

## The Effect of Penicillin on Bacterial Interference in Vivo

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The study was designed to analyse the effect of penicillin on interference between alfa-streptococci and group A streptococci in vivo. Tissue cages were implanted subcutaneously in 9 rabbits and inoculated with  $10^5$  cfu of alfa- and beta-streptococci together as well as separately. Both streptococci were recovered 96 h after the inoculation in the untreated rabbits. Alfa-streptococci inhibiting the growth of beta-streptococci in vitro retained this capacity under the experimental in vivo conditions. Higher penicillin concentration did not increase the killing rate of beta-streptococci grown separately while a faster killing was observed with low penicillin levels in the presence of inhibiting alfa-streptococci, indicating synergistic effect.

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### INTRODUCTION

It has been shown that alfa-streptococci of the normal throat flora can interfere with the colonization and growth of beta-hemolytic streptococci group A (1-4). It is thus likely that this interference phenomenon contributes to the defence against infections in the throat. In an earlier study (5) we isolated from individuals—seemingly resistant to streptococcal tonsillitis—alfa-hemolytic streptococci with strong inhibitory capacity on beta-streptococci, while from patients with repeated tonsillitis no or low numbers of inhibiting alfa-streptococci were demonstrated. Other authors (6) have made similar observations in patients resistant to colonization by group A streptococci (GAS). Furthermore, it has been demonstrated (7) that the oral flora was replaced by noninhibitory bacteria in penicillin treated subjects. The biological consequences of this ecological disturbance was illustrated by Sprunt et al. (8) who found that elimination of alfa-streptococci from the throat during intensive antibiotic treatment resulted in overgrowth of Enterobacteriaceae spp. The significance of the interplay between pathogens, the normal flora and antibiotics was strengthened in an in vitro study (16). Thus a synergistic effect was demonstrated between penicillin and inhibitory alfa-streptococci on the killing rate of beta-streptococci on the assumption that the penicillin level was above the minimum inhibitory concentration (MIC) for the beta-streptococci but below that of the alfa-streptococcal strain.

The aim of the present study was to test the validity of the above-mentioned in vitro finding under in vivo conditions using a tissue cage model (10, 11) employing single and mixed bacterial cultures in the presence and absence of penicillin.

### MATERIALS AND METHODS

**Bacterial strains.** Beta-hemolytic streptococci group A (Lancefield strain T4-95-R65), T-type 4 ( $\beta$ T4lab) and a clinical isolate of group A recovered from the throat flora of a tonsillitis patient, T-type 4 (15179) were used. The alfa-streptococci were strain  $\alpha$ 1—a *Streptococcus mitis* strain and  $\alpha$ 2155—a *Streptococcus sanguis* II strain isolated from patients with beta-streptococcal tonsillitis.

**MIC.** The MICs against benzylpenicillin for the beta-streptococci, the  $\alpha$ 1 and  $\alpha$ 2155 strains were 0.02, 0.1 and 0.2 mg/l respectively. The MIC determination was made in TY-broth (12) with a final inoculum of  $2 \times 10^5$ – $5 \times 10^5$  cfu/ml.

**Culture media and growth.** The streptococci were grown in TY-broth (12) at 37°C for about 3 h to reach an optical density of 0.3 (500 nm) corresponding to  $10^6$  cfu/ml. The bacteria were washed and resuspended in PBS pH 7.0 to reach OD 0.3 before use.



**Antibiotics.** The animals were given 10 or 0.5 mg/kg body weight of benzylpenicillin (ASTRA, Sweden) intramuscularly, twice a day for 5 days.

**Determination of penicillin activity.** All samples were tested using the agar well method. TG-agar (Bacto-agar, Difco with yeast extract, Trypticase peptone BBL and 0.2% glucose) with *Bacillus stearothermophilus* as an indicator strain (ATCC 3032) were used. Agar wells (6 mm diam.) were filled with 30 µl samples from tissue cage fluids, serum or standard dilutions of benzylpenicillin (prepared in 50% rabbit serum). The plates were incubated at 56°C overnight. The sensitivity limit of the method was 0.01 mg/l. All analyses were made in duplicates.

**Animals.** 9 rabbits from improved breed French hydraulic ram/chinchilla, weighing 3–4 kg were used. They were fed rabbit pellets (FORS, Sweden) ad lib. and given free access to drinking water.

**The animal model.** The tissue cage model has been described earlier (10) and the contents of the tissue cage fluid analysed (11). Briefly, 4 steel net chambers were implanted subcutaneously in the 9 rabbits. Four weeks later the chambers were covered with a layer of connective tissue and filled with a slightly yellow fluid—the tissue cage fluid (tcf). 0.1 ml of each of the bacterial suspensions was injected into the chambers. In the two cages on the right side the alfa- and beta-streptococci were injected separately, while on the left side a mixture of these strains were injected simultaneously into each of the cages (Table I). Samples (0.2 ml) were drawn from the chambers during the following 5 days. On day 1, samples were taken within 10 min after the injection and benzylpenicillin was given i.m. Then sampling was made after 1, 2, 4, 6 and 8 h. The following days (24–96 h) only 1 sample was taken per day just before the morning dose of penicillin. Samples were used undiluted for determination of penicillin and diluted (if necessary) for counting of bacteria. Blood agar plates containing penicillinase (Penase, 1000 U/ml) were used for cfu counting.

## RESULTS

The concentration of penicillin reached in the chambers varied slightly between individual rabbits but the differences between the chambers in the same animal were small. The mean benzylpenicillin concentration in tcf from the rabbits treated with 0.5 mg/kg and 10 mg/kg is shown in Table II. Rabbits given the low dose (0.5 mg/kg) reached 0.026 mg/l at 120 min as the highest value while rabbits given the high dose (10 mg/kg) attained a peak value of 0.25 mg/l at 60 min.

Alfa-streptococci ( $\alpha$ 2155)—in the absence of penicillin—were recovered from the tissue cage fluid in approximately the same number after 96 h as when initially injected (Fig. 1a). In contrast the beta-streptococci ( $\beta$ T4lab) decreased already at an early stage (8–48 h) but were still demonstrable after 96 h. In the mixture a significant decrease of beta-streptococci was noted during the first days and only few bacteria were demonstrated 72 h after the inoculation. At 96 h no beta-streptococci (<10 cfu/ml) could be isolated from the cages although the alfa-streptococci were still recovered in high numbers.

Table I. Inoculation and treatment scheme of animals with tissue cages

$\alpha$ 1 = Strept. mitis strain;  $\alpha$ 2155 = Strept. sanguis II strain;  $\beta$ T4lab = GAS, Lancefield strain T4-95-R65;  $\beta$ T4clin = GAS, type 4 strain

Rabbit	Right front	Right rear	Left front	Left rear	Penicillin treatment
A	$\alpha$ 2155	$\beta$ T4lab	Mixed	Mixed	No
B	$\alpha$ 2155	$\beta$ T4lab	Mixed	Mixed	0.5 mg/kg
C	$\alpha$ 2155	$\beta$ T4lab	Mixed	Mixed	10 mg/kg
D	$\alpha$ 2155	$\beta$ T4clin	Mixed	Mixed	No
E	$\alpha$ 2155	$\beta$ T4clin	Mixed	Mixed	0.5 mg/kg
F	$\alpha$ 2155	$\beta$ T4clin	Mixed	Mixed	10 mg/kg
G	$\alpha$ 1	$\beta$ T4lab	Mixed	Mixed	No
H	$\alpha$ 1	$\beta$ T4lab	Mixed	Mixed	0.5 mg/kg
I	$\alpha$ 1	$\beta$ T4lab	Mixed	Mixed	10 mg/kg



In rabbit B given 0.5 mg/kg of penicillin the alfa-streptococci were still present after 96 h of treatment (Fig. 1b) while the beta-streptococci gradually decreased in number and could not be demonstrated after 48 h when grown alone. In the presence of the alfa-streptococci the beta-streptococci declined even earlier and no beta-streptococci were detected 24 h after the inoculation.

No bacteria were isolated from any of the chambers 72 h after the start of treatment with 10 mg/kg of penicillin (Fig. 1c). The killing rate of beta-streptococci was not influenced by the presence of alfa-streptococci or vice versa.

An experiment performed in an identical way using a clinical isolate of GAS T-type 4 ( $\beta$ T4clin) but the same  $\alpha$ 2155 strain is shown in Fig. 2. In rabbit D—not given penicillin—the  $\alpha$ 2155 strain behaved in a similar way as in rabbit A while the beta-streptococcal strain was still present in high number after 96 h (Fig. 2a). The  $\alpha$ 2155 strain was not capable of killing the  $\beta$ T4 clinical strain within the time of observation although a significant decline from  $5 \times 10^5$  to  $10^3$  was noted. In rabbit E given the low penicillin dose (0.5 mg/kg), the alfa-streptococci were recovered in the same number as initially inoculated after 96 h of treatment (Fig. 2b). This alfa-streptococcal strain also showed a synergistic effect with low penicillin concentration and killed the beta-streptococci after 24 h, while the beta-streptococci when grown alone were not killed until 48 h. In rabbit F (Fig. 2c) no beta-streptococci were recovered 24 h after the start of the penicillin treatment (10 mg/kg) in the presence as well as the absence of alfa-streptococci. Also the alfa-streptococci were influenced by the penicillin concentration and were killed within 72 h of therapy.

The results of testing the interaction between alfa- and beta-streptococci in vivo in a third combination in absence and in presence of low and high concentration of benzylpenicillin are depicted in Fig. 3. In rabbit G the  $\alpha$ 1 strain was still recovered from the tissue cages in high numbers 96 h after the start of the experiment in absence of penicillin (Fig. 3a). In the single culture, the  $\beta$ T4lab strain decreased gradually in the tissue cage fluid but its presence could still be demonstrated at 96 h. However, in the presence of the  $\alpha$ 1 strain no beta-streptococci could be isolated at the sampling performed after 72 h. In rabbit H (Fig. 3b), given a low dose of penicillin, the alfa-streptococci could not be demonstrated in the control cage after 4 days of treatment, although the penicillin concentration was below the MIC for the alfa-strain. The beta-streptococci were killed after 48 h without the presence of alfa-streptococci, but together with the  $\alpha$ 1 strain the beta-streptococci were killed already at 24 h. In this mixture the  $\alpha$ 1 decreased the first 6 h but could still be

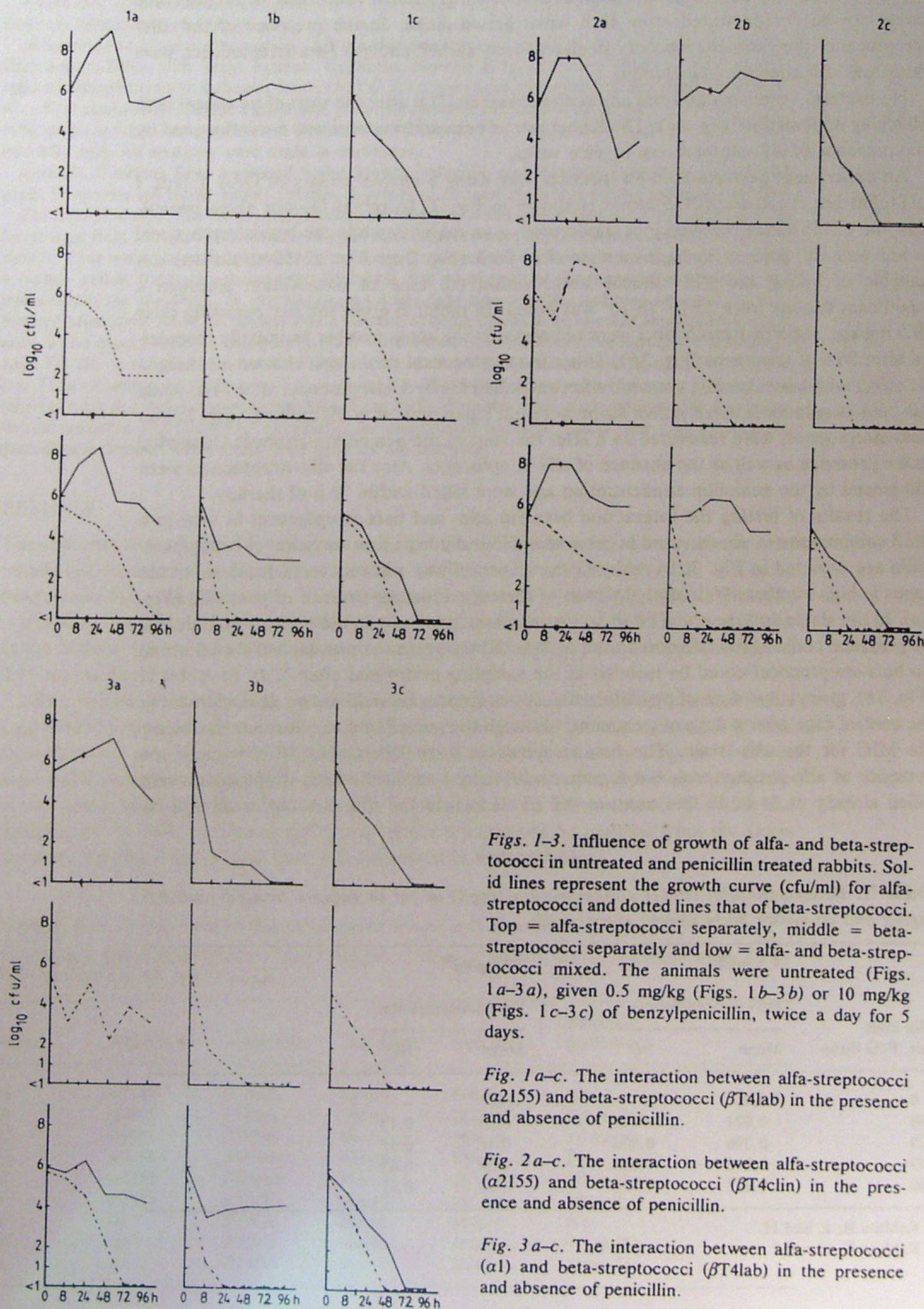
Table II. Benzylpenicillin (PcG) concentration (mg/l) in tcf in rabbits treated with 0.5 mg/kg and 10 mg/kg body weight respectively

Min after i.m. PcG dose	0.5 mg/kg <sup>a</sup>		10 mg/kg <sup>b</sup>	
	PcG concentration		PcG concentration	
	Mean	SD	Mean	SD
0	<0.01	—	<0.01	—
60	0.021	0.009	0.25	0.19
120	0.026	0.006	0.17	0.17
240	0.016	0.003	0.08	0.05
360	0.012	0.002	0.04	0.02

<sup>a</sup> Rabbits B, E and H.

<sup>b</sup> Rabbits C, F and I.





Figs. 1-3. Influence of growth of alfa- and beta-streptococci in untreated and penicillin treated rabbits. Solid lines represent the growth curve (cfu/ml) for alfa-streptococci and dotted lines that of beta-streptococci. Top = alfa-streptococci separately, middle = beta-streptococci separately and low = alfa- and beta-streptococci mixed. The animals were untreated (Figs. 1a-3a), given 0.5 mg/kg (Figs. 1b-3b) or 10 mg/kg (Figs. 1c-3c) of benzylpenicillin, twice a day for 5 days.

Fig. 1a-c. The interaction between alfa-streptococci (a2155) and beta-streptococci (betaT4lab) in the presence and absence of penicillin.

Fig. 2a-c. The interaction between alfa-streptococci (a2155) and beta-streptococci (betaT4clin) in the presence and absence of penicillin.

Fig. 3a-c. The interaction between alfa-streptococci (a1) and beta-streptococci (betaT4lab) in the presence and absence of penicillin.

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demonstrated after 96 h. When the same strains were used in rabbit I (Fig. 3c), given 10 mg/kg of penicillin, none of the two streptococci were recovered in the separately infected cages after 72 h and similar results were obtained in the mixed infection. Although the penicillin concentration in the tcf was above the MIC for the beta-streptococcal strain during the whole period, the killing rate for the beta-streptococcal strain was not substantially shorter in the presence of high levels of penicillin than it was in the presence of only the  $\alpha 1$  strain (Fig. 3a+3c low). Fig. 3b (low) furthermore indicates that a more rapid killing of beta-streptococci was achieved in the presence of low penicillin levels and inhibiting alfa-streptococci than at higher penicillin concentration.

## DISCUSSION

In the present study both beta-streptococcal strains survived in the tcf of the untreated rabbits during the 5 days of experiment although the  $\beta T4lab$  strain spontaneously decreased substantially. The two alfa-strains were also recovered from the tcf but in numbers close to those of the inoculation doses. A strong interfering capacity of alfa-streptococci resulting in killing of the  $\beta T4lab$  strain under in vivo condition could be documented using either of the two alfa-streptococcal strains showing in vitro inhibitory capacity for the beta-streptococci (16). Also the  $\beta T4clin$  strain was clearly inhibited but not killed as demonstrated by repeated sampling. The killing capacity of the two alfa-streptococcal strains on the  $\beta T4lab$  strain was equivalent to that registered with penicillin (dose 10 mg/kg) and no beta-streptococci could be isolated from tcf 48 h after the inoculation. In contrast the  $\beta T4clin$  strain was killed within 24 h in the presence of penicillin and no additional effect was afforded by the alfa-strain compared to the controls.

The penicillin doses (10 mg/kg and 0.5 mg/kg) were chosen on the basis of the results of an earlier study (16). The aim was to reach a concentration above the MIC of the beta-streptococcal strain (0.02 mg/l) but below that of the inhibitory alfa-streptococcal strains (0.1–0.2 mg/l) since a synergistic effect between penicillin and inhibiting alfa-streptococci on the killing rate of beta-streptococci was observed in these studies. Using 10 mg/kg body weight of benzylpenicillin twice a day the desired level for the beta-streptococci was successfully surpassed in all rabbits. However, also the MIC for the alfa-streptococci was reached during 1–2 h post injection in tcf in the 3 penicillin treated rabbits. This short period was obviously enough to kill the alfa-streptococci within the following 48–72 h and thus no synergistic effect between penicillin and living alfa-streptococci on the killing rate of the beta-streptococci like the one earlier noted in vitro could be demonstrated.

Using a lower dose of penicillin (0.5 mg/kg), we reached a concentration below the MIC for the alfa-streptococci in the tcf, and near the MIC for the beta-streptococci. In accordance with our in vitro results (16) we could then observe a synergistic effect between penicillin and living alfa-streptococci, as illustrated by a faster killing rate of  $\beta T4lab$ .

The inoculation dose ( $10^5$  cfu/ml) was chosen on the basis of tissue cage experiments with beta-streptococci earlier described (13). These authors demonstrated that after inoculation of  $10^3$  cfu/ml of streptococci in untreated rabbits, streptococci declined spontaneously and no viable bacteria could be found after 48 h. However, after a dose of  $10^5$ – $10^9$  cfu/ml, streptococci could still be recovered for several days from the tcf in approximately the same number as originally inoculated. Also in other tissue cage experiments a dose of  $10^5$  cfu/ml of streptococci or more has been successfully used (14, 15). The fluctuations in bacterial counts sometimes observed in a series of analyses (e.g. Fig. 3a, middle row) is probably due to difficulties in recovering enough representative material for the plating procedure. It should be added that various strains of beta-streptococci were used in these



experiments representing different serologic types but they all seemed to behave similarly concerning growth, spontaneous killing and influence of penicillin therapy.

We have earlier reported on the pharmacokinetics of penicillin in mixed human saliva after oral tablet intake in doses commonly used in treatment of acute tonsillitis (9). It was demonstrated that a very high initial penicillin concentration was reached in saliva during the first minutes after the administration due to release of penicillin from the tablet. From this high initial peak, which may amount to more than 2 mg/l saliva, a rapid decrease was noted but a second salivary penicillin peak appeared after 60–90 min. The initial peak is thus well above the MIC for beta- as well as alfa-streptococci. Also the second peak of 0.1 mg/l is high enough to influence both streptococcal species. Roos et al. (17) recently reported in a study of 267 patients with tonsillitis a salivary concentration above 0.03 (the MIC for GAS) in 63% of the patients 60 min after administration of 12.5 mg/kg body weight of phenoxymethylpenicillin. More than 30% of the patients reached the level 0.1 mg/l which is the MIC for most of the alfa-streptococci in the throat flora. It thus seems reasonable to assume that the penicillin tablet initially but also in a second saliva peak evokes concentrations which highly influence the normal oral flora and thus also the defence system offered by the interference between inhibiting alfa- and beta-streptococci (16). The present results and those earlier reported indicate that also the presence of high penicillin levels during a short time should be avoided in order to protect the individual against ecological disturbances with profound biological effects.

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