HOST RESPONSE TO INFECTION

The chemotactic response of blood neutrophils and monocytes to strains of *Escherichia coli* with different virulence characteristics

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Summary. Chemotactic responses of blood neutrophils and monocytes to media conditioned by eight strains of *Escherichia coli* with different virulence characteristics were measured in modified Boyden assay chambers to determine if these characteristics were associated with differences in leucocyte mobility. Responding neutrophils and monocytes were prepared on conventional density gradients, and in three instances, the chemotaxis of eosinophils isolated on metrizamide gradients was also studied. Media conditioned by enteroinvasive and non-enteroinvasive *E. coli* strains were tested as chemo-attractants and compared to the formylated peptide standard attractant. Chemotactic activity of neutrophils was greater than that of monocytes and eosinophils, and migration by all populations was significantly greater to conditioned media than to the control medium. Chemotactic responses to media conditioned by non-enteroinvasive *E. coli* and strains lacking virulence factors was greater than to media conditioned by plasmid- and Sereny-positive enteroinvasive organisms. The results suggest that virulence factors of *E. coli* that determine invasiveness did not augment the chemotactic responses of the leucocyte populations tested *in vitro*, and give no support to the hypothesis that they induce mucosal inflammation by directly increasing chemotaxis *in vivo*.

Introduction

The leucocyte population of the normal human colon mucosa consists of lymphocytes, plasma cells, macrophages and variable numbers of eosinophils and mast cells. In health, there is a notable absence of neutrophils. In acute colitis caused by invasive enteric bacteria, a modest increase in the numbers of resident leucocytes is seen, but the predominant response is margination of neutrophils in dilated capillaries of the lamina propria and their migration in large numbers to the interstitium, with ultimate invasion of the epithelium and the formation of crypt abscesses.^{1,2} The initial chemotactic response of the neutrophils and other leucocytes to invasive organisms might be expected to influence the degree of inflammatory infiltration and thus the severity of the colitis. The leucocyte chemotactic response to non-invasive bacteria, which constitute normal colonic flora but which also may breach the epithelial barrier with injury, might logically be expected to be different from the leucocyte responses to their invasive counterparts. This possibility was tested by measuring chemotaxis

by blood neutrophils and monocytes in response to *Escherichia coli* strains representing organisms with different virulence characteristics, such as the presence or absence of a 140-MDa invasive plasmid and a positive or negative Sereny test. In several experiments, eosinophil chemotactic responses were also documented. Statistical analysis evaluated both the system and biological variability of the in-vitro chemotactic assays and the response of leucocytes to the various bacterial chemo-attractants.

Materials and methods

Chemotactic assays

Chemotaxis was assayed in 48-well plates (Neuro Probe, Cabin John, MD, USA) by the method of Falk *et al.*³ Responding leucocytes suspended in Gey's Balanced Salt Solution (GBSS; Gibco, Grand Island, NY, USA) containing bovine serum albumin (BSA; Sigma, MO, USA) 1% were placed in the upper, and the bacteria-conditioned media in the lower portion of the well, separated by a polycarbonate filter (Neuro Probe) with 3- μ m pores for neutrophils, 5- μ m pores for monocytes and 8- μ m pores for eosinophils. Plates were incubated at 37°C in CO₂ 5% for 30 min

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(neutrophils) or 90 min (monocytes and eosinophils). Assays were conducted in triplicate with GBSS-BSA and Mueller-Hinton (M-H) bacterial culture medium (Scott Laboratories, Fiskeville, RI, USA) as negative controls and 10^{-5} M N-formyl-met-phe (FMP; Sigma) as a standard chemo-attractant in each plate. After incubation, filters were drained, air dried and stained with Diff-Quik (AHS del Caribe Inc., Aguada, PR) for microscopic enumeration of migrating cells. Cells moving through the filter were counted in 30 microscopic fields under high power and averaged. Mean values for cells responding to the GBSS-BSA and M-H broth were subtracted from the means responding to the test chemo-attractants and FMP. Results were expressed as the mean number of cells migrating, and also as the percentage of the response to FMP. Checkerboard experiments were performed with 100%, 20%, 10% and 5% concentrations of the five chemo-attractants (A, B, C, D, H; table I) above and below the filters to differentiate random migration of neutrophils from directed chemotaxis.⁴

Isolation of responding leucocytes

Neutrophils, monocytes and eosinophils were separated from 50 ml of heparinised blood from normal volunteers. Erythrocytes were sedimented by the addition of dextran (Macrodex, Pharmacia) 6% and monocytes were separated from neutrophils by density gradient sedimentation on Ficoll-sodium diatrizoate (Histopaque-1077; Sigma) at 300 *g* for 30 min at 24°C. Residual erythrocytes were cleared from the neutrophil pellet by the addition of ammonium chloride lysing buffer.⁵ Eosinophils from three subjects were isolated from neutrophils by gradient centrifugation on metrizamide (Sigma) 22% at 1200 *g* for 45 min.⁶ Cell preparations were washed, resuspended and counted in a haemocytometer. Cyto-centrifuge preparations were stained with Diff-Quik and examined microscopically for cell purity.

Bacterial chemo-attractants

The eight bacteria listed in table I were used to generate conditioned medium for testing the chemotactic responses of the leucocyte populations. The first five organisms were isolated from patients with acute dysentery. *E. coli* strains 497-78, 978-77, 5898-71 and 3189-75 were obtained from I. Kaye Wachsmuth, Centers for Disease Control, Atlanta, GA, where the Sereny tests were done. Strains 4608-58 and its isogenic plasmid-free mutant 4608-50A4 were obtained from T. L. Hale, Walter Reed Army Institute for Research, Washington, D.C. A. Wanger, University of Texas, Houston, TX, kindly performed the plasmid analysis of the other organisms. The *E. coli* strain O6-H1 was capsulate and contained a haemolysin. Strain ON-2 was obtained from P. K. Peterson, University of Minnesota, Minneapolis, MN. Conditioned media were produced by inoculating 10^7 bacteria into separate

culture flasks containing M-H broth and culturing for 16 h to stationary phase. The bacteria were then removed by centrifugation and the supernate was passed through a 200-nm filter; it was subdivided into convenient samples and frozen until use in the chemotactic assays. In a number of experiments ($n = 4-8$) chemo-attractant activity of each undiluted conditioned medium was compared to that of a 1 in 10 dilution.

Statistical analysis

To identify the various factors influencing the results of the chemotactic assays, chemotactic responses of neutrophils and monocytes to GBSS-BSA and FMP were tested by analysis of variance with a nested design⁷ to examine inter-subject, intra-subject, plate-to-plate and well-to-well variation.

The inter- and intra-subject variations reflected biological factors, and the plate-to-plate and well-to-well variations were of a technical nature. Results from 29 subjects, each contributing from 1 to nineteen assays, were used for the analysis. For each assay, from 1 to 3 plates were employed and each test variable was run in three adjacent wells. The total sample size for neutrophil experiments was 398 and for monocytes 577.

In the above nested design, the error factor for testing hypotheses is determined by the factor which is nested. For example, intra-subject variation was the error factor for inter-subject differences; plate-to-plate variation was used to test intra-subject differences and well-to-well variation was the plate-to-plate error term. Thus, the method of analysis needs to be considered when interpreting the results.

A randomised block design⁷ was used to test for significant differences of neutrophil and monocyte responses to the eight *E. coli*-conditioned media. Cells from a single subject were tested in one plate with three wells for each chemo-attractant. Eight subjects were tested in all. Separate statistical analyses were conducted for the number of cells migrating and for the number expressed as a percentage of the response to FMP. Eosinophil chemotaxis results were not analysed statistically because cells from only three donors were assayed.

Results

The chemotactic responses of neutrophils and monocytes to each bacterial chemo-attractant were significantly greater ($p < 0.05$) than to GBSS-BSA and unconditioned M-H broth (table II) by Duncan's multiple range test.⁷ Neutrophil chemotaxis in response to conditioned media ranged from 65.9% to 157.8% of the response to FMP, and monocyte responses varied from 92.9% to 114.8% of those to the FMP standard (table III). As regards the invasive *E. coli* strains, medium conditioned by strain 5898-71

Table I. Characteristics of *E. coli* used to produce media with chemo-attractant activity

Serotype	Strain no.	140-MDa plasmid	Sereny test
A. 028 acNM	497-78	+	+
B. 029 NM	978-77	+	—
C. 0124 H30	5898-71	+	+
D. 0124 HNT	3189-75	+	—
E. 0143	4608-58	+	NT
F. 0143	4608-58A4	—	NT
G. 25922 ATCC	O6-H1	—	NT
H. O22.H16	ON-2	—*	NT

NT, not tested.

* 120-MDa plasmid.

Table II. Leucocyte migration in response to conditioned culture media, controls and FMP standard

Medium	Number of subjects	Mean (SD) number of migrating	
		neutrophils	monocytes
497-78	8	126.1 (61.5)	114.8 (77.1)
978-77	8	152.3 (85.4)	126.7 (112.6)
5898-71	8	205.1 (96.7)	145.6 (110.4)
3189-75	8	142.2 (88.9)	142.2 (132.8)
4608-58	4	197.8 (64.3)	191.7 (175.9)
4608-58A4	4	167.0 (84.2)	202.3 (67.1)
O6-H1	8	275.8 (132.7)	161.1 (150.6)
ON-2	8	245.2 (117.7)	155.7 (154.4)
GBSS-BSA	12	22.1 (8.4)	21.8 (13.2)
M-H broth	12	69.5 (36.3)	66.8 (33.4)
FMP	12	212.8 (128.1)	153.1 (125.8)

Table III. Chemotaxis expressed as a percentage of cells migrating to FMP

Medium	Number of subjects	Mean (SD) percentage of migrating	
		neutrophils	monocytes
497-78	8	65.9 (28.9)	92.9 (37.3)
978-77	8	80.5 (46.7)	94.8 (41.2)
5898-71	8	103.3 (38.1)	107.8 (52.0)
3189-75	8	88.0 (36.6)	101.5 (52.9)
4608-58	4	157.8 (89.6)	94.6 (10.8)
4608-58A4	4	145.1 (81.1)	109.4 (33.2)
O6-H1	8	141.4 (83.3)	114.8 (56.9)
ON-2	8	126.1 (48.9)	111.3 (46.9)

was the most chemo-attractive for neutrophils, but some of the most marked neutrophil chemotactic activity occurred toward organisms not associated with enteric invasion and also to one of the invasive organisms stripped of its invasion plasmid. For example, the response of neutrophils to the non-invasive strains ON2 and O6H1 was distinctly greater than to the enteroinvasive strains 497-78, 978-77 and 3189-71. Monocytes exhibited somewhat less variability in their chemotactic activity.

The mean numbers of monocytes and neutrophils attracted by undiluted conditioned media from the eight bacteria were invariably greater than attracted

Table IV. Chemotaxis of eosinophils in response to conditioned culture medium

Medium	Number of subjects	Mean (SD) number of cells migrating	Number of subjects	Mean (SD) percentage of FMP response
497-78	3	38.6 (19.8)	2	63.2 (10.0)
978-77	3	42.3 (27.9)	2	78.0 (21.9)
5898-71	3	60.7 (21.1)	2	93.8 (26.5)
3189-75	3	42.4 (24.5)	2	73.0 (17.3)
O6-H1	3	46.2 (26.0)	2	80.0 (20.1)
ON-2	3	35.6 (17.4)	2	58.7 (9.1)
GBSS-BSA	3	15.0 (5.4)	...	...
FMP	2	81.7 (19.5)	...	...
M-H broth	3	18.8 (7.7)	...	...

by the 1 in 10 dilutions, but the differences were not statistically significant. Checkerboard experiments indicated directed chemotaxis in response to each conditioned medium tested, but high concentrations invoked some random movement. When all checkerboard experiments are considered, approximately one-third of cell migration was random, and two-thirds was directed. Eosinophil responses were less than those of neutrophils and monocytes (table IV). Except for a somewhat more active response to broth conditioned by virulent strain 5898-71, eosinophils did not discriminate between the media conditioned by the various bacteria.

For experiments evaluating system variations, the mean chemotactic responses of neutrophils to GBSS-BSA was 25.3 cells ($n = 133$) and that of monocytes was 19.5 cells ($n = 192$). The mean number of neutrophils migrating in response to the 10^{-5} M FMP standard was 255.3, and of monocytes 168.7 cells. Thus the response to GBSS-BSA for neutrophils was 10.1% and for monocytes 11.6% of the respective responses to FMP. On the basis of these data, statistical analysis (tables V and VI) indicated that differences in intra-subject responses of neutrophils and monocytes accounted for most of the variability in the chemotaxis assay system. Therefore, we have not included replicate data from the same subject in the analysis of responses to bacterial chemo-attractants. The next most important factor was plate-to-plate variation, which led us to express results as a percentage of the response to the FMP standard of each plate in addition to indicating the absolute number of cells migrating. Variation due to the technical factor of well-to-well differences, and the biological effects of person-to-person variations were negligible relative to intra-subject variations.

Discussion

Certain methodological aspects of this study deserve mentioning. In preliminary work we tested the chemo-attractant properties of bacterial lysates, and found them considerably less active than the culture supernates. We also found that M-H broth was somewhat

Table V. Analysis of variance of the number of leucocytes migrating to buffer (GBSS)

Source of variation	df	Variance	F	p value
<i>Neutrophils</i>				
Subject-to-subject	26	3161.8	1.00	0.490
Intra-subject	39	3146.5	9.15	< 0.001
Plate-to-plate	64	343.9	11.99	< 0.001
Well-to-well	259	28.7	...	...
<i>Monocytes</i>				
Subject-to-subject	28	304.8	0.49	0.979
Intra-subject	55	618.3	7.57	< 0.001
Plate-to-plate	103	81.7	5.07	< 0.001
Well-to-well	373	16.1	...	...

df, degrees of freedom; variance, mean square of the differences from the means; F, F statistic.

chemo-attractive, therefore all results with culture supernates have the M-H activity subtracted. The statistical analysis indicated considerable variation in chemotaxis of leucocytes of the same person studied at different times relative to the variation between different people. Thus, the variation in chemotactic responses of leucocytes from the same person at different times must be taken into consideration in any analysis of such data. In addition, plate-to-plate variation in similar assays should be eliminated by expressing the results of each plate in terms of its standard.

These studies documented the in-vitro chemotactic responses of blood neutrophils, monocytes and eosinophils to media conditioned by various strains of *E. coli* with different virulence characteristics. The organisms tested included two enteroinvasive strains that produced kerato-conjunctivitis (Sereny positive) in guinea-pigs and two others that did not, and a virulent strain with a 140-MDa invasion plasmid and its non-invasive isogenic mutant stripped of its plasmid. The two other organisms tested were also not enteroinvasive. The greatest in-vitro chemotactic activity was in response to medium from bacteria lacking in an important invasion characteristic or to those not known to be enteroinvasive. Conversely, the neutrophils, monocytes and to some extent eosinophils

Table VI. Analysis of variance of the number of leucocytes migrating to FMP

Source of variation	df	Variance	F	p value
<i>Neutrophils</i>				
Subject-to-subject	26	44061.1	1.03	0.456
Intra-subject	42	42672.2	6.82	< 0.001
Plate-to-plate	64	6252.8	15.30	< 0.001
Well-to-well	265	408.8	...	...
<i>Monocytes</i>				
Subject-to-subject	28	13210.8	0.69	0.857
Intra-subject	57	19267.3	23.29	< 0.001
Plate-to-plate	107	828.2	9.33	< 0.001
Well-to-well	384	88.8	...	...

df, degrees of freedom; variance, mean square of difference from the mean; F, F statistic.

exhibited less chemotaxis to the more virulent organisms. If these in-vitro results can be extrapolated to in-vivo responses, factors other than the virulence characteristics of bacteria govern the magnitude of the chemotactic response of neutrophils, monocytes and eosinophils in inflammation due to invasive *E. coli*. The role of these agents in the secretion of the various chemotactic cytokines⁸ would be of interest.

In summary, active chemotaxis was exhibited by blood neutrophils, monocytes and eosinophils toward each *E. coli*-conditioned medium. However, chemotaxis was usually less vigorous toward media conditioned by enteroinvasive *E. coli* strains with recognised virulence factors, than to media conditioned by non-enteroinvasive strains. It is possible that an invading organism with diminished chemo-attractant properties might encounter a diminished or delayed leucocyte response, and thus have an advantage over more chemo-attractive bacteria. These experiments do not support the hypothesis that virulence factors of enteroinvasive *E. coli* contribute to mucosal inflammation by directly augmenting chemotactic responses by neutrophils, monocytes and eosinophils.

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