Phenotypically Determined Resistance of *Neisseria gonorrhoeae* to Normal Human Serum: Environmental Factors in Subcutaneous Chambers in Guinea Pigs

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Some gonococci obtained from human urethral exudate or from subcutaneously implanted chambers in guinea pigs show a resistance to killing by human serum which is lost on subculture *in vitro* after a few generations. The environmental factors which may influence the phenotypic expression of resistance to serum killing were investigated in guinea pig chambers and in chamber fluid *in vitro*.

The redox potential in chambers before and after infection was lower than that of heart blood but conditions were not anaerobic; H_2O_2 increased the redox potential but did not decrease gonococcal serum resistance. The chambers were slightly alkaline before and after infection. When the concentration of glucose (depleted in infected chambers by the abundant polymorphonuclear cells) was restored to excess, the serum resistance of the gonococci was unaffected. Concentrations of free amino acids in chambers changed little during infection. Gonococci adapted to growth in chambers and subsequently rendered serum-sensitive by growing once on agar reverted to serum-resistance after 0·5 to 1 h incubation in chamber fluid *in vitro* at 37 °C but not at 25 °C or 4 °C. After 16 to 24 h growth at 37 °C, resistance was again lost. The reversion to serum resistance did not occur in a complex laboratory medium. Examination of the chamber fluid after growth of gonococci *in vitro* showed depletion of lactate, glutamine and proline.

INTRODUCTION

The environment *in vivo* affects the production of the determinants of microbial pathogenicity by selection of genotypes and through phenotypic change. Recent studies on gonococcal pathogenicity provide excellent examples of these types of influence. A selective process occurs for organisms resistant to killing by phagocytes whilst phenotypic change underlies their resistance to the bactericidal action of human serum (Penn *et al.*, 1977*a*).

The loss of resistance to killing by human serum on subculture was first detected in strains of gonococci isolated from urethral pus (Ward *et al.*, 1970). A similar resistance to human serum, maintained by passaging gonococci serially through human serum *in vitro*, was lost rapidly on subculture on normal solid media (McCutchan *et al.*, 1976). We observed that gonococci adapted to growth *in vivo* in plastic chambers implanted subcutaneously in guinea pigs were resistant to killing by human serum but became susceptible on subculture

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in vitro (Penn *et al.*, 1976). These various observations were made on cultures grown for 24 h or more and the loss of serum resistance may therefore have been due to mutation and selection. However, Rittenberg *et al.* (1977) showed that the resistance of guinea pig chamber gonococci to human serum was lost after only a few generations *in vitro* both in culture medium and in chamber fluid. This supports the hypothesis of a phenotypic change resulting from a deficiency of the environment *in vitro*.

Some strains of gonococci found in urethral pus (Ward *et al.*, 1970), in guinea pig chambers (Penn *et al.*, 1977*b*) and, notably, in cases of disseminated gonorrhoea (Schoolnik *et al.*, 1976) have a resistance to killing by human serum which is stable to subculture *in vitro*. However, this paper is concerned only with the type of resistance which is phenotypically controlled and particularly with that expressed in guinea pig chambers. This resistance may well be relevant to the pathogenesis of gonorrhoea since gonococci in urethral pus reacted similarly to those adapted to growth in guinea pig chambers.

Research on the biochemical basis for the phenotypic resistance to killing by human serum would be facilitated by a medium for the production of large numbers of serum-resistant gonococci *in vitro*, and investigations of the environmental factors within the guinea pig chambers might help in the design of such a medium. Previous studies (Rittenberg *et al.*, 1977) have emphasized that after 24 h growth *in vivo* the glucose concentration was low ($\leqslant 0.5$ mmol l^{-1}) and the population was limited to 1×10^6 to 5×10^6 organisms ml^{-1} in chamber fluid. The concentration of glucose fell dramatically because of usage by cells involved in the inflammatory response which began 5 to 8 h after infection. With such a striking glucose depletion, it seemed important to monitor environmental factors within the chamber which might influence energy sources utilized by gonococci (pH, redox potential, lactate, acetate and amino acid concentrations) in an attempt to assess, and subsequently to influence, the limiting conditions which might alter the serum susceptibility of the gonococci. Lactate or acetate may serve as alternative energy sources in certain circumstances (Holten, 1976; Hebeler & Morse, 1976), as may amino acids, depending on the availability of glucose (Holten, 1973; Hebeler & Morse, 1976; M. A. Chan & M. Goldner, personal communication). The changes taking place in the same environmental factors concurrent with the reversion of gonococci from serum sensitivity to resistance when cultured in chamber fluid *in vitro* were also studied.

METHODS

Organism. *Neisseria gonorrhoeae* strain BS4 (a laboratory strain, BS, which had been passaged four times through guinea pig chambers) was used immediately after removal from the animal or after dilution 10- to 20-fold in cryo-protective medium followed by storage in liquid nitrogen. After one subculture on agar the strain was called BS4 (agar) (Penn *et al.*, 1976).

Implantation of chambers in guinea pigs. The techniques for implanting, inoculating and sampling of chambers were as described by Veale *et al.* (1975), except that tincture of iodine was used to disinfect the skin before implantation.

Media, cultural and counting procedures. These were as described previously (Veale *et al.*, 1975; Penn *et al.*, 1976, 1977*b*; Rittenberg *et al.*, 1977). Liquid cultures were grown in 0.5 to 2.0 ml medium contained in loosely capped 7.5 ml Bijou bottles incubated at 37 °C in candle jars.

Alteration of chamber environment. The redox potential in a chamber was increased by serial injections of 0.1 ml 1 % (v/v) H$_2$O$_2$ (100 vol.), and the glucose concentration was increased by serial injections of 0.2 ml glucose (800 mmol l^{-1}).

Chamber fluid for cultures in vitro. Pooled fluid from at least five chambers (which had been *in situ* for 6 to 10 weeks) was centrifuged at 1000 *g* for 5 min to remove cells and used either immediately or after storage at -20 °C for not more than 2 weeks.

Measurement of resistance to killing by normal human serum. A pool of freshly collected human serum from about 10 subjects was stored in 0.5 ml portions at -70 °C. It was thawed at room temperature immediately before use, diluted 1 in 4 in barbitone buffer pH 7.5 [consisting of (mmol l^{-1}): NaCl, 150; sodium barbitone, 1.82; barbitone, 3.13; MgCl$_2$, 0.5; CaCl$_2$, 0.15] and dispensed as duplicate 0.1 ml samples into wells in polystyrene microtitre trays (Flow Laboratories, Scotland). Trypticase Soy Broth (TSB; BBL) was also dispensed in duplicate wells, as controls. The test culture was diluted in TSB to contain about 10^4 colony-

forming units (c.f.u.) ml^{-1} and vortex mixed with 3 mm diam. glass beads for 30 s to disperse any clumps; then 10 μl was added to each well and the contents were mixed by repeated expulsion from the micropipette. After incubation at 37 °C for 30 min, a 100 μl sample from each well was taken for viable count. The count in the experimental sample containing fresh human serum expressed as a percentage of that in the control sample containing TSB was taken as the measure of serum resistance. Control tests showed that survival in TSB was comparable with that in heated (56 °C, 30 min) human serum. A culture of a known susceptible strain (BSSH; Penn *et al.*, 1977*b*) was included in every test to ensure that the serum was active, i.e. that resistance of this strain was less than 20%.

Measurement of redox potential within chambers in vivo. Redox potential was measured with a platinum electrode against a calomel reference electrode. The platinum electrode (wire) was cleaned in chromic acid before use and inserted into the chambers of anaesthetized guinea pigs through fine polypropylene micro-pipette tips which had been pushed through needle punctures in the overlying skin and connective tissue. A second micropipette tip containing agarose gel saturated with KCl was also inserted, and a calomel reference electrode (K4112; Radiometer, Copenhagen, Denmark) was connected by contact with the agarose salt bridge in the broad end of the micropipette tip. The potential difference (mV) between electrodes was measured with a pH/mV meter (Radiometer PHM 62). The electrode function was checked by immersing the electrodes in a saturated solution of quinhydrone in 0·1 M-phosphate buffer, pH 7·0, which always gave a reading within ± 10 mV of the theoretical value of 44 mV. Readings obtained were added to the potential of the reference electrode (244 mV) to give values on the hydrogen scale. For comparison, the redox potential of heart blood and of caecal contents of guinea pigs was measured immediately after death by cervical dislocation.

pH of chamber fluid. Fresh samples (0·3 to 0·4 ml) of chamber fluid were centrifuged at 1000 *g* for 5 min and then the pH was measured using a small combination electrode and pH meter (Radiometer PHM 62).

Analysis of chamber fluid. Glucose was determined by the glucose oxidase method of Werner *et al.* (1970), using the Test-combination Glucose (Boehringer). L(+)-Lactate was determined by the lactate dehydro-genase–NAD$^+$ method of Gutman & Wahlefeld (1974), using the Test-combination Lactate (Boehringer). Acetate was determined by gas–liquid chromatography using a Pye series 104 chromatograph (Pye Unicam) with a Tenax AD column, N$_2$ as the carrier gas (30 ml min^{-1}) and a flame ionization detector; all temperatures 125 °C, attenuation × 50, range 0·5 to 9·5 mV, sample volume 0·8 μl. Amino acids were determined on a Locarte autoanalyser (Locarte, London) after removal of protein by the addition of an equal volume of 10% (v/v) perchloric acid.

RESULTS

Redox potential, pH, lactate, acetate and amino acid concentrations in chambers in vivo before and after infection with gonococci

The redox potentials of five uninfected chambers (340, 310, 317, 250 and 349 mV) were lower than that of left ventricular heart blood (442 mV in one experiment). After infection for 24 h, the redox potentials were decreased to 248, 229, 263, 159 and 281 mV, respectively, but the chambers were not anaerobic. Redox potentials of the caecal contents of two guinea pigs were − 76 and − 26 mV.

The pH values of four chambers before infection and 24 h after infection were, respectively, 7·6 and 7·5, 7·7 and 7·6, 7·9 and 7·7, and 8·0 and 7·8.

Glucose, lactate and acetate concentrations in the chambers were followed during infection. As reported previously (Rittenberg *et al.*, 1977), glucose, present initially at 4 to 5 mmol l^{-1}, was reduced to 0·4 mmol l^{-1} during the first 24 h. Concurrently, as might be expected from glycolysis in phagocytes, the lactate concentration doubled to about 8 mmol l^{-1} (Fig. 1). The acetate concentration remained almost constant at about 2·5 mmol l^{-1}. There was little further change in the concentrations of glucose, lactate and acetate up to 10 and 20 d infection.

Amino acid concentrations were measured in two chambers before and after infection. No marked changes occurred. Asparagine was not found, presumably due to the presence of asparaginase in guinea pig serum (Broome, 1961).
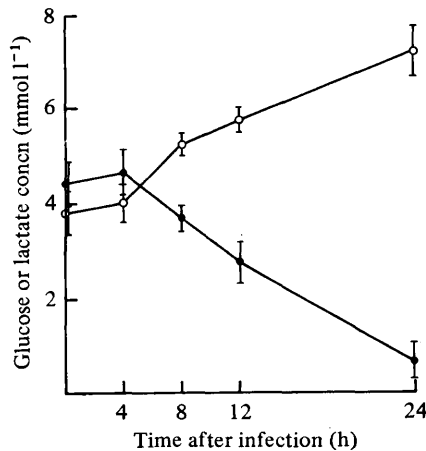
Fig. 1. Changes in the concentrations of glucose (●) and lactate (○) in guinea pig chambers during infection with *N. gonorrhoeae*. The results are the mean of six experiments; the bars indicate the standard error of the mean.

Table 1. *Effect of increasing the redox potential within an infected chamber on the viable count and serum resistance of serum-resistant gonococci* (BS4) *present in the chamber*

The results are typical of three similar experiments.

Time after infection (h)	Chamber conditions		Gonococci	
	H_2O_2 treated*	Redox potential (mV)	No. of c.f.u. ml^{-1}	Serum resistance† (%)
24	−	248	5×10^6	60
24	+	418	ND	ND
26	+	278	7×10^6	> 90
28	+	390	1×10^7	> 90

ND, Not determined.

* Where indicated, 0·1 ml 1 % (v/v) H_2O_2 (100 vol.) was injected at 24 h and then at 0·5 h intervals up to 27·5 h.

† Serum resistance was defined as the count after treatment with serum for 30 min at 37 °C expressed as a percentage of the count of control organisms in Trypticase Soy Broth at 37 °C.

Serum resistance of gonococci from chambers in which the redox potential or glucose concentration had been artificially increased

Raising the redox potential in the chamber with H_2O_2 did not reduce the resistance to human serum of the gonococci growing in the chamber (Table 1).

The rate of multiplication, the population at 24 h and the resistance to human serum during that time of gonococci growing in chambers with added glucose were no different from those of organisms growing in normal chambers (Table 2). Similarly, the omission of glucose from AG broth (Veale *et al.*, 1975; Penn *et al.*, 1976; Rittenberg *et al.*, 1977) [apart from the residual amount (1 to 1·5 mmol l^{-1}) contributed by the newborn calf serum] did not alter the sensitivity to human serum of BS4 (agar) organisms, i.e. reversion to serum resistance, as occurs when such organisms are cultured in chambers *in vivo* (Rittenberg *et al.*, 1977), did not take place.

Growth of serum-sensitive BS4 (agar) in chamber fluid in vitro: effect on serum sensitivity of gonococci and chamber fluid composition

Serum-sensitive gonococci BS4 (agar) incubated in chamber fluid *in vitro* at 37 °C remained sensitive for 0·5 to 1 h and then became resistant. At 25 °C and 4 °C the organisms

Table 2. *Effect of added glucose on serum resistance of the serum-resistant gonococci* (BS4) *during their growth in guinea pig chambers*

Results are for a typical experiment.

Time after inoculation of chamber (h)	Glucose-treated chamber*			Normal chamber		
	Glucose concn (mmol l^{-1})	Gonococci		Glucose concn (mmol l^{-1})	Gonococci	
		No. of c.f.u. ml^{-1}	Serum resistance† (%)		No. of c.f.u. ml^{-1}	Serum resistance† (%)
0	4·7	$2·5 \times 10^3$	ND	7·6	$2·5 \times 10^3$	> 90
0·75	44	$2·5 \times 10^3$	> 90	7·9	$2·5 \times 10^3$	> 90
4	21	2×10^4	> 90	4·8	2×10^4	> 90
6	40	8×10^4	> 90	4·6	6×10^4	> 90
12	27·7	4×10^5	> 90	3·1	2×10^5	> 90
24	3·3	2×10^6	> 90	0·23	2×10^6	> 90

ND, Not determined.
* Glucose (0·2 ml, 800 mmol l^{-1}) was injected at 0, 0·75, 4, 6 and 12 h after inoculation. Samples for assay of glucose were taken immediately before injection of glucose.
† Expressed as in Table 1.

Table 3. *Reversion of serum-sensitive gonococci* [BS4 (agar)] *to resistance during incubation in chamber fluid in vitro*

Two different batches of chamber fluid were used in these experiments.

Length of incubation (h)	Gonococci incubated at:					
	37 °C		25 °C		4 °C	
	No. of c.f.u. ml^{-1}	Serum resistance* (%)	No. of c.f.u. ml^{-1}	Serum resistance* (%)	No. of c.f.u. ml^{-1}	Serum resistance* (%)
Experiment 1						
0	2×10^4	< 5	1×10^5	< 5	1×10^5	< 5
0·5	2×10^4	< 5	1×10^5	< 5	4×10^4	< 5
1	$2·5 \times 10^4$	> 90	1×10^5	< 5	$2·5 \times 10^4$	< 5
3	6×10^4	> 90	1×10^5	< 5	1×10^4	< 5
24	3×10^7	< 5	ND	ND	ND	ND
Experiment 2						
0	1×10^4	< 5	1×10^5	< 5	1×10^5	< 5
0·5	1×10^4	< 5	1×10^5	< 5	1×10^5	< 5
1	1×10^4	> 90	1×10^5	< 5	5×10^4	< 5
3	$3·5 \times 10^4$	> 90	1×10^5	< 5	1×10^4	< 5
24	8×10^6	< 5	ND	ND	ND	ND

ND, Not determined.
* Expressed as in Table 1.

remained sensitive for at least 3 h (Table 3). Although the number of c.f.u. inoculated was 10-fold greater at 25 °C and 4 °C than at 37 °C, to allow for a decline in numbers during the course of the experiments, this was not the reason for the lack of reversion to resistance at the lower temperatures, since in another experiment an inoculum of equal magnitude incubated at 37 °C reverted just as rapidly as did the smaller ones.

In further experiments, observations at between 12 and 24 h revealed that reversion to serum sensitivity occurred when the number of c.f.u. ml^{-1} had increased by 10^3, i.e. after 10 generations. The time taken for this increase ranged from 16 to 24 h. Organisms grown at 37 °C in AG broth (Rittenberg *et al.*, 1977) did not show a similar temporary reversion to resistance.

In marked contrast to chamber fluid *in vivo*, there was only a small decrease (0 to 30%) in

Table 4. *Changes in concentrations of glucose, lactate, alanine, aspartate, glutamine and proline in chamber fluid in vitro after growth of serum-sensitive gonococci* [BS4 (agar)]

The results are typical of three similar experiments with different batches of chamber fluid.

	Concn (mmol l^{-1}) in chamber fluid:		
Component	Initially	After 24 h at 37 °C (control)	After 24 h growth of BS4 (agar)*
Glucose	4·50	5·04	3·06
Lactate	5·92	6·22	0·1
Alanine	0·31	0·37	0·65
Aspartate	0·02	0·02	0·10
Glutamine	0·41	0·36	0·01
Proline	0·34	0·26	0·04

* The number of c.f.u. ml^{-1} at 24 h was 3×10^7.

the concentration of glucose in the chamber fluid *in vitro* after the serum-sensitive BS4 (agar) had been growing in it for 24 h. However, the reverse was true for lactate, there being a considerable (60 to 100%) reduction in concentration. As in chamber fluid *in vivo*, acetate concentrations did not change significantly. Of the amino acids, only the concentrations of glutamine and proline were decreased and those of aspartate and alanine increased (Table 4). Addition of asparagine (0·11 mmol l^{-1}) to the chamber fluid produced no significant change except that the concentration of aspartate was further increased.

The decrease in lactate was interesting, but preliminary experiments suggested that it was not solely responsible for the late loss of serum resistance. First, in some experiments, although lactate was metabolized, an appreciable amount (4 mmol l^{-1}) was still present in the chamber fluid when serum resistance was lost. Second, addition of more lactate (6 mmol l^{-1}) at about 12 h did not prolong the expression of resistance until 24 h.

DISCUSSION

The failure of the gonococcal population in guinea pig chambers to reach more than about 5×10^6 organisms ml^{-1} may have been due to anaerobic conditions in the chambers (Rittenberg *et al.*, 1977). The method of measuring redox potential described here may not have been accurate and the results were somewhat variable. However, in comparison with heart blood and caecal contents, the chambers were not anaerobic even when infected, in agreement with the results of Novotny *et al.* (1977). Nevertheless, when H_2O_2 was added to the chambers, both the redox potential and the gonococcal population increased. Hence, whatever substrate was providing the energy requirements, oxygen availability in the chamber may have contributed to the limitation of growth. However, increasing the redox potential did not decrease serum resistance (Table 1).

The pH of the chambers was slightly alkaline before infection and had fallen only marginally after 24 h despite the intense inflammatory response which might have been expected to cause acidic conditions (Hays & Mandell, 1974). This slightly alkaline pH may have had an influence in maintaining serum resistance if the metabolism of the factors responsible were affected by changes in pH, as is the metabolism of glucose (Morse & Hebeler, 1978).

During infection of normal chambers, serum resistance of the gonococci was expressed during the exponential phase of growth (Table 2) as well as at 24 h. By this time the population had reached the maximum it attains even after days of infection (Veale *et al.*, 1975). The stationary phase might not have been reached at 24 h, because gonococci will have

been removed by phagocytosis and by migration into the surrounding tissue. However, large numbers of gonococci were not present either in phagocytes or in the surrounding tissue (Veale *et al.*, 1975; Chandler *et al.*, 1976) and therefore it is probable that the growth rate at 24 h was slow. Thus, in the guinea pig chambers, serum resistance seems to be expressed regardless of growth phase, unlike the greater resistance of exponential phase, egg-grown gonococci (Gibbs & Roberts, 1975) and the variable serum resistance of other Gram-negative bacteria during different growth phases (Muschel & Fong, 1977).

Previous investigations (Rittenberg *et al.*, 1977) had raised the possibility that the low glucose concentrations in infected subcutaneous chambers might be responsible for the serum resistance of gonococci in these chambers. However, this appears not to be so. Addition of glucose to the chambers so that the concentration was comparable with that found in the laboratory medium (AG broth) in which chamber-grown organisms became susceptible to serum killing after incubation *in vitro* (Rittenberg *et al.*, 1977) did not abolish the serum resistance of the chamber-grown organisms or influence their growth (Table 2). Also growth of gonococci *in vitro* in AG broth of low glucose content did not confer serum resistance. The relatively low counts of gonococci in the chambers (Rittenberg *et al.*, 1977) were not due to low glucose concentrations. Under these conditions gonococci may utilize carbon and energy sources other than glucose; one such substance is lactate (Hebeler & Morse, 1976). As might have been expected because of glycolysis by the large number of phagocytes (10^7 ml^{-1}) in the inflammatory response, the lactate concentration in the chambers increased during infection (Fig. 1) and this substrate could have been utilized by the gonococci. On the other hand, concentrations of amino acids remained relatively constant during infection, the demand by the relatively small numbers of gonococci (5×10^6 ml^{-1}) and any requirements of the phagocytes being satisfied by homeostatic mechanisms of the guinea pig.

Rittenberg *et al.* (1977) showed that chamber-derived organisms retained their serum resistance when grown in chamber fluid *in vitro* but not in AG broth. However, even in chamber fluid the serum resistance was lost after 12 to 24 h culture. We have shown here that serum-sensitive BS4 (agar) organisms regain their serum resistance after 0·5 to 1 h in chamber fluid at 37 °C and maintain it for about 10 generations when there is a reversion to serum susceptibility after 12 to 18 h incubation. Adsorption of chamber fluid constituents to the gonococcal surface was not the reason for acquiring serum resistance since treatment of gonococci with chamber fluid at 25 °C and 4 °C, temperatures at which adsorption but not metabolic modification or multiplication would take place, did not confer serum resistance. Thus, expression of serum resistance may depend on the utilization of specific substrates which are limited in chamber fluid *in vitro* (when the organisms become susceptible) but are replaced as they are used *in vivo*. The specific nature of the substrates is indicated by the fact that acquisition of resistance by BS4 (agar) organisms did not occur in the complex medium used by Rittenberg *et al.* (1977). The situation may be akin to the recent demonstration (Taylor, 1978) of the effect of growth conditions on the serum resistance of strains of *Escherichia coli* from the urinary tract.

The depletion of lactate, glutamine and proline in chamber fluid *in vitro* after growth of gonococci for 24 h (Table 4) does not occur *in vivo* where serum resistance is maintained. Thus, one or more of these compounds may be involved in maintaining serum resistance, although loss of resistance *in vitro* does not correspond with complete usage of any one of them (Tables 3 and 4). The most marked effect of gonococcal growth was the decreased concentration of lactate, but addition of more lactate during the late-exponential growth phase of gonococci in chamber fluid *in vitro* did not prolong expression of serum resistance. However, serum resistance may rely on the correct balance of the three nutrients that appear to be used by gonococci and on other factors not yet recognized; hence further study is required.

The surface components which may account for the phenotypically expressed serum

resistance of gonococci grown in chamber fluid have not been investigated. Adaptation to growth in guinea pig chambers appears to produce capsule-like structures on gonococci which can be retained during minimal subculture *in vitro* (Hormaeche *et al.*, 1978; Ward *et al.*, 1978). Ward *et al.* (1978) reported that chamber-adapted organisms obtained from a parent strain P9 (PS) were resistant to killing by rabbit antibody to lipopolysaccharide and complement in fresh human serum; this resistance (and the capsule-like structure) was maintained after subculture *in vitro* in a medium described by Swanson (1977) and hence a stable serum-resistant genotype may have been selected by the conditions in guinea pig chambers. The capsular material or possibly the production of O chains on the lipopolysaccharide of the gonococci (Ward *et al.*, 1978) may have protected these organisms from serum killing. Whether such explanations apply to the phenotypically expressed resistance to human serum described by us is a matter for future research.

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