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Endotoxin liberation by strains of *N. meningitidis* isolated from patients and healthy carriers

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SUMMARY

The main objective of this study was to assess whether the capacity of *Neisseria meningitidis* to release endotoxin depends upon the type of strain or upon bacterial mass. Endotoxin release was studied in 32 strains isolated from patients with meningococcal infections and in 49 from asymptomatic carriers, using a quantitative test (limulus test with a chromogenic substrate). The results show that the strains from patients release significantly higher amounts of endotoxin than strains from carriers regardless of serogroup and isolation site. No correlation was found between stage of bacterial growth and the amount of endotoxin liberated. These findings suggest that endotoxin liberation is a characteristic of certain strains of *N. meningitidis* and is not determined simply by bacterial mass.

INTRODUCTION

It is known that the endotoxins of *Neissevia meningitidis* are released during the growth phase of the microorganism [1–3]. Although it is not completely clear which mechanisms cause the sequence of events in the fulminant disease sometimes produced by *N. meningitidis*, studies on laboratory animals [3, 4] point to an origin in the endotoxins liberated by the meningococci.

Epidemiological analyses of the clinical evolution of meningococcal infections reveal that sepsis and shock, when they occur, peak during the first 24 h after the onset of the disease. This has been related to the endotoxin liberating capacity of the meningococci [5–8].

The present study attempts to assess and compare liberation of endotoxins in strains producing the disease and in strains from asymptomatic carriers, and to suggest an explanation for their different behaviours in the host.

Because shock may occur within 24 h of infection [6, 9, 10], we studied quantitatively the sequence of endotoxin liberation throughout a 24-h period of bacterial growth, sampling at 4, 8, 12, and 24 h. These time periods correspond

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respectively to the beginning of exponential growth, to its maximum, and to the start of bacterial autolysis.

MATERIAL AND METHODS

Bacterial isolates

A total of 81 strains of *N. meningitidis* was used. Thirty-two had been isolated by the Department of Microbiology from CSF and blood samples from patients with meningococcal infections admitted to the University of Granada Hospital between June 1985 and December 1987. Most patients were small children. The remaining 49 strains were isolated from asymptomatic carriers from throat swabs taken at the out-patient pediatric unit of the Hospital or from students of the Schools of Medicine and Nursing. Both the isolations and the subsequent identification of the serogroups were by standard techniques (Table 1).

Colony forming units (c.f.u.) were assessed by a dilution set of the inoculum in medium 199 read by spectrophotometry at 620 nm. Medium 199 was used as the blank.

Technique of endotoxin liberation

The methodology described by Andersen and Solberg [2] was followed. Strains were cultured in a protein-free medium (medium 199) at 37 °C for 24 h. The meningococci precultured for 18 h on blood agar at 37 °C in an atmosphere of 5% CO_2, were washed three times in 0·9% saline. A bacterial inoculum was prepared at an optical density of 0·6 at 620 nm, the equivalent of 10^8–10^9 c.f.u./ml. Seven hundred and fifty microlitres of the bacterial suspension were transferred to test-tubes containing 20 ml of medium 199. They were shaken and then incubated at 32 °C for 24 h. Growth was observed by turbidometry with a spectrophotometer. From the onset of exponential bacterial growth, four samples were taken, at 4, 8, 12, and 24 h of culture. Each was divided in two (to duplicate observations) and filtered using sterile 0·45 μm Millipore filters to avoid passage of bacteria, and were refrigerated at −70 °C until assayed for endotoxin. All reagents, media, and other liquids were pyrogen-free.

Endotoxin assay

The limulus with a chromogenic substrate (endotoxin Coatest, Kabivitrum, Sweden) was used, which permits the quantitative determination of endotoxins [8]. As the number of samples was large, culture filtrates were stored at −70 °C. All the samples were processed in one batch.

Prior to the assay, the samples and the reagents (diluted in pyrogen-free water at appropriate concentrations) were maintained at room temperature for a few minutes. The test is based on the capacity of the lipidic portion – lipid A – of the endotoxins to activate a proenzyme in the amoebocyte lysate of the limulus *polyphemus* (ALAL). The activated enzyme, in turn, has an amidase activity and catalyses the liberation of P-nitroanaline from the substrate, producing a yellow coloration the intensity of which is proportional to the concentration of endotoxins.

The assay was carried out by adding 75 μl of the ALAL to 75 μl of each of the samples, gently shaking and incubating for 10 min at 37 °C. Then 150 μl of a

Table 1. *Strains of* N. meningitidis: *distribution by serogroup*

Host	Serogroup	No. strains (%)
Patients	A	5 (15·6)
	B	27 (84·4)
		32 100·0
Carriers	A	11 (22·4)
	B	13 (26·5)
	C	8 (16·3)
	29E	4 (8·2)
	Pol.	6 (12·2)
	Aut.	4 (8·2)
	X	2 (4·1)
	Z	1 (2·0)
		49 100·0

chromogenic buffer-substrate solution was added, and the samples incubated for an additional 3 min. The endotoxin concentration was measured by spectrophotometry at 405 nm.

The endotoxin standard was that of *Escherichia coli* O 111:B4 (Coatest endotoxin, Kabivitrum, Sweden). The bacteria-free medium was used as blank.

Endotoxin isolation

To ascertain if the endotoxin liberation capacity depends on the amount of lipopolysaccharide (LPS) in the bacterial wall, endotoxin was extracted and purified from six clinical isolates of *N. meningitidis*, randomly chosen, using the phenol—water method of Westphal and Jahn [11].

RESULTS

Each sample and its duplicate yielded similar results. The percentage of variation was in every case less than 5%. The mean of both determinations was taken as the end result.

The strains obtained from patients showed endotoxin liberation ranging from 0·1 to 1·00 ng/ml, the mean values being 0·302, 0·319, 0·324, and 0·317 ng/ml, respectively, at 4, 8, 12, and 24 h. Liberation was lower than 0·2 ng/ml with the strains from asymptomatic carriers, with mean values of 0·12, 0·114, 0·108, and 0·119 ng/ml, respectively (Table 2).

A comparison of the mean values of endotoxins liberated in patients and carriers for the four time periods established shows that the former groups present a significantly higher liberation ($P < 0·0001$) at each of the time periods, regardless of the origin of the isolate, CSF or blood, (Table 3), and of serogroup (Table 4).

Bacterial growth (measured as c.f.u.) did not show any relationship to the amount of endotoxin liberated in either patient or carrier strains, although a low, but significant, correlation was observed for patient strains when they were incubated for 4 h (Table 5). Patient strains were further analysed by dividing them into two groups, strains with a high capacity for endotoxin liberation and strains without that characteristic. The results remained unchanged (data not shown). Thus endotoxin liberation has no direct relationship with the quantity of

Table 2. *Endotoxin liberation in strains of* N. meningitidis *from patients and carriers*

Time (h)	Patient strains m ± s (ng/ml)	Carrier strains m ± s (ng/ml)	*P**
4	0·302 ± 0·207	0·123 ± 0·051	< 0·0001
8	0·319 ± 0·229	0·117 ± 0·046	< 0·0001
12	0·324 ± 0·224	0·108 ± 0·050	< 0·0001
24	0·317 ± 0·215	0·110 ± 0·055	< 0·0001

* Significance, after using the Student's *t* test.
There were 32 patient strains and 49 carrier strains.

Table 3. *Endotoxin liberation in patient strains of* N. meningitidis *by isolation site*

Site	No. strains	Endotoxin m ± s (ng/ml)
CSF	19	0·273 ± 0·093
Blood	13	0·344 ± 0·101

Student's *t* test was non-significant.

Table 4. *Endotoxin liberation by* N. meningitidis *serogroup*

Host	Serogroup	Endotoxin mean (ng/ml)	*P*
Patients	A	0·320	ns*
	B	0·313	
Carriers	A	0·104	< 0·01†
	B	0·132	
	C	0·097	
	E29	0·151	
	Poly.	0·068	
	Aut.	0·128	

* Student's *t* test non-significant.
† ANOVA I. Significance due to the difference between serogroups Poly. and E29.

Table 5. *Correlation between bacterial growth (c.f.u.) and amount of endotoxin liberated*

Time	c.f.u. × 10^8 (mean)	Endotoxins mean (ng/ml)	Correlation coefficient	*P*
		Patient strains		
4	1·64	0·302	0·433	< 0·01
8	3·89	0·319	0·204	ns
12	5·75	0·327	0·178	ns
24	1·24	0·317	0·213	ns
		Carrier strains		
4	2·17	0·123	0·077	ns
8	5·50	0·117	−0·109	ns
12	8·17	0·108	0·035	ns
24	1·28	0·110	−0·068	ns

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Table 6. *Endotoxin liberation and amount of LPS in bacterial wall*

Strain	Dry weight of bacteria (g)	Extract (mg)	Extraction efficiency (%)	LPS in extract (%)	Endot. liberation (mean) (ng/ml)
E2	1·81	2·00	1·1	94	0·924
E11	2·50	2·80	1·1	72	0·389
E12	2·00	2·00	1·0	89	0·264
E15	1·86	1·80	1·0	91	0·167
E0371	2·35	1·70	0·7	91	0·610
E0366	1·95	1·55	0·8	93	0·518

All the strains were serogroup B.
Efficiency was estimated dividing the extract by the dry weight.
No significant correlation coefficient was observed among these data.

microorganisms (Fig. 1). The differences in the amount of endotoxin liberated between the different time periods was evaluated for both patient and carrier strains but were not found to be significant in either group.

In Table 6, the results regarding endotoxin extraction, the amount of LPS found in the extract, and the amount of endotoxin liberated from six strains are presented. The efficiency of the phenol–water method range between 0·07 and 0·11%. The amount of LPS in the extracts ranged between 72 and 94%. No relationship could be demonstrated between this variable and the amount of endotoxin liberated. None of the linear regression coefficients was significant.

DISCUSSION

Meningococci are able to release complexes from the cell wall which contains endotoxins, which play an important role in the pathogenesis of the meningococcal infection [9, 12]. However, experimental studies [3] have shown that this capacity varies among strains. Our study evaluates the capacity of strains of *N. meningitidis* isolated from two types of host, patients with meningococcal infections (meningitis or septicaemia) and healthy carriers, to liberate endotoxins in order to relate possible differences to virulence.

The method used was the limulus test using a chromogenic substrate the sensitivity and specificity of which have been assessed as 93 and 95%, respectively [13, 14]. Our results indicate that variation is low, less than 5%.

The results indicate that patient strains liberate significantly higher amounts of endotoxins than do the carrier strains, at each of the time periods studied (4, 8, 12, and 24 h). A 'liberation' ratio between the two groups yielded a mean value of 2·8 indicating that the bacteria isolated from patients liberate nearly three times as much endotoxin as the meningococci isolated from carriers. These findings agree with those of Andersen and Solberg [3]. They observed a greater degree of liberation of endotoxin in patient strains than we did although their methods of quantification differed from ours.

A potential bias could arise if the isolation site (CSF, blood, or throat) of meningococcal strains has an effect on the strains' capacity to liberate endotoxin, although no relevant studies could be found in the literature. There were no significant differences in the amount of endotoxin liberated between the patient

strains isolated from blood and those isolated from CSF. Serogroup was not found to influence the amount of endotoxin liberated by the patient strain, and only minor differences were found between the carrier strains. These findings differ from those obtained by Andersen [8]. Since the methods used were the same as those of this author, the reason for such differences remains obscure unless geographical distribution may be a factor.

In light of the possibility that a greater liberation of endotoxin by patient strains could be associated with a faster and more substantial bacterial growth in the culture medium, the amount of endotoxin in relation to the number of c.f.u. for each time span studied was compared. The results show that the two types of strains exhibit similar growth patterns and no statistical correlation could be established between the c.f.u. number and the amount of endotoxin liberated. This observation agrees with the data of other authors [3, 8] who point out that endotoxin liberation is only partly a function of bacterial growth, and that this liberation is not proportional to the stage of bacterial growth. However, Andersen and Solberg [15], in a study of 123 strains, found a direct relationship between bacterial growth and the amount of endotoxin liberated. The methods used by us are similar to those performed by them, and again, no explanation has been found for this discrepancy.

The data shown in Table 6 establish that no relationship can be demonstrated between the amount of endotoxin liberated and the amount of endotoxin in the bacteria, and reinforce the suggestion that endotoxin liberation is a property of each meningococcal strain.

Summarizing, it is apparent that the capacity to liberate endotoxin may be a specific property of a bacterial strain and as it is greater in strains isolated from patients with meningococcal infections, a relationship with a greater virulence may exist. In addition, no association can be shown between endotoxin liberation and the origin of the isolate (CSF or blood), serogroup, and bacterial growth.

REFERENCES

1. Zollinger WD, Kasper DL, Vetri RY, Artenstein MS. Isolation and characterization of a native cell wall complex from *N. meningitidis*. Infect Immun 1972; **6**: 835–51.
2. Andersen BM, Solberg O. Liberation of endotoxin during growth of *N. meningitidis* in a chemically defined medium. Acta Pathol Microbiol Scand (B) 1978; **86**: 275–81.
3. Andersen BM, Solberg O. Endotoxin liberation and invasivity of *N. meningitidis*. Scand J Infect Dis 1984; **16**: 247–52.
4. Holbein BE. Enhancement of *N. meningitidis* infection in mice by addition of iron bound to transferrin. Infect Immun 1981; **34**: 120–5.
5. Hawiger J, Hawiger A, Steckiey S. Membrane changes in human platelets by lipopolysaccharide endotoxin. J. Hematol 1977; **35**: 285–99.
6. De Voe LW. The interaction of polymorphonuclear leukocytes and endotoxin in meningococcal disease: a short review. Can J Microbiol 1980; **26**: 729–40.
7. Tubbs HR. Endotoxin in meningococcal infections. Arch Dis Child 1980; **55**: 808–19.
8. Andersen BM. Disease and mortality caused by *N. meningitidis*: the role of endotoxin liberation as a virulence factor. J Oslo City Hosp 1983; **33**: 37–68.
9. Harthug S, Bjorkatn B, Osterud B. Quantitation of endotoxin in blood from patients with meningococcal disease using a limulus lysate in combination with chromogenic substrate. Infection 1983; **11**: 192–5.
10. De Voe LW. The *meningococcus* and mechanism of pathogenicity. Microbial Rev 1982; **46**: 162–90.

11. Westphal O, Jahn K. Bacterial lipopolysaccharides. Extraction with phenol–water and further applications of the procedure. In: Whistler RL, BeMiller JN, eds. Methods in carbohydrate chemistry. Volume 5. New York: Academic Press, 1965: 83–91.
12. Russell RPB. Free endotoxin: a review. Microb Lett 1976; **2**: 125–35.
13. Dwelle TL, Dunkle LM, Blair L. Correlation of cerebrospinal fluid endotoxin-like activity with clinical and laboratory variables in gram-negative bacterial meningitis. J Clin Microbiol 1987; **25**: 856–8.
14. Sabolle MA. Chromogenic limulus amebocyte lysate assay as an aid in the diagnosis of meningitis. In: Cate JW, Büller H, Sturk A, Levin J, eds. Progress in clinical and biological research. New York: Alan R. Liss, 1985: 369–84.
15. Andersen BM, Solberg O. Endotoxin liberation associated with growth, encapsulation and virulence of *N. meningitidis*. Scand J Infect Dis 1988; **20**: 21–31.