CHEMOTAXIS OF HUMAN POLYMORPHONUCLEAR LEUKOCYTES TOWARD *NEISSERIA GONORRHOEAE*

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SUMMARY. Chemoattractive properties of *Neisseria gonorrhoeae* were studied by measuring leukocyte migration in agarose gel. Human serum albumin (0.5%) was present in the gel and normal human serum was excluded from all components of the assay. Viable cell populations and lysates of colonial types F62T1, F62T2 and F62T3 induced migration of polymorphonuclear leukocytes. Chemotactic activity of the lysate was not altered by heating at 100°C for 10 min and was retained in the 12 100 g supernatant fraction of the heated lysate. Fractionation of the supernate by Sephadex G-100 chromatography showed that the chemotactic activity was associated primarily with an absorbance peak at 280 nm of relatively low mol. wt. The chemotactic activity of this fraction was lost after dialysis and the peak was no longer present in the Sephadex G-100 elution profile of the dialysed supernate. The gonococcal leukotaxins were sensitive to digestion by trypsin, pronase and amyloglucosidase, but insensitive to treatment with RNAase, DNAase or lipase at pH 5.7-7.1.

INTRODUCTION

Symptomatic gonorrhoea results from infiltration of polymorphonuclear leukocytes (PMNL) into the foci of gonococcal colonisation, presumably by a process of chemotactic attraction (Ward and Watt, 1972; Ward, Watt and Robertson, 1974). The basis for the attraction of PMNL induced by *Neisseria gonorrhoeae* is not understood. James and Williams (1978) showed that when 16 clinical isolates of *N. gonorrhoeae* were grown in chemically defined medium, 13 of the culture filtrates were chemotactic for PMNL. Watt and Medlen (1978) suggested that, in the early stages of gonococcal infection, interaction of gonococci with plasma proteins generates chemotaxins.

This report presents preliminary findings of an in-vitro study of the ability of whole cells and fractionated components of *N. gonorrhoeae* to cause PMNL leukotaxis.

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MATERIALS AND METHODS

Production of bacterial chemotactic factors. *Escherichia coli* was obtained from Dr H. Hill, University of Utah School of Medicine, Salt Lake City, UT, 84132. The *E. coli* bacterial culture filtrate (BF) containing chemotactic factor was prepared according to the method of Hill *et al.* (1975) and stored at -70°C in 1-ml portions without detectable loss of activity. *E. coli* BF is elaborated into liquid medium 199 (M199) (Keller and Sorkin, 1967; Ward, Lepow and Newan, 1968; Baum, Mowat and Kirk, 1971; Hill *et al.*, 1975; Nelson, Quie and Simmons, 1975). Similar filtrates could not be used in this study because *N. gonorrhoeae* failed to grow in M199. Growth was achieved in the medium of Gerhardt and Heden (1980) or GC broth base but the recovered gonococci displayed all four variations in colonial type, T1, T2, T3 and T4 (see below). Furthermore, the uninoculated media were chemotactic for PMNL in our experiments. Because stability of colony type was present after growth on solid agar, gonococci were harvested from agar plates for studies of chemotactic properties.

Strains of *N. gonorrhoeae*. Virulent *N. gonorrhoeae* strain F62 exhibiting the colonial type variations designated as T1 and T2, and the non-virulent strain F62T3 were obtained from Dr D. Kellogg, Communicable Disease Center, Atlanta, GA, 30333. The strains were grown on GC agar base supplemented with Isovitalex (BBL, Cockeysville, MD) and transferred daily with precaution to maintain the stability of the colonial types, as described by Kellogg *et al.* (1963). Plates exhibiting greater than 1% variation in colonial type were not used. Bacterial growth was collected after flooding each plate with 2 ml of Gey's buffer without glucose (Wilkinson, 1974). The agar surface was scraped gently with the edge of a sterile microscope slide, the bacterial suspension decanted, centrifuged at 12 100 *g* for 20 min at 4°C and the supernate discarded. The pellet was washed twice and resuspended in Gey's buffer at a density of $c. 1 \times 10^{10}$ cfu/ml, confirmed by viable plate count. The suspensions of viable cells were tested for chemotactic activity.

The gonococcal cell suspensions were disrupted by immersion in acetone-dry ice and transfer to a warm water bath (10 repetitions). The resulting lysate was heated for 10 min in a boiling water bath and centrifuged at 12 100 *g* for 20 min at 4°C . The pelleted fraction was resuspended in an original volume of Gey's buffer. Both the supernatant and pellet fractions were tested for chemotactic activity.

Column chromatography. Sephadex G-100 (Pharmacia, Uppsala, Sweden) was allowed to swell in Gey's salt solution (GSS) which had been diluted to 1 in 50 with sterile distilled water. GSS contained NaCl 8 g/L, KCl 0.377 g/L, $\text{Na}_2\text{PO}_4 \cdot 7\text{H}_2\text{O}$ 0.25 g/L, and KH_2PO_4 0.025 g/L. A 1.5×25 cm column was prepared and samples of the supernatant fraction of gonococcal lysate (0.1–0.25 ml) were applied. The column was eluted with diluted GSS, 1-ml fractions were collected and absorbance at 280 and 260 nm determined for each fraction. Protein content was assayed by the method of Hartree (1972) and carbohydrate was measured by the method of Dubois *et al.* (1956).

The supernatant fraction was dialysed for 36 h against three 1-L changes of Gey's buffer at 4°C . The fluid retained in the dialysis bag was tested in the chemotaxis assay and fractionated by Sephadex G-100 column chromatography as described above.

Fractions obtained by column chromatography were pooled as indicated by roman numerals in the figure and stored at -70°C . Before testing for chemotactic activity, the collected pools were concentrated in a flash evaporator (Buchi Rotavapor, Buchi, Switzerland) to the original volume of the supernatant fraction.

Ultrafiltration. The supernatant fraction (0.1 ml) was diluted 1 in 10 in 10% Gey's buffer and ultrafiltered in an Amicon model 12 apparatus (Amicon Corporation, Lexington, MA) with nitrogen as the propellant and a UM-10 membrane with a mol. wt retention of 10 000 was used. The collected effluent was concentrated by flash evaporation before testing for chemotactic activity.

Enzyme treatment. Equal volumes of enzyme and pool IV were mixed and incubated as indicated below, whereupon the reaction mixture was immersed in a boiling-water bath for 2 min to stop the reaction. The enzyme concentrations, digestion time, and temperature of incubation were as follows: trypsin (1 in 250, Difco), 2.4 g/L, 20 min, 35°C (Rick, 1965); pronase (Calbiochem), 5 g/L, 60 min, 35°C (Klipstein and Engert, 1976); amyloglucosidase (Grade II, Sigma),

31.25 g/L, 60 min, 35 °C (Pazur and Kleppe, 1962); lipase (Type VII, Sigma), 1 g/L, 60 min, 35 °C (Bier, 1955); RNAase (Type IIA, Sigma), 1 g/L, 60–90 min, room temperature (Kunitz, 1946, cited in Biochemica Information, 1974); and DNAase (Type I from bovine pancreas, Sigma), 1 g/L, 60–90 min, room temperature (Kunitz, 1950, cited in Biochemica Information, 1974). Standard substrates were used to monitor the activity of trypsin, RNAase and DNAase.

Preparation of human leukocytes. Normal volunteers with no past history of gonorrhoea donated blood for these experiments. Peripheral blood (20 ml) was collected by venipuncture into a syringe containing 100 units of heparin. After standing for 45 min at room temperature the leukocyte-rich plasma was expressed into a sterile tube. The white blood cells were counted, washed three times in 5 ml of Eagle's Modified Medium (MEM) containing penicillin 60 mg/L, streptomycin 100 mg/L, and 200 mM L-glutamine. The leukocytes were resuspended in MEM plus Earle's Salts (Flow Laboratories, Rockland, MD) and adjusted to a concentration of 2.5×10^7 leukocytes/ml.

Chemotaxis assay. The migration of leukocytes under agarose gel was measured as described by Nelson *et al.* (1975) with slight modification. Human serum albumin (Hyland Laboratories, Cosa Mesa, CA) 0.5% was added in the agarose gel as a substitute for normal human serum. Although Keller and Sorkin (1967) studied chemotaxis in Gey's solution with 2% human serum albumin, this concentration of albumin was inhibitory to PMNL migration in our assay. In brief, six rows of three round wells were extracted mechanically from an agarose gel in a small petri plate. Each well received 15 μ l of a fluid substance. The central well received the adjusted leukocyte suspension, the inner well received either Gey's buffer or GSS (serving as negative controls) and the outer well received *E. coli* BF (positive control) or the test solution. After the incubation period for migration, the leukocytes were fixed and stained. Migration was measured under 20 $\times$ magnification. The difference between the distance of migration to the *E. coli* BF or the test solution and the negative control was calculated.

RESULTS

Leukotactic activity of N. gonorrhoeae

PMNL responded to Gey's buffer with random migration, as shown in table I, whereas the PMNL response to intact whole cells of *N. gonorrhoeae* strain F62T1 was

TABLE I
PMNL migration toward *N. gonorrhoeae* strain F62T1

Test substance	Distance migrated* by PMNL towards		Difference in migration distance (T-C)
	T†	C‡	
<i>E. coli</i> BF	2.13 $\pm$ 0.20	0.85 $\pm$ 0.07	1.28 $\pm$ 0.24
Gey's buffer	0.54 $\pm$ 0.12	0.61 $\pm$ 0.06	-0.07 $\pm$ 0.09
Strain F62T1			
Viable cells	2.18 $\pm$ 0.14	0.63 $\pm$ 0.11	1.54 $\pm$ 0.18
Cell lysate	1.74 $\pm$ 0.25	0.70 $\pm$ 0.09	1.04 $\pm$ 0.26
Cell lysate (heated)	1.71 $\pm$ 0.28	0.68 $\pm$ 0.09	1.03 $\pm$ 0.33
Supernate	1.93 $\pm$ 0.36	0.78 $\pm$ 0.11	1.15 $\pm$ 0.29
Pellet	1.12 $\pm$ 0.12	0.53 $\pm$ 0.06	0.58 $\pm$ 0.10

* Values represent the mean of six replicate well $\pm$ SD in cm measured after 20-fold magnification. The results of a representative experiment are shown.

† Outer well containing the test substance.

‡ Inner well containing the control substance (in this case, the MEM used to suspend the leukocytes).

comparable with the response to *E. coli* BF. The distance of migration in response to the lysate of strain F62T1 was *c.* 70% of the response to intact viable cells. Although leukotactic responses of PMNL showed some variation from day to day, the response to F62T1 lysate relative to viable F62T1 was highly reproducible. Migration distance decreased linearly with each 10-fold dilution of the cell suspension or lysate for four successive dilutions but diminished rapidly thereafter. Additionally, two clinical isolates of *N. gonorrhoeae* were obtained. The colonial growth was determined to be of the T1 designation and was capable of eliciting the PMNL migrational response with equal strength to that of the *E. coli* BF control (data not shown).

The chemotactic activity of the F62T1 lysate was stable to heating at 100°C for 10 min and to storage at -10°C or -70°C. When the F62T1 lysate was heated at 100°C for 10 min and centrifuged at 12 100 *g*, a two-fold greater PMNL response was elicited by the supernatant fraction than the pellet fraction (table I). The PMNL response to the pellet fraction of unheated lysate was twice the response to the supernatant fraction (not shown). Chemotactic responses were also elicited by viable cells of gonococcal colony types F62T2 and F62T3 (table II) and their respective cell lysates (not shown).

TABLE II
PMNL migration toward strains of *N. gonorrhoeae*

Test substance	Distance migrated by PMNL towards		Difference in migration distance (T-C)
	T	C	
<i>E. coli</i> BF	2.69 ± 0.24	1.24 ± 0.14	1.41 ± 0.33
Gey's buffer	1.03 ± 0.20	1.03 ± 0.21	0.00 ± 0.09
F62T1 viable cells	2.08 ± 0.49	1.09 ± 0.10	0.99 ± 0.53
F62T2 viable cells	1.83 ± 0.06	0.94 ± 0.12	0.88 ± 0.13
F62T3 viable cells	1.59 ± 0.37	0.96 ± 0.13	0.63 ± 0.42

Footnotes as in table I.

Fractionation of F62T1 supernatant fraction

When the supernatant fraction of F62T1 was subjected to Sephadex G-100 chromatography, two peaks of absorbance at 280 nm were eluted, as shown in the figure, and a corresponding profile of absorbance at 260 nm was obtained (not shown). Identical profiles were obtained when measuring protein (Hartree, 1972) or carbohydrate (Dubois *et al.*, 1956). In the supernate of lysate originally containing 10¹⁰ cfu/ml, total protein was 5.03 ± 0.51 mg and total carbohydrate 0.398 ± 0.15 mg (mean of three determinations of each).

Eluted fractions were pooled as indicated in the figure. Each pool was flash evaporated to the original volume of the sample applied to the column before testing for leukotactic activity. The results are shown in table III. The strongest chemotactic response was elicited by pool IV; pools II and III were not consistently chemotactic and pools I and V were never chemotactic (not shown).

FIG. —Sephadex G-100 elution profiles of the supernatant fraction of a gonococcal lysate after centrifugation (O) and after dialysis (●) for 36 h at 4°C. The profile begins at the point of elution of blue dextran 2000 during prior calibration of the column. Ovalbumin (mol. wt 43 000, left arrow) gave an elution peak at fraction 15 and cytochrome c (mol. wt 14 000, right arrow) gave an elution peak at fraction 30. The profile ends at the point immediately preceding elution of dinitrophenol (mol. wt 184).

TABLE III

PMNL migration toward sephadex G-100 fractions of the supernate from N. gonorrhoeae strain F62T1 cell lysate

Test substance	Distance migrated by PMNL towards		Difference in migration distance (T-C)
	T	C	
<i>E. coli</i> BF	2.79 ± 0.16	1.02 ± 0.08	1.78 ± 0.18
GSS	0.64 ± 0.24	0.67 ± 0.13	-0.03 ± 0.21
F62T1			
Supernate	1.88 ± 0.22	0.83 ± 0.14	1.06 ± 0.14
Pool II	0.99 ± 0.20	0.64 ± 0.15	0.35 ± 0.15
Pool III	1.18 ± 0.12	0.77 ± 0.10	0.42 ± 0.09
Pool IV	2.16 ± 0.10	0.94 ± 0.10	1.22 ± 0.17

Footnotes as in table I

When the supernatant fraction of F62T1 lysate was dialysed and fractionated by Sephadex column chromatography, the second peak seen in the figure was absent and the loss of absorbance at 280 nm corresponded approximately to the fractions combined in pool IV. The supernatant fraction of the F62T1 lysate also showed no chemotactic activity after dialysis.

A 0.1-ml supernatant fraction of F62T1 lysate was diluted 1 in 10, ultrafiltered on a UM-10 membrane and reconstituted to its original volume. The retentate elicited 69% of the PMNL migration observed with the original supernatant fraction. The eluate was not attractive for PMNL.

Leukotactic activity of enzymatic digests of pool IV

Table IV shows the effects of enzyme treatment on the chemotactic activity of pool IV. Parallel experiments with *E. coli* BF are shown for comparison. PMNL were unresponsive to each enzyme, whether in an active or inactive state. Trypsin treatment of pool IV and *E. coli* BF caused a decrease of *c.* 50% in chemotactic response

TABLE IV

Effect of enzyme treatment on chemotactic factor from E. coli or N. gonorrhoeae strain F62T1

Treatment	Migration (percentage of untreated control in response to)	
	<i>E. coli</i> BF	F62T1 pool IV
Untreated	100	100
Trypsin	43	43
DNAase	108	119
RNAase	110	100
Pronase	29	56
Amyloglucosidase	53	33
Lipase pH 5.7	103	84
Lipase pH 6.4	112	93
Lipase pH 7.1	90	85
Lipase pH 7.8	97	65

compared with the untreated control substances (table IV). RNAase and DNAase treatment had no effect on PMNL migration in response to pool IV or *E. coli* BF. Treatment of both leukoattractants with pronase or amyloglucosidase decreased the migration response of PMNL. However, PMNL migration was less toward pronase-treated *E. coli* BF than toward pronase-treated pool IV. Amyloglucosidase treatment of pool IV promoted less PMNL migration than amyloglucosidase treatment of *E. coli* BF. At pH 7.8, lipase caused a reduction in chemotactic activity of pool IV. Lipase treatment had little effect on pool IV at lower pH and had no effect on *E. coli* BF at any pH tested.

DISCUSSION

N. gonorrhoeae strains F62T1, F62T2, and F62T3 that showed no variation in

colonial type or viability were chemotactic for PMNL. Phase variation between opaque and transparent gonococci was not considered in this study because only the general phenomenon of the migration stimulus was pursued.

The chemotactic activity of the viable gonococci was retained after the bacteria were disrupted by freezing and thawing. When heated, chemotactic activity of the gonococcal lysate was present predominantly in a 12 100 *g* supernatant fraction. The major leukotaxin of the supernate was associated with a low-mol. wt peak (pool IV) obtained by Sephadex G-100 chromatography. Chemotactic activity of the supernate was retained by Amicon ultrafiltration with a membrane retention cutoff of 10 000 but was lost during dialysis, which suggests a mol. wt < 12 000. Chemotactic factors from *Pseudomonas aeruginosa* and *E. coli* have also been reported to be heat stable and dialysable (Ward *et al.*, 1968; Schiffmann *et al.*, 1975).

Chemotactic activity of pool IV was sensitive to the effects of trypsin, pronase, amyloglucosidase and lipase treatment at pH 7.8, but not to DNAase or RNAase. With the exception of lipase, enzyme treatment of *E. coli* BF showed a similar pattern of effects on chemotactic activity. Schiffmann *et al.* (1975) reported that *E. coli* BF is pronase sensitive, insensitive to DNAase or RNAase and unaffected by trypsin. Our finding that both pronase and amyloglucosidase reduced leukoattractant activity suggests that glycopeptide may be important in the composition of *E. coli* BF and gonococcal chemotaxin. The activity of trypsin suggests that L-lysyl or L-arginyl bonds within the glycopeptide may be integral in the composition as well.

Watt and Medlen (1978) propose that gonococci must interact with natural antibody and complement in plasma to generate leukotaxins. Their proposal is supported by the observation that PMNL are attracted to gonococci suspended in normal human plasma, but not to gonococci suspended in tissue culture medium or 24-h culture filtrates in the absence of plasma. However, James and Williams (1978) showed that filtrates of laboratory gonococcal strains and 13 of 16 clinical isolates grown in chemically defined medium were chemotactic for PMNL, which suggests that a leukoattractant acts independently of serum factors. We have shown that serological factors are not required for the in-vitro response of PMNL to viable gonococci or a soluble chemotactic substance obtained from gonococci. This may also be true *in vivo*, although binding and activation of plasma components may contribute to the severity of the acute inflammatory response. We concur with James and Williams (1978) that the chemotaxin of gonococci does not appear to be related to virulence, because F62T3 gonococci attract PMNL, as do the virulent F62T1 and F62T2 colonial types.

The evidence suggests that the elaboration of gonococcal chemotaxin may stimulate the inflammatory response leading to symptomatic gonorrhoea. The evidence of James and Williams (1978) suggests that most but not all clinical isolates of gonococci produce the chemotaxin. If so, chemotaxin-negative strains may be associated with the phenomenon of asymptomatic gonorrhoea. This phenomenon may be reflected in the observation of a higher proportion of women than men who have asymptomatic gonorrhoea. However, undefined environmental influences may inhibit the expression *in vivo* of the chemotaxin-positive phenotype in these individuals. *In vitro*, the chemotaxin positive phenotype of the organism may be expressed as suggested by James and Williams (1978). Further investigation of the phenomenon of PMNL migration toward gonococci may lead to a better understanding of the pathogenesis of this disease.

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REFERENCES

- Baum J, Mowat A G, Kirk J A 1971 A simplified method for the measurement of chemotaxis of polymorphonuclear leukocytes from human blood. *Journal of Laboratory and Clinical Medicine* 19:501-509.
- Bier M 1955 Lipases. In: Colowick S P, Kaplan N O (eds) *Methods in enzymology*, vol. I. Academic Press, New York, pp 627-642.
- Dubois M, Gilles K A, Hamilton J K, Rebers P A, Smith F 1956 Colorimetric method for determination of sugars and related substances. *Analytical Chemistry* 28:350-356.
- Gerhardt P, Hedén C G 1960 Concentrated culture of gonococci in clear liquid medium. *Proceedings of the Society for Biology and Medicine* 105:49-51.
- Hartree E F 1972 Determination of protein: a modification of the Lowry method that gives a linear photometric response. *Analytical Biochemistry* 48:422-427.
- Hill H R, Hogan N A, Mitchell T G, Quie P G 1975 Evaluation of a cytocentrifuge method for measuring neutrophil granulocyte chemotaxis. *Journal of Laboratory and Clinical Medicine* 86:703-710.
- James A N, Williams R P 1978 Chemotactic effect of *Neisseria gonorrhoeae* on polymorphonuclear leukocytes. In: Brooks G F *et al* (eds) *Immunobiology of Neisseria gonorrhoeae*. American Society for Microbiology, Washington, DC, pp 236-238.
- Keller H U, Sorkin E 1967 Studies on chemotaxis. V. On the chemotactic effect of bacteria. *International Archives of Allergy and Applied Immunology* 31:505-517.
- Kellogg D S, Peacock W L, Deacon W E, Brown L, Pirkle C 1963 *Neisseria gonorrhoeae*. I: Virulence genetically linked to clonal variation. *Journal of Bacteriology* 85:1274-1279.
- Klipstein F A, Engert R F 1976 Purification and properties of *Klebsiella pneumoniae* heat stable enterotoxin. *Infection and Immunity* 13:373-381.
- Kunitz M 1946 RNAase A. In: *Biochemica Information—1974*. Boehringer Mannheim, Mannheim, West Germany, pp 156-157.
- Kunitz M 1950 DNAase I. In: *Biochemica Information—1974*. Boehringer Mannheim, Mannheim, West Germany, p 80.
- Nelson R D, Quie P G, Simmons R L 1975 Chemotaxis under agarose: A new and simple method for measuring chemotaxis and spontaneous migration of human polymorphonuclear leukocytes and monocytes. *Journal of Immunology* 115:1650-1656.
- Pazur J H, Kleppe K 1962 The hydrolysis of α -D-glucosides by amyloglucosidase from *Aspergillus niger*. *Journal of Biological Chemistry* 237:1002-1006.
- Rick W. 1963 Trypsin. In: Bergmeyer H U (ed) *Methods of enzymatic analysis*. Academic Press, New York, pp 807-818.
- Schiffmann E, Showell H V, Corcoran B A, Ward P A, Smith E, Becker E L 1975 The isolation and partial characterization of neutrophil chemotactic factors from *Escherichia coli*. *Journal of Immunology* 114:1831-1837.
- Ward M E, Watt P J, 1972 Adherence of *Neisseria gonorrhoeae* to urethral mucosa cells: An electron microscopical study of human gonorrhea. *Journal of Infectious Diseases* 126:601-605.
- Ward M E, Watt P J, Robertson J N 1974 The human fallopian tube: A laboratory model for gonococcal infection. *Journal of Infectious Diseases* 129:650-659.
- Ward P A, Lepow I H, Newman L J 1968 Bacterial factors chemotactic for polymorphonuclear leukocytes. *American Journal of Pathology* 52:725-736.
- Watt P J, Medlen A R 1978 Generation of chemotaxins by gonococci. In: Brooks G F *et al* (eds) *Immunobiology of Neisseria gonorrhoeae*. American Society for Microbiology, Washington, DC, pp. 239-241.
- Wilkinson P C 1974 Outline of a method for measuring chemotaxis (Appendix). In: *Chemotaxis and inflammation*. Churchill Livingstone, Edinburgh, pp 168-172.