Effects of Penicillin G In Vitro On Hemophilus ducreyi

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During the course of experiments at the Venereal Disease Research Laboratory on the treatment and prophylaxis of chancroid induced in human volunteers (*1*) and in animals, it was observed that therapy with oral penicillin G (Bicillin) did not aid recovery.

Previous reports have indicated *Hemophilus ducreyi* to be sensitive to penicillin in vitro (*2–4*) while in vivo experiments have demonstrated equivocal findings.

Deacon and associates (*1*), trying to confirm Willcox's theory that penicillin might be of value if given in such a manner as to produce and maintain high serum concentrations (*5*), found that penicillin G administered orally in five chancroid infected volunteers produced mean serum levels of only 0.025 penicillin G units per milliliter of serum. This concentration was not effective as a prophylactic agent for *H. ducreyi* infections. Treatment with orally administered penicillin V in five volunteers, however, resulted in a mean serum level of 0.232 penicillin G units per milliliter of serum, and lesions were definitely controlled during this treatment. Furthermore, if penicillin V was administered orally prior to inoculation with *H. ducreyi*, levels of penicillin G units per milliliter of serum reached a mean level of 0.492, and the volunteers either failed to develop lesions or developed very few lesions.

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In an effort to correlate Deacon's findings with in vitro results, the present study was undertaken. Our purpose was to determine the concentrations at which penicillin G inhibited the growth of *H. ducreyi* and the length of time at which concentrations of penicillin G inhibited growth.

Materials and Methods

Inoculum for all experiments was prepared by inoculating 5 ml. of casitone-saline medium (*6*) (1 percent Difco bacto casitone, 0.85 percent NaCl, plus 5 percent sterile rabbit serum), contained in 150 mm. x 20 mm. screw-cap test tubes (Kimble No. 45066), with a loopful of 24-hour *H. ducreyi*, strain CH1A (*7*), which is representative of virulent strains in our laboratory, grown in the same medium. Incubation of these cultures was at 34° C. for 16 to 24 hours. At the end of the incubation period, cultures were checked for purity by means of a Gram stain and subculture upon nutrient blood agar (*7*) (Difco nutrient agar 1.5 percent plus 5 percent defibrinated rabbit blood) plates. These plates were incubated at 34 ° C. in candle jars for 48 hours. The culture used as the inoculum was the same as used by Deacon and co-workers (*1*), had been checked previously for its ability to produce typical lesions in volunteers (*1*), was of known pathogenicity, and during manipulation did not lose pathogenicity.

To determine the concentration at which penicillin G inhibits the growth of *H. ducreyi*, nutrient blood agar plates containing concentrations of penicillin G from 0 to 20 units per milliliter of agar were inoculated with 0.5 ml. inoculum and the surface of the agar spread with sterile bent glass rods. These plates were incubated at 34° C. in candle jars for 48 hours. Colonies were observed, and Gram stains were made in order to observe morphology.

It was also desired to determine the length of time at which concentrations of penicillin G inhibited growth. Screw-capped test tubes, 150 mm. x 20 mm., with 10 ml. of casitone-saline medium and containing graded amounts of penicillin G were inoculated with a loopful of the *H. ducreyi* culture. One milliliter of 1,000 units of Difco bacto-penase (penicillinase) was added to each tube of each penicillin G concen-

tration medium at 0, 2, 4, and 6 hours. Separate tubes were employed for the penicillinase addition. Therefore, five tubes were used for each penicillin G concentration. The fifth tube was a control containing no penicillinase. The concentration of penicillinase used was sufficient to inhibit all concentrations of penicillin G used in this experiment and did not inhibit the growth of the organism when penicillin was absent or present.

Results and Discussion

Penicillin G inhibited the growth of *H. ducreyi* strain CH1A, at concentrations over 0.1 unit per milliliter of agar. Gram stains of cells of the 0.1-unit plates showed cells that were swollen and elongated to several times their normal size, which indicates that this concentration constitutes a sublethal dose (*8*). Growth was not inhibited, and subcultures of these organisms on nutrient blood agar without the drug resulted in normal appearing *H. ducreyi*. These results are shown in the following tabulation:

Penicillin G (units/ml. agar)	*Type of cells*
0	Normal chains.
.001	Do.
.01	Do.
.1	Swollen and elongated.
.2	No growth.
.3 to 20.0	Do.

Efforts to transfer normal and elongated cells to gradually increased amounts of the drug resulted in no detectable resistant organisms.

The results as shown in the following table indicate that inhibition is a relationship of concentration of penicillin G and the length of time *H. ducreyi* is in contact with the drug. Consequently, 0.1 unit of penicillin G per milliliter of casitone-saline medium did not inhibit growth after 24 hours; 0.2 units of penicillin G per milliliter of medium inhibited growth after 6 hours' contact; 0.3 units of penicillin G per milliliter of medium inhibited growth after 4 hours' contact; and concentrations of penicillin G of 0.4 units or more per milliliter of medium inhibited growth in less than 2 hours' contact.

In the liquid medium no swollen and elongated cells were observed at any concentration of the drug used.

Time at which concentrations of penicillin G inhibit growth of Hemophilus ducreyi, strain CH1A, grown in casitone-saline medium for 24 hours

Penicillin G (units/ml. medium)	Hours at which penicillinase was added				No penicillinase added
	0	2	4	6	
0	+	+	+	+	+
.1	+	+	+	+	+
.2	+	+	+	+	—
.3	+	+	+	—	—
.4	+	—	—	—	—
.5	+	—	—	—	—
.6	+	—	—	—	—
.7	+	—	—	—	—
.8	+	—	—	—	—
.9	+	—	—	—	—
1.0	+	—	—	—	—

+ indicates growth and no inhibition.
— indicates inhibition of growth.

All tubes were incubated an additional 24 hours and were read, with no changes in the results. Wetherbee and co-workers (*2*) cultured *H. ducreyi* in tryptose phosphate serum broth with various concentrations of penicillin G and subcultured the organism 24 and 48 hours on chocolate tryptose agar plates. They found that penicillin G inhibited at 0.1 to 0.5 units per milliliter at 24 hours but that there was less inhibition at 48 hours. This suggested to Wetherbee that penicillin prolongs the lag phase but is not significantly bacteriostatic. While Wetherbee and his associates did not demonstrate any significant bacteriostatic effect of penicillin G upon *H. ducreyi*, our results indicate actual bacteriostatic and bactericidal effects based upon concentration of the penicillin G and the length of time the drug had to act upon the organisms. In our laboratory, avirulent strains of *H. ducreyi* showed different sensitivities to penicillin G and other antibiotics than did *H. ducreyi*, strain CH1A, or other virulent strains. Therefore, it seems probable that the strains Wetherbee and co-workers used were avirulent.

Eagle (*9*) states that penicillin G is inactivated by serum in vitro. Our experiments showed that 5-percent rabbit serum did not inhibit the effect of penicillin G upon the organisms. This, of course, may be more apparent

than real. Some of the penicillin G may have been bound by some component of the serum, but the bactericidal effect is there, nevertheless.

Consequently, the sensitivity of this organism to penicillin G is an expression of the amounts which must be present in the medium in order to effect a binding with some cell constituent or constituents. Eagle and co-workers (*10*) interpret the binding of penicillin by bacteria to be determined by a single essential component, which is functionally inactivated by that combination which is normally present in significant excess. Schepartz and Johnson (*11*) propose that "binding occurs by cleavage of the beta lactam ring of penicillin and combination of the carbonyl or imino group of that ring with the binding component."

Our in vitro results can be compared with the in vivo results of Deacon and co-workers (*1*) in that, if the serum level of penicillin G is about 0.2 units per milliliter, that concentration should be sufficient to inhibit growth of *H. ducreyi*, strain CH1A, less than 24 hours after the patient's blood has attained this penicillin level. More immediate response would be expected if 0.3 or 0.4 units of penicillin G per milliliter of blood could be attained. Eagle and associates (*12*) state that the therapeutic action of penicillin is in a large part measured by the aggregate time for which it remains at effective levels at the focus of infection, that these effective levels in vivo are of the same order of magnitude as those effective in vitro, and that the effective penicillin time in the serum is usually a reasonable approximation of the penicillin time in tissue fluids.

While penicillin G can inhibit the growth of *H. ducreyi* in vitro, Deacon and co-workers (*1*) have shown that the serum penicillin G level does not attain the necessary concentration. Penicillin V, however, by increasing the serum penicillin G level, shows some usefulness in treatment of chancroid.

Summary

Penicillin G is bactericidal for *Hemophilus ducreyi*, strain CH1A, at concentrations over 0.1 unit per milliliter of medium. Inhibition of growth is dependent upon the concentration of the drug and the length of time at which the organism is in contact with the penicillin G.

A blood level of 0.3 or 0.4 units of penicillin G per milliliter should inhibit the growth of virulent *H. ducreyi* in 2 to 6 hours, and a level of 0.2 units of penicillin G per milliliter should inhibit the growth of this organism in less than 24 hours.

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