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Relationships between Bacterial Resistance to Serum and Penicillin. (23658)

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Despite numerous studies on differential resistance of bacterial species and strains to bactericidal effects of normal sera, only little information is available on the genetic aspects of bacterial sensitivity or resistance to serum factors(1-3). Where such information has been collected, antigenic alterations of the bacteria usually accompanied alterations in "serum resistance." Recently, there has been a resurgence of interest in bactericidal systems, due to recognition of the role of properdin(4-5) in such phenomena. Therefore, a renewed effort has been made to study certain genetic aspects of bacterial resistance with particular emphasis on closely related strains of similar antigenicity. In the course of these studies, changes in serum resistance without qualitative antigenic alterations and an inverse correlation between resistance to penicillin and to bactericidal factors of normal human serum were observed. These observations form the subject of the present report.

Methods. Smooth cultures of *Shigella dysenteriae* #377, and *Escherichia coli* SPI, sensitive to the properdin system(4), were obtained from Dr. R. J. Wedgwood. These cultures proved extremely heterogeneous on the basis of colonial morphology when inspected by oblique lighting(6). Single colony isolates were used to establish substrains with greater colonial homogeneity, displaying identical antigenic* and fermentation characteristics. The susceptibility of these strains to normal human serum, obtained from a single donor, was determined by exposing a known number of bacteria (approx. 5000) for various periods of time at 37°C to serum diluted with ABA buffer (Michaelis buffer, pH 7.5, containing albumin(4)). The concentration of serum in buffer varied from 1:6 to 1:2.5 depending upon the sensitivity of the strain tested, but the total volume during exposure time was always 1.2 ml. Bacteria for testing were harvested from 18-hour tryptose agar slants and plated on tryptose agar (in 0.1 ml aliquots) following exposure to serum. Prior to plating and prior to incubation at 37°C the bacterial suspensions in serum were kept in an ice-bath. Sera were stored at –40°C. All tests were done at least in duplicate and duplicate samples of replicate cultures were assayed.

Results. Six colonial types were recognized when the original culture of *Sh. dysenteriae* was inspected on McConkey agar. Four of these yielded stable subcultures differing in their resistance to the bactericidal effects of normal human serum; the other 2 isolates proved to be unstable in regard to colonial morphology and antigenic characteristics and were not studied further. Table I demonstrates typical differences in serum resistance between the most sensitive (S_3) and most resistant (S_1) of the stable substrains isolated from the original heterogeneous culture.

When attempts were made to convert mem-

* Based on reactions with specific antiserum and acriflavine.

TABLE I. Resistance of Closely Related Strains of *Sh. dysenteriae* to Normal Human Serum (Serum Sample #H2).

Strain	Serum conc.	% survival after 15′	30′	60′	ET_{50}(10)	Sensitivity to penicillin (units/ml)
S_1	1:6	94 ± 5.2	92 ± 5.4	76 ± 4.2	162′	12.5
S_3	"	95 ± 3.5	24 ± 3.7	3.6± .5	18′	25
S_1-pr_{12}*	"	47 ± 6.5	22 ± 3.4	2.8± 2.0	12′	700
S_1	1:2.5	67 ± 4.3	45 ± 7.1	28 ± 1.5	33′	12.5
S_1-pr_5	"	41 ± 5.5	22 ± 2.2	13 ± 1.8	13′	100
S_1-pr_8	"	34 ± 6.8	13 ± 2.2	5.3± 1.3	10′	200

* Mutant isolated after 12 transfers of S_1 on gradient plates containing penicillin.

bers of these two representative strains into protoplasts by exposure to penicillin + sucrose(7), it was noted in the sucrose-free penicillin-containing (2000 units/ml) control suspensions that S_1 cells were lysed far more rapidly than S_3 cells. Subsequent measurements of penicillin resistance of the strains differing in serum resistance revealed that increased resistance to one was associated with decreased resistance to the other (Table I).

To verify the generality of this relationship, mutants with increased resistance to penicillin then were isolated from the sensitive S_1 strain by transfers on gradient plates(6). The serum resistance of the recovered mutants, showing stepwise increases in penicillin resistance, was assayed following one or more transfers on penicillin-free media and, as illustrated in the lower part of Table I, confirmed the inverse correlation between the 2 resistance characteristics. Resistance to normal serum factors decreased as resistance to penicillin increased. Similar observations were made with *E. coli* strains.

Penicillin is known to interfere with the synthesis of bacterial cell-wall components (8). Therefore, it could be expected that compensating alterations in the cell-wall of resistant mutants are responsible for the altered sensitivity to bactericidins. To provide a basis for further elucidation of such relationships, the serum resistance of genetically identical cells was compared following 18 hours of pregrowth in plain broth or in broth containing noninhibitory concentrations of penicillin. As shown in Table II, prior exposure to penicillin did produce significant phenotypic changes in serum resistance; bacteria pregrown in sufficiently low concentrations of penicillin proved significantly more resistant to serum than their sister cells grown in plain media. Possible causes for such effects will be discussed below. An interesting zonal effect also was observed: pregrowth in 0.5 or 5 units of penicillin per ml protected against subsequent serum effects, pregrowth in 1 or 2 units/ml did not (the actual units differ depending upon the strain employed). Also, pregrowth in penicillin concentrations that are close to the inhibitory level (*e.g.* S_1 in 10 units) have effects opposite to those just described; they render the cells more sensitive to subsequent serum treatment. This can be explained on the basis of piling one injurious effect upon another, similar to the synergistic effects of other antibiotics and serum noted by others(2) during

TABLE II. Effect of Pregrowth in Penicillin-Containing Media upon Subsequent Susceptibility of *Sh. dysenteriae* Cells to Normal Human Serum (Serum Sample #H3).

Strain	Penicillin in pregrowth medium (units/ml)	Serum conc.	% survival after 30′	60′	90′	ET_{50}	Sensitivity to penicillin (units/ml)
S_1		1:2.5	54 ± 6.2	23 ± 3.1	3.9 ± 1.1	32′	12.5
"	5	"	75 ± 3.9	63 ± 4.1	48 ± 2.3	84′	12.5
S_1-pr_{12}		1:6	33 ± 3.4	12 ± 1.6	3.9 ± .6	19′	700
"	100	"	67 ± 4.2	40 ± 3.6	21 ± 2.8	42′	700

simultaneous exposures. In additional studies with *Sh. dysenteriae* strains from other sources (Walter Reed Army Hospital), it was observed that occasionally differences in serum resistance occur that are not correlated in a simple fashion† with differences in sensitivity to penicillin. This may indicate that there are at least two different mechanisms leading to altered bacterial resistance to serum. Finally, it has been determined that the presence of 20% sucrose, but not of 20% lactose, prevents all bactericidal effects of serum. The nature of this protection is now being studied, and it remains to be seen whether or not it is related to the carbohydrate effects previously observed by Maaløe (1).

Discussion. The foregoing observations demonstrate that considerable heterogeneity in regard to resistance to bactericidal effects of normal human serum can exist in cultures believed to be relatively homogeneous on the basis of antigenic characteristics. Differences in serum resistance that occur independently of detectable antigenic changes have been noted before(1), but the recognition and control of such genetic alterations in stock-cultures of bacteria used for the testing of bactericidal systems has been generally neglected. In the case of *Sh. dysenteriae* and *E. coli*, cultural heterogeneity in regard to serum resistance now has been shown to be detectable by checking colonial morphology with the aid of oblique lighting on McConkey agar. Various other media have been tested but failed to produce comparable differentiation. Nevertheless, it is probable that entirely different media may be required to detect such differences in still other species.

More data are required to elucidate the actual nature of the alteration in penicillin-resistant cells that renders such cells more sensitive to serum and is modifiable by pregrowth in noninhibitory concentrations of the antibiotic. The alteration is presumably in the cell-wall, since penicillin is known to interfere with cell-wall synthesis(8), and since the cell wall represents the initial locus of reaction for serum bactericidins. It is generally assumed that each cell may possess several reaction sites for serum factors, that a minimum number of these must be affected for cidal effects, and that the bactericidins distribute themselves among the exposed cells according to the binomial distribution. Therefore, the change occurring with increased resistance to penicillin may either effect 1) a change in total number of reactive sites per cell, 2) chemical reactivity of these sites, or 3) a change in the minimum number of reactions per cell required for killing. Differences in the killing end-points, obtained by extrapolating the present data to infinite time, appear too great to support the simplest assumption, namely, that penicillin resistance and serum resistance have been altered merely by a quantitative change in cell-wall material and reactive sites. The possible involvement of qualitative changes may become apparent as additional titration data become available.

Finally, the observation of an inverse correlation between resistance to penicillin and resistance to serum may help to explain the known occurrence of two general groups of penicillin-resistant pathogens, detected primarily in studies with staphylococci(9). It will be recalled that resistant organisms isolated from clinical cases as a rule owe their resistance to the production of penicillinase, whereas resistant mutants isolated *in vitro* fail to produce penicillinase and generally are of decreased virulence. The low survival value *in vivo* of the latter group of mutants now may be attributed to their increased sensitivity to normal serum bactericidins. In contrast, mutants resistant to penicillin by virtue of penicillinase production can be assumed to possess unaltered resistance to serum and thus a higher survival value *in vivo*.

Summary. Substrains isolated on the basis of colonial morphology from antigenically homogeneous cultures of *Sh. dysenteriae* and *E. coli* displayed stable genetic differences in resistance to the bactericidal effects of normal human serum. This serum resistance was inversely correlated with penicillin resistance: with increased resistance to the antibiotic there was an increased susceptibility to serum.

† There is some preliminary evidence for differences among these strains in rate of lysis at a given penicillin concentration.

Mechanisms that may be responsible for this relationship, and their bearing on certain problems of *in vivo* penicillin resistance, have been discussed.

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Distribution of Terminal Temperatures in Hypothermic Dogs. (23659)

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Anesthetized dogs subjected to acute progressive hypothermia succumb in one of 3 ways(1). Under pentobarbital anesthesia the majority (60%) terminate in ventricular fibrillation (VF) at heart temperatures of 25°-19°C, terminus usually being preceded by extrasystolic activity. A proportion (15%) terminate in asystole at 18°-15°C. The remainder (25%) exhibit a period of asystole of variable duration (5-80 sec.) or an exceedingly slow heart rate (less than 16 b.p.m.) either of which is followed by VF. The latter type of death has been termed "asystolic fibrillation" (AF) and occurs at temperature range intermediate between the 2 former groups, *i.e.* 20-17°C(1).

It would be of interest to discover whether each group represented a distinct segment of the population or whether each type of death was a direct consequence of the terminal temperatures which were otherwise distributed along a predictable curve. Statistically the question is whether the population of animals employed was homogeneous regarding susceptibility to VF, *i.e.* whether terminal temperatures are uniformly distributed as opposed to a distribution exhibiting bi- or trimodalities. This could be determined only after a large group of "control" experiments had accumulated. Such a series of animals, similarly treated, is now available to make the analysis possible.

Methods. Results from a total of 110 mongrel dogs of both sexes, weighing between 8-18 kg, are included. All animals were anesthetized with pentobarbital (30-35 mg/kg) and cooled to terminus by immersion in an iced bath. Details of the method have been previously reported(2). Heart temperatures were measured thermoelectrically with a catheter thermocouple inserted into the right auricle via the right jugular vein. Rectal temperatures were measured with a rectal thermocouple. The accuracy of the temperature recording instrument is within $\pm$ 0.5°C. All animals were allowed to breathe spontaneously until a heart temperature of 25 $\pm$ 1°C was reached. At lower temperatures, they were respired artificially by means of a positive pressure respirator.

Results. When all terminal heart temperatures were plotted in a frequency nomogram, it became apparent that they deviated greatly from a Gaussian distribution. Nevertheless there was no evidence of bimodality to suggest that the animals terminating in asystole or asystolic fibrillation were derived from a different population from that showing extrasystoles followed by VF.

A simple transformation of the terminal