Resistance of *Neisseria gonorrhoeae* Grown *in vivo* to Ingestion and Digestion by Phagocytes of Human Blood

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(Received 25 February 1976)

SUMMARY

Attempts to study quantitatively the phagocytosis of gonococci from urethral pus failed because of the small numbers of organisms and technical difficulties. However, gonococci from chambers implanted subcutaneously in guinea pigs, which were similar to gonococci from urethral pus in their resistance to killing by human serum, were obtained in sufficient quantities for comparison in phagocytosis tests with the *in vitro* grown strain from which they were derived.

Microscopic and viable counts of gonococci in phagocytes showed that *in vivo* grown organisms (strain BSV) were readily phagocytosed by human polymorphonuclear phagocytes. There was little difference between BSV organisms and the *in vitro* grown organisms (strain BS) in resistance to ingestion. There was, however, a marked difference in the intracellular survival of strains BSV and BS during the first hour of phagocytosis. Whereas BSV organisms survived well, many BS organisms were killed. Subsequently, strain BSV and the survivors of the strain BS inoculum responded similarly to the intracellular bactericidins. These results were supported by electron microscopy of infected phagocytes.

Resistance of gonococci *in vivo* to ingestion and digestion by human phagocytes seem to be important facets of the pathogenesis of gonorrhoea.

INTRODUCTION

Strongly staining gonococci are seen both extracellularly and intracellularly in urethral pus from patients with gonorrhoea. The presence of extracellular organisms suggests overloading of phagocytic capacity and indicates that some gonococci *in vivo* may resist phagocytosis. However, the presence of intracellular organisms shows that phagocytosis occurs and, further, that gonococci may resist the intracellular bactericidins. Thus resistance to ingestion and digestion by polymorphonuclear phagocytes (PMN phagocytes), the predominant cell type in urethral pus, would be important in acute infection; and survival inside the fewer mononuclear phagocytes (MN phagocytes) could be important in chronic infection.

Pilated, small-colony gonococcal types resembling the virulent Kellogg types 1 and 2 (Kellogg *et al.*, 1963, 1968) seem to be less easily ingested by phagocytes than non-pilated, large-colony types resembling the avirulent Kellogg types 3 and 4 (Punsalang & Sawyer, 1973; Ofek, Beachey & Bisno, 1974; Dilworth, Hendley & Mandell, 1975; Gibbs & Roberts, 1975; Witt, Veale & Smith, 1976), with the exception of the non-pilated large-colony type described by Swanson *et al.* (1974) which is of unknown virulence for man. In some reports a

clear distinction has been made between intracellular and cell-attached gonococci, e.g. by electron microscopy (Witt *et al.*, 1976), by phase-contrast microscopy (Gibbs & Roberts, 1975) and by fluorescent antibody staining (Dilworth *et al.*, 1975). Thus, resistance to ingestion appears to be correlated with the virulence for man of the Kellogg types, but whether or not pili are responsible for the resistance is still a matter of dispute (Swanson *et al.*, 1974).

With regard to resistance to intracellular killing, appreciable numbers of a pilated, small-colony-forming strain of gonococcus (BS) resembling Kellogg type 2 survived and multiplied within human buffy-coat phagocytes (predominantly PMN phagocytes) over 15 h and also survived for at least 6 h in the few MN phagocytes that could be separated from the buffy coat (Veale *et al.*, 1976). A non-pilated, large-colony-forming strain (AL) resembling Kellogg type 4 survived at a much lower level than the BS strain and was destroyed in 10 to 12 h (Veale *et al.*, 1976). Thus, with strains BS and AL, resistance to digestion could be correlated with infectivity for guinea-pig chambers (Veale *et al.*, 1975) and, by analogy with the Kellogg types, with potential virulence for man. These results are in accord with electron microscopic observations of human urethral pus (Novotny, Short & Walker, 1975) that intracellular survival and growth occurs in gonorrhoea. However, the above phagocytosis tests were conducted with gonococci grown *in vitro* and there is increasing evidence that gonococci grown *in vivo* are different.

Urethral pus gonococci were more resistant to killing by human serum and complement than the same strains grown *in vitro* (Ward, Watt & Glynn, 1970). They also had different walls and were less pilated (Novotny *et al.*, 1975). When our BS strain was passaged three times through plastic chambers implanted subcutaneously into guinea pigs after the method of Arko (Arko, 1972; Veale *et al.*, 1975), its infectivity was increased about 100-fold and it formed markedly smaller colonies. It appeared to produce some different antigens in gel-diffusion and, like urethral pus organisms, it became resistant to killing by human serum (Penn *et al.*, 1975, 1976). These observations prompted us to examine the phagocytosis of gonococci grown *in vivo*.

METHODS

Gonococcal strains. Strain BS (pilated, small-colony-forming, MID₅₀ for subcutaneously implanted guinea-pig chambers about 10³, resembling Kellogg type 2) was derived, stored and cultured as described by Veale *et al.* (1975).

Strain BS grown *in vivo* (BSV) was prepared as follows. Strain BS organisms were passaged three times through guinea-pig chambers (Veale *et al.*, 1975; Penn *et al.*, 1975); the chamber contents of the third passage were diluted about 1 to 40 with 1 % (w/v) Proteose Peptone (Difco) and 8 % (v/v) glycerol in water and stored in 0.5 ml aliquots in liquid nitrogen. For an individual phagocytosis test, a thawed aliquot (containing about 1 × 10⁴ organisms) was diluted with 0.5 ml saline and 0.25 ml amounts were introduced into 3 to 4 guinea-pig chambers (Veale *et al.*, 1975), from each of which 0.25 ml of fluid had been removed for 24 h sterility tests. After 24 h, 2 ml of fluid (about 10⁶ gonococci/ml) were removed from each chamber and the bulked sample (6 to 8 ml) was centrifuged at 380 g for 10 min to remove blood cells. Fluid (0.25 ml) was withdrawn from each of 12 fresh guinea-pig chambers for 24 h sterility tests and replaced by the infected exudate (0.25 ml). After another 24 h, infected exudate (about 2 ml/chamber) was collected from chambers which had been sterile when inoculated. The bulked exudate (20 to 25 ml) was centrifuged at 380 g for 10 min to remove blood cells, and the supernatants were centrifuged at 2600 g for 1 h. The deposited organisms were resuspended in Parker medium 199 (BDH) containing 10 % (v/v) heated

human serum (Witt *et al.*, 1976). Helber chamber counts of gonococci could not be made because of other particles. Hence, the total number of gonococci per ml was estimated by comparing the number of stained gonococci in 40 fields of a standard smear (1 cm²) made from one loopful of suspension (Smith *et al.*, 1961) with the corresponding number in fields of a similar smear from a suspension of BS organisms of known concentration. The suspension was then diluted for the phagocytosis tests. Subsequent viable counts on suspensions of BSV organisms were 50 to 140 % of the total counts.

Culture media. These were described by Veale *et al.* (1975) and Witt *et al.* (1976).

Human phagocytes. Populations consisting predominantly of PMN phagocytes (about 80 %) were obtained as described by Witt *et al.* (1976).

Phagocytosis tests. Modified forms of the tests for assessing the relative abilities of gonococcal strains to resist ingestion and digestion by phagocytes described by Witt *et al.* (1976) and Veale *et al.* (1976) were used. The ratio of gonococci to PMN phagocytes was reduced from between 20:1 and 100:1 to approximately 1:1 and particular attention was paid to total and viable counts of gonococci found in phagocyte deposits that attach to glass during the first 1 h ingestion period. The essential steps were as follows. A suspension of gonococci (0.5 ml; about 2×10^6 /ml) and a suspension of PMN phagocytes (0.5 ml; about 2×10^6 /ml) in Parker medium 199 containing 10 % (v/v) isologous heated (56 °C; 30 min) human serum were mixed in Leighton tubes. Control tubes contained gonococci but no PMN phagocytes. The tubes were gassed (5 % CO₂/95 % air) and incubated at 37 °C to allow phagocytosis of organisms to occur, and for the cells to become attached to the glass. One h after mixing (all experiments), and also at 15 and 30 min after mixing in some experiments, tubes were taken and the viable organisms in the supernatants and in the deposits attached to the glass were counted as described by Veale *et al.* (1976). From the total number of phagocytes attached to the glass, the proportion of them containing gonococci, and the average number of gonococci ingested by them (Witt *et al.*, 1976), the total gonococci visibly associated with phagocytes per slide was estimated. After 1 h ingestion, the supernatant was removed from the remaining tubes and the cell deposits were washed (Earle's saline; 3 × 5 ml) before a bactericidal medium (0.5 ml/tube) containing fresh human serum (50 %, v/v) and penicillin (0.4 µg ml⁻¹) in Parker medium 199 was added. The tubes were incubated for up to 6 h for assessment of intracellular survival by viable counts of gonococci in the phagocyte deposits attached to the glass. Incubation was not continued as strain BS was known to survive and multiply during 15 h under similar conditions (Veale *et al.*, 1976). The controls for the phagocytosis test and its interpretation were as described by Witt *et al.* (1976).

Electron microscopy. Possible differences between strains BS and BSV in their resistance to destruction within human phagocytes were investigated. We are indebted to Mr B. Dowsett of the Microbiological Research Establishment, Porton, Wiltshire, for taking electron micrographs of ultra-thin sections of the infected (BS and BSV) PMN phagocyte deposits after 1 h ingestion in the standard phagocytosis test described above. Cells containing gonococci were fixed [1 % (w/v) osmium tetroxide; 4 °C; overnight], embedded in agar, dehydrated in ethanol, embedded in Epon resin, sectioned using a Reichert OMU2 ultra-microtome, stained with uranyl acetate [0.2 % (w/v); 30 min] and lead citrate [0.04 (w/v); 1 min] and examined under a Philips EM 300 electron microscope (accelerating voltage 60 kV). Many fields were examined and gonococci associated with phagocytes (160 for strain BS and 299 for strain BSV) were classified as follows:

1. Intracellular disintegrated gonococci. These included an intact membrane with less than 10 % of the cytoplasm staining, and pieces of membrane with or without attached cytoplasm inside phagosomes (Fig. 1a). These gonococci were undoubtedly dead.

Fig. 1. Electron micrographs of sections of PMN phagocytes in deposits attached to glass 1 h after mixing with gonococci. (a) Disintegrated gonococci (short arrows) and healthy looking, whole gonococci (long arrows). The latter show a well-defined wall and stained cytoplasm occupying the whole of the bacterial sections. (b) Two, less healthy looking gonococci with paler cytoplasm receding from less well defined walls; at least one of these gonococci was probably dead at the time of sampling (arrow).

2. Intracellular whole gonococci. The appearance of these organisms varied between two extremes: from those with a complete layered membrane and the whole area of the cytoplasm well stained (Fig. 1*a*) to others in which, although the majority of cytoplasm was stained, there was shrinkage from the edges near the wall or incompletely stained areas in the centre, and sometimes the membrane was crinkly and less completely layered (Fig. 1*b*). Some of the less healthy looking whole gonococci were probably dead at the time of sampling but they had not disintegrated.

3. Extracellular attached gonococci.

The relative proportions of organisms in categories 1 and 2 indicated the degree of destruction of strains BSV and BS. The proportion of organisms in category 3 indicated the degree of extracellular adherence of the strains.

RESULTS

Attempts to compare gonococci from urethral pus with strain BS in phagocytosis tests

Urethral pus could be collected from male patients but only in 0.1 to 0.2 ml amounts containing 10^4 to 10^6 organisms. The test for comparing the ingestion of strains by obtaining phagocytic indices from visual counts (Witt *et al.*, 1976) was modified by using a chamber holding one tenth of the volume and reducing the numbers of strain BS organisms to those in the pus samples. However, in six tests the numbers of gonococci were so few that only 1 to 2 % of the phagocytes became infected and quantitative work was impossible because counts in experimental tubes were not significantly above background counts of stainable particles in uninfected phagocytes. Two types of tests were used to follow intracellular behaviour. First, gonococci from frozen and thawed pus were mixed with either fresh buffy-coat phagocytes (mainly PMN phagocytes) or buffy-coat phagocytes incubated for 24 h on Leighton tubes (mainly MN phagocytes) in modified versions of the tests described in Methods, using lower numbers of pus and strain BS organisms. After 1 h phagocytosis only a few viable gonococci (about 200 per tube) were detected in the phagocyte deposits attached to the glass even before adding the human serum and penicillin and these disappeared after 1 to 2 h incubation. In the second test, fresh urethral pus was diluted with medium containing fresh human serum and penicillin and incubated in Leighton tubes: PMN phagocytes (some with associated gonococci) settled on the glass surface and viable counts were made at intervals. This test also failed to detect intracellular survival for more than 2 to 3 h because so few organisms were present initially in the phagocyte deposits and because extracellular pus-grown organisms were more resistant to the killing action of the penicillin/fresh serum medium than BS organisms grown *in vitro*. Thus, the concentration of penicillin was either too small (about $0.4 \mu\text{g ml}^{-1}$) to kill all the extracellular organisms so that viable counts on phagocyte deposits did not necessarily indicate intracellular gonococci, or it was large enough (2 to $4 \mu\text{g ml}^{-1}$) to penetrate the phagocytes and kill intracellular gonococci (Veale *et al.*, 1976). The results were similar to those reported by Ward, Glynn & Watt (1972) for incubation of pus with a medium containing penicillin at $1 \mu\text{g ml}^{-1}$.

Microscopic and viable counts of gonococcal strains BS and BSV in the phagocyte deposits attached to the glass during phagocytosis by human PMN phagocytes

Figure 2 shows the viable counts and estimates of the total numbers visually associated with the PMN phagocytes for the two strains during the first 2.5 h of a typical experiment. By electron microscopy, the majority of the cell-associated gonococci were intracellular (Table 3; see also Witt *et al.*, 1976). The light microscopic counts indicated that strain BSV

Fig. 2. Viable counts (○, ●) and light microscopic counts (□, ■) of strain BS (○, □) and strain BSV (●, ■) gonococci in PMN phagocyte deposits attached to glass during the first hour of ingestion by human PMN phagocytes and 1.5 h after incubation with penicillin ($0.4 \mu\text{g ml}^{-1}$) and fresh human serum. Light microscopic counts were estimated from the total numbers of PMN phagocytes in the deposits, the proportion infected and the average number of gonococci in the infected PMN phagocytes. Inocula before phagocytosis were 6.8×10^5 BS organisms and 9.0×10^5 BSV organisms.

might be less easily phagocytosed than strain BS at the beginning of the ingestion period but appreciable ingestion of strain BSV took place in 1 h in this and other experiments (Table 1). The striking difference between the two strains was in the relation between the microscopic and viable counts during the first hour of phagocytosis (Fig. 2). For strain BS, the viable counts were markedly below the corresponding microscopic counts, indicating that many of the intracellular BS organisms were dead. In contrast, the numbers of viable BSV organisms were similar to the numbers visibly associated with the PMN phagocytes indicating that strain BSV was not killed within the PMN phagocytes as rapidly as was strain BS. Furthermore, the viable count at 1 h was markedly greater than that at 30 min for strain BSV but not for strain BS. Results similar to those shown in Fig. 2 were obtained in other experiments (Table 1). Because of ingestion of gonococci, the viable counts in the supernatants from experimental tubes after 1 h were consistently less than those in the control tubes for both strain BSV and strain BS, although the control counts for strain BSV showed a two to three-fold increase over the inoculum whereas those for strain BS showed a reduction to 40 to 80 % of the inoculum. When the supernatants containing strains BS or BSV from experimental tubes after 1 h phagocytosis were incubated for a further 1 h after removal from the phagocyte deposit, there was an increase not a decrease in viable counts, indicating that bactericidins had not been liberated from the PMN phagocytes.

Intracellular survival of strains BS and BSV when PMN phagocytes were incubated with a bactericidal mixture of penicillin ($0.4 \mu\text{g ml}^{-1}$) and fresh human serum

Although the numbers of strain BSV surviving in the PMN phagocyte deposits after 1 h ingestion were higher than those of strain BS from a similar inoculum (Fig. 2; Table 2), the two strains behaved similarly during subsequent incubation of the PMN phagocytes with the bactericidal mixture of penicillin and fresh human serum (Table 2). The numbers of viable strain BSV in the PMN phagocyte deposits remained higher than those of strain BS

Table 1. *Viable and microscopic counts of strain BS (grown in vitro) and strain BSV (grown in vivo) gonococci in PMN phagocyte deposits attached to glass during the first hour of ingestion by human PMN phagocytes*

Counts of gonococci in PMN phagocyte deposits are expressed per 10^4 of the original viable inoculum. Microscopic counts were estimated from counts of the total number of PMN phagocytes deposited in Leighton tubes, the proportion of PMN phagocytes infected and the average number of gonococci in the infected PMN phagocytes (see Witt *et al.*, 1976).

Blood sample	Inoculum; $10^{-6} \times$ viable count	Ingestion time (min)	Counts of gonococci		
			Viable	Microscopic	Viable Microscopic
KG	BS; 0.68	15	2	36	0.06
		30	3	54	0.06
		60	3	96	0.03
	BSV; 0.90	15	14	4	3.5
		30	44	31	1.3
		60	150	410	0.37
CW	BS; 0.40	15	5	35	0.14
		30	8	110	0.07
		60	8	340	0.02
	BSV; 0.60	15	10	42	0.24
		30	150	180	0.83
		60	590	870	0.68
KG	BS; 1.6	15	2	28	0.07
		30	2	810	0.002
		60	3	720	0.004
	BSV; 1.5	15	11	0.4*	(28)
		30	22	24	0.92
		60	170	310	0.55
CW	BS; 1.6	60	1	260	0.004
	BSV; 0.45	60	740	930	0.79
CW	BS; 0.70	60	6	82	0.07
	BSV; 0.60	60	1100	750	1.5
CW	BS; 1.0	60	2	93	0.02
	BSV; 0.44	60	740	1700	0.44

* Gonococci were rarely seen in the stained film.

but there was an initial reduction in numbers of both strains, usually followed by an increase – a pattern already observed for strain BS (Veale *et al.*, 1976). The extent of the decrease and increase was similar for both strains. Strain BSV was more resistant than strain BS to fresh human serum alone (Penn *et al.*, 1975) and in the supernatants strain BSV seemed to be more resistant than strain BS to the bactericidal mixture containing penicillin (Table 2). In most cases, however, the supernatant counts at 2 to 4 h were so much lower than the deposit counts that the latter could only represent intracellular organisms (see Veale *et al.*, 1976).

Electron microscopy of strains BS and BSV in sections of infected PMN phagocyte deposits

Observations on sections of infected PMN phagocyte deposits 1 h after phagocytosis of strains BS and BSV are summarized in Table 3. The results support those already described. Of strain BSV organisms, 36 % had not disintegrated after the 1 h phagocytosis compared

Table 2. *Recovery of strain BS (grown in vitro) and strain BSV (grown in vivo) gonococci after ingestion by human PMN phagocytes and incubation with a medium containing penicillin and fresh human serum*

Gonococci and PMN phagocytes were mixed in the ratio of about 1:1 in Leighton tubes. After 1 h phagocytosis, 10^4 to 10^5 cells (about 80 % PMN phagocytes) were attached to the glass and 50 to 80 % of these remained on the glass after 4 to 6 h. Viable counts of gonococci in the phagocyte deposit, and in the corresponding supernatant (figures in parentheses), were made after the initial ingestion and at intervals after incubation with a bactericidal medium containing fresh human serum and penicillin ($0.4 \mu\text{g ml}^{-1}$). Results are expressed as the number of gonococci per tube: each value is the average of duplicate determinations. Note that these are not progressive counts on the same infected phagocyte population.

Expt; blood sample	Inoculum; $10^{-6} \times$ viable count	Viable gonococci* after ingestion	Viable gonococci† after incubation for:				
			1 h	1.5 h	2 h	4 h	6 h
1; KG	BS; 0.68	200	—	5(< 5)	—	—	—
	BSV; 0.90	13 800	—	2 275(520)	—	—	—
2; CW	BS; 0.40	300	—	—	5(< 5)	904(14)	—
	BSV; 0.60	35 625	—	—	953(250)	2 328(120)	—
3; KG	BS; 1.6	456	—	—	314(30)	2 313(15)	—
	BSV; 1.5	25 750	—	—	1 050(8)	4 812(410)	—
4; CW	BS; 1.6	125	—	—	33(14)	138(4)	5(< 5)
	BSV; 0.45	33 125	—	—	825(130)	1 263(40)	750(< 5)
5; CW	BS; 3.5	6000	—	—	16(< 5)	10(< 5)	30(< 5)
	BS; 0.70	450	—	—	< 5(< 5)	< 5(< 5)	< 5(< 5)
	BSV; 0.60	65 500	—	—	220(< 5)	30(< 5)	880(< 5)
6; CW	BS; 1.0	175	< 5(< 5)	—	5(< 5)	20(< 5)	—
	BSV; 0.44	32 750	925(128)	—	88(< 5)	80(< 5)	—

* Electron microscopy indicated that most of these organisms were intracellular.

† Supernatants and deposits in control tubes (no PMN phagocytes) were sterile (< 5 viable gonococci)

Table 3. *Gonococci in electron microscope sections of PMN phagocytes in deposits attached to glass 1 h after mixing with strain BS or strain BSV*

Strain	No. of infected PMN phagocytes examined	Total no. of gonococci	Intracellular gonococci		Attached gonococci
			Disintegrated*	Whole*	
BSV	119	299	188	107	4
BS	97	160	139	21	0

* See Methods.

with 13 % of BS organisms. Viable counts indicated that many of the whole organisms were alive. Counts of gonococci in the electron micrographs showed that almost all the cell-associated bacteria in the phagocyte deposits were intracellular (Table 3; see Witt *et al.*, 1976).

DISCUSSION

Despite much effort it proved impossible to conduct satisfactory phagocytosis experiments with gonococci from urethral pus. Therefore, the organisms from guinea-pig chambers were used because they were obtained in larger quantity.

Some gonococci are ingested *in vivo* by human PMN phagocytes, as was evident from smears of urethral pus, and the gonococci from guinea-pig chambers were ingested to some

extent by human PMN phagocytes in the tests *in vitro* (Fig. 2; Table 1). Any difference between strains BSV and BS in their resistance to phagocytosis was not marked. Although the microscopic counts of cell-associated gonococci in the early stages of phagocytosis indicated less ingestion of strain BSV than strain BS (Fig. 2), this was not consistent (expt. 2, Table 2).

Strain BSV resisted intracellular killing by PMN phagocytes during 1 h phagocytosis more than did strain BS (Fig. 1; Table 1). This could also be seen from electron microscopy of the infected phagocyte deposits 1 h after phagocytosis (Table 3). There was a greater proportion of whole gonococci in the sections of the BSV-infected phagocytes than in those infected with BS. After the first hour of phagocytosis, the subsequent intracellular behaviour of strains BSV and BS was similar (Fig. 1; Table 2). The absolute numbers of strain BSV within the PMN phagocytes were greater than those of strain BS because of the greater resistance of BSV to killing during the first hour. When the infected PMN phagocytes were incubated with the mixture of human serum and penicillin, the counts for both strains first decreased and then increased. This had previously been observed with strain BS in experiments where the ratio of gonococci to PMN phagocytes was higher and where there were more intracellular organisms in the phagocyte deposits before incubating with penicillin (Veale *et al.*, 1976). In the first hour of phagocytosis many BS organisms were ingested and destroyed leaving a residual population with a resistance to intracellular bactericidins similar to that of the gonococci grown *in vivo*.

On the assumption that the behaviour of gonococci grown *in vivo* in guinea-pig chambers is similar to that in urethral pus, the results reported here, coupled with previous work by ourselves and others (see Introduction), emphasize that both extracellular and intracellular gonococci are present in urethral pus and scrapings. In the natural disease, virulent gonococci partially resist ingestion by human phagocytes. Some, however, are ingested and a proportion of these survive and multiply intracellularly. Thus, resistance to ingestion and digestion are important facets of the pathogenesis of gonococci. The next step is to identify the aggressins involved.

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