A Determinant of Resistance of *Neisseria gonorrhoeae* to Killing by Human Phagocytes: an Outer Membrane Lipoprotein of about 20 kDa with a High Content of Glutamic Acid

By N. J. PARSONS, A. A. A. KWAASI, P. V. PATEL, C. A. NAIRN
AND H. SMITH*

Department of Microbiology, University of Birmingham, PO Box 363, Birmingham B15 2TT, UK

(Received 25 March 1986; revised 22 July 1986)

A protein of about 20 kDa was extracted by sodium cholate (1%, w/v) from outer membranes of a strain of *Neisseria gonorrhoeae*, BS4 (agar), which is resistant to killing by human phagocytes. When the protein was purified by repeated fractionation on Sephadex G75, contamination with other outer-membrane proteins and lipopolysaccharide was negligible. The protein contained a full complement of amino acids, with high levels of glutamic acid. Carbohydrate, detected by the anthrone method and by sugar and hexosamine analysis, was present, but at very low levels. There was a significant content of fatty acids (about 5.7% of the protein), indicating a lipoprotein. The 20 kDa lipoprotein: (1) neutralized the ability of antiserum against whole organisms of BS4 (agar) to reduce the resistance of this strain to phagocyte killing; (2) evoked in mice an antiserum which reduced this resistance and immunoblotted only with 20 kDa lipoprotein in the cholate extract of outer membranes; and (3) promoted resistance to intracellular killing of an otherwise phagocyte susceptible gonococcal strain (BSSH). This is strong evidence that it is a determinant of gonococcal resistance to phagocyte killing.

INTRODUCTION

An important aspect of the pathogenicity of *Neisseria gonorrhoeae* is that a proportion of gonococci in a particular population resist killing by human phagocytes and grow intracellularly (Parsons *et al.*, 1985*b*). This is clear from observations on the intracellular behaviour of gonococci *in vivo*, and in phagocytosis tests conducted *in vitro* but using a strain selected from laboratory cultures by growth *in vivo* (Parsons *et al.*, 1985*b*). These observations have more relevance to the situations *in vivo* than conclusions drawn from phagocytosis tests, which do not distinguish between extracellular and intracellular killing and which are conducted with subcultured laboratory strains (Rest *et al.*, 1982; Mezzatesta & Rest, 1983; Virji & Heckels, 1985, 1986; Ross & Densen, 1985), although even in these tests indications of intracellular survival are seen (Mezzatesta & Rest, 1983; Ross & Densen, 1985; Virji & Heckels, 1986). Indeed, in recent phagocytosis experiments (Casey *et al.*, 1986) conducted with the laboratory strain FA19 but with meticulous care to ensure that intracellular gonococci were distinguished from extracellular organisms, intracellular survival of some gonococci for 165 min was detected unequivocally and at a level (about 2% of ingested bacteria) comparable to those shown by us previously for laboratory-grown strains (Parsons *et al.*, 1985*b*).

In previous work on the determinant concerned (Parsons *et al.*, 1981, 1982, 1985*a*), the resistance to killing by human phagocytes of an *in vivo* selected strain, BS4 (agar), was shown to be associated not with pili, lipopolysaccharide (LPS) or the principal outer membrane proteins (OMPs), but with a membrane protein of about 20 kDa. The putative determinant neutralized

Abbreviations: KDO, 2-keto-3-deoxyoctonate; LPS, lipopolysaccharide; OMP, outer membrane protein; OMV, outer membrane vesicles.

the capacity of an antiserum raised against live organisms of strain BS4 (agar) to abolish the resistance of this strain to intracellular killing by human phagocytes. Also, antisera raised against the putative determinant directly nullified the resistance of strain BS4 (agar) (Veale *et al.*, 1978; Parsons *et al.*, 1981). It was a reasonable working hypothesis (see Veale *et al.*, 1978; Parsons *et al.*, 1981) that the determinant of gonococcal resistance to killing by phagocytes might be detected by these methods.

The 20 kDa protein was detected by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of an outer membrane fraction of strain BS4 (agar) called fraction 1b. The latter was obtained by gel filtration on Sephadex G75 of outer membrane vesicles (OMV) dissolved in an alkaline buffer containing sodium cholate (Parsons *et al.*, 1985a). SDS-PAGE showed that fraction 1b contained proteins of about 60 kDa and some principal OMPs as well as the 20 kDa protein. When the areas of the gel containing the proteins of about 60 kDa, the principal OMPs and the 20 kDa protein, and the gel between the 20 kDa protein and the solvent front, were extracted, only the 20 kDa protein had the biological activity described above (Parsons *et al.*, 1985a). Also, a fraction equivalent to 1b, separated from the closely related phagocyte susceptible strain BSSH, showed no evidence of the 20 kDa protein on SDS-PAGE (Parsons *et al.*, 1985a). In relation to final purification of the 20 kDa protein, it was probable that in fraction 1b before treatment with SDS, some of the 20 kDa protein was complexed with the other proteins and LPS due to incomplete dissociation of the OMV by the sodium cholate (Parsons *et al.*, 1985a).

This paper describes the purification of the 20 kDa protein from fraction 1b without treatment with SDS, and its chemical and biological properties.

METHODS

Neisseria gonorrhoeae. Strain BS4 (agar) is relatively resistant to killing by human phagocytes. It was selected from a laboratory strain, BS (Kellogg type 2, small colony forming, pilate) by four passages through plastic chambers implanted subcutaneously in guinea pigs and then cultured once on agar medium (Penn *et al.*, 1976, 1977; Parsons *et al.*, 1979). Strain BSSH is susceptible to killing by phagocytes. It constituted the majority of the population of strain BS from which it was selected *in vitro* by its 'single highlight' (SH) colony morphology (Penn *et al.*, 1977). Both strains were grown, stored and counted as described previously (Veale *et al.*, 1975; Penn *et al.*, 1976, 1977).

Extraction of crude OMV with sodium cholate. OMV were prepared from strain BS4 (agar) (100 ml ; 10^{11} gonococci ml^{-1}) in 1 M -lithium chloride and extracted with glycine/NaOH buffer (0.1 M , pH 9.5) containing sodium cholate (1% , w/v) as described previously (Parsons *et al.*, 1982, 1985a). If not fractionated immediately, extracts were stored at $-20\text{ }^{\circ}\text{C}$.

Preparation of fraction 1b from cholate extracts. This was as described previously (Parsons *et al.*, 1985a), but larger amounts ($8\text{--}10\text{ ml}$ rather than $3\text{--}4\text{ ml}$) of the cholate extract (about $1\text{ mg protein ml}^{-1}$ and derived from about 5×10^{11} gonococci ml^{-1}) were applied to the column of Sephadex G75 (Pharmacia; $1\text{ m} \times 25\text{ mm}$). Fractions (2 ml) corresponding to fraction 1b (Parsons *et al.*, 1985a) were pooled and, in earlier work, concentrated by negative pressure dialysis. Later this was done by freeze drying, dissolving in water (4 ml) and desalting on a Sephadex G25 column ($1\text{ m} \times 25\text{ mm}$) with water elution. The final volume of the pooled fractions was adjusted to that of the original extract.

The protein profiles on SDS-PAGE of OMV of strain BS4 (agar), their sodium cholate extract and fraction 1b are shown in Fig. 2 of Parsons *et al.* (1985a) and they form the basis of much of the biochemical work in this paper.

Refractionation of fraction 1b on Sephadex G75. Fraction 1b ($8\text{--}10\text{ ml}$, derived from 5×10^{11} gonococci ml^{-1}), whose protein profile on SDS-PAGE is shown in Fig. 2 (lanes 4 and 5) of Parsons *et al.* (1985a), was refractionated on a column of Sephadex G75 ($1\text{ m} \times 15\text{ mm}$) with glycine/NaOH buffer (0.1 M , pH 9.5) containing sodium cholate (1% , w/v) as eluant as described for the preparation of fraction 1b (Parsons *et al.*, 1985a). Elution was followed by monitoring A_{279} . The region where materials of 20 kDa would elute was indicated by preliminary experiments under the same conditions with the following marker proteins: ribonuclease, chymotrypsinogen A, ovalbumin and bovine serum albumin (BSA) (13.7 , 25.0 , 43.0 , 67.0 kDa respectively) (Fig. 1). Fractions of the gonococcal extract in the 20 kDa region were pooled, and concentrated by freeze drying and desalting on Sephadex G25 as described above. The volume of the final material was adjusted with phosphate buffered saline (PBS, Penn *et al.*, 1976) to $1/25$ of the volume of the original cholate extract. Several batches contained $1\text{--}1.5\text{ mg protein ml}^{-1}$, representing about 5% of the protein in the original cholate extract of OMV (see above).

Purified pili from strain BS4 (agar). These were two of the four preparations described by Parsons *et al.* (1981) and contained about 1.0 and $1.2\text{ mg protein ml}^{-1}$ respectively.

SDS-PAGE. A modification (Parsons *et al.*, 1982) of the method of Laemmli (1970) was used with a 12.5% (w/v) acrylamide gel for the gels stained with Coomassie blue (Fig. 2) and for the immunoblotting experiments (see later). A 50 µl sample of the solution of 20 kDa protein (containing 50–75 µg protein) was applied to the gels and other materials (amounts see text) were applied in the same volume.

For the gels stained with the more sensitive silver stain (Fig. 3), SDS-PAGE was conducted with a 15% (w/v) acrylamide gel: 2.5 µl of the solution of 20 kDa protein (containing 2.5 µg protein) and of a solution of fraction 1b (containing 0.125 and 0.25 µg protein) were applied to the gels. LPS (0.2 µg) was also applied to the gels. The gels were stained with silver by the following modification of the method of Hitchcock & Brown (1983). The gels were fixed overnight at room temperature in 25% (v/v) 2-propanol in water containing 7% (v/v) acetic acid and then treated with an oxidizing solution of sodium periodate (1.05 g), 2-propanol (1 ml), acetic acid (0.28 ml) and water (to 150 ml) for 5 min at room temperature with gentle agitation. They were then washed in deionized water (one wash in 200 ml followed by eight washes of 30 ml each) and immersed in freshly prepared silver reagent [silver nitrate 1 g, NaOH (0.1 M) 28 ml, ammonia solution (sp.gr. 0.88) 2 ml and deionized water to 180 ml] for 10 min at room temperature with gentle agitation. After four washes in deionized water (each wash for 10 min in 200 ml), stained components were developed in a solution of citric acid (50 mg) and formaldehyde (0.25 ml) in water (250 ml). When the colour of components reached a suitable intensity, development was stopped by addition of a 25% (v/v) methanol solution containing 7% (v/v) acetic acid. Gels were photographed within 24 h.

Estimation of contamination with foreign protein. This was achieved by SDS-PAGE using both Coomassie blue (Fig. 2) and silver (Fig. 3) staining.

The maximum amount of the final preparation which showed no bands other than in the 20 kDa region with Coomassie blue staining was ascertained. Also dilutions of a solution of fraction 1b of known total protein concentration were run on SDS-PAGE with Coomassie blue staining to obtain the minimum amount that would show the 20 kDa protein band and which would provide clearly detectable bands corresponding to the principal OMPs of gonococci (about 30–35 kDa) (Parsons *et al.*, 1985a). These amounts of 20 kDa protein and fraction 1b were then run on the same gel (Fig. 2). Thus contamination with those proteins most likely to be present in the 20 kDa protein was checked. Precise figures could not be obtained because the contents of the different proteins in fraction 1b were not known. Nevertheless these amounts would have been much less than that indicated by the total protein content. Because of the lack of knowledge of the precise amounts of various proteins in fraction 1b, an additional indication of the limits of detection of foreign protein by Coomassie blue was obtained by also running a mixture of weighed minimal detectable quantities of three known proteins (chymotrypsinogen A, ovalbumin and serum albumin) side by side with the 20 kDa protein and fraction 1b (Fig. 2).

A similar comparison of the 20 kDa protein with fraction 1b (but not the three known proteins) was carried out with the more sensitive silver stain (Fig. 3); the amounts of both materials used were about 20-fold less than those for the Coomassie blue staining.

Chemical analysis. Protein (Lowry method), 2-keto-3-deoxyoctonate (KDO) and carbohydrate (anthrone method) were assayed as described by Parsons *et al.* (1982, 1985a) using BSA, 2-keto-3-deoxyoctonic acid and glucose, respectively, as standards.

We are indebted to Professor J. N. Hawthorne and Dr C. Simpson of the Department of Biochemistry, University of Nottingham Medical School, for the analysis of fatty acids. This was done by gas-liquid chromatography (GLC) of fatty acid methyl esters prepared as described by Christie (1973) after extraction of the lipid by the method of Griffin & Hawthorne (1978). Butylated hydroxytoluene was included in the extraction mixture as an antioxidant and as an internal standard for quantification.

After acid hydrolysis, amino acids (except cysteine), hexosamines and sugars were analysed by Dr J. E. Fox, Macromolecular Analysis Service, University of Birmingham, using the methods described by Patel *et al.* (1984).

Two indirect tests for the determinant of resistance to intracellular killing. These two tests and various control experiments on the intracellular habitat of the test organisms and the negligible influence of extracellular organisms were described in detail by Parsons *et al.* (1985a). Only the principles are summarized here together with a description of the antisera used. The results presented in the Tables are representative of numerous tests; the variable viability of gonococci in phagocyte deposits from different donors on different days preclude presentation of mean values and standard errors and statistical analysis (Parsons *et al.*, 1985a).

In the first test, the capacity of strain BS4 (agar) to survive intracellularly in human phagocytes (see below) was markedly reduced by pretreatment with a rabbit antiserum prepared against live organisms of strain BS4 (agar), but not if the antiserum had been absorbed previously with extracts of BS4 (agar) which contained the putative determinant of resistance to intracellular killing (Parsons *et al.*, 1981, 1982, 1985a). Absorption of antiserum and subsequent pretreatment of gonococci before the phagocytosis tests were described by Parsons *et al.* (1985a). Solutions of the 20 kDa protein used for absorption of the antisera contained 0.5–1.5 mg protein ml⁻¹.

The second test was to see if an antiserum against the putative determinant abolished the capacity of strain BS4 (agar) to survive intracellularly, as did antisera against whole organisms (Parsons *et al.*, 1985a). Mouse antiserum to the 20 kDa protein was raised by five intraperitoneal injections at 14 d intervals of 100 µg protein per mouse, given as an emulsion in Freund's adjuvant (0.1 ml per mouse; complete adjuvant for the first injection, incomplete

adjuvant for subsequent injections) and, 7 d after the final intraperitoneal injection, 100 µg protein per mouse given intravenously. One week later, blood was collected, allowed to clot for 4 h at room temperature and serum separated. Three separate batches of sera were used for the phagocytosis tests, which included controls with normal, pre-immunization mouse serum in place of the immune serum.

Test for the resistance of gonococci to intracellular killing by human phagocytes. The test, a modification of that used to detect intracellular survival of gonococci selected *in vivo* (Witt *et al.*, 1976*a, b*; Penn *et al.*, 1977; Veale *et al.*, 1978; Parsons *et al.*, 1981), was described in detail by Parsons *et al.* (1985*a*). In summary, 10^6 gonococci of strain BS4 (agar), either pretreated with antiserum as described above or control, untreated organisms, were mixed in Leighton tubes with equal numbers of PMN phagocytes freshly prepared from human peripheral blood. After incubation for 1 h at 37 °C, phagocytes attached to the flat surface of the tubes were thoroughly washed to remove extracellular gonococci. The phagocytes containing gonococci were lysed mechanically to quantify both the number of adherent phagocytes (by measuring DNA: Parsons *et al.*, 1982) and the number of viable gonococci (by plating out). Coverslips from replicate tubes were removed and stained for microscopic determination of the phagocytic index, i.e. the number of gonococci per phagocyte. The percentage of the cell associated gonococci (the product of the number of phagocytes and the phagocytic index) per tube that were viable after the 1 h incubation was a measure of the resistance of gonococci to intracellular killing.

Direct test for the determinant of resistance to intracellular killing. In some phagocytosis tests, attempts were made to enhance the resistance to intracellular killing of the otherwise susceptible strain, BSSH, by pretreating it with the 20 kDa protein separated from the phagocyte resistant strain BS4 (agar). The effect of treatment with purified pili separated from strain BS4 (agar) was also assessed as a control. The phagocytosis tests were done as described for strain BS4 (agar) (see above and Parsons *et al.*, 1985*a*) but substituting strain BSSH and pretreating it (10^6 organisms ml⁻¹) with 50–75 µg 20 kDa protein ml⁻¹ or purified pili (100–120 µg protein ml⁻¹) in Parker's medium 199 with 10% (w/v) heated human serum for 1 h at 37 °C. The organisms were centrifuged (1500 g, 10 min, room temperature) and washed three times with the medium before being used in the phagocytosis tests. Control organisms were treated similarly, but the protein was omitted.

Immunoblotting of a sodium cholate extract of OMV with rabbit antiserum to fraction 1b and mouse antiserum to the 20 kDa protein. A modification of the method of Towbin *et al.* (1979) was used. Immediately after SDS-PAGE of a sodium cholate (1%, w/v) extract of OMV from strain BS4 (agar) (about 25 µg total protein, equivalent to 50 µl of a suspension of 2.5×10^{11} gonococci ml⁻¹; see lane 2 of Fig. 2 Parsons *et al.*, 1985*a*), the protein components were transferred electrophoretically (positive to negative) to nitrocellulose membranes (0.45 µm pore size, Sartorius) in an EC Transphoelectrophoresis transfer cassette and electrophoresis unit (Applied Corporation) in 25 mM-Tris/192 mM-glycine buffer pH 8.2, containing 25% (v/v) methanol, at a current of 0.4 A for 16 h. The membranes were treated for 90 min at room temperature with 50 ml PBS containing 3% (w/v) BSA to saturate all remaining binding sites, washed quickly twice with PBS (50 ml) and reacted with either rabbit antiserum to fraction 1b (prepared as described by Parsons *et al.*, 1985*a*) or mouse antiserum to the 20 kDa protein (0.2 ml in 5 ml PBS containing 0.1% BSA) for 45 min at room temperature. Controls were treated with either normal, pre-immunization rabbit serum or normal, pre-immunization mouse serum. The membranes were washed three times, each for 5 min, with PBS (50 ml) and incubated for 1 h with 50 µl of either sheep anti-rabbit IgG conjugated to alkaline phosphatase (Sigma) or anti-mouse IgG conjugated to alkaline phosphatase (Sigma), as appropriate, each diluted in 5 ml PBS containing 0.1% BSA. They were then washed twice with PBS (10 ml) and placed in 0.1 M-Tris buffer pH 8.2 (10 ml). To visualize the antigen-antibody complexes, the membranes were placed in 10 ml 0.2 M-Tris buffer pH 8.2 containing 4 mg sodium naphthol AS-MX phosphate (Sigma) until the desired intensity of colour was reached. Development was stopped by transferring the membranes to 0.1 M-Tris buffer pH 8.2 (10 ml).

RESULTS

Separation of a 20 kDa protein from fraction 1b of OMV by gel filtration on Sephadex G75

These results should be viewed in relation to the original separation of fraction 1b from the OMV of strain BS4 (agar) and particularly the protein profiles on SDS-PAGE of the OMV, their sodium cholate (1%, w/v) extract and fraction 1b (Fig. 2 of Parsons *et al.*, 1985*a*); fraction 1b contained several proteins in the 60 kDa region and some principal OMPs as well as the 20 kDa protein.

The elution profile (Fig. 1) of a typical re-fractionation of fraction 1b shows that most of the material absorbing at 279 nm was eluted from Sephadex G75 well before the region in which molecules of 20 kDa should elute as indicated by preliminary experiments with marker proteins (see Methods and Fig. 1). Fractions collected from the marked region were pooled and concentrated as described in Methods. Several batches of the final solution in PBS (volume 1/25 that of the original cholate extract from which fraction 1b was separated) contained 1–1.5 mg protein ml⁻¹, representing about 5% of the protein in the original cholate extract (see Methods).

Fig. 1. Typical profile (A_{279}) of the separation on Sephadex G75 of fraction 1b from the OMV of strain BS4 (agar). The eluting buffer was glycine/NaOH, 0.1 M, pH 9.5, containing sodium cholate (1%, w/v). Fractions were collected from the region marked 20 kDa; this region had been designated by preliminary experiments with marker proteins (B, BSA; O, ovalbumin; C, chymotrypsinogen A; R, ribonuclease).

Fig. 2

Fig. 3

Fig. 2. SDS-PAGE gels (12.5%, w/v, acrylamide) stained with Coomassie blue; lane 1, the purified 20 kDa protein (65 μ g); lane 2, fraction 1b (4 μ g protein); lane 3, a mixture of chymotrypsinogen A (2 μ g), ovalbumin (2 μ g) and BSA (2 μ g).

Fig. 3. SDS-PAGE gels (15%, w/v, acrylamide) treated with silver stain: lane 1, the purified 20 kDa protein (2.5 μ g); lanes 2 and 3, fraction 1b (0.25 μ g and 0.125 μ g respectively); lane 4, LPS (0.2 μ g). Lane 5 is an unloaded channel to show the two lines produced by the bromophenol marker which were just above the solvent front.

Table 1. *Intracellular killing of strain BS4 (agar) by human phagocytes: effect of antiserum [to BS4 (agar)] absorbed with the 20 kDa protein from OMV of BS4 (agar)*

The results are typical of six similar tests.

Expt no (phagocyte donor)	Antiserum*	Absorbed† with 20 kDa protein batch no. (mg ml ⁻¹)	Viable gonococci in phagocyte deposits after 1 h at 37 °C‡ (% of total microscopic count)
1. (JK)	—	—	22.4
	+	—	<0.5
	+	1 (1.0)	23.7
	+	2 (1.5)	24.0
2. (WS)	—	—	23.6
	+	—	4.3
	+	3 (0.8)	9.5
	+	—	—
3. (JH)	—	—	12.6
	+	—	3.3
	+	4 (0.7)	6.3
	+	5 (0.7)	8.0

* Treatment of gonococci with rabbit antiserum against BS4 (agar) before mixing with phagocytes as described by Parsons *et al.* (1981, 1985a).

† Absorption was done as described in the text with the concentration of 20 kDa protein shown in parentheses.

‡ The phagocytosis test was as described in Methods. Suspensions of human phagocytes (10⁶, about 80% PMN) were mixed with 10⁶ BS4 (agar) organisms (>60% viable) in Leighton tubes and incubated for 1 h at 37 °C. Viable and visual counts of gonococci and counts of total number of phagocytes were made as described by Parsons *et al.* (1981, 1985a).

Fig. 2 shows patterns on SDS-PAGE with Coomassie blue staining for the final preparation of 20 kDa protein, fraction 1b and a mixture of three marker proteins in amounts just above their limit of detection with this stain (see Methods). The track containing the purified preparation was overloaded (65 µg), yet showed only a single band in the 20 kDa region (lane 1). In lane 2, fraction 1b (4 µg total protein) showed, as before (lanes 4 and 5 of Fig. 2 of Parsons *et al.*, 1985a), a faint band of the 20 kDa protein and strong bands in the principal OMP region (about 30–35 kDa). In lane 3, a mixture of 2 µg of each of the three marker proteins showed clearly discernible bands. Thus, contamination of the 20 kDa protein (65 µg examined) with foreign proteins was very low: (a) because of the strength of the bands of the protein constituents of fraction 1b, each of which was present in an amount much less than the total protein content of the fraction 1b examined (4 µg); and (b) because of the strength of the bands of the marker proteins examined in about 3% of the quantity of the purified 20 kDa protein preparation. The results summarized in Fig. 2 were obtained for three different batches of the final preparation.

When the more sensitive silver stain was used, similar results were obtained. A 2.5 µg sample of the purified protein produced only one strong band in the 20 kDa region (Fig. 3, lane 1) and no other bands either in the higher M_r region or in the low M_r region between the 20 kDa protein and the solvent front (lane 5 is an unloaded gel channel showing the two lines of the bromophenol marker which were just above the solvent front). On the other hand, much smaller quantities of fraction 1b (0.25 µg and 0.125 µg; lanes 2 and 3) produced many bands in the higher M_r regions and a strong band in the lower M_r region which appeared to be LPS (see marker LPS; lane 4) which was not revealed by Coomassie blue staining (Fig. 2). Thus, for the reasons discussed in the preceding paragraph, silver staining also indicated that any contamination of the 20 kDa protein with other proteins and LPS derived from fraction 1b was much less than 5% (calculated from the relative quantities of the materials used).

The amount of KDO in two batches of the 20 kDa protein was <0.08% and <0.01% of the total protein content, indicating that contamination with LPS was less than 0.8% and 0.1% respectively, since a purified preparation of LPS from BS4 (agar) contained 11.8% of KDO (Tan *et al.*, 1986).

Table 2. Intracellular killing of strain BS4 (agar) by human phagocytes: effect of mouse antisera against the 20 kDa protein

These results are typical of five similar tests.

Expt no. (phagocyte donor)	Gonococci [BS4 (agar)] pretreated* with			Viable gonococci in phagocyte deposits after 1 h at 37 °C† (% of total microscopic counts)
	Rabbit anti- BS4 (agar) serum	Normal mouse serum	Mouse anti- 20 kDa protein serum (batch no.)	
1. (WS)	—	—	—	9.6
	+	—	—	1.7
	—	+	—	15.5
	—	—	+ (1)	1.1
	—	—	+ (2)	1.2
2. (CP)	—	—	—	29.9
	+	—	—	6.3
	—	+	—	34.8
	—	—	+ (1)	15.0
	—	—	+ (3)	11.2

* Gonococci [strain BS4 (agar)] were incubated in media containing rabbit antiserum against live BS4 (agar) or normal pre-immunization mouse serum or one of three batches (1, 2, 3) of mouse antisera against the 20 kDa protein (see Methods and Parsons *et al.*, 1985a).

† See footnote ‡ to Table 1.

Biological activity of the 20 kDa protein

The results in Table 1 show that several batches of the final preparation neutralized the capacity of rabbit antiserum against whole BS4 (agar) organisms to reduce the intracellular survival of strain BS4 (agar).

The results in Table 2 show that, in two experiments, three different batches of mouse antiserum raised against the purified 20 kDa protein reduced the resistance of strain BS4 (agar) to killing by phagocytes in a manner similar to that for rabbit antiserum against whole BS4 (agar) organisms. The mouse antiserum raised against the 20 kDa protein reacted only with that protein. Thus, Fig. 4 records the results of immunoblotting experiments with a SDS-PAGE gel of a crude sodium cholate (1%, w/v) extract of OMV of strain BS4 (agar) which contained many proteins including proteins I and II of the principal OMPs (lane 2 of Fig. 2 of Parsons *et al.*, 1985a). A rabbit antiserum raised against fraction 1b which was obtained from the cholate extract (Parsons *et al.*, 1985a) and from which the 20 kDa protein was separated (see above) showed many lines in immunoblotting (lane 1 of Fig. 4) including one corresponding to the 20 kDa protein (this rabbit antiserum also neutralized the resistance of strain BS4 (agar) to phagocyte killing: Parsons *et al.*, 1985a). In contrast, mouse antiserum against the 20 kDa protein produced only one line corresponding to the 20 kDa protein (lane 2; Fig. 4). Immunoblotting with normal pre-immunization rabbit serum (lane 3) and mouse serum (lane 4) produced no lines.

The results of experiments 1 and 2 in Table 3 show that pretreatment of the otherwise phagocyte susceptible gonococcal strain BSSH with the 20 kDa protein conferred on it significant resistance to killing by phagocytes as judged by comparison of the results with those obtained with control BSSH and BS4 (agar) organisms. In contrast, pili purified from strain BS4 (agar) (Parsons *et al.*, 1981) conferred no resistance (experiments 3 and 4 of Table 3).

Chemical nature of the 20 kDa protein

Two batches of the final purified preparation contained the following amino acids respectively (% of total recovered amino acids): Asp 11.2, 10.7; Thr 5.4, 5.4; Ser 7.3, 7.3; Glu 22.4, 28.1; Pro 7.4, 8.1; Gly 6.3, 6.4; Ala 4.8, 4.1; Val 5.8, 5.6; Met 1.5, 1.4; Ile 4.1, 3.9; Leu 6.2, 6.7; Tyr 2.8, 2.8; Phe 3.4, 4.0; His 4.1, 4.1; Lys 4.7, 4.4; and Arg 2.7, 3.1.

Fig. 4. Immunoblots of SDS-PAGE gels of a sodium cholate (1%, w/v) extract of OMV of strain BS4 (agar) (about 25 µg total protein, equivalent to 50 µl of a suspension of 2.5×10^{11} gonococci ml⁻¹; see lane 2 of Fig. 2 of Parsons *et al.*, 1985*a*, for protein profile) with a rabbit antiserum against fraction 1b (lane 1), a mouse antiserum against the 20 kDa protein (lane 2), normal rabbit serum (lane 3) and normal mouse serum (lane 4). See Methods for details.

The total carbohydrate (calculated as glucose) content of the two batches was 1.2% and 1.3% of the total protein content; the corresponding contents of sugars estimated after hydrolysis with trifluoroacetic acid (Patel *et al.*, 1984) were: glucose 0.6%, 0.6%; galactose 0.4%, 0.2%; mannose 0.1%, 0.2%; and other sugars <0.1%, <0.1% respectively. The glucosamine content of the two batches was 0.2% and 0.4% of the total protein content, and the galactosamine content 0.1% and 0.1%.

Analysis of two samples of the final purified preparation for fatty acids by GLC of their methyl esters showed the same three unidentified methyl esters in each sample. The total fatty acid content was estimated as equivalent to 5.7% of the protein content.

DISCUSSION

The 20 kDa protein in fraction 1b had been identified in previous work as a putative determinant of gonococcal resistance to phagocyte killing (Parsons *et al.*, 1985*a*) by SDS-PAGE in which the SDS would have liberated the protein. Indeed, fraction 1b separated from cholate-solubilized OMV of strain BS4 (agar) by gel filtration on Sephadex G75 (Parsons *et al.*, 1985*a*), but not treated with SDS, still contained much complexed material of high molecular mass as shown by the main peak in its re-fractionation on Sephadex G75 (Fig. 1). Nevertheless, fraction 1b contained some uncomplexed 20 kDa protein. Using marker proteins to designate the region in the elution profile where this protein would be eluted (Fig. 1), a sufficient quantity of adequately pure material was obtained for relevant biological and chemical tests.

Table 3. *Intracellular killing by human phagocytes of a susceptible strain of N. gonorrhoeae (BSSH) with and without pretreatment by the 20 kDa protein or purified pili from the resistant strain BS4 (agar)*

Tests 1 and 2, done with different batches of 20 kDa protein, are typical of six tests with different batches of material. Tests 3 and 4, done with different batches of purified pili, are typical of four tests with different batches of material. The phagocyte resistant strain BS4 (agar) was included for comparison with the untreated and treated susceptible strain BSSH.

Expt no. (phagocyte donor)	Organism	Pretreatment* with		Viable gonococci in phagocyte deposits after 1 h at 37 °C† (% of total microscopic count)
		20 kDa protein ($\mu\text{g ml}^{-1}$)	Pili protein ($\mu\text{g ml}^{-1}$)	
1. (AC)	BSSH	—	—	6.1
	BSSH	+ (75 μg)	—	11.5
	BS4 (agar)	—	—	12.8
2. (CP)	BSSH	—	—	4.6
	BSSH	+ (50 μg)	—	13.5
	BS4 (agar)	—	—	18.4
3. (CP)	BSSH	—	—	4.2
	BSSH	—	+ (100 μg)	4.0
	BS4 (agar)	—	—	10.8
4. (NP)	BSSH	—	—	8.4
	BSSH	—	+ (120 μg)	6.3
	BS4 (agar)	—	—	14.0

* Gonococci (strain BSSH; 10^6 ml^{-1}) were incubated with 20 kDa protein (50–75 $\mu\text{g ml}^{-1}$) or purified pili (100–120 $\mu\text{g protein ml}^{-1}$) for 1 h at 37 °C and then washed three times with medium before use in the phagocytosis test.

† See footnote ‡ to Table 1 [10^6 BSSH or BS4 (agar) organisms were used].

The results of SDS-PAGE with Coomassie blue staining (Fig. 2) on large amounts of the final preparation and small amounts of fraction 1b and of a mixture of three marker proteins indicated that contamination with foreign proteins was negligible. These results were confirmed using the more sensitive silver stain (Fig. 3). The gels of the 20 kDa protein in Fig. 3 also show that there was no contamination with low M_r proteins running between the 20 kDa protein and the solvent front. Also, the LPS found in fraction 1b (Parsons *et al.*, 1985a) could not be detected in the 20 kDa protein preparation.

In seeking to identify the bacterial determinants of interference with host defences, it is relatively easy to reach the first stage, namely associating certain bacterial components with relevant biological activity by comparing different strains biologically and chemically. Many studies are left at this stage because it is much harder to obtain evidence of causation. Indeed, in most cases, the best that can be done is to improve on mere association and provide evidence of strong implication in the particular facet of pathogenicity (Smith, 1983). We feel that such evidence has been provided here. First, the 20 kDa protein has been isolated and purified to a stage where contamination with other outer membrane components is negligible. Second, it is active in relevant biological tests. The final purified preparation neutralized the capacity of antiserum against strain BS4 (agar) to reduce the resistance of that strain to intracellular killing by human phagocytes (Table 1). Also, mouse antisera raised against the final preparation, like the antiserum against whole BS4 (agar) reduced the resistance of BS4 (agar) to intracellular killing (Table 2). The mouse antiserum was specific for the 20 kDa protein. Immunoblotting against a crude extract of OMV of strain BS4 (agar) which contained many proteins including proteins I and II of the OMPs (Parsons *et al.*, 1985a) showed that the mouse antiserum reacted only with the 20 kDa protein, in contrast to a rabbit antiserum raised against fraction 1b which reacted with many proteins (Fig. 4). Finally, the protein had the positive effect of promoting resistance to phagocytic killing of an otherwise susceptible strain, BSSH, in contrast to the negative effect of purified pili (Table 3), which had been shown in previous work (Parsons *et al.*,

1981) neither to neutralize the above effect of antiserum against BS4 (agar) nor to produce an antiserum which would reduce the resistance of BS4 (agar) to phagocyte killing. All three biological tests, and particularly the third, are strong evidence that the 20 kDa protein is a determinant of gonococcal resistance to killing by phagocytes (Smith, 1983).

Turning to the chemical nature of the protein, its being a glycoprotein with a substantial carbohydrate content might have explained its poor staining ability in SDS-PAGE. However, the carbohydrate content as indicated by the anthrone method and by sugar and hexosamine analysis after acid hydrolysis was no more than 1.5% of the total protein content. It probably represented a slight contamination of the protein with other cell-wall constituents such as LPS, and possibly peptidoglycan fragments, but the possibility that it, or some of it, might be an inherent constituent of this protein cannot be ruled out. There was a significant content of fatty acids (about 5.7% of the protein) and three components were seen in the GLC of fatty acid methyl esters. Clearly, the protein is a lipoprotein and, if allowance is made for glycerol and other possible groups, the lipid content may approach 10%. The lipoprotein contained a full complement of amino acids, with a strikingly high content of glutamic acid. Whether this high content is connected with the biological activity of the protein in inhibiting the bactericidal activities of the phagocytes is a matter for speculation.

The 20 kDa lipoprotein described here might have been the same as the H8 antigen described by Cannon *et al.* (1984). However, in eight tests, a monoclonal antibody which recognized the H8 antigen (Cannon *et al.*, 1984), kindly supplied by Dr Janne Cannon (Department of Microbiology and Immunology, University of North Carolina, Chapel Hill, N. Carolina, USA), failed to reduce the resistance of gonococcal strain BS4 (agar) to killing by phagocytes. This was in contrast to the effect of rabbit antiserum to BS4 (agar) (Table 1) and mouse antiserum to our protein (Table 2). The fact that our 20 kDa lipoprotein contains three components in its fatty acid content, a very high amount of glutamic acid compared with other amino acids, and significant quantities of aromatic amino acids, also suggests that it is different from the H8 antigen. H8 appears to contain only two lipid components, no strikingly high content of glutamic acid compared with other amino acids and no aromatic acids (Strittmatter & Hitchcock, 1986). Proteins with molecular masses of about 20 kDa were seen in recent isolates from an outbreak of infection caused by a single gonococcal strain (Schwalbe *et al.*, 1985) and in a gonococcal strain grown *in vitro* with human serum (Britigan & Cohen, 1985). The occurrence of the 20 kDa protein in fresh gonococcal isolates and of antibodies to it in patients are being investigated.

REFERENCES

- BRITIGAN, B. & COHEN, M. S. (1985). Effect of growth in serum on uptake of *Neisseria gonorrhoeae* by human neutrophils. *Journal of Infectious Diseases* **152**, 330-338.
- CANNON, J. G., BLACK, W. J., NACHAMKIN, I. & STEWART, P. W. (1984). Monoclonal antibody that recognises an outer membrane antigen common to the pathogenic *Neisseria* species but not to most non-pathogenic *Neisseria* species. *Infection and Immunity* **43**, 944-949.
- CASEY, S. G., SHAFER, W. M. & SPITZNAGEL, J. K. (1986). *Neisseria gonorrhoeae* survive intraleucocytic oxygen dependent antimicrobial capacities of anaerobic and aerobic granulocytes in the presence of pyocin lethal for extracellular gonococci. *Infection and Immunity* **52**, 384-389.
- CHRISTIE, W. W. (1973). *Lipid Analysis*. Oxford: Pergamon Press.
- GRIFFIN, H. D. & HAWTHORNE, J. N. (1978). Calcium-activated hydrolysis of phosphatidyl-myoinositol 4-phosphate and phosphatidyl-myoinositol 4,5-bisphosphate in guinea-pig synaptosomes. *Biochemical Journal* **176**, 541-552.
- HITCHCOCK, P. J. & BROWN, T. M. (1983). Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver stained polyacrylamide gels. *Journal of Bacteriology* **154**, 269-277.
- LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, London* **227**, 680-685.
- MEZZATESTA, J. R. & REST, R. F. (1983). Phagocytic killing of *Neisseria gonorrhoeae* by human monocytes. *Infection and Immunity* **42**, 99-105.
- PARSONS, N. J., PENN, C. W., VEALE, D. R. & SMITH, H. (1979). More than one antigen contributes to the immunogenicity of *Neisseria gonorrhoeae* in the guinea pig subcutaneous chamber model. *Journal of General Microbiology* **113**, 97-104.
- PARSONS, N. J., KWAASI, A. A., TURNER, J. A., VEALE, D. R., PERERA, V. Y., PENN, C. W. & SMITH, H. (1981). Investigation of the determinants of the survival of *Neisseria gonorrhoeae* within human polymorphonuclear phagocytes. *Journal of General Microbiology* **127**, 103-112.
- PARSONS, N. J., KWAASI, A. A., PERERA, V. Y., PATEL, P. V., MARTIN, P. M. V. & SMITH, H. (1982).

- Outer-membrane proteins of *Neisseria gonorrhoeae* associated with survival within human polymorphonuclear phagocytes. *Journal of General Microbiology* **128**, 3077–3081.
- PARSONS, N. J., KWAASI, A. A. A., PATEL, P. V., MARTIN, P. M. V. & SMITH, H. (1985*a*). Association of resistance of *Neisseria gonorrhoeae* to killing by human phagocytes with outer-membrane proteins of about 20 kilodaltons. *Journal of General Microbiology* **131**, 601–610.
- PARSONS, N. J., PATEL, P. V., MARTIN, P. M. V., GOLDNER, M. & SMITH, H. (1985*b*). Gonococci *in vivo* and *in vitro*: studies of the host and bacterial determinants of gonococcal resistance to killing by human serum and by phagocytes. In *The Pathogenic Neisseriae*, pp. 487–494. Edited by G. K. Schoolnik, G. F. Brooks, S. Falkow, C. E. Frasch, J. S. Knapp, J. A. McCutchan & S. A. Morse. Washington, DC: American Society for Microbiology.
- PATEL, P. V., VEALE, D. R., FOX, J. E., MARTIN, P. M. V., PARSONS, N. J. & SMITH, H. (1984). Fractionation of guinea pig serum for an inducer of gonococcal resistance to killing by human serum; active fractions containing glucopeptides similar to those from human red blood cells. *Journal of General Microbiology* **130**, 2757–2766.
- PENN, C. W., SEN, D., VEALE, D. R., PARSONS, N. J., SMITH, H. & WITT, K. (1976). Morphological, biological and antigenic properties of *Neisseria gonorrhoeae* adapted to growth in guinea pig subcutaneous chambers. *Journal of General Microbiology* **97**, 35–43.
- PENN, C. W., VEALE, D. R. & SMITH, H. (1977). Selection from gonococci grown *in vitro* of a colony type with some virulence properties of organisms adapted *in vivo*. *Journal of General Microbiology* **100**, 147–158.
- REST, R. F., FISCHER, S. H., INGHAM, Z. Z. & JONES, J. T. (1982). Interactions of *Neisseria gonorrhoeae* with human neutrophils: effects of serum and gonococcal opacity on phagocyte killing and chemiluminescence. *Infection and Immunity* **36**, 737–744.
- ROSS, S. C. & DENSEN, P. (1985). Opsonophagocytosis of *Neisseria gonorrhoeae*: interaction of local and disseminated isolates with complement and neutrophils. *Journal of Infectious Diseases* **151**, 33–41.
- SCHWALBE, R. S., SPARLING, P. F. & CANNON, J. G. (1985). Variation of *Neisseria gonorrhoeae* protein II among isolates from an outbreak caused by a single gonococcal strain. *Infection and Immunity* **49**, 250–252.
- SMITH, H. (1983). The elusive determinants of bacterial interference with non-specific host defences. *Philosophical Transactions of the Royal Society* **B303**, 99–113.
- STRITTMATTER, W. & HITCHCOCK, P. (1986). Isolation and characterisation of the gonococcal H8-antigen. *Abstracts of the 1986 Annual Meeting of the American Society for Microbiology*, B-3. Washington, DC: American Society for Microbiology.
- TAN, E. L., PATEL, P. V., PARSONS, N. J., MARTIN, P. M. V. & SMITH, H. (1986). Lipopolysaccharide alteration is associated with induced resistance of *Neisseria gonorrhoeae* to killing by human serum. *Journal of General Microbiology* **132**, 1407–1413.
- TOWBIN, H., STAHELIN, T. & GORDON, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy of Sciences of the United States of America* **76**, 4350–4354.
- VEALE, D. R., SMITH, H., WITT, K. & MARSHALL, R. B. (1975). Differential ability of colonial types of *Neisseria gonorrhoeae* to produce infection and an inflammatory response in subcutaneous perforated plastic chambers in guinea pigs and rabbits. *Journal of Medical Microbiology* **8**, 325–335.
- VEALE, D. R., PENN, C. W., SUTTON, S. & SMITH, H. (1978). The effect of specific antiserum on the resistance of *Neisseria gonorrhoeae* to intracellular killing by phagocytes of human blood. *Journal of General Microbiology* **106**, 129–136.
- VIRJI, M. & HECKELS, J. E. (1985). Role of anti-pilus antibodies in host defence against gonococcal infection studied with monoclonal anti-pilus antibodies. *Infection and Immunity* **49**, 621–628.
- VIRJI, M. & HECKELS, J. E. (1986). The effect of protein II and pili on the interaction of *Neisseria gonorrhoeae* with human polymorphonuclear leucocytes. *Journal of General Microbiology* **132**, 503–512.
- WITT, K., VEALE, D. R., FINCH, H., PENN, C. W., SEN, D. & SMITH, H. (1976*a*). Resistance of *Neisseria gonorrhoeae* grown *in vivo* to ingestion and digestion by phagocytes of human buffy coat. *Journal of General Microbiology* **96**, 341–350.
- WITT, K., VEALE, D. R. & SMITH, H. (1976*b*). Resistance to ingestion and digestion of *Neisseria gonorrhoeae* by phagocytes of human buffy coat. *Journal of Medical Microbiology* **9**, 1–12.