Effects of Actinobolin on Growth and Some Metabolic Activities of Cariogenic Streptococci In Vitro and In Vivo

BERNARD B. KEELE, JR., HUBERT L. POWELL, JR., JUAN M. NAVIA, AND JERRY McGHEE

Institute of Dental Research, Department of Microbiology, and Department of Biochemistry, School of Dentistry, University of Alabama, Birmingham, Alabama 35233

Received for publication 11 February 1971

Actinobolin, a known inhibitor of protein synthesis, has been shown not to interfere selectively with acid production or dextranase activity in a cariogenic streptococcus when the antibiotic is added to a concentration of 500 μg/ml. It has also been shown that actinobolin does not alter the total in vivo flora of the oral cavity of the rat when tested in a rat caries model system. A culture of cariogenic streptococci, adapted to in vitro growth in the presence of 1 mg of actinobolin per ml, has also been isolated.

The use of antibiotics for the control of dental caries was proposed many years ago (9) and has been tested in several laboratories (8). Because of the inconsistent results obtained in clinical caries studies of the prolonged and continuous use of any antibiotic, and the possible ill effects which may result, this method of caries control has not received widespread acceptance. However, attention has again focused on the use of antibiotics for dental caries control, since actinobolin, an antibiotic absorbed to a limited extent and not otherwise used in disease therapy, was recently demonstrated to inhibit growth of cariogenic streptococci in vitro (7) and to reduce in vivo rat caries (6).

Because the bacteria in dental plaque are believed to be present under resting conditions (4, 5, J. M. Tanzer et al., Abstr. Genl. Mtg. Int. Ass. Dent. Res., 47th, Houston, 1969), a cariostatic agent, to be effective, must either stop all key metabolic processes that are potentially caries causative or inhibit bacterial growth (cell division) of cariogenic organisms.

Actinobolin has been shown to inhibit protein synthesis in streptococci (D. E. Hunt et al., Abstr. Gen. Mtg. Int. Ass. Dent. Res., 48th, New York, 1970), and therefore it is possible that it might inhibit other cellular processes. However, if a cell possessed a full complement of the enzymes necessary for cariogenic processes, inhibition of protein synthesis alone would only convert a growing cell into a resting cell that would remain capable of acid production and dextran formation, thus continuing the carious process.

In view of these findings, it seemed desirable to investigate the effects of actinobolin upon some metabolic activities that have been implicated as playing a vital role in caries production by the cariogenic streptococci. The object of this paper is to evaluate the in vivo effect of actinobolin on certain components of the rat molar flora and the in vitro effects of actinobolin on acid production, dextranase (EC 2.4.1.5) activity, and total soluble dextran production of both a wild-type strain and an actinobolin-adapted strain of cariogenic streptococci.

MATERIALS AND METHODS

A cariogenic streptococcus termed Streptococcus mutans OMZ176, obtained from E. Newbrun, School of Dentistry, University of California Medical Center, San Francisco, was maintained on NIH Thioglycollate Broth (Difco) with added calcium carbonate. After an 18-hr incubation at 37 C in 1 atm of 95% N₂ and 5% CO₂, the cultures were stored at −10 C until needed. For the experiments, the frozen cultures were thawed, transferred into APT broth (BBL), and then incubated for 10 to 12 hr at 37 C in 1 atm of 95% N₂ and 5% CO₂. Bacterial growth was measured at 620 nm in a Gilford model 2000 spectrophotometer (Gilford Instrument Co., Oberlin, Ohio).

The adapted culture of S. mutans OMZ176 was obtained by transferring the wild-type strain into tubes containing APT broth; with each transfer, an increased concentration of actinobolin was added to the culture, and the culture was allowed to grow for at least 24 hr before being transferred to the next highest concentration. The concentrations of actinobolin used were 0, 1, 5, 10, 25, 50, 100, 200, 400, 600,
and 1,000 μg/ml. Dale E. Hunt kindly provided the actinobolin sulfate (80% base, lot no. X8061; Parke, Davis & Co., Detroit, Mich.). All cultures were incubated at 37 C in 1 atm of 95% N₂ and 5% CO₂. The adapted culture grew to an absorbance of 1.4 to 1.5 in the presence of 1 mg of actinobolin per ml. Under the same conditions, no growth of the wild-type S. mutans strain was observed. Purity of all cultures used in this study was checked by growth on Mitis-Salivarius agar (Difco), by growth on blood-agar, and by Gram stains. The ρH of bacterial cultures was determined with a Radiometer pH-26 meter (Radiometer, Inc., Copenhagen, Denmark) with a combination electrode.

Dextranase activity was determined by the method of Carlsson et al. (1), except that reducing sugar was measured by the method of Park and Johnson (11). One unit of activity was that amount of enzyme that would transform 1 μmole of sucrose/min, i.e. 20.5 mg of sucrose/hr at 37 C and ρH 6.8. Total non-cell-associated dextran production was determined by the addition of two volumes of cold (4 C) absolute ethanol to one volume of cold (4 C) culture supernatant fluid from cultures in late exponential-phase growth. After incubation at 4 C for 1 to 2 hr, the mixture was centrifuged in a refrigerated centrifuge at 8,000 × g for 10 min. The precipitate was washed twice with 30 volumes of distilled water, and the mixture was centrifuged. After drying at 110 C for 24 hr, the alcohol-precipitated material was weighed.

For the in vivo experiments, four groups of 10 COBS male rats (5 days old) from Charles River Laboratories were offered the following dietary treatments (10): group A, a high-sucrose cariogenic purified diet no. 200; group B, diet 200 plus 37.6 ppm of actinobolin; group C, diet 200 plus 37.6 ppm of actinobolin (actinobolin added to the diet 200 offered to rats starting at 22 days of age); group D, diet 200 plus 37.6 ppm of tetracycline. All animals were offered the diet and distilled water ad lib., and were weaned at 19 days of age. At 22 and 42 days of age, rats in each group were sacrificed, the mandibles were dissected, and the first and second mandibular molars were aseptically extracted. These molars were first broken in a glass homogenizer containing 0.067 m potassium phosphate (pH 7.0) sterile buffer and then sonicated at 100 w for 10 sec to disperse the bacteria. Samples of dilutions made in the above buffer were then plated on: (i) blood-brain heart infusion-cysteine-yeast extract (BCY) medium, (ii) Rogosa SL medium, (iii) Mitis-Salivarius (MS) medium, and (iv) eosin methylene blue (EMB) medium (Difco). The BCY medium had the following percentage composition (H. J. Sandham, personal communication): brain heart infusion, 5.2; yeast extract, 0.5; Trypticase, 0.4; cysteine, 0.05; sheep blood, 5.0. Incubation of all plates except the EMB plates was under 1 atm of 95% N₂ and 5% CO₂ for 24 hr at 37 C. The EMB plates were incubated at 37 C for 24 hr. Only those colonies showing typical coliform morphology were counted.

RESULTS

In vivo experiments. To evaluate the effect of actinobolin on some components of the rat molar flora, an age interval was used which coincided with the period of maximal caries development. The total bacterial counts obtained in different selective media are shown in Table 1. Although the levels of antibiotics used (37.6 ppm) have been shown repeatedly to be cariostatic for rats, supplementation of the diets with these antibiotics gave no drastic differences in the total counts on any media for any group. This was true for rats sacrificed at 22 or 42 days of age. Typical S. mutans cells were found in only 2 of 10 animals in group A, and none was observed in groups B, C, and D (H. J. Sandham, personal communication).

In vitro experiments. To determine the length of time necessary for actinobolin to exert a bacteriostatic effect, the antibiotic was added at various time intervals during the growth of wild-type S. mutans. Figure 1 shows that cells in logarithmic growth changed, from logarithmic to stationary, approximately 1.5 hr after the addition of ac-

| Medium                            | Age of rat (days) | No. of viable cells/lowe r molars | Group A (control) | Group B (actinobolin) | Group C (actinobolin at 22 days of age) | Group D (tetracycline) |
|-----------------------------------|-------------------|----------------------------------|-------------------|-----------------------|---------------------------------------|------------------------|
| Blood-cysteine-yeast extract-agar| 22                | 4.5 × 10⁸ ± 0.55                 | 5.9 × 10⁸ ± 0.75  | 2.6 × 10⁸ ± 0.14     | 3.9 × 10⁸ ± 0.36                      | 6.3 × 10⁸ ± 0.83       |
|                                   | 42                | 6.8 × 10⁸ ± 0.83                 | 4.7 × 10⁸ ± 0.59  | 7.6 × 10⁸ ± 0.84     | 4.3 × 10⁸ ± 0.72                      | 4.1 × 10⁸ ± 0.81       |
| Rogosa SL Agar                    | 22                | 3.2 × 10⁸ ± 0.31                 | 4.7 × 10⁸ ± 1.01  | 2.3 × 10⁸ ± 0.17     | 2.5 × 10⁸ ± 0.14                      | 3.8 × 10⁸ ± 0.52       |
|                                   | 42                | 3.2 × 10⁸ ± 0.61                 | 3.7 × 10⁸ ± 0.48  | 5.1 × 10⁸ ± 0.92     | 5.6 × 10⁸ ± 1.45                      | 6.4 × 10⁸ ± 1.02       |
| Mitis-Salivarius Agar             | 22                | 9.3 × 10⁸ ± 3.43                 | 3.4 × 10⁸ ± 0.50  | 2.5 × 10⁸ ± 0.14     | 8.6 × 10⁸ ± 2.30                      | 3.6 × 10⁸ ± 1.31       |
|                                   | 42                | 6.3 × 10⁸ ± 0.87                 | 4.2 × 10⁸ ± 0.59  | 5.6 × 10⁸ ± 1.45     | 6.6 × 10⁸ ± 2.29                      | 6.9 × 10⁸ ± 1.90       |
| Eosin methylene blue agar         | 22                | 9.6 × 10⁸ ± 3.88                 | 6.9 × 10⁸ ± 0.19  | 8.6 × 10⁸ ± 2.30     | 3.6 × 10⁸ ± 1.31                      | 6.9 × 10⁸ ± 1.90       |
|                                   | 42                | 7.6 × 10⁸ ± 0.20                 | 4.1 × 10⁸ ± 0.87  | 6.6 × 10⁸ ± 2.29     | 6.9 × 10⁸ ± 1.90                      | 6.9 × 10⁸ ± 1.90       |

* Both drugs were given as a dietary supplement at 37.6 ppm.

* Mean ± standard error for 10 animals (two plates/animal).
Figure 4 shows the effects of actinobolin on growth of an actinobolin-adapted *S. mutans* strain. During logarithmic growth of this adapted culture, the addition of 500 μg of actinobolin per ml did not appreciably affect the growth of the culture. This contrasts with the marked inhibition of the wild-type *S. mutans* strain (Fig. 1). To determine whether this adapted culture was a

Figure 1. Effect on growth of Streptococcus mutans OMZ176 by actinobolin added at various times during the growth curve. Flasks of APT broth were inoculated at zero time and 500 μg of actinobolin per ml was added as indicated by the arrows. The flasks were incubated at 35°C under constant sparging with 95% N₂ and 5% CO₂. Samples were removed aseptically at time intervals for absorbance readings. Solid line: control. Dashed lines: □, added at 0 hr; Δ, added at 2.5 hr; ○, added at 3.5 hr; ●, added at 4.5 hr.

Figure 2. Effect on the pH of cultures of Streptococcus mutans OMZ176 of actinobolin added at various times during the growth curve. Conditions are the same as for Fig. 1. Solid line: control. Dashed lines: □, added at 0 hr; Δ, added at 2.5 hr; ○, added at 3.5 hr; ●, added at 4.5 hr.

Figure 3. Effect of actinobolin on dextranase activity and pH of Streptococcus mutans OMZ176. Two flasks of APT broth were inoculated at the same time and 500 μg of actinobolin per ml was added to one flask at 1.5 hr. The flasks were incubated at 37°C under constant sparging with 95% N₂ and 5% CO₂. Samples were removed aseptically at intervals for determination of pH and dextranase activity. For activity of dextranase: ○, control; ●, actinobolin added. For pH: ○, control; Δ, actinobolin added.
Fig. 4. Effect of actinobolin on growth of adapted Streptococcus mutans OMZ176. Conditions are the same as in Fig. 1. Symbols: ○, adapted culture; ○, adapted culture with 500 μg of actinobolin/ml added.

Fig. 5. Effect of actinobolin on growth of adapted Streptococcus mutans OMZ176 which had been transferred six times in the absence of actinobolin. Conditions are the same as in Fig. 1 except that 1 mg of actinobolin/ml was added as indicated by the arrow. Symbols: ○, adapted culture; ○, adapted culture with actinobolin added.

Fig. 6. Effect of actinobolin on pH change in adapted Streptococcus mutans OMZ176. Conditions are the same as in Fig. 5. Symbols: ○, adapted culture; ○, adapted culture with actinobolin added.

Table 2. Effect of actinobolin on total soluble dextran production by normal and adapted cultures of Streptococcus mutans OMZ176

| Sample                                      | Dextran production* |
|---------------------------------------------|---------------------|
| Wild-type culture                           | 72                  |
| Wild-type culture + actinobolin (500 μg/ml) | 39                  |
| Adapted culture                             | 45                  |
| Adapted culture + actinobolin (500 μg/ml)   | 46                  |
| Adapted culture + actinobolin (1 mg/ml)     | 23                  |

* Milligrams (dry weight) of dextran per 300 ml of culture supernatant fluid.

mutation, the adapted culture was transferred six times in APT broth without added actinobolin. After the transfers, growth of this culture in the presence and in the absence of actinobolin was determined (Fig. 5). The only observable effect was a slight lag in growth after the addition of 1 mg of actinobolin per ml. Figure 6 shows the pH change of this culture during growth. The pH of both the control and actinobolin-treated culture reached almost the same minimum, but as in the growth experiment (Fig. 5) actinobolin slowed this drop in pH.

Table 2 exhibits the ability of actinobolin to interfere with dextran production. As can be seen, a decrease in total soluble dextran production was caused by the addition of 500 μg of actinobolin per ml to the wild-type S. mutans culture. Though less dextran was produced in the adapted culture without added actinobolin, the addition of 500 μg
of antibiotic per ml did not reduce the total dextran produced by the extracellular enzyme dextran sucrase. However, at the level of 1 mg of actinobolin per ml, there was a 50% reduction in dextran production.

**DISCUSSION**

We have observed that the addition of actinobolin to a growing culture causes a change in the growth rate of the culture, but it does not cause any apparent cell lysis. A decrease in pH was directly associated with the cessation of growth, as reflected by the time of addition of actinobolin. Unless actinobolin was added very early in the growth curve, the pH of the culture that contained actinobolin continued to decrease to what has been termed a critical level for caries initiation (2, 3). These data suggest that, if actinobolin is to be an effective agent in the control of caries, it should be used as a preventive agent after the initial cell population on the enamel surface of the tooth has been reduced.

When the dextran sucrase activity of cultures was assayed in either the presence or the absence of actinobolin, no difference could be detected in the profile or dextran sucrase activity versus time. The decrease in enzyme activity shown in Fig. 3 can possibly be attributed to proteolytic enzyme action in the extracellular solution.

One problem always associated with the use of antibiotics is the acquisition of resistance or adaptation by the treated bacterial population. Since it was reported that resistance to actinobolin by cariogenic streptococci is of no significance (D. E. Hunt et al., Abstr. Genl. Mtg. Int. Ass. Dent. Res., 49th, Chicago, 1971), it appeared pertinent to see whether cariogenic streptococci resistant to actinobolin could be obtained. We have observed in this study that *S. mutans* adaptation to actinobolin can be achieved in vitro and that this adaptation is not lost after transfer in media without actinobolin present. This appears to be a very important consideration in the effectiveness of actinobolin as a cariostatic agent.

Though less soluble dextran was produced in the adapted culture than in the wild-type *S. mutans* culture, without actinobolin added, the addition of 500 µg of actinobolin per ml to the adapted culture produced no decrease in total dextran produced. However, at the level of 1 mg of actinobolin per ml, there was a 50% reduction in dextran production.

If one assumes that smooth surface caries initiation requires both dextran and acid production, and that the bacteria in dental plaque are in an essentially nongrowing state (4, 5; J. M. Tanzer et al., Abstr. Genl. Mtg. Int. Assoc. Dent. Res., 47th, Houston, 1969), actinobolin would seem useful as a cariostatic agent if a large proportion of the cariogenic bacteria present are removed before the use of actinobolin. In any contemplation of the use of this antibiotic, it should be considered that resistant or adapted streptococci have been shown to develop in vitro.

Actinobolin as a supplement in a high-sucrose cariogenic diet has been found to be effective in preventing the development of carious lesions in the rat. No colonies of *S. mutans* were observed in samples taken from molars of rats in the control or experimental group, thus indicating that the typical *S. mutans* is not necessary for caries development in these rats. Total counts obtained on different media did not show major differences between groups. Reductions in numbers of specific cariogenic bacteria immediately after eruption are difficult to detect; yet, they may be of sufficient magnitude to prevent the initiation of caries at this early susceptible stage. Inhibition of cariogenic activity at this time may be sufficient to retard caries and thus allow other factors, such as the maturation process of the rodent enamel, to maintain the integrity of the tooth. The bacteria present in the rat molars of 42-day-old animals may represent a microbial population resistant to the antibiotic or noncariogenic types of bacteria which have taken the place of cariogenic bacteria inhibited by the antibiotic without affecting the total count. Another possibility exists that actinobolin exerts its cariostatic effect through a chelating effect at the enamel-plaque interface. The answers to these questions are not available at this time.

The data presented in this investigation suggest that actinobolin affects cariogenic organisms by decreasing their growth and acid-producing capacity, and that accommodation to the drug can take place. This indicates that the effectiveness of actinobolin in the control of caries is better when it is used as a preventive rather than as a therapeutic agent.

**ACKNOWLEDGMENTS**

The technical assistance of Brenda Cummings is greatly appreciated.

This investigation was supported by Public Health Service grant DE-02670 from the National Institute of Dental Research.

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