Cytidine 5'-Monophospho-*N*-acetylneuraminic Acid or a Related Compound is the Low M_r Factor from Human Red Blood Cells Which Induces Gonococcal Resistance to Killing by Human Serum

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A low- M_r factor which induces gonococcal resistance to complement-mediated serum killing has been partially purified from lysates of mixed red and buffy coat cells from human blood. The lysates were dialysed against Tris buffer for 24 h at 25 °C with the diffusate being continuously recycled through a column of QAE-Sephadex A25. After elution in an NaCl gradient, the active fractions were both desalted and further purified on Sephadex G10. A second fractionation on QAE-Sephadex A25 and desalting with Sephadex G10 preceded further purification by repeated high-pressure liquid chromatography (HPLC) using a DEAE anion exchange column and desalting with Sephadex G10. Less than 500 µg of material showing one peak in HPLC was obtained from 1 litre of blood. After NMR had indicated the possible presence of pyrimidine nucleotide, carbohydrate and *N*-acetyl groups, nanogram quantities of a commercial preparation of cytidine 5'-monophospho-*N*-acetylneuraminic acid (CMP-NANA) were shown to induce gonococci to serum resistance. The synthetic CMP-NANA also co-eluted with the preparation from blood cells in HPLC, and the two materials were indistinguishable in their patterns of acid and heat lability. Furthermore, the resistance-inducing activity of both materials was inhibited by cytidine monophosphate, which is known to inhibit sialylation reactions by CMP-NANA. It appears therefore that the resistance-inducing factor is CMP-NANA or a closely related compound. If the factor is CMP-NANA, biological activities indicated that the cell lysate from 1 litre of blood contained about 40 µg, and the most purified preparation contained only about 1%. With this minute amount in a mixture, the presence of CMP-NANA or a closely related analogue could not be established unequivocally by NMR.

INTRODUCTION

Gonococci obtained from patients and examined without subculture are resistant to complement-mediated serum killing (Parsons *et al.*, 1985). Many strains lose the resistance on culture *in vitro* (Ward *et al.*, 1970) and we have been trying to explain this unstable type of resistance using initially a guinea-pig model. Gonococci were resistant to killing by serum after being passaged four times through subcutaneous plastic chambers in guinea-pigs (strain BS4) but not after subsequent growth on agar [strain BS4(agar)] (Penn *et al.*, 1976). The loss of resistance *in vitro* was phenotypic (Rittenberg *et al.*, 1977) and could be reversed by incubation for 3 h at 37 °C *in vitro* with guinea-pig chamber fluid, with guinea-pig serum, or in a defined medium supplemented with an ultrafiltrate of the latter (Veale *et al.*, 1981; Patel *et al.*, 1984b). An acid- and heat-labile, low- M_r resistance-inducing factor (RIF) of about 1000 Da was then

Abbreviations: CMP-NANA, cytidine 5'-monophospho-*N*-acetylneuraminic acid; NANA, *N*-acetylneuraminic acid; RBC, red blood cells; RIF, resistance inducing factor.

separated from the ultrafiltrate into still impure fractions which appeared to contain one or more glucopeptides with glutamic acid, glycine and cysteine present amongst other amino acids (Patel *et al.*, 1984*b*).

Human urogenital secretions and about 20% of tested sera also had resistance-inducing activity (Martin *et al.*, 1981, 1982). In addition, the resistance-inducing activities of sera correlated with some patterns of female gonorrhoea (Martin *et al.*, 1984). Thus, host-induced resistance to complement-mediated killing may be important in gonorrhoea. On ultrafiltration of human serum, some resistance-inducing activity was found in low- M_r fractions but, in contrast to guinea-pig serum, far more activity remained in high- M_r fractions. The low- M_r RIF from humans was similar to the guinea-pig material in ultrafiltration characteristics and acid and heat lability (Martin *et al.*, 1981). Furthermore, two glucopeptide fractions containing glutamic acid, glycine and cysteine and other amino acids obtained from extracts of human red blood cells (RBC) had a small resistance-inducing activity (Weiss *et al.*, 1971; Patel *et al.*, 1984*b*). Lysates of guinea-pig and human RBC were 30- to 60-fold more active in inducing resistance than the corresponding sera (Patel *et al.*, 1984*a*). As for the sera, however, almost all the guinea-pig RIF was low- M_r , whereas the human factors were predominantly, but not exclusively, high- M_r (Patel *et al.*, 1984*a*). Some low- M_r RIF is present in human RBC lysates and this paper describes its fractionation and identification. Initially, extraction from RBC and fractionation were based on the methods of Weiss *et al.* (1971, and private communication) and Patel *et al.* (1984*b*). This preliminary work provided information about the properties of low- M_r RIF and of potential impurities which was used in designing the method described.

METHODS

Bacterial strain and growth conditions. *Neisseria gonorrhoeae* [strain BS4(agar)], media, culturing and counting procedures were as described previously (Penn *et al.*, 1976; Veale *et al.*, 1981; Patel *et al.*, 1984*b*).

Generation of serum-resistant gonococci, test for resistance to killing by fresh human serum and an approximate assay of RIF activity. These were as described by Patel *et al.* (1984*b*). One RIF unit is the amount of inducing factor which, under standard conditions previously described (Patel *et al.*, 1984*b*), will convert 50% of gonococci [2×10^4 strain BS4(agar)] to resistance to killing by fresh human serum.

Release of low- M_r resistance-inducing activity from blood cells. Packed RBC and buffy coat cells, obtained from 1 litre of human peripheral blood by centrifugation for 15 min at 4 °C and 2000 g, were resuspended in approximately 400 ml 10 mM-Tris/HCl buffer, pH 6.5. The mixture was gently shaken for 1 h at 37 °C, transferred in 25–30 ml portions to 1.9 cm diameter dialysis bags and dialysed for 24 h at 25 °C against the smallest possible volume (typically 800 ml) 10 mM-Tris/HCl buffer, pH 6.5. The long period of dialysis at elevated temperature was appropriate because control experiments had established that the release of low- M_r RIF, possibly from a high- M_r precursor, was both time- and temperature-dependent.

Chromatography on QAE-Sephadex A25 and gel filtration. A 2.6 × 70 cm column of QAE-Sephadex A25 was equilibrated at 4 °C with 10 mM-Tris/HCl, pH 6.5, at a flow rate of 60 ml h⁻¹. The diffusate from the lysed blood cells was then loaded onto the column at 25 °C by continuous recycling during the 24 h dialysis process with a flow rate of 60 ml h⁻¹. Most of the low- M_r RIF adsorbed to the column, but a significant amount (about 30% of total recovery) remained in the diffusate. This material was pre-loaded onto the QAE-Sephadex A25 column at 4 °C before the next batch of diffusate was processed. After the adsorption stage, the column was transferred to a cold room at 4 °C and bound material was eluted at 60 ml h⁻¹ with a 1.44 litre linear gradient of 0–0.3 M-NaCl in 10 mM-Tris/HCl, pH 6.5. After the gradient, any material remaining on the column was eluted with 0.3 M-NaCl in 10 mM-Tris/HCl, pH 6.5. The eluate was monitored at 279 nm and 5 ml fractions were tested without concentration for RIF, carbohydrate and Folin-positive peptide. Active fractions from the QAE-Sephadex A25 column were pooled, freeze dried, redissolved in 2–5 ml distilled water and desalted by filtration through a 1.5 × 160 cm column of Sephadex G10. The buffer was 1 mM-Tris/HCl, pH 6.5, and the flow rate 12 ml h⁻¹. Fractions (4 ml) were assayed for RIF, conductivity, carbohydrate and peptide.

Active fractions from the Sephadex G10 column were freeze dried, dissolved in 2 ml 100 mM-Tris/HCl, pH 6.5, and applied to a second QAE-Sephadex A25 column (1.0 × 70 cm) which had been equilibrated at 4 °C with 100 mM-Tris/HCl, pH 6.5. The column was washed with 80 ml 100 mM-NaCl in 100 mM-Tris/HCl, pH 6.5, prior to elution of the bound material with a 400 ml linear gradient of 0.1–0.3 M-NaCl in 100 mM-Tris/HCl, pH 6.5, at a flow rate of 25 ml h⁻¹. The eluate was monitored at 279 nm and 4 ml fractions were assayed without concentration for RIF, carbohydrate, peptide and glutathione. Active fractions were pooled, freeze dried, dissolved in 2 ml water and desalted by filtration through a column of Sephadex G10 as described above.

Purification of RIF by high-pressure liquid chromatography (HPLC). The freeze-dried material from the Sephadex G10 column was dissolved in 800 µl water and applied in sixteen 50 µl aliquots to a DEAE 5PW HPLC column (7.5 mm internal diameter × 7.5 cm) which was protected by a 6 mm internal diameter × 1 cm guard column (Toyo Soda Manufacturing Co.). Two LDC model III dual-head pumps were driven by an LDC gradient master (Laboratory Data Control). The column was equilibrated with 10 mM-NaCl in 1 mM-Tris/HCl, pH 7.0, and the aliquot was eluted at a flow rate of 1 ml min⁻¹ with a 30 ml linear gradient prepared from 15 ml of the same buffer and 15 ml of 90 mM-NaCl in 1 mM-Tris/HCl, pH 5.0. The eluate was monitored at 210 nm and 500 µl fractions were collected. The column was then washed at 1 ml min⁻¹ for 4 min with 90 mM-NaCl in 1 mM-Tris/HCl, pH 5.0, and re-equilibrated with 10 mM-NaCl in 1 mM-Tris/HCl, pH 7.0, for 15 min before the next aliquot was applied.

The least-contaminated fractions with appreciable RIF from 12 of the 16 aliquots were combined, desalted by passage through a 1.5 × 160 cm column of Sephadex G10 in 1 mM-NaH₂PO₄/Na₂HPO₄ buffer, pH 6.8, freeze dried and redissolved in 400–500 µl distilled water. This sample was then applied in two portions to the same DEAE 5PW HPLC column which had been equilibrated with 30 mM-NaCl in 1 mM-Tris/HCl, pH 6.5, and eluted isocratically at 1 ml min⁻¹ with the same buffer. Fractions which contained least-contaminated RIF activity were combined from both portions, desalted by filtration through Sephadex G10 and equilibrated with 1 mM-NaH₂PO₄/Na₂HPO₄ buffer, pH 6.8. The samples containing active material were bulked, freeze dried, and repeatedly re-dissolved in deuterium oxide and freeze dried in preparation for NMR spectroscopy.

Comparisons of the acid and heat labilities of low-M_r RIF from blood and cytidine 5'-monophospho-N-acetylneuraminic acid (CMP-NANA). The acid labilities of low-M_r RIF from blood and CMP-NANA were compared by adding 5 µl samples containing approximately equal numbers of RIF units or a mixture of 2.5 µl of each material to 245 µl 10 mM-citric acid/sodium citrate buffers which had been adjusted to pH 4.0, 4.5, 5.0 and 6.5. After 1 h at 37 °C, the pH of each sample was adjusted to 6.5 with 0.5 M-NaOH. Each sample was then diluted with an equal volume of defined medium (DM; Veale *et al.*, 1981) and assayed for RIF activity.

Heat labilities were compared by adding 10 µl of sample (low-M_r RIF or CMP-NANA, each containing approximately equal numbers of RIF units) or 5 µl RIF plus 5 µl CMP-NANA to 490 µl 10 mM-citrate buffer pH 6.5 and incubating at 75 °C. Aliquots (100 µl) were removed after 0, 15, 45 and 60 min, immediately cooled on ice, diluted with an equal volume of DM and assayed for RIF activity.

Effect of cytidine monophosphate (CMP) on the RIF activity of CMP-NANA and the low-M_r RIF from blood. Samples of low-M_r RIF or CMP-NANA (each containing approximately equal numbers of RIF units in 100 µl) were added to 100 µl of 1 mM-CMP, 0.1 mM-CMP, 0.01 mM-CMP or distilled water and tested for RIF activity. A control with 1 mM-CMP but no low-M_r RIF or CMP-NANA was also included.

NMR spectroscopy. We are indebted to Drs J. Feeney and T. Frenkel at the Biomedical NMR Centre, National Institute for Medical Research, Mill Hill, London, UK, for examining the 500 MHz ¹H NMR spectra of the preparation from blood: measurements were made on the Bruker AM 500 spectrometer. The freeze-dried preparation was dissolved in deuterium oxide (0.5 ml) and contained approximately 40 mM-sodium phosphate.

Chemical analysis. Total peptide was measured by the Lowry method using bovine serum albumin as standard and total carbohydrate by the anthrone method with glucose as standard. Amino acids, except cysteine, were analysed by the methods described by Patel *et al.* (1984b) after 6 M-HCl hydrolysis under vacuum for 24 h at 100 °C. After this treatment, little cysteine was detected (as cystine). Hence, if any cysteine had been detected by this method, it was assayed accurately as cysteic acid after hydrolysis and oxidation of a separate sample by performic acid. Sugar analysis was as described by Patel *et al.* (1984b). Glutathione, measured by the method of Bernt & Bergmeyer (1974), could be detected at 0.5 µg ml⁻¹.

Compounds containing pyrimidine nucleotides. CMP-NANA, CMP, uridine diphosphate (UDP), uridine diphospho-N-acetylglucosamine and uridine diphospho-N-acetylgalactosamine were obtained from Sigma. Cytidine diphosphoribitol was kindly supplied by Professor R. Archibald and Dr I. C. Hancock of the Department of Microbiology, University of Newcastle upon Tyne, UK.

RESULTS

Initial extraction of low-M_r RIF from blood cells and preliminary characterization

Preliminary experiments were done in the belief that low-M_r RIF might be a glucopeptide containing glutamic acid, glycine, cysteine and possibly other amino acids (see Introduction). Hence, the low-M_r RIF was extracted from human RBC with saline EDTA essentially as described by Weiss *et al.* (1971; and private communication). The dilute extract was then fractionated by a combination of the methods used by the same authors and by Patel *et al.* (1984b) for low-M_r RIF from guinea-pig serum and from lysates of guinea-pig RBC (unpublished observations). High-M_r RIF was removed by filtration through XM 50 Diaflo

membranes (Amicon; nominal cut-off 50000 Da) or HIP 30 hollow-fibre cartridges (Amicon; nominal cut-off 30000 Da). Low- M_r RIF passed through a YM5 membrane (nominal cut-off 5000 Da) but was retained on a YCO5 membrane (nominal cut-off 500 Da). About 70% of the total RIF activity was recovered from ultrafiltration, of which about 70% was high- M_r and 30% low- M_r . Further purification of the YCO5 retentate was achieved by gel filtration through Biogel P2 and anion-exchange chromatography on QAE-Sephadex A25. This procedure indicated that the active component had an M_r of about 500–1000 and contained acid groups. The RIF activity was associated with substantial quantities of glucose, glycine, glutamic acid and cysteine, and with smaller quantities of aspartic acid and serine. This analysis was similar to that of the fractions obtained from guinea-pig serum and RBC lysates (Patel *et al.*, 1984*b*, and unpublished observations), and from human RBC by Weiss *et al.* (1971, and private communication). At this point, appreciable quantities of glutathione were detected in the fractions, explaining the relatively high content of glutamic acid, glycine and cysteine. However, neither reduced nor oxidized glutathione was active in the RIF assay, even at concentrations as high as 1 mg ml⁻¹. Glutathione was removed from the low- M_r RIF as described below. RIF activity was heat- and acid-labile (after 1 h at 75 °C, pH 6.5, and 37 °C, pH 4, respectively). At room temperature and at 37 °C it was stable at pH 6.5, the pH of the medium used for induction of serum resistance (Veale *et al.*, 1981; Patel *et al.*, 1984*b*). It was unaffected by incubation for 18 h at 37 °C and pH 5 with agarose-bound neuraminidase (Sigma).

Samples of RIF of comparable activity and acid lability, and again associated with sugar, glutathione and other amino acids, were also obtained by fractionating water lysates (16 h, 4 °C) of RBC that had previously been extracted with saline EDTA. This indicated that the extraction of low- M_r RIF by saline EDTA was incomplete. The purification protocol subsequently used was therefore designed to release as much low- M_r RIF as possible, rapidly, and in a form suitable for immediate anion-exchange chromatography. In the later stages of the preliminary fractionation, some low- M_r RIF activity passed through YCO5 membranes. Hence, Sephadex G10 was used both to desalt and to fractionate RIF after each ion-exchange procedure. A long column of QAE-Sephadex A25 and a moderately slow flow rate were selected to optimize the separation of RIF from contaminating glutathione and carbohydrate-containing components by exploiting differences in both size and charge.

Release and purification of low- M_r RIF

During initial dialysis of the blood-cell lysate for 24 h at 25 °C the diffusate was continuously loaded onto the QAE-Sephadex A25 column. About 60% of the total RIF activity of the lysate was retained as high- M_r RIF in the dialysate (Table 1). About 30% of the total recovered diffused low- M_r RIF remained unadsorbed on the QAE-Sephadex A25 column at the end of the dialysis period (Table 1) and was processed with the next batch of diffusate (see Methods).

The elution profile of the diffusate on the first QAE-Sephadex A25 column using a linear gradient of NaCl in 10 mM-Tris/HCl, pH 6.5, is shown in Fig. 1(a). RIF activity was eluted by about 0.26 M-NaCl. Considerable amounts of peptide (27 mg) and carbohydrate (270 mg) were removed in early fractions (1–75) before the gradient was applied. The combined active fractions contained about 70% of the total recovered low- M_r RIF with some peptide (5.6 mg) and carbohydrate (2.9 mg) (Table 1).

The active material from the first QAE-Sephadex A25 column was separated from several impurities that absorbed at 279 nm during filtration through Sephadex G10 (Fig. 1b). The recovery of activity in the combined active fractions was 70% (Table 1). Salt was removed and the eluate contained less peptide and far less carbohydrate than the QAE-Sephadex A25 eluate (Table 1).

Residual glutathione and further carbohydrate were then removed by a second QAE-Sephadex A25 column (Fig. 1c). The recovery of activity in the bulked active fractions was 61% (Table 1). Desalting with Sephadex G10 was accomplished with 82% recovery of activity and no change in peptide and carbohydrate contamination (Table 1).

The pooled and desalted active material from the second QAE-Sephadex A25 column was fractionated on the first HPLC column in 16 aliquots. The multicomponent elution profile of one

Fig. 1. Elution profiles (279 nm) of: (a) the diffusate from a RBC lysate (from 1 litre of blood) on QAE-Sephadex A25 equilibrated with 10 mM-Tris/HCl, pH 6.5, and eluted with a gradient of NaCl in the same buffer; (b) the desalting and fractionation of the active material from (a) on Sephadex G10 equilibrated and eluted with 1.0 mM-Tris/HCl, pH 6.5; and (c) the fractionation of the active material from (b) on a second QAE-Sephadex A25 column equilibrated with 100 mM-Tris/HCl, pH 6.5, and eluted with a gradient of NaCl in the same buffer. Fractions containing RIF are indicated by the hatched boxes on the bottom axis. ---, Conductivity; ●---●, glutathione.

Table 1. *RIF activity, peptide content and carbohydrate content of the active fractions at progressive stages in the fractionation of low- M_r RIF from the diffusate of the RBC lysate from 1 litre of blood*

Fractionation stage	Total content in fraction			RIF recovery (%) [*]	Specific activities	
	RIF (units)	Peptide (mg)	Carbohydrate (mg)		RIF units (mg peptide) ⁻¹	RIF units (mg carbohydrate) ⁻¹
Lysate	210000	ND	ND			
Dialysate (high- M_r RIF)	130000	ND	ND			
Diffusate†						
QAE-Sephadex A25	24000	5.61	2.91		4278	8247
Sephadex G10	16700	2.58	0.54	70	6472	30925
QAE-Sephadex A25	10100	1.28	0.09	61	7890	112222
Sephadex G10	8300	1.07	0.08	82	7757	103750

ND, Not determined.

^{*} Recoveries (%) are calculated with respect to the immediate preceding stage: any losses include the activity in small samples taken for biological and chemical assay.

† The diffusate was continuously recirculated through the column of QAE-Sephadex A25; 10000 RIF units remained in the diffusate when circulation ceased; this material was preloaded onto the column before fractionation of the next batch of diffusate.

aliquot is shown in Fig. 2(a). The RIF activity coincided with a shoulder preceding a major contaminant which was eluted in the gradient by about 50 mM-NaCl. Less separation of components occurred with four of the aliquots: for the remaining 12, fractions with the highest ratio of RIF activity to contaminant (fractions 8 and 9 in Fig. 2a) were pooled, desalted by filtration through a column of Sephadex G10, freeze dried and redissolved in 0.5 ml water. The material was applied to the second HPLC column in two portions, and eluted isocratically (Fig. 2b). Fractions 6–8 of both portions were combined, and desalted by filtration through Sephadex G10 which had been equilibrated with a phosphate buffer, pH 6.8. The samples containing the active material were bulked (20 ml), freeze dried and prepared for NMR spectroscopy. Only a single absorption peak at 210 nm was detected when a sample of the final RIF preparation was applied to and eluted isocratically from the HPLC column in 30 mM-NaCl, 1 mM-Tris/HCl, pH 6.5 (Fig. 2c).

Major losses of activity occurred in the two HPLC fractionations because of the need to select only the leading RIF-active fractions (Fig. 2a, b), leaving much activity in later neighbouring fractions. In the first separation from 1 litre of blood, the preliminary stages of which are summarized in Table 1, the final preparation had 1400 RIF units in 0.5 ml deuterium oxide. In a second separation from 2.5 litres of blood, the final preparation had 7500 RIF units in 0.5 ml deuterium oxide.

Examination of the preparation from blood by NMR and UV spectroscopy

NMR spectroscopy, done mainly on the sample containing 7500 RIF units, indicated that at least four components were present. The major component appeared to be a sugar (possibly related to fructose). Two further components were pyrimidine nucleotides and were subsequently shown to have ¹H spectra characteristic of uridine diphospho-*N*-acetylglucosamine and uridine diphospho-*N*-acetylgalactosamine. An additional compound contained aliphatic groups. There was no evidence for guanine, adenine, thymine, glycine, cysteine, glutamic acid, threonine, aspartic acid or other amino acids in the major components detected. The strength of the major signals indicated a concentration of approximately 1 mM for the main components, equivalent to about 300 µg of each (assuming an M_r indicated by gel filtration and ultrafiltration of approximately 600) in the 0.5 ml deuterium oxide. Thus, with several components present, a very rough estimate of the total material was of the order of 1 mg.

The absorbancy maximum of 260 nm in the UV spectrum of the final preparation was in accord with the presence of pyrimidine nucleotides.

Fig. 2. Elution profiles (210 nm) of: (a) an aliquot of the desalted (Sephadex G10) active material from the second QAE-Sephadex A25 column on the first DEAE 5PW HPLC column eluted with a linear gradient of 10–90 mM-NaCl in 1 mM-Tris/HCl with a pH gradient from pH 7.0 to pH 5.0 [RIF activity (▨) was in fractions 7–12]; (b) selected active fractions (8 and 9) from (a) after pooling and desalting (Sephadex G10) on the second HPLC column eluted with 30 mM-NaCl in 1 mM-Tris/HCl, pH 6.5 [RIF activity (▨) was in fractions 5–12]; and (c) the final preparation obtained by pooling and desalting (Sephadex G10) selected active fractions (6, 7 and 8) from (b).

The high resistance-inducing activity of CMP-NANA: comparison of its properties with those of the preparation from blood

The NMR results and the extreme acid lability of RIF guided the subsequent experiments. Uridine diphospho-*N*-acetylglucosamine and uridine diphospho-*N*-acetylgalactosamine, the presence of which had been demonstrated by NMR, were much less acid-labile than RIF and were inactive when tested at 100 μ M in the RIF assay. However, CMP-NANA is an extremely acid-labile pyrimidine nucleotide and a synthetic (enzymically prepared) commercial sample proved active in the RIF assay at 0.2 μ M. In contrast, CMP, UDP, cytidine diphosphoribitol and *N*-acetylneuraminic acid (NANA) were inactive at 100 μ M. Two different commercial samples of CMP-NANA contained 525 and 550 RIF units per nmol (0.6 μ g), i.e. approximately 1 RIF unit per ng.

After being examined by NMR, during which the sample was frozen and thawed several times, the preparation from blood was compared with CMP-NANA in various tests. When applied to the same HPLC column that had been used for the fractionation and eluted with 30 mM-NaCl, 1 mM-Tris/HCl, pH 6.5, its profile at 210 nm (Fig. 3a) showed a main peak (retention time 12.7 min, coinciding with RIF activity) and a small one (retention time 14.3 min). Clearly, some degradation had taken place since the original separation (Fig. 2c). Under the same conditions, the profile of CMP-NANA (20 μ l of 0.1 mM) showed a single peak (retention time 12.8 min) (Fig. 3b). When CMP-NANA and the preparation from blood were mixed together, the profile (Fig. 3c) showed a single peak (retention time 12.7 min) with a slight shoulder on the trailing end.

Fig. 3. Elution profiles (210 nm) of the final preparation from blood, and of authentic CMP-NANA, from a DEAE 5PW HPLC column with 30 mM-NaCl, 1 mM-Tris/HCl, pH 6.5, as the eluant. (a) The final preparation from blood (Fig. 2c; 150 RIF units) examined after NMR during which the sample had been frozen and thawed several times; (b) CMP-NANA, 20 μ l of 0.1 mM; and (c) a mixture of (a) and (b).

Table 2. Acid and heat inactivation of the RIF activities of the preparation from human blood and of CMP-NANA

Treatment*	RIF activity (units ml ⁻¹) in solutions of:		
	(1) Blood preparation	(2) CMP-NANA (0.2 μ M)	(1) and (2) equal mixture
Incubation (37 °C, 1 h) at pH:			
6.5	95	100	91
5.0	55	45	31
4.5	13	25	24
4.0	<1	<1	<1
Incubation (75 °C, pH 6.5) for:			
0 min (control, room temp.)	111	133	105
15 min	45	69	55
45 min	27	31	36
60 min	6	8	10

* A 10 mM-citrate buffer was used (see Methods).

The preparation from blood, CMP-NANA, and a mixture of the two were indistinguishable in the extents to which their RIF activities were reduced at pH 5.0, 4.5 and 4.0 compared with controls at pH 6.5 after 1 h at 37 °C (Table 2). Similarly, the extents to which their activities were reduced after 15, 45 and 60 min at 75 °C and pH 6.5 were almost identical (Table 2). Even more significant, the resistance-inducing activities of both CMP-NANA and the preparation from blood were inhibited in an almost identical fashion by increasing concentrations of CMP (Table 3). Such inhibition is characteristic of sialylation reactions in which CMP-NANA is the donor substrate (Bernacki, 1975).

Table 3. Inhibition of the RIF activity of the preparation from human blood and of CMP-NANA by CMP

Solutions (100 μ l) of the blood preparation or CMP-NANA (0.4 μ M) were mixed with 100 μ l water, or 0.01, 0.1 or 1 mM-CMP. The RIF activity of 500 μ M CMP alone was <1 RIF unit ml⁻¹.

CMP concn (μ M)	RIF activity (unit ml ⁻¹)* in solutions of:	
	Blood preparation	CMP-NANA (0.2 μ M)
0	125	100
0	44 (64)	38 (62)
50	10 (92)	8 (92)
500	<1 (100)	<1 (100)

* Percentage inhibition values are given in parentheses.

If RIF is CMP-NANA, then the relative resistance-inducing activities indicated that only about 1% of the final preparation from blood (estimated around 1 mg from NMR, see above) would have been CMP-NANA. Re-examination of the original ¹H spectrum indicated some very small aromatic signals which could be assigned to a small amount of cytidine nucleotide, but at the prevailing signal-to-noise ratio, unequivocal evidence for CMP-NANA could not be obtained from this sample.

DISCUSSION

Identification of the low-*M_r* RIF in lysates of human blood cells has been difficult because of the minute quantities that are present. Initially, extraction and fractionation were based on previous work (Weiss *et al.*, 1971 and private communication; Patel *et al.*, 1984*b*) in the belief that low-*M_r* RIF might be a glucopeptide containing glutamic acid, glycine, cysteine and possibly other amino acids. These studies showed that human low-*M_r* RIF had an *M_r* between 500 and 1000, contained acid groups and was very acid- and heat-labile. They also showed that the relatively high contents of glutamic acid, glycine and cysteine of the fractionated material were due to contaminating, biologically inactive, glutathione which co-eluted with low-*M_r* RIF from anion-exchange columns. Carbohydrate contamination was also present. Corresponding material from guinea-pig serum or RBC lysates was not examined for glutathione and carbohydrate contamination because effort was concentrated on the human material. It was probable, however, that they were present in the fractions previously described (Patel *et al.*, 1984*b*) and that the biological activity was due to the same cause as that of the human material (see below).

The process for extracting RIF activity from blood was redesigned after first demonstrating that low-*M_r* RIF was released in blood cell lysates in a time- and temperature-dependent manner. A prolonged dialysis of lysates at 25 °C was used to obtain adequate amounts of activity in the low-*M_r* form (Table 1). Fractionation on two QAE-Sephadex A25 columns with intermediate desalting by Sephadex G10 columns produced considerable purification of low-*M_r* RIF with reasonable recovery of activity at each stage (Fig. 1, Table 1). Glutathione was eluted from the second column just before the low-*M_r* RIF but separate from it (Fig. 1*c*). HPLC revealed a complex mixture (Fig. 2*a*) from which, after two separations, a fraction showing only one peak was eventually obtained (Fig. 2*c*) by selecting leading RIF active fractions (Fig. 2*a*, *b*).

There was insufficient material for conventional analysis. NMR spectroscopy showed that despite extensive purification and the elution of RIF activity as a single symmetrical peak during HPLC, the final preparation was still a mixture. NMR evidence indicating the presence of pyrimidine nucleotides was supported by the UV absorption maximum of 260 nm. The extreme acid lability of low-*M_r* RIF guided the choice of CMP-NANA as a possible candidate. Fortunately, commercial synthetic (enzymically prepared) material was available and it proved very active in inducing gonococcal resistance to killing by human serum. In contrast, several other relatively acid-stable pyrimidine nucleotides, notably uridine diphospho-*N*-acetylglucos-

amine and uridine diphospho-*N*-acetylgalactosamine, whose presence had been indicated by NMR and whose adsorption maxima are about 260 nm, were inactive, as was NANA itself.

The low- M_r RIF from blood and CMP-NANA were identical in six properties. They co-eluted from HPLC (Fig. 3) and they were indistinguishable in their patterns of acid and heat inactivation (Table 2). More importantly, the resistance-inducing activity of both was inhibited by CMP (Table 3), which is known to inhibit the sialylation reaction of CMP-NANA (Bernacki, 1975). Also, treatment of crude low- M_r RIF with neuraminidase did not destroy its activity: CMP-NANA, unlike most other NANA-containing compounds, is resistant to this enzyme (Gottschalk, 1972). Finally, when CMP-NANA or low- M_r RIF from blood were used to induce resistance in gonococci, the accompanying profound changes in their lipopolysaccharide (LPS) patterns were indistinguishable (Parsons *et al.*, 1988). While identity in any one of these properties could be fortuitous, identity in all six is overwhelming evidence that CMP-NANA or some closely related analogue is probably the low- M_r RIF in blood cell lysates. If it is, biological activities indicate that only about 40 μ g CMP-NANA was present in the cell lysate from 1 litre of blood and the fractionated preparation was only about 1% pure. With this minute amount in a mixture, the presence of CMP-NANA in the preparation from blood could not be established unequivocally by NMR, although some consistent signals were obtained. We cannot, therefore, exclude the possibility that low- M_r RIF may not be CMP-NANA or a closely related analogue but another compound with identical biological properties.

CMP-NANA is present in mammalian tissue and bacteria including meningococci; its main function appears to be as the donor of NANA to glycoproteins, carbohydrates and gangliosides under the influence of sialyltransferases (Gottschalk, 1972; Brackmann *et al.*, 1983). It is synthesized from CTP and NANA and split into CMP and NANA by synthetases and hydrolases, respectively, both enzymes being widespread (Gottschalk, 1972; Brackmann *et al.*, 1983). It has been found in many types of mammalian cells (Gottschalk, 1972; Cary & Hirschberg, 1979; Igarashi *et al.*, 1985; Ferwerda *et al.*, 1983), including human cervical epithelial cells (Scudder & Chantler, 1981). As far as the authors are aware, it has not been demonstrated before in RBC nor in buffy coat phagocytes, which contain high RIF activity (Patel *et al.*, 1988).

Is CMP-NANA available to gonococci *in vivo*? It is primarily intracellular and could be used by gonococci which sometimes grow within cells (Parsons *et al.*, 1985). Only minute quantities would be present in blood and body fluids because it does not readily pass across cell membranes (Brackmann *et al.*, 1983). However, only small amounts would be needed to stimulate gonococcal resistance to serum killing. It must be remembered that the majority of RIF activity in blood-cell lysates is in high- M_r form. On the reasonable assumption that high- and low- M_r RIF are connected, it appears that CMP-NANA or a related precursor may be held on some large carrier molecule. This molecule may be intracellular, but it could be in the membrane of the cell or on the surface, or even be in the process of being transported across the membrane. Thus, it may allow access of CMP-NANA or a related precursor to any gonococci near or attached to the cells. The nature of the carrier molecule and the availability of CMP-NANA to gonococci in this form must be investigated.

Sialylation is probably the basis of resistance induction since the latter was abolished for both CMP-NANA and the preparation from blood by CMP, a known inhibitor of sialylation by CMP-NANA (Bernacki, 1975). The most likely acceptor of the sialic acid groups is LPS, and there is clear evidence that LPS is changed when gonococci are induced to serum resistance. In previous work with impure low- M_r RIF from guinea-pig blood, pyocin sensitivities and silver-stained LPS patterns of proteinase K digests on SDS-PAGE gels were different for susceptible and resistant organisms (Winstanley *et al.*, 1984; Tan *et al.*, 1986). Also, as mentioned above, striking differences in LPS patterns of such digests occurred when gonococci were induced to resistance by either low- M_r RIF from human blood or CMP-NANA.

It is probable that these changes are responsible for the acquisition of serum resistance. LPS is normally the target antigen in gonococci for the bactericidal antibody (mostly IgM) of fresh human serum (Glynn & Ward, 1970; Schoolnik *et al.*, 1979; Rice *et al.*, 1980; Apicella *et al.*, 1986). Resistance or susceptibility to fresh human serum is determined to a considerable degree

by LPS structure, which can vary with both strain and growth conditions (Guymon *et al.*, 1978, 1982; Apicella *et al.*, 1987; Griffiss *et al.*, 1987; Stephens & Shafer, 1987). Sialylation of LPS by CMP-NANA would lead to a change of structure which may affect target sites for bactericidal IgM.

If conversion of gonococci to resistance by CMP-NANA occurs in the host, the LPS of *in vivo*-grown gonococci might be expected to contain NANA residues. Such gonococci have not been examined, and investigations of *in vitro*-grown gonococci do not provide a clear answer. Wiseman & Caird (1977) concluded that NANA was present in the LPS of 38 strains on the basis of gas-liquid chromatography and colour reactions. Other studies mention glucose, galactose, *N*-acetylglucosamine, *N*-acetylgalactosamine, and a heptose, but not NANA (Perry *et al.*, 1975; Griffiss *et al.*, 1987). NANA is present in the capsular polysaccharides of group B streptococci, K1-type *Escherichia coli* and *Neisseria meningitidis* (Robbins *et al.*, 1980). Studies with CMP-NANA radiolabelled in the NANA moiety should show whether or not sialylation of the LPS occurs during resistance induction.

It is generally agreed that the host can influence the production of virulence determinants (Brown & Williams, 1985; Smith, 1988). However, particular aspects of the environment *in vivo* which lead to the production of specific virulence determinants have not been identified except for the recognition that iron deprivation of some Gram-negative pathogens induces new outer-membrane proteins (Brown & Williams, 1985). Our work provides a much-needed specific example of this important general principle of pathogenicity.

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