Letters in Applied Microbiology 2000, **31**, 294–298

The influence of different concentrations of melatonin on the cell surface hydrophobic characteristics of *Neisseria meningitidis*

J. Uberos, A. Molina, J. Liébana[1], M.C. Augustin and A. Muñoz

Grupo de Investigación CTS-190, Departamento de Paediatría, Hospital Clínico Universitario 'San Cecilio' and [1]Departamento de Microbiología, Facultad de Medicina, Granada, Spain

180/2000: received 9 May 2000 and accepted 20 June 2000

J. UBEROS, A. MOLINA, J. LIÉBANA, M.C. AUGUSTIN AND A. MUÑOZ. 2000. The cell surface hydrophobicity of micro-organisms is a characteristic that has been associated with the colonization of mammalian epithelia and with their capacity to induce diseases. Melatonin is a hormone produced by the pineal gland that affects the immune response mechanism. This study investigated, as an expression of the virulence of *Neisseria meningitidis*, how its hydrophobic characteristics were affected by exposure to increasing concentrations of melatonin. An increase in the cell surface hydrophobicity of *N. meningitidis* was found at concentrations of 1 mmol l^{-1}, while lower concentrations of melatonin did not significantly affect this particular cell surface characteristic of the micro-organism. It may be concluded that melatonin clearly influences the cell surface hydrophobicity of *N. meningitidis*, a circumstance that should be taken into account in future studies to determine whether this hormone plays a role in the variable pathogenicity of the bacteria in different hosts.

INTRODUCTION

The cell surface hydrophobicity of bacteria is considered to be a characteristic that is related to the colonization of epithelia in mammals (Stendahl *et al.* 1973; Rosenberg *et al.* 1980; Rosenberg 1984a) and to their susceptibility to phagocytosis (Stendahl *et al.* 1973; Ferreiros *et al.* 1989; Svanborg *et al.* 1984). Nevertheless, despite its theoretical importance in the virulence of *Neisseria meningitidis*, no clear parallel has yet been found between this cell surface characteristic and the clinical evidence of the micro-organism, which may remain in the oropharynx of asymptomatic subjects or which may become evident as a disease with systemic extent and a fatal sepsis (Ferreiros *et al.* 1986). We are led to conclude that other factors inherent to the host, and not just the micro-organism, may be involved in the varying patterns of *N. meningitidis.*

In recent years there have been important advances in our knowledge of the immune-affecting role of melatonin (Attia *et al.* 1991; Lissoni *et al.* 1991) and of its participation in the stabilization processes of oxidative stress (Hardeland *et al.* 1993; Reiter *et al.* 1993; Reiter 1994; Reiter *et al.* 1997) and membrane stabilization (Reiter *et al.* 1995; Garcia *et al.* 1998).

Melatonin is a hormone in the pineal gland which has been related to the immune response mechanism (Rodríguez *et al.* 1997). The influence of melatonin on the non-specific immune response mechanism and its repercussions on the virulence of certain bacteria have not yet been clearly identified. Therefore, our aim in this study was to evaluate the possible relationship between melatonin and the cell surface characteristics of *N. meningitidis*. In addition, as iron is a factor that has been widely linked to the virulence of *N. meningitidis* and other micro-organisms (Criado *et al.* 1993; Lachica and Zink 1984) we tested whether the different concentrations of iron in the culture medium also modified the cell surface characteristics of this particular micro-organism.

Correspondence to: J. Uberos, Grupo de Investigación CTS-190, Departamento de Paediatría, Hospital Clínico Universitario 'San Cecilio', Granada, Spain.

MATERIALS AND METHODS

Bacterial strains and culture conditions

We studied six strains of *N. meningitidis*, two of which were isolated from the nasopharynx of healthy subjects (P391, ungroupable, autoagglutinable, serotype 15 and P000, serogroup B, serotype 15) and four (V002, serogroup B, serotype 15; V021, serogroup B, non typable; V019, serogroup B, serotype 1 and V111, serogroup C) which were

isolated from the cerebrospinal fluid or blood of patients suffering an invasive meningococcal disease. Strains P000, P391, V019, V002 and V021 were provided by Dr C. Ferreiros and Dra. M.T. Criado (University of Santiago, Spain) and strain V111 was isolated in the Microbiology Department of this hospital. The strains were stored at $-30\,^{\circ}C$ until use and recovered by isolation on blood agar plates (5–10% CO_2). For each experiment, the different strains were cultured in tubes containing 4 ml Mueller-Hinton broth (MH) and incubated at $36 \pm 1\,^{\circ}C$ for 24 h before processing. The iron content of the culture medium was modified by adding 39 μmol l^{-1} ethylene-diamino-dihydroxyphenyl-acetic acid (Sigma-Aldrich, Madrid, Spain) to 100 ml MH (EDDA-MH, iron restriction) or ferric-ammonium-citrate (1 μg Fe ml^{-1}; Sigma) (FAC-MH, iron excess).

We prepared 1 mmol l^{-1}, 50 μmol l^{-1}, 500 and 5 nmol l^{-1} solutions of melatonin (Sigma) in sterile distilled water. After incubating the strains of *N. meningitidis* for 12 h at $36 \pm 1\,^{\circ}C$ (logarithmic growth phase) 1 ml of each of the solutions was added to each tube of the culture medium. Incubation was continued for 12 h at $36 \pm 1\,^{\circ}C$ until the process was complete.

Cell surface hydrophobicity

The cell surface hydrophobicity was determined according to the method described by Rosenberg (1984b). In brief, the organisms were incubated at $36 \pm 1\,^{\circ}C$ for 24 h in the culture medium, after which they were centrifuged at 3500 rev min^{-1} for 10 min. The supernatant fluid was then discarded and the cells washed three times with phosphate-buffered saline (PBS). After centrifugation, 0·4 ml buffer-urea (PUM; pH 7·1) (Rosenberg *et al.* 1980) and 0·8 ml 0·2 mol l^{-1} ammonium sulphate (Sigma) were added, having adjusted the suspension to an absorbance of 0·6, measured with a spectrophotometer at 550 nm. The absorbance was then measured in a spectrophotometer at 560 nm, following which 200 μl *p*-xylene (Sigma) was added to 1·2 ml of the bacterial suspension. The mixture was shaken vigorously for 2 min to achieve a homogeneous suspension of bacteria and hydrocarbon. The suspension was then allowed to settle for 30 min, in order for the aqueous phase to separate completely from the hydrocarbon. A glass Pasteur pipette was used to extract 1 ml of the aqueous phase and the absorbance was measured by spectrophotometer at 550 nm and $36 \pm 1\,^{\circ}C$. The hydrophobicity was calculated as $1-(Abs_2/Abs_1)$, where Abs_1 is the absorbence measured before addition of the hydrocarbon and Abs_2 is the subsequent absorbence.

Adherence to nitrocellulose filters

We applied the technique described by Lachica and Zink (1984) in which the organisms were incubated at 37 °C for 24 h in a culture medium and then centrifuged at 3500 rev min^{-1} for 10 min. Following this, the supernatant fluid was discarded and the cells washed three times with PBS. The micro-organisms were then resuspended in a PUM buffer with ammonium sulphate (1 : 2, v/v). After adjusting the absorbance to 0·6 at 540 nm, 3 ml of this suspension were passed through a nitrocellulose filter of 25 mm and 8-μm pore diameter (Millipore). The absorbance of the filtrate was then determined. Results are expressed as the percentage of adherence to the nitrocellulose filter.

Statistical methods

We first performed the Kolmogorov normality study. Having determined the absence of normality in the population distributions, we carried out the Wilcoxon paired data test, the Kendall agreement test and Spearman's correlation analysis.

RESULTS

The concentration of iron in the culture medium did not significantly alter the cell surface hydrophobicity of *N. meningitidis*; only when concentrations of 50 μmol l^{-1} melatonin were used during incubation did we observe any differences between the hydrophobicity of *N. meningitidis* in MH and FAC-MH, and at concentrations of 1 mmol l^{-1} for differences between MH and EDDA-MH. When *N. meningitidis* was incubated with melatonin at concentrations of less than 1 mmol l^{-1}, the cell surface hydrophobicity was unaltered. However, when incubation was carried out with a melatonin concentration of 1 mmol l^{-1}, we observed a significant increase in the cell surface hydrophobicity of *N. meningitidis*, irrespective of the concentration of iron in the culture medium. Although the overall trend was towards an increase in the cell surface hydrophobicity at higher concentrations of melatonin, certain strains of *N. meningitidis*, such as P000, did not evidence any variation in any of the culture media regardless of the melatonin concentration applied. An identical behaviour pattern was observed for V019 in FAC-MH.

There was a slight increase in the adherence of *N. meningitidis* to nitrocellulose filters after incubation with melatonin at concentrations of 1 mmol l^{-1}, although these variations were only statistically significant when the culture medium presented an excess of iron (Table 1). Although the behaviour of the strains of *N. meningitidis* examined in this study was fairly consistent, P391 pre-

Table 1 Cell surface hydrophobicity and adherence to nitrocellulose filters of *Neisseria meningitidis* incubated with increasing concentrations of melatonin, for a Mueller-Hinton broth (MH), for iron excess (FAC-MH) and for iron restriction (EDDA-MH)

Melatonin MH	Hydrophobicity (%)			Adherence to nitrocellulose (%)		
	EDDA-MH*	FAC-MH†	MH	EDDA-MH*	FAC-MH†	
0 nmol l^{-1}	57·5 (30·8)	57·3 (37·03)	52·9 (31·8)	89·9 (15·9)	87·6 (10·8)	91·7 (10·4)
5 nmol l^{-1}	51·1 (30·5)	49·8 (35·6)	52·4 (33·4)	89·0 (21·1)	94·0 (3·04)	93·6 (4·6)
500 n mol l^{-1}	45·6 (31·8)	60·1 (33·3)	61·3 (28·7)	88·6 (23·3)	91·6 (6·4)	89·9 (14·2)
50 μmol l^{-1}	50·3 (32·6)	58·8 (32·4)	60·4 (28·3)‡¶	89·9 (19·8)	96·2 (2·6)	89·2 (16·5)
1 mmol l^{-1}	71·8 (25·1)‡	78·8 (27·1)‡§	73·5 (21·2)‡	98·6 (2·5)	97·1 (3·3)	99·2 (1·9)‡

*MH broth + ethylene-diamino-dihydroxyphenilacetic acid.
†Ferric ammonium citrate + MH broth.
‡$P < 0{\cdot}05$, comparison with hydrophobicity, melatonin 0 nmol l^{-1}.
§$P < 0{\cdot}05$, comparison with hydrophobicity, MH and melatonin 1 mmol l^{-1} (Wilcoxon test).
¶$P < 0{\cdot}05$, comparison with hydrophobicity, MH and melatonin 50 μmol l^{-1}.

sented a 40% lower level of adherence to the filters when incubated with melatonin at concentrations of 50 μmol l^{-1}.

No significant relationship was observed between cell surface hydrophobicity and adherence to nitrocellulose filters at any of the melatonin concentrations investigated.

The cell surface hydrophobicity of *N. meningitidis* incubated without melatonin correlated significantly (r_0 Spearman) with the cell surface hydrophobicity observed after incubation at increasing concentrations of melatonin, from 5 nmol l^{-1} to 1 mmol l^{-1}, when the culture medium presented an iron excess (FAC-MH) or iron restriction (EDDA-MH). When the culture medium contained a normal proportion of iron (MH), no significant correlation was observed between the melatonin-absent cell surface hydrophobicity and that obtained after incubation with melatonin at the concentrations used in this study. The adherence of *N. meningitidis*, incubated without melatonin, to the nitrocellulose filters did not correlate significantly with that of *N. meningitidis* incubated with melatonin at concentrations of 5 nmol l^{-1} to 1 mmol l^{-1} when growth occurred in a culture medium with iron excess (FAC-MH). When the concentration of iron was normal or restricted, correlations were highly variable.

DISCUSSION

The first stage in the colonization of an epithelium by *N. meningitidis* is determined by the ability of the bacteria to adhere to the colonized cells, which in turn depends on the cell surface electrical charge and the hydrophobicity of the micro-organism (Ferreirós & Criado 1984). Factors such as the formation of pili or specific adhesins also contribute to the adhesion to epithelia (Ferreirós & Criado 1980; Gibbons 1996). While the adhesion to cells is fundamental for the colonization of a cell tissue, bacterial growth and the expression of surface antigens are also key elements in recognition and phagocytosis by the mononuclear–phagocyte system, which prevent the subsequent extension of the micro-organism and limit its capacity to provoke disease. Tests carried out previously (Ferreiros *et al.* 1986; Pintor *et al.* 1993) have shown conclusively that various factors apart from the micro-organism itself must intervene in the development of invasive meningococcic disease, as the necessary adherence of the bacteria to the pharyngeal epithelium, determined by the adhesins, becomes a factor that prevents the subsequent extension of the bacteria, as these very adhesins are easily recognized by circulating phagocytes. Furthermore, it has been observed that the presence or absence of iron in the culture medium may be a key factor in determining the expression of surface proteins with an affinity for transferrin (Pintor *et al.* 1993; Gómez *et al.* 1996).

Several studies have demonstrated the importance of melatonin in activating the immune response processes of the organism (Maestroni 1993; Lissoni *et al.* 1994; Di Stefano & Paulesu 1994) and that it may be an agent leading to an improved prognosis in the treatment of endotoxic shock (Maestroni 1996). The mechanisms by which melatonin is able to alter the immune system have not been clearly identified; it has been speculated that these lymphokines may be mediators of the observed effects of melatonin on the immune system. Melatonin also has a potent action of neutralizing free radicals (Reiter *et al.* 1995).

Several studies (Rodríguez *et al.* 1997) have revealed that there is a reduction in chemotaxis and the release of free radicals in the phagocytes after exposure to 100 μmol l^{-1}

melatonin, while lower doses have no apparent effect. These results could be extrapolated to our study, as the observed alterations in cell surface hydrophobicity were only apparent when the micro-organism was incubated with concentrations of 1 mmol l^{-1} melatonin, a level which is much higher than that occurring in physiological situations. Such increases in cell surface hydrophobicity occured irrespective of the supplementation or restriction of iron in the culture medium. As iron restriction seems to be related to the greater expression of proteins binding to transferrin 1 and 2 (Pintor *et al*. 1993; Criado *et al*. 1990), we deduce that alterations in the cell surface hydrophobicity of *N. meningitidis* arising from high concentrations of melatonin and an excess of iron are not determined by these proteins. On the other hand, iron excess does seem to provoke a slight increase in cell surface hydrophobicity after incubation with μmol l^{-1} and mmol l^{-1} concentrations of melatonin. Similarly, after incubating *N. meningitidis* with melatonin at a concentration of 1 mmol l^{-1}, we observed a significant increase in adhesion to nitrocellulose filters. To determine whether there is a relationship between observed hydrophobic characteristics and adherence to nitrocellulose filters, we performed the Kendall agreement analysis. No association was observed between the two sets of results. Thus, the variations in the cell surface hydrophobicity and adherence to nitrocellulose filters must arise from unrelated characteristics of the bacterial cell surface.

In the presence of iron excess, the cell surface hydrophobicity of *N. meningitidis* after incubation without melatonin was strongly correlated with that obtained after incubation with increasing concentrations of melatonin to 1 mmol l^{-1}. This conclusion was not valid when the culture medium contained normal or restricted concentrations of iron; doubtless, the existence of iron in the culture medium together with an antioxidant and eliminator of free radicals (scavenger), i.e. melatonin, had a synergistic effect on certain surface components of *N. meningitidis*, which was apparent as an increase in cell surface hydrophobicity.

On considering the above results, it is difficult to reach a practical conclusion, as physiological concentrations of melatonin are well below those considered useful in our study. Nevertheless, the discovery of such effects provoked by an endogenous antioxidant, a hormone, should provide a basis for further investigation to clarify the relations between the redox metabolism of the bacteria, its virulence potential and the redox mechanisms of the host.

ACKNOWLEDGEMENTS

The authors thank Dr C. Ferreiros and Dr M.T. Criado for their thought-provoking questions and long-standing encouragement of our hopes and dreams.

REFERENCES

Attia, A.M., Reiter, R.J., Stokkan, K.-A., Mostafa, M.H., Soliman, S.A. and El-Sebae, A.-K. (1991) Parathion (O,O-dimethyl-O-*p*-nitrophenyl phosphorothionate) induces pineal melatonin synthesis at night. *Brain Research Bulletin* **26**, 553–557.

Criado, M.T., Del Río, M.C., Ferreirós, C.M., Pintor, M., Sainz, V. and Carballo, J. (1990) Iron and outer membrane proteins in the susceptibility of *Neisseria meningitidis* to human serum. *FEMS Microbiological Letters* **58**, 145–150.

Criado, M.T., Pintor, M. and Ferreiros, C.M. (1993) Iron uptake by *Neisseria meningitidis*. *Research Microbiology* **144**, 77–82.

Di Stefano, A. and Paulesu, L. (1994) Inhibitory effect of melatonin on production of IFN gamma or TNF alpha in peripheral blood mononuclear cells of some blood donors. *Journal of Pineal Research* **17**, 164–169.

Ferreirós, C.M. and Criado, M.T. (1980) Adhesive properties associated with the K99 antigen of *Escherichia coli*. *IRCS Medical Science* **10**, 917–918.

Ferreirós, C.M. and Criado, M.T. (1984) Expression of surface hydrophobicity encoded by R-plasmids in *Escherichia coli* laboratory strains. *Archives of Microbiology* **138**, 191–194.

Ferreiros, C.M., Criado, M.T., Sainz, V., Carballo, J., del Rio, C. and Suarez, B. (1986) Evaluation of hydrophobicity and adherence of *Neisseria meningitidis* strains and a study of their correlation by analysis of alterations induced by antibiotics. *Annals of Institute Pasteur Microbiology* **137B**, 37–45.

Ferreiros, C.M., Criado, M.T., Sainz, V., Suarez, B., Carballo, J. and Del Rio, M.C. (1989) Changes in surface hydrophobicity and charge in *Neisseria meningitidis* and their correlation with the association to phagocytic cells. *Revista Española de Fisiologia* **45**, 105–109.

Garcia, J.J., Reiter, R.J., Ortiz, G.G., Oh, C.S., Tang, L., Yu, B.P. and Escames, G. (1998) Melatonin enhances tamoxifen's ability to prevent the reduction in microsomal membrane fluidity induced by lipid peroxidation. *Journal of Membrane Biology* **162**, 59–65.

Gibbons, R.J. (1996) Role of adhesion in microbial colonization of host tissues: a contribution of oral microbiology. *Journal of Dental Research* **75**, 866–870.

Gómez, J.A., Agra, C., Ferrón, L., Powell, N., Pintor, M., Criado, M.T. and Ferreirós, C.M. (1996) Antigenicity, cross-reactivity and surface exposure of the *Neisseria meningitidis* 37 kDa protein (Fbp). *Vaccine* **14**, 1340–1346.

Hardeland, R., Reiter, R.J., Poeggeler, B. and Tan, D.-X. (1993) The significance of the metabolism of the neurohormone melatonin: antioxidative protection and formation of bioactive substances. *Neuroscience Biobehaviour Review* **17**, 347–357.

Lachica, R.V. and Zink, D.L. (1984) Plasmid-associated cell surface charge and hydrophobicity of *Yersinia enterocolitica*. *Infection and Immunity* **44**, 540–543.

Lissoni, P., Tisi, E., Brivio, F., Ardizzoia, A., Crispino, S., Barni, S., Tancini, G., Conti, A. and Maestroni, G.J.M. (1991) Modulation of interleukin-2-induced macrophage activation in cancer patients by the pineal hormone melatonin. *Journal of Biological Regulation Homeostatics Agents* **5**, 154–156.

Lissoni, P., Barni, S., Ardizzoia, A., Tancini, G., Conti, A. and Maestroni, G. (1994) A randomized study with the pineal hormone melatonin versus supportive care alone in patients with brain metastases due to solid neoplasms. *Cancer* **73**, 699–701.

Maestroni, G.J. (1993) The immunoneuroendocrine role of melatonin. *Journal of Pineal Research* **14**, 1–10.

Maestroni, G.J. (1996) Melatonin as a therapeutic agent in experimental endotoxic shock. *Journal of Pineal Research* **20**, 84–89.

Pintor, M., Ferreiros, C.M. and Criado, M.T. (1993) Characterization of the transferrin-iron uptake system in *Neisseria meningitidis*. *FEMS Microbiological Letters* **112**, 159–165.

Reiter, R.J. (1994) Free radicals, melatonin, and celullar antioxidative defense mechanisms. In *Pathophysiology of Immune-Neuroendocrine Communications Circuit* ed. Gupta, D., Wollmann, H.A. and Fedor-Fregbergh, P.G. pp. 135–160. Stuttgart: Mattes-Verlag.

Reiter, R.J., Poeggeler, B., Tan, D.-X., Chen, L.-D., Manchester, L. and Guerrero, J.M. (1993) Antioxidant capacity of melatonin: a novel action not requiring a receptor. *Neuroendocrinological Letters* **15**, 103–116.

Reiter, R.J., Melchiorri, D., Sewerynek, E., Poeggeler, B., Barlowwalden, L., Chuang, J.I., Ortiz, G.G. and Acunacastroviejo, D. (1995) A review of the evidence supporting melatonins role as an antioxidant. *Journal of Pineal Research* **18**, 1–11.

Reiter, R.J., Tang, L., Garcia, J.J. and Muñoz-Hoyos, A. (1997) Pharmacological actions of melatonin in oxygen radical pathophysiology. *Life Science* **60**, 2255–2271.

Rodríguez, A.B., Ortega, E., Lea, R.W. and Barriga, C. (1997) Melatonin and the phagocytic process of heterophils from the ring dove (*Streptopelia risoria*). *Molecular Cell Biochemistry* **168**, 185–190.

Rosenberg, M. (1984a) Isolation of pigmented and nonpigmented mutants of *Serratia marcescens* with reduced cell surface hydrophobicity. *Journal of Bacteriology* **160**, 480–482.

Rosenberg, M. (1984b) Ammonium sulphate enhances adherence of *Escherichia coli* J5 to hydrocarbons and polystyrene. *FEMS Microbiological Letters* **25**, 41–45.

Rosenberg, M., Gutnick, D. and Rosenberg, E. (1980) Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surface hydrophobicity. *FEMS Microbiological Letters* **9**, 29–33.

Stendahl, O., Tagesson, C. and Edebo, M. (1973) Partition of *Salmonella typhimurium* in a two-polymer aqueous phase system in relation to liability to phagocytosis. *Infection and Immunity* **8**, 36–41.

Svanborg, E.C., Bjursten, L.M., Hull, R., Hull, S., Magnusson, K.E., Moldovano, Z. and Leffler, H. (1984) Influence of adhesins on the interaction of *Escherichia coli* with human phagocytes. *Infection and Immunity* **44**, 672–680.