOMYCIN

ARTHUR BOAND AND MILAN NOVAK

Department of Bacteriology, College of Medicine, University of Illinois, Chicago, Illinois

Received for publication February 9, 1949

Actinomycosis in the human being is a chronic, suppurative disease resulting from infection with the fungus *Actinomyces bovis*. Prior to the advent of the sulfonamides and later penicillin and streptomycin, the prognosis of patients suffering from actinomycotic infections was not too promising.

There are many reports in the literature concerning the successful use of penicillin as a chemotherapeutic agent (Decker, 1946; Farris and Douglas, 1947), but there is little in the literature on the clinical use of streptomycin in the treatment of actinomycosis. Costigan (1947) and Jacobson and Cloward (1948) have reported success in using this antibiotic in these infections; however, Pulaski and Seeley (1948) reported a case of actinomycosis treated with streptomycin that failed to respond to therapy and that was associated with the development of a highly resistant organism. The etiological agent in this case, however, was *Nocardia asteroides*, an organism closely related to *Actinomyces bovis*, which is not frequently associated with actinomycotic infections.

In view of the chronic nature of the disease, long therapeutic periods have been indicated with the time of treatment varying from 5 days (Costigan, 1947) to 1 year or longer (Emmons, 1938). Since there are a number of reports concerning the rapid development of bacterial resistance to the antibiotics, especially to streptomycin, in which Miller and Bohnhoff (1946) and Seligman and Wassermann (1947) report that resistance may attain a 2,000- to 50,000-fold increase, it is well to consider the possibility that resistant strains of *Actinomyces bovis* may develop during a long therapeutic period.

METHODS

Throughout this work thioglycolate medium was used; and though it is known that this medium contains factors that are antagonistic to the antibiotics, it was chosen because it presented a simple method of culturing these organisms and the results of growth could be easily read. With this medium the several strains of anaerobic *Actinomyces* could be grown at 37 C. The results were read on the basis of visible growth alone.

Because of the small amounts of penicillin and streptomycin required to inhibit the growth of these organisms, it was necessary to prepare the various dilutions in large quantities in order to obtain as high a degree of accuracy as possible. One-hundred-ml amounts of thioglycolate medium were prepared and a suitable amount of the antibiotic was added to give the desired concentration. The medium containing the antibiotic was then well mixed and transferred aseptically to sterile test tubes, about 10 ml per tube. These were then incubated

overnight at 37 C to eliminate any contaminated tubes. Each tube was then inoculated with 0.1 ml of a young growing culture and incubated at 37 C for the desired time.

Since the strains of *Actinomyces* used in these experiments had been adapted to thioglycolate medium, growth was usually observed within 24 to 48 hours. In all cases the control tube containing no antibiotic grew in this time, but in some instances those containing the antibiotic required a longer period for growth. If growth did not occur within 5 days, the tubes were considered negative. There was no marked change in growth between 5 and 10 days.

The six strains of *Actinomyces* used in this work were of several origins. Four were isolated from cases of human actinomycosis; two were of bovine origin, being isolated from cases of lumpy jaw in cattle. Three of the human strains were obtained from the Mayo Clinic through the kindness of Dr. F. R. Heilman. All strains presented typical morphology, though slight growth differences were noted in thioglycolate, as is typical of these organisms.

PENICILLIN

Since there are certain factors in thioglycolate medium that antagonize the effect of penicillin to a certain extent, and since penicillin is somewhat unstable at 37 C, it was essential to determine how much of the activity was lost in this medium at 37 C over a 10-day period. A concentration of 1.0 unit per ml was prepared in thioglycolate medium and was incubated over the 10-day period. Samples were taken every 24 hours and titrated to determine the loss of activity, with *Staphylococcus aureus* as a test organism. A similar test was run with 1.0 unit per ml in water in order to determine whether any loss of activity was due to the thioglycolate medium or to the temperature of incubation, or to a combination of these factors. It is realized that this type of test does not show the effect of the metabolic processes of bacterial growth, but it does demonstrate the inactivation of the antibiotic by the components of the medium and the temperature of incubation. It was found that there was no loss of activity through 48 hours, but a slight loss was noted at 72 hours. No further loss was noted in thioglycolate medium until the seventh day. Penicillin in water, however, steadily declined in potency until no activity remained after 4 days. It is evident that penicillin in thioglycolate medium at 37 C is relatively stable. It was therefore unnecessary to add additional antibiotic during incubation since growth occurred in controls within 5 days. The sodium salt of penicillin was used throughout this work. Solutions were prepared in sterile distilled water as previously described.

On determining the initial penicillin sensitivity of the various strains, it was found that all strains grew in less than 0.05 units per ml within 24 hours. At and above that level the individual strains seemed to vary somewhat in their sensitivities (table 1). Two strains were inhibited by 0.05 units per ml, two strains were inhibited by 0.1 unit per ml, and two strains were inhibited by 0.5 units per ml. One strain showed growth in one of the four tubes at 0.5 units per ml. The growth in this tube may have been due to a larger number of

organisms in the inoculum since this was difficult to control accurately because of the nature of the organisms.

In an effort to determine whether the various strains would become resistant to penicillin *in vitro*, all strains were inoculated into thioglycolate medium containing the maximum amount of penicillin that would allow visible growth in 72 hours. In all cases only slight growth was noted at this time; this growth was then transferred to a medium containing the same concentration of penicillin in an effort to adapt the organisms to growth in the presence of the antibiotic. After 7 such transfers, all strains grew in 0.1 unit per ml in 72 hours. After 12 transfers, one strain grew in 0.2 units per ml, and two strains grew in 0.15 units

TABLE 1

Initial penicillin sensitivity of Actinomyces bovis strains

CONCENTRATION	H1	H3	M	D	P	Y
*Control**	++++	++++	++++	++++	++++	++++
0.05 u/ml	----	++++	++++	----	++++	++++
0.10 u/ml	----	----	---+	----	--++	--++
0.50 u/ml	----	----	----	----	--+-	----

* Control showed growth in 24 hours.

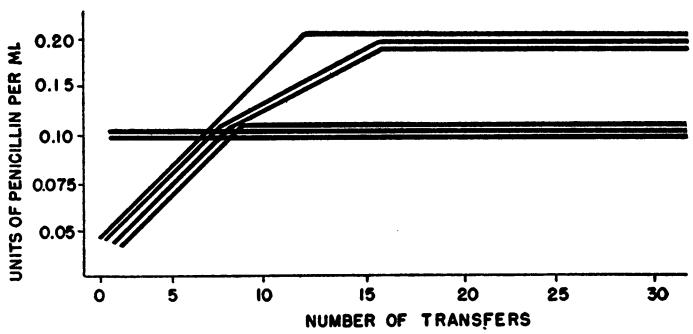

Figure 1. Increase of penicillin tolerance by six strains of *Actinomyces bovis* on repeated subculture to increasing concentrations of penicillin in thioglycolate medium.

per ml. After 16 transfers, three strains were able to grow in 0.2 units per ml, whereas the other three strains failed to grow in concentrations above 0.1 unit per ml. Repeated transfers were made in the maximum amount of penicillin that allowed growth, but, after 32 transfers over a period of 3 months, there was no further change in resistance from that which had been encountered on the sixteenth transfer (figure 1).

Although it was found that four of the strains developed a 2- to a 4-fold resistance to penicillin, it is evident that, *in vitro*, the strains of *Actinomyces bovis* tested did not become readily adapted, or resistant, to the antibiotic effect of penicillin.

STREPTOMYCIN

Owing to the numerous factors that are inhibitory to the action of streptomycin, a brief study of the loss of streptomycin activity under the conditions of the test was made. A test similar to that performed with penicillin was run. Thioglycolate medium was prepared so as to contain 50 units per ml of streptomycin and was incubated over a 10-day period at 37 C, with samples being titrated daily.

It was found that streptomycin lost no activity in thioglycolate medium at 37 C for a period of 48 hours, but beyond that time a rapid loss occurred. It became totally inactive after 120 hours' incubation under these conditions. In water, however, streptomycin was relatively stable at 37 C, as no loss was noted through 96 hours, and at 10 days only a slight loss had occurred. Throughout this work the calcium chloride complex of streptomycin was used. It was diluted in sterile distilled water and mixed with thioglycolate medium as described.

The streptomycin sensitivity of these organisms was found to be between 20 to 30 units per ml, since all strains were inhibited by 30 units per ml, but grew

TABLE 2

Initial streptomycin sensitivity of Actinomyces bovis strains

CONCENTRATION	H1	H3	M	D	P	Y
*Control**	++++	++++	++++	++++	++++	++++
20 u/ml	++++	++++	++++	++++	++++	++++
30 u/ml	----	----	----	----	----	----
50 u/ml	----	----	----	----	----	---+
100 u/ml	----	----	----	----	---+	----

* Control showed growth at 24 hours.

slightly in 20 units per ml (table 2). Again it was noted that one human strain showed slight growth in 50 units per ml and one bovine strain grew in 100 units per ml. Since in each case this occurred in only one of four tubes, it may have been due to the number of organisms in the inoculum or to the presence by chance of naturally resistant variants.

Since only slight growth was noted in 96 hours in 20 units per ml, it was presumed that most of the sensitive organisms were inhibited and that only the more resistant forms were able to grow. This growth was then inoculated into media containing 25 units per ml and growth was noted in 72 hours. The next transfer into 30 units per ml grew in 48 hours. Subsequent transfers were made into 35, 50, 100, 200, 500, 750, 1,000, and 5,000 units per ml. All of these tubes showed growth within 24 hours after transfer, as did the control tubes containing no streptomycin. There was no evidence indicating that streptomycin dependence was developed along with resistance.

Since all strains had readily developed a high degree of resistance (figure 2), higher concentrations were not employed. Penicillin sensitivities run on the streptomycin-resistant strains indicated that the development of streptomycin resistance did not alter the penicillin sensitivity of the organisms.

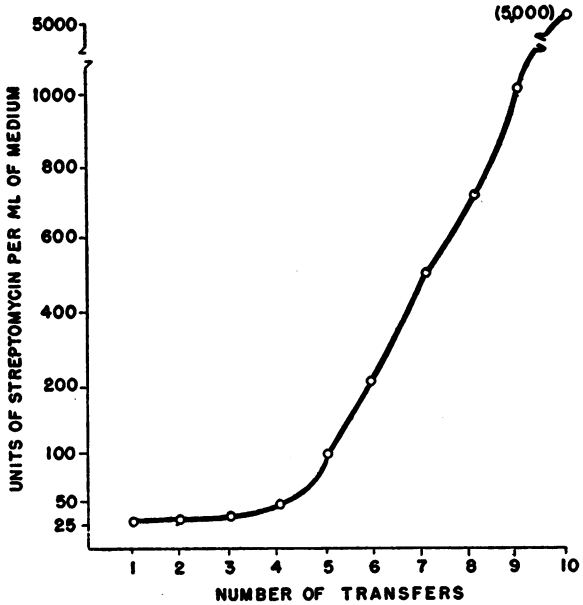

Figure 2. Uniform increase of streptomycin resistance by all six strains of *Actinomyces bovis* on continued transfer in increasing concentrations of streptomycin.

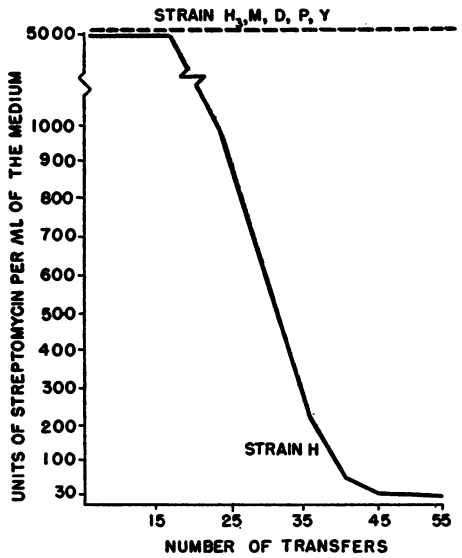

Figure 3. Loss of streptomycin resistance on repeated subculture in streptomycin-free thioglycolate medium. Only strain H showed a loss, whereas strains H_3, M, D, P, and Y maintained their acquired resistance.

It was of interest to determine whether this rapidly acquired resistance was a permanent characteristic or one of a temporary nature that might be lost through successive subcultures in streptomycin-free medium. The six resistant strains

were transferred daily in thioglycolate medium as growth was abundant within 24 hours. After 20 transfers all strains had retained their resistance, growing in 5,000 units per ml, but after 25 transfers one strain began to lose its resistance. After 45 transfers the one strain had returned to its original sensitivity, being inhibited by 30 units per ml. All other strains showed no signs of losing their acquired resistance after 54 transfers in streptomycin-free medium (figure 3).

DISCUSSION

It appears from a review of the literature that there is no clear-cut evidence for the use of a particular antibiotic in well-defined quantities in the therapy of actinomycosis. The two antibiotics, penicillin and streptomycin, have been found to be of value in the several cases in which they have been employed, though the intensity and duration of the treatment seems to vary greatly with the individual case. Because of the long duration of antibiotic therapy usually employed in the treatment of these cases, the possibility of the development of drug-fast microorganisms is constantly present.

Although penicillin resistance did not develop to any appreciable extent with the strains of *Actinomyces bovis* tested, nevertheless four of the six strains did develop a slight degree of tolerance. It appears that this was developed in a stepwise manner, possibly as the result of gene mutations, as Demerec (1948) believes is the case. Two strains showed a three-step increase, one strain a two-step increase, and one strain a one-step increase. The other two strains showed no increase whatsoever.

Since the resistance that developed occurred during the first few transfers, it would seem that, in the clinical use of penicillin in the treatment of actinomycosis, large doses of penicillin might be indicated to prevent the survival of any first-step mutants that could result in the development of highly resistant strains. There is, however, at present no evidence to indicate that this would happen since it does not occur *in vitro* even when the organisms are grown in sublethal concentrations of the antibiotic over long periods of time.

In contrast to penicillin, streptomycin-resistant strains are quite likely to develop during therapy. This was shown to occur rapidly in the *in vitro* studies of this work and has also been reported in at least one case of actinomycosis caused by *Nocardia asteroides*.

Streptomycin-resistant variants seemingly are caused by gene mutations as resistance was developed in a stepwise manner. The evidence indicated that this resistance is a permanent characteristic since five of the six resistant strains retained their resistance after 54 transfers in streptomycin-free medium. The one strain that returned to its original sensitivity after 45 transfers did so in a stepwise manner, indicating that reverse mutation may also occur.

Although it is evident that continued growth in streptomycin stimulated the development of highly resistant strains, it is quite possible that naturally resistant forms were originally present and that the resistance was merely greatly increased. The changes in time required for growth indicated that only a few organisms become resistant and that a longer time to show visible growth was required in the first few transfers.

A brief study of the resistant strains indicated that no morphological changes had occurred, but evidence was presented showing that the development of streptomycin resistance may have been accompanied by changes in the metabolic processes of the organisms. The resistant strains showed the production of acid from glucose and sucrose, although the strains originally showed acid production only from glucose. This, however, may have little significance since acid production was measured only by color change in the indicator employed, and detailed studies of this were not made.

In view of the evidence in this and other work, it is evident that streptomycin-resistant strains may not be avoided merely by the use of high initial doses of the antibiotic, since naturally resistant forms may occur and highly resistant forms may be obtained rapidly. The therapeutic value of this antibiotic then lies in its ability to inhibit the growth of the great majority of the organisms, leaving the natural body defenses to deal with the few remaining less sensitive organisms.

CONCLUSIONS

The effect of penicillin and streptomycin has been tested on six strains of *Actinomyces bovis in vitro*. Under the conditions of the test it was found that:

Penicillin was inhibitory in concentrations varying from 0.05 to 0.5 units per ml.

Four strains showed the development of a slight stepwise increase in resistance to penicillin after 16 transfers, but did not develop further resistance after 32 transfers over a period of 3 months.

Two strains showed no increase in penicillin resistance.

Although the resistance developed to penicillin was somewhat higher than the original sensitivity of the organisms, the strains did not become readily adapted or resistant to the antibiotic effect of penicillin.

All six strains tested were originally inhibited by 30 units per ml of streptomycin.

The six strains all developed at least a 250-fold increase in streptomycin resistance in 10 transfers.

The resistance of five strains was retained after 54 transfers in streptomycin-free medium. One strain reverted to its original sensitivity after 45 transfers, however. Streptomycin dependence did not develop.

There were no morphological changes in the resistant strains.

Slight biochemical changes in the metabolic processes of the resistant strains occurred. No detailed studies of this were conducted.

Streptomycin resistance does not affect the penicillin sensitivity of the various strains.

Penicillin appears to be more effective *in vitro* than does streptomycin on the six strains of *Actinomyces bovis* used in these experiments.

REFERENCES

COSTIGAN, J. 1947 A case of actinomycosis treated with streptomycin. Can. Med. Assoc., **56**, 431.

DECKER, C. L. 1946 The treatment of thoracic actinomycosis by penicillin and sulfa drugs. J. Thoracic Surg., **15**, 430–440.

DEMEREC, M. 1948 Origin of bacterial resistance to antibiotics. J. Bact., **56**, 63–73.

EMMONS, C. W. 1938 Isolation of *Actinomyces bovis* from tonsillar granules. U. S. Pub. Health Service, Pub. Health Repts., **53**, 1967.

FARRIS, E. M., AND DOUGLAS, R. V. 1947 Abdominal actinomycosis. Arch. Surg., **54**, 434–444.

JACOBSON, J. R., AND CLOWARD, R. B. 1948 Actinomycosis of the central nervous system. J. Am. Med. Assoc., **137**, 769–771.

MILLER, C. P., AND BOHNHOFF, M. 1946 Streptomycin resistance of gonococci and meningococci. J. Am. Med. Assoc., **130**, 485–488.

PULASKI, E. J., AND SEELEY, S. F. 1948 Further experience with streptomycin therapy in U. S. Army hospitals. J. Lab. Clin. Med., **33**, 7–15.

SELIGMAN, E., AND WASSERMANN, M. 1947 Induced resistance to streptomycin. J. Immunol., **57**, 351.