The Incidence of Penicillin-sensitive Variant Colonies in Penicillinase-producing Strains of *Staphylococcus pyogenes*

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SUMMARY: Penicillinase-producing strains of *Staphylococcus pyogenes* isolated from human infections and kept under various conditions were plated out and fifty colonies from each plate tested for penicillin-sensitivity to determine the permanence of their resistance to penicillin.

Of 32 strains kept in Lemco broth and tested once, 5–12 months after isolation, two yielded only penicillin-sensitive colonies and fifteen others yielded a proportion of such.

Of six strains preserved by the gelatin-ascorbic acid drying process and kept for over a year, only one yielded penicillin-sensitive colonies.

Of six strains kept in Lemco broth with and without regular subculture and tested at intervals for 9 months, all gave rise to at least one penicillin-sensitive variant colony and more than half the colonies of one strain became penicillin-sensitive.

The natural tendency of these strains to yield penicillin-sensitive variant colonies was not appreciably accelerated by treatment with X-rays or by growing them with other organisms.

From many studies on penicillin-resistant staphylococci it is clear that the cocci display two fundamentally different types of resistance; this was first pointed out by Spink, Hall & Ferris (1945) and has been confirmed by many workers since. First, there is the type of resistance acquired *in vitro* by serial subculture of staphylococci in increasing concentrations of penicillin. The organisms often show gross changes in morphology, cultural characteristics and virulence, and tend to revert to the typical penicillin-sensitive variety after repeated subculture in the absence of penicillin. The resistance is not usually associated with the production of penicillinase. Bellamy & Klimek (1948), however, describe a strain trained *in vitro* to grow in penicillin '4 mg./ml.', which produced small amounts of penicillinase, but only when grown in the presence of penicillin. This strain had lost many of the properties of a staphylococcus. It consisted of Gram-negative cocci and rods and had lost the ability to ferment lactose, sucrose, maltose, mannitol and galactose. The second type of resistance occurs in strains isolated from infective processes and human carriers; these strains do not differ from typical penicillin-sensitive strains of *Staphylococcus pyogenes* in morphology, cultural characteristics or virulence. As first pointed out by Kirby (1944), this type of resistance is characterized by the production of a powerful penicillin-inactivator. In reports by Spink *et al.* (1945) Bondi & Dietz (1945), Gots (1945), Barber (1947*a, b*) and Barber & Rozwadowska-Dowzenko (1948), all penicillin-resistant strains of *Staph. pyogenes* isolated *in vivo* were shown to destroy penicillin.

During the past two years penicillin-resistant strains of *Staph. pyogenes*

were isolated at Hammersmith Hospital from 111 patients with staphylococcal infection, and a number were obtained from nasal and skin carriers (Barber, 1947*a, b*; Barber & Rozwadowska-Dowzenko, 1948). All these strains were able to destroy penicillin. Single colonies of penicillin-resistant cocci appearing on the original culture plates of human material were subcultured into Lemco broth and maintained in the laboratory, many of them for from 9 to 18 months. In the present investigation forty-four of these cultures were studied to determine the permanence or impermanence of their resistance to penicillin.

METHODS

Penicillin ditch-plates. Ditches were cut at one side of Lemco agar plates and filled with the same medium containing approximately 10 units penicillin/ml. The ditch-plates were prepared 24 hr. or more before use to allow some penicillin to diffuse out of the ditch. As a control, the Oxford staphylococcus was streaked across every ditch-plate at the time of its use.

Penicillinase production was tested for by the method of Barber (1947*a*).

Typing by bacteriophage. Parent and variant cultures were phage-typed by Dr Allison by the method of Wilson & Atkinson (1945).

RESULTS

Variants from thirty-two strains kept in Lemco broth for 5–12 months. Thirty-two strains kept, with occasional subculture, in Lemco broth were plated out on Lemco agar plates and from each plate fifty colonies were picked off and streaked across penicillin ditch-plates. The results are summarized in Table 1. Of the 32 strains tested seventeen gave penicillin-sensitive colonies and from

Table 1. *Distribution of percentage of colonies of penicillin-sensitive Staphylococcus pyogenes in thirty-two penicillin-resistant strains (fifty colonies of each strain tested.)*

No. of strains	Percentage
2	100
5	> 50
3	22–50
3	10–20
1	4
3	2
15	0

two cultures all fifty colonies tested were penicillin-sensitive. All the penicillin-sensitive colonies appeared to be as sensitive as the Oxford staphylococcus and all the resistant colonies grew across the ditch (Pl. 1, fig. 1). No intermediate degrees of resistance were encountered. The penicillin-sensitive colonies bred true on subculture and proved to have lost their capacity to destroy penicillin.

The parent broth cultures were then plated directly on to penicillin ditch-plates. The two cultures from which only penicillin-sensitive colonies were obtained (Pl. 1, fig. 2) showed no penicillin-resistant colonies even by this method, which can detect as few as one in 10,000 resistant colonies (see Barber,

1947*b*). The fifteen cultures yielding a mixture all resembled wholly penicillin-resistant strains and no colonial variation was detectable (Pl. 1, fig. 3). This is presumably because a few resistant organisms produce penicillinase sufficient to permit growth of the penicillin-sensitive organisms. In artificial mixtures of penicillin-sensitive and penicillin-resistant organisms the ratio of sensitive to resistant had to be at least 100:1 if the sensitive organisms were to be detected on a penicillin ditch-plate (Pl. 1, fig. 4). One interesting point is that whereas penicillin-sensitive colonies get smaller as the penicillin is approached, the penicillin-resistant colonies get larger. This is particularly well seen in Pl. 1, fig. 4.

Variants from six strains preserved by the gelatin-ascorbic acid drying process. Six strains kept for over a year after drying by Stamp's (1947) method yielded far fewer variants. From five of the cultures no penicillin-sensitive colonies were isolated and one strain yielded only three.

Table 2. *Incidence of penicillin-sensitive variants in six strains kept under controlled conditions for 9 months*

	No. of penicillin-sensitive colonies out of fifty tested											
	D3R		D6R		P193R		P268R		Sp249R		P5155RR	
Tested after	<i>a</i>	<i>b</i>	<i>a</i>	<i>b</i>	<i>a</i>	<i>b</i>	<i>a</i>	<i>b</i>	<i>a</i>	<i>b</i>	<i>a</i> *	<i>b</i>
6 weeks	0	0	0	0	0	0	1	0	13	5	6	1
3 months	0	0	0	1	0	0	0	0	9	3	5	0
4 months	0	0	0	1	0	0	0	0	28	12	3	0
5 months	0	0	0	0	0	0	3	1	25	20	5	0
6 months	0	0	0	1	0	0	0	1	36	25	4	3
9 months	0	1	0	—	1	0	0	0	34	40	41	6
12 months	0	0	0	—	0	0	0	—	42	31	50	11

a = Subcultured twice a week in Lemco broth.

b = Left on bench in Lemco broth and subcultured every 3 months.

— = Not tested.

* This culture became contaminated with a bacillus between the fourth and fifth months.

Variants from six strains kept under controlled conditions for 9 months. Six cultures were studied more carefully over a period of 9 months; five of these had been isolated within a few weeks of starting the experiment and one, P5155RR, was a penicillin-resistant colony from a culture isolated 7 months previously and at that time yielding a mixture of sensitive and resistant colonies. One culture of each strain was subcultured twice a week in Lemco broth, and one was left on the bench in the same medium and subcultured once during the period. Each of the two series was plated out after 1½, 3, 4, 5, 6, 9 and 12 months; and, except for two strains left without subculture that had died at 9 and 12 months, fifty colonies from each strain were tested on the ditch-plates. In all 600–700 colonies were tested from each of the twelve cultures (Table 2). From all six strains penicillin-sensitive variant colonies were isolated, although two yielded only a single variant and two more only a few. Strain Sp249R yielded an increasing number of penicillin-sensitive

colonies and subculture seemed to increase the rate of conversion. For the first 6 months strain P 5155 RR, when subcultured, gave a few variant colonies at each test. At the ninth month, however, there were forty-one colonies and at the twelfth fifty. This specimen became contaminated with a bacillus between the fourth and fifth months. When left on the bench the strain yielded only one variant in the first 5 months and then at subsequent tests a steadily increasing proportion.

As in the previous experiment, nearly all the colonies tested were either as sensitive as the Oxford staphylococcus or grossly resistant, growing right across the penicillin ditch. Occasionally, however, colonies showed an intermediate degree of resistance; but when such streaks were emulsified in broth, plated on plain agar and fifty colonies again tested, a mixture of penicillin-sensitive and resistant organisms was found. As before, penicillin-sensitive colonies bred true and had lost the capacity to destroy penicillin.

Attempts to stimulate variation

Two agents were tested for power to accelerate the natural tendency of penicillinase-producing staphylococci to give rise to penicillin-sensitive variants.

The effect of X-radiation. A dose of 1,000,000 r. (about the maximum dose leaving any surviving staphylococci) was used on strains D3 and D6, which naturally produced only occasional penicillin-sensitive variants. The irradiated cultures were plated out after 1, 2, 6, 12, 20 and 26 subcultures and fifty colonies examined. Of the 300 colonies tested from each after X-raying, none was penicillin-sensitive.

The effect of mixing cultures with Streptococcus pyogenes. Following the work of Voureka (1948), *Streptococcus pyogenes* (Group A) was mixed with penicillin-resistant staphylococci. Three strains of *Strep. pyogenes*, including the strain Milne used by Voureka, and ten strains of staphylococci, some of which were producing many penicillin-sensitive variants and some only producing a few, were used. The streptococci were grown for 18–24 hr. in Hartley broth and the cultures then seeded with two drops of a broth culture of one of the staphylococci. The mixtures were incubated for 24 hr., plated and fifty staphylococcal colonies tested. Control cultures of Hartley broth, seeded with a staphylococcus only, were similarly treated. There was (Table 3) no obvious difference in the number of variants occurring in the mixed cultures and in the controls.

The effect of mixing penicillin-resistant and penicillin-sensitive strains of Staph. pyogenes. A penicillin-sensitive (S) strain and a penicillin-resistant (R) strain (D3) were mixed in the following proportions: S/R 1:1, 1:100, 100:1. The mixtures were divided into two parts, one subcultured twice a week in Lemco broth and the other left on the bench in the same medium. At varying intervals during 5 months the mixtures were tested (Table 4). The S/R 1:1 mixture subcultured twice a week yielded penicillin-sensitive colonies in proportions varying from 1:1 to 4:1, whereas the culture left on the bench gave an increasing number of penicillin-sensitive colonies until all fifty tested were sensitive. Even at this stage, however, when the culture was plated directly on

to a penicillin ditch-plate, a few resistant colonies were isolated. The S/R 1:100 mixture when subcultured became and remained a nearly equal mixture and when left on the bench yielded a few sensitive colonies for the first 4 months and suddenly at the fifth month became predominantly penicillin-sensitive. With

Table 3. *Incidence of penicillin-sensitive variants after mixing with Streptococcus pyogenes*

Strain	No. of penicillin-sensitive colonies out of fifty tested				
	Mixed with <i>Strep. pyogenes</i> , strain			Control cultures	
	Richards	C203	Milne	1	2
D3R	1	0	0	0	0
D6R	0	0	0	0	0
Sp249R	43	41	32	36	44
P5155RR	15	10	15	10	9
P6395R	6	4	—	8	—
B7007R	14	16	—	18	—
P7004R	29	26	—	21	—
P7108R	12	20	—	33	—
P268R	—	—	0	0	—
P193R	—	—	0	1	—

Table 4. *Incidence of penicillin-sensitive colonies in mixtures of penicillin-sensitive and penicillin-resistant strains of Staphylococcus pyogenes*

	No. of penicillin-sensitive colonies out of fifty tested						
	S/R 1:1		S/R 1:100		S/R 100:1		S/R 1:3
Tested after	<i>a</i>	<i>b</i>	<i>a</i>	<i>b</i>	<i>a</i>	<i>b</i>	X-irradiation
(Immediately)	19	34	4	—	49	—	49
2 weeks	—	—	2	1	50	50	39
4 or 6 weeks	29	47	23	3	44	50	16
2 months	17	48	34	6	41	50	20
3 months	10	49	26	3	49	39	34
					48	39	
4 months	14	50	20	7	35	14	48
					34	11	
5 months	16	50*	21	42	50*	30	50

a Subcultured twice a week in Lemco broth.

b = Left on bench in Lemco broth.

— = Not tested.

* Penicillin-resistant colonies isolated when whole culture was plated on a ditch-plate.

the S/R 100:1 mixture once again there appeared to be an increase in the strain originally in the minority. The results obtained at the third and fourth months were surprising and were therefore repeated, but with similar results. The results with these mixtures are difficult to interpret. One explanation would be that the two strains grow at different rates. It is, however, possible that a transference of properties with regard to penicillin-sensitivity takes place.

The S/R 1:1 mixture was also subjected to X-irradiation of 1,000,000 r. to see whether this would affect one strain more than the other. The X-irradiated

mixture was then subcultured twice a week in Lemco broth for 6 months. It will be seen from Table 4 that the number of resistant colonies was diminished immediately, that it increased until the second month, after which it steadily decreased until at 6 months no resistant colonies were detected even when the culture was plated directly on to a penicillin ditch-plate. It seems probable that the sensitive strain was originally more susceptible to the lethal action of irradiation, but that serial subculture, as with the S/R 100:1 mixture not irradiated, led to the sensitive colonies, originally in the minority, eventually becoming predominant.

Typing by bacteriophage

Since the cultures used in these experiments were derived originally from single colonies, not single cells, the purity of eight strains was checked by comparing the phage reaction of parent cultures, the variants, and cultures obtained from penicillin-resistant descendant colonies. In all eight strains, the variants were of the same phage type as the parent, and as the penicillin-resistant daughter colonies.

Colonial variations

A number of variations in colonial appearance was observed during the course of these experiments, especially in cultures which had been in the laboratory for some time. The variant colonies were best seen when plates were incubated for 18–24 hr. and then left on the bench for a few days. Gross variations in colour and opacity occurred and were apparently favoured by repeated subculture, so that a single plate from a subcultured strain often consisted of typical golden, white, semi-transparent and transparent colonies. Size variations were also very common and were more marked in plates from the cultures that had been left on the bench without subculture, many consisting of a large number of small pin-point colonies with a few larger and more typical ones (Pl. 1, fig. 5). Another, less frequent variant occurring in several strains was a colony with an irregular and crenated edge, a rough or matt surface and a depressed centre (Pl. 1, fig. 6). All these colonial variants retained their capacity to clot citrated or oxalated plasma. Occasionally coagulase-negative colonies were encountered, but these were discarded as probable air contaminants. There was no association between colonial appearance and sensitivity to penicillin.

DISCUSSION

Studies on the permanence of resistance to penicillin of penicillinase-producing staphylococci have in many cases been invalidated by the assumption that the individual organisms making up a culture act uniformly in their behaviour to penicillin. Thus Blair, Carr & Buchman (1946) and Spink & Ferris (1947), as a result of testing the whole culture, claimed that cultures of penicillinase-producing staphylococci were permanently resistant to penicillin. But, since a few penicillinase-producing cocci may protect many penicillin-sensitive cocci in the culture from the action of penicillin (see Pl. 1, fig. 3), testing the whole culture does not give an accurate picture of what is really happening.

The present study makes it clear that penicillinase-producing strains of *Staph. pyogenes* tend to give rise to penicillin-sensitive variants when kept in the laboratory. It is, however, obvious that the method used is not accurate enough for much reliance to be placed on the actual number of variants obtained at any one time. Because single colonies, not single cells, were the starting-point for all experiments, it is possible that the original cultures consisted of a mixed population. The identity of phage-type of parent and variant colonies of eight strains, however, make it almost certain that at least in these strains, the penicillin-sensitive colonies were true variants of a penicillin-resistant parent.

I have not yet found a method of accelerating the natural variation, but this line of investigation is being continued. Vourekka (1948) claims to have rendered both types of penicillin-resistant staphylococci sensitive to penicillin by mixing them with other organisms, such as the Milne streptococcus. My results (Table 3) do not confirm this. Bennison & Schwabacher (1948) have also been unable to repeat Vourekka's results. What happens when two strains of *Staph. pyogenes* are mixed is not clear. In both S/R1:100 and S/R100:1 mixtures there was an increase in the minority strain. It is, however, possible that the strain in the majority was giving rise to variant colonies.

Until we discover the mode of origin of penicillin-destroying strains of staphylococci, it is not possible to understand the mechanism whereby they give rise to penicillin-sensitive variant colonies. Three explanations have been put forward. The first is that this type of resistance is truly acquired by contact with penicillin. As pointed out by Luria (1947), this would represent an example of the inheritance of an acquired characteristic, and therefore is contrary to the 'general outlook of modern biological thought'. The second, suggested by Barber (1947*a*), is that a few strains of *Staph. pyogenes* have always been penicillin-resistant and are now increasing in frequency by a simple process of selection. This view is supported by the fact that so far it has not proved possible to produce this type of resistance to penicillin *in vitro*. The frequency, however, with which these strains yield penicillin-sensitive variants is against this hypothesis. The third view, first put forward by Demerec (1945) to explain the origin of 'acquired' penicillin-resistance *in vitro* by staphylococci, and later by Spink & Ferris (1947) as an explanation for the origin of penicillinase-producing strains, is that these resistant organisms arise originally as rare mutants independent of the presence or absence of penicillin. In the presence of the antibiotic, however, these mutants are favoured at the expense of the penicillin-sensitive parent and thus increased in frequency by differential selection. If this hypothesis be correct then the penicillin-sensitive variants recorded here must be regarded as reverse mutants. Their frequency, in view of the difficulty of demonstrating the direct mutation, is perhaps surprising, but as Luria (1947) says 'very mutable characters often present rates of reverse mutations higher than the direct mutation rates'.

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EXPLANATION OF PLATE

Penicillin ditch-plates ($\times \frac{1}{2}$)

- Fig. 1. Typical example of strain showing a mixture of penicillin-resistant and sensitive colonies. Central streak = Oxford staphylococcus.
- Fig. 2. Upper half plated with culture P6652, penicillin-resistant on primary isolation now sensitive, and lower half with the Oxford staphylococcus.
- Fig. 3. Strain giving nine out of fifty penicillin-sensitive colonies—mixture undetectable by this method. Central streak = Oxford staphylococcus.
- Fig. 4. Mixture of penicillin-sensitive and resistant staphylococci in proportion of 100 sensitive to 1 resistant. Note gradual fading out of sensitive colonies as penicillin is approached and large resistant colonies near penicillin. Central streak = Oxford staphylococcus.

Colonial variations

- Fig. 5. One typical-sized colony and a number of small colony variants from same culture, $\times 20$.
- Fig. 6. Two typical smooth colonies of *Staph. pyogenes* and three 'rough' variants, $\times 18$.

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Figs. 1-6