Letters in Applied Microbiology 2001, **32**, 303–306

Comparative study of the influence of melatonin and vitamin E on the surface characteristics of *Escherichia coli*

J. Uberos[1], C. Augustin[1], J. Liébana[2], A. Molina[1] and A. Muñoz-Hoyos[1]
[1]*Research Group CTS-190, Department of Paediatrics, 'San Cecilio' University Clinical Hospital, and*
[2]*Department of Microbiology, School of Medicine, Granada, Spain*

2000/58: received 29 January 2001 and accepted 9 February 2001

J. UBEROS, C. AUGUSTIN, J. LIÉBANA, A. MOLINA AND A. MUÑOZ-HOYOS. 2001.

Aims: Melatonin is a hormone produced by the pineal gland and that affects the response of various cell membranes to an oxidative stimulus.

Methods and Results: The present study evaluates the hydrophobic characteristics of *Escherichia coli* in response to melatonin (100 nmol l^{-1}, 200 μmol l^{-1}) and to vitamin E (5 mg dl^{-1}). A reduction was found in the surface hydrophobicity of *E. coli* at concentrations of 200 μmol l^{-1} melatonin in a Müeller-Hinton (MH) broth. These effects were modified when a protein synthesis inhibitor (chloramphenicol) was added at sub-lethal concentrations to the broth. Vitamin E produced a greater diminution in surface hydrophobicity than melatonin. The adherence of *E. coli* to nitrocellulose filters increased in the presence of melatonin + chloramphenicol, and vitamin E. The effects observed were independent of the concentration of iron in the broth.

Conclusions: Oxidative stress plays an important role in modifying the surface characteristics of *E. coli*, which could affect the micro-organism's capacity to adhere to epithelia.

Significance and Impact of the Study: We think that the oxide reduction potential of the host may be a determinant factor in the bacterial colonization of animal tissue.

INTRODUCTION

The first stage in the colonization of an epithelium by *Escherichia coli* is determined by the bacteria's ability to adhere to the epithelial surface, a capability that is initially dependent on non-specific adhesion mechanisms such as the electrical charge at the surface of the micro-organism or the surface hydrophobicity (Ferreirós and Criado 1984). Other specific factors, such as the formation of pili or specific adhesins, subsequently contribute to the adhesion of the micro-organism to epithelia (Ferreirós and Criado 1980; Gibbons 1996). The relative importance of specific or non-specific adhesion mechanisms varies between micro-organisms, and also depends on their adaptation to the colonization of specific epithelia (Gibbons 1996). Melatonin, which is a hormone found in the pineal gland, plays a stabilizing and regulatory role in humans and is also significant in sleep regulation, the immune response, the elimination of free radicals and the stabilization of cell membranes (Li and Xu 1997; Rodríguez *et al.* 1997; Reiter *et al.* 1997).

The present study evaluates possible relations between melatonin, vitamin E and the surface hydrophobic characteristics of *E. coli*. In addition, as iron has been shown to be an important factor in the virulence of many micro-organisms (Criado *et al.* 1993; Stojiljkovic *et al.* 1995), it was determined whether the different concentrations of iron in the culture medium, in conjunction with melatonin or vitamin E, modify the surface characteristics of this micro-organism. The initial focus of the study is the hypothesis that the oxide reduction potential of the host may be a determinant factor in the bacterial colonization of animal tissue.

Correspondence to: Dr José Uberos, Dpto. Paediatría, Hospital Universitario, Granada, Spain (e-mail: med018111@nacom.es).

MATERIALS AND METHODS

Bacterial strains and culture conditions

Eleven strains of *E. coli* were obtained from the Spanish Collection of Standard Cultures (CECT; Valencia, Spain)

(ATCC 25922, ATCC 11775, Rowe E8775-A, CCUG 20570, CGSC 6512, ATCC 23985, ATCC 8739, ATCC 12407, NCIMB 9482, ATCC 33780, Starlinger 1045). Twelve strains of *E. Coli* (Lac^+), isolated from upper pathway urinary infections in the microbiology laboratory at this Hospital (629, 695, 787, 472, 471, 697, 595, 753, 795, 792, 760, 469), were also used.

In each test, *E. coli* was cultivated in 4 ml Müeller-Hinton (MH) broth (Fluka) and incubated at 36 ± 1°C for 24 h before being processed. The iron content of the culture medium was modified by adding 39 μmol l^{-1} ethylene-diamine-dihydroxiphenyl-acetic acid (Sigma) to 100 ml MH (EDDA-MH, divalent cations restriction), or by adding ferric-ammonium-citrate (Sigma) (1 μg ml^{-1}) (FAC-MH, iron excess).

Solutions of melatonin (Sigma; 1 mmol l^{-1} and 500 nmol l^{-1}) were prepared in sterile distilled water. After incubating the strains of *E. coli* for 12 h at 36 ± 1°C (logarithmic growth phase), 1 ml of each of the solutions was added to each tube of the culture medium (4 ml), resulting in a final concentration of 200 μmol l^{-1} and 100 nmol l^{-1}. Incubation was continued for 12 h at 36 ± 1°C until the process was complete.

Surface hydrophobicity

The surface hydrophobicity was determined by Rosenberg's method (Rosenberg *et al.* 1980). The organisms were incubated at 36 ± 1°C for 24 h in a culture medium and then recovered by centrifugation. The supernatant fluid was removed and the sediment washed three times with saline phosphate buffer (PBS). The sediment was centrifuged again and then mixed with 0·4 ml urea buffer tampon (PUM) (pH 7·1) and 0·8 ml 0·2 mol l^{-1} ammonium sulphate (Sigma). The suspension was adjusted to an optical density (O.D. 560 nm) of 0·6; 200 μl p-xylene (Sigma) were added to 1·2 ml of the bacterial suspension, which was then stirred vigorously for 2 min to obtain a homogeneous mixture of the suspension and the hydrocarbon. This was allowed to stand for 30 min in order for the aqueous phase to separate completely from the hydrocarbon. Using a glass Pasteur pipette, 1 ml of the aqueous phase was then removed and the absorbance measured (560 nm) at a temperature of 36 ± 1°C. Hydrophobicity was calculated as $1 - (A_2/A_1)$, where A_1 and A_2 represent the absorbance before and after the hydrocarbon was added, respectively.

Adherence to nitrocellulose filters

The procedure described by Lachica and Zink was used (Lachica and Zink 1984). The organisms were placed in a culture broth for 24 h at 36 ± 1°C and centrifuged at 3500 *g* for 10 min. The supernatant fluid was removed and the sediment washed three times with phosphate buffer (PBS). The micro-organisms were resuspended in PUM buffer and ammonium sulphate (1 : 2, v/v), after the absorbance had been adjusted to 0·6 at 560 nm. Then, 3 ml of this suspension were passed through a nitrocellulose filter of 8 μm pore diameter (Millipore). The O.D. of the filtrate was determined and the results expressed as a percentage of the adherence to the nitrocellulose filter.

Incubation with vitamin E

The disodium salt (250 mg) of alpha-tocopherol phosphate (Sigma) was dissolved in 50 ml distilled water. The micro-organisms were incubated at 36 ± 1°C for 12 h in tubes containing 4 ml of the MH, EDDA-MH and FAC-MH media. Then, 40 μl of the vitamin E solution were added, thus obtaining a concentration of 5 mg dl^{-1} of vitamin E in the culture broth. The incubation was continued for a further 12 h until analysis.

Incubation with chloramphenicol at sub-lethal concentrations

Chloramphenicol at sub-lethal concentrations (100 μg ml^{-1}) inhibits protein synthesis by micro-organisms (Fu *et al.* 1989). Before each test, solutions of sodium succinate chloramphenicol (Sigma) (1 mg ml^{-1}) were prepared. After incubating the bacteria for 12 h in MH, EDDA-MH and FAC-MH media, 400 μl of the chloramphenicol solution and 1 ml of the melatonin solution (see above) were added to each tube of culture medium (4 ml). Incubation was completed at 36 ± 1°C for 12 h.

Statistical methods

A Kolmogorov normality study was carried out. After observing a lack of normality in the distributions, the Wilcoxon comparison test for paired data and the Kendall agreement test were performed.

RESULTS

Table 1 gives the mean values for surface hydrophobicity and adherence to nitrocellulose filters obtained for *E. coli* incubated with melatonin 1 mmol l^{-1}, melatonin 1 mmol l^{-1} + chloramphenicol and vitamin E in Müeller-Hinton broth (MH, normal iron), MH with ferric-ammonium citrate (FAC-MH, 1 g Fe ml^{-1}, iron-excess) and with 20 μmol l^{-1} ethylene-diamino-dihydroxyphenylacetic (EDDA-MH, iron restriction). The concentration of iron in the culture broth had no significant effect on the surface hydrophobicity, or on the adherence to nitrocellulose filters, of *E. coli*.

Table 1 Cell-surface hydrophobicity and adherence to nitrocellulose filters of *Escherichia coli* incubated with melatonin (aMT), vitamin E and chloramphenicol + aMT, for a Müeller-Hinton broth (MH), for iron excess (FAC-MH) and for iron restriction (EDDA-MH)

	% Hydrophobicity (S.D.)			% Adherence to nitrocellulose (S.D.)		
	MH†	EDDA-MH‡	FAC-MH‡‡	MH†	EDDA-MH‡	FAC-MH‡‡
No aMT	16·9 (21·3)	17·6 (19·7)	15·3 (19·6)	81·4 (19·2)	78·9 (24·2)	84·9 (13·7)
aMT (100 nmol l^{-1})	16·1 (19·0)	17·9 (19·7)	15·5 (19·8)	83·5 (16·5)	79·8 (16·3)	84·1 (16·5)
aMT (200 μmol l^{-1})	6·5* (11·6)	10·1 (13·9)	8·5 (12·9)	77·8 (19·1)	71·8 (24·9)	72·9** (24·7)
aMT (200 μmol l^{-1}) + Chloramphenicol	11·2 (19·1)	11·9 (17·2)	12·4 (20·9)	88·6§ (11·7)	83·3 (18·1)	88·6§§ (11·6)
Vitamin E	0·23¶¶ (1·1)	4·1¶ (17·7)	0¶¶ (0·0)	89·2¶ (10·8)	86·3¶ (13·6)	90¶¶ (11·1)

†Müeller-Hinton broth; ‡Mueller-Hinton broth + ethylene-diamino-dihydroxyphenilacetic acid; ‡‡ferric ammonium citrate + Mueller-Hinton broth.; (*) $P < 0{\cdot}05$ (**) $P < 0{\cdot}01$, comparison between melatonin 0 mmol l^{-1} and melatonin 200 μmol l^{-1}; (¶) $P < 0{\cdot}05$ (¶¶) $P < 0{\cdot}01$, comparison between melatonin 200 μmol l^{-1} and vitamin E.; (§) $P < 0{\cdot}05$ (§§) $P < 0{\cdot}01$, comparison between melatonin 200 μmol l^{-1} and melatonin 200 μmol l^{-1} + chloramphenicol.

The surface hydrophobicity of *E. coli* diminished significantly after incubation with melatonin at a concentration of 200 μmol l^{-1} in MH broth. There was a similar reduction in the rate of adherence to nitrocellulose in FAC-MH broth. The addition of sub-lethal concentrations of chloramphenicol + melatonin (200 μmol l^{-1}) to the broth did not produce significant changes in surface hydrophobicity. Under these conditions, the percentage of adherence to nitrocellulose filters increased significantly when *E. coli* was incubated in the presence of iron, as the addition of a divalent cation chelating agent (ethylene-diamino-dihydroxyphenylacetic acid) to the broth reduced the percentage of adherence to nitrocellulose.

Vitamin E produced a significant reduction in the surface hydrophobicity of *E. coli*, much greater than that obtained with melatonin 200 μmol l^{-1}. The percentage of adherence of *E. coli* to nitrocellulose filters increased significantly when the micro-organism was incubated in the presence of vitamin E. In these cases, the changes in surface hydrophobicity and adherence to nitrocellulose were greater when the culture broth was supplemented with iron.

The Kendall agreement test between adherence to nitrocellulose and surface hydrophobicity only revealed agreement between the two measurement methods ($T = 0{\cdot}52$; $P = 0{\cdot}001$) when *E. coli* was incubated with chloramphenicol and melatonin (200 μmol l^{-1}) in EDDA-MH.

DISCUSSION

The first stage in the colonization of an epithelium by *E. coli* depends on the bacteria's ability to adhere to the cells of the host; initially, this ability is determined by the electrical charge at the surface of the micro-organism and by its surface hydrophobicity (Ferreirós and Criado 1984), and subsequently, by factors such as the formation of pili and specific adhesins (Gibbons *et al.* 1976; Ferreirós and Criado 1980; Gibbons and Hay 1989).

Previous studies (Uberos *et al.* 2000) have shown that melatonin at a concentration of 200 μmol l^{-1} significantly increases the surface hydrophobicity of *Neisseria meningitidis* and its adherence to nitrocellulose, and that the concentration of iron in the culture broth is not a significant factor in such differences. These conclusions, together with those of the present study, lead to the belief that the effects of melatonin on the surface characteristics of micro-organisms vary between different micro-organisms.

Several studies (Ellis and Vorhies 1976; Hogan *et al.* 1993; Friedman *et al.* 1998) have shown how the administration of high concentrations of vitamin E to animals is associated with higher levels of antibodies against *E. coli*. It is not known whether this finding bears any relation to the reduction in surface hydrophobicity revealed in the present study, although in theory a reduction in the surface hydrophobicity of *E. coli* would impede the colonization of epithelia, both gastrointestinal and genito-urinary. Furthermore, the adjuvant effect described for vitamin E (Hogan *et al.* 1993) in the induction of the humoral immune response could be a consequence of the altered surface hydrophobicity characteristics of *E. coli* incubated with vitamin E, namely, the reduction in surface hydrophobicity and the increase in the percentage of adherence to nitrocellulose.

Chloramphenicol at inhibitory concentrations inhibits protein synthesis by micro-organisms (Fu *et al.* 1989).

The present study included incubation with chloramphenicol and melatonin (200 μmol l^{-1}) to test whether the effects observed on the surface characteristics of *E. coli* were due to protein synthesis *de novo*. It was found that melatonin leads to a reduction in the surface hydrophobicity of *E. coli*, a reduction which is unaltered by incubation with chloramphenicol + melatonin. Therefore, it is concluded that the effect of melatonin on the surface hydrophobicity of *E. coli* is due to the synthesis *de novo* of surface proteins of the micro-organism. When *E. coli* (in the presence of iron) was incubated with melatonin + chloramphenicol, the adherence to nitrocellulose filters increased significantly to levels close to those found when vitamin E was included. The inhibition of protein synthesis by chloramphenicol was very probably responsible for the more significant effect of melatonin on the organism, which approached that observed when *E. coli* was incubated with vitamin E.

A Kendall agreement test was applied to determine whether any parallelism exists between the hydrophobic characteristics of *E. coli* and its adherence to nitrocellulose filters. The test revealed no association between the two groups of results in any case, except when chloramphenicol + melatonin was applied in the EDDA-MH culture broth. Therefore, the variations in surface hydrophobicity and adherence to nitrocellulose filters must be determined by surface characteristics of the bacteria that are interrelated when *E. coli* is incubated in EDDA-MH.

Although the incubation of *E. coli* with or without melatonin did not produce significant differences in surface hydrophobicity, a tendency was observed towards a reduction of the latter in the presence of melatonin. This reduction was greater when *E. coli* was incubated with another antioxidant, vitamin E. It is therefore concluded that in *E. coli*, the antioxidant effect of vitamin E is reflected as a significant reduction in surface hydrophobicity. It is noteworthy that *E. coli* did not respond in the same way to melatonin, which in most situations is a powerful antioxidant (Daniels *et al.* 1995; Poeggeler *et al.* 1995). This could be related to the genito-urinary origin of most of the *E. coli* studied, as well as to the adaptation of the micro-organism to the periodic presence of the hormone in the urine, bearing in mind that a reduction in the surface hydrophobicity of *E. coli* in the presence of endogenous melatonin (normally eliminated in the urine) would mean that it would be eliminated more readily from colonized urinary epithelia.

REFERENCES

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

Daniels, W.M.U., Reiter, R.J., Melchiorri, D., Sewerynek, E., Pablos, M.I. and Ortiz, G.G. (1995) Melatonin counteracts lipid peroxidation induced by carbon tetrachloride but does not restore glucose 6 phosphatase activity. *Journal of Pineal Research* **19**, 1–6.

Ellis, R.P. and Vorhies, M.W. (1976) Effect of supplemental dietary vitamin E on the serologic response of swine to an *Escherichia coli* bacterin. *Journal of the American Veterinary Medicine Association* **168**, 231–232.

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.

Friedman, A., Bartov, I. and Sklan, D. (1998) Humoral immune response impairment following excess vitamin E nutrition in the chick and turkey. *Poultry Science* **77**, 956–962.

Fu, K., Hassett, D.J. and Cohen, M.S. (1989) Oxidant stress in *Neisseria gonorrhoeae*: adaptation and effects on L-(+)lactate dehydrogenase activity. *Infection and Immunity* **57**, 2173–2178.

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.

Gibbons, R.J. and Hay, D.I. (1989) Adsorbed salivary acidic proline-rich proteins contribute to the adhesion of *Streptococcus mutans* JBP to apatitic surfaces. *Journal of Dental Research* **68**, 1303–1307.

Gibbons, R.J., Spinell, D.M. and Skobe, Z. (1976) Selective adherence as a determinant of the host tropisms of certain indigenous and pathogenic bacteria. *Infection and Immunity* **13**, 238–246.

Hogan, J.S., Weiss, W.P., Smith, K.L., Todhunter, D.A., Schoenberger, P.S. and Williams, S.N. (1993) Vitamin E as an adjuvant in an *Escherichia coli* J5 vaccine. *Journal of Dairy Science* **76**, 401–407.

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

Li, J.C. and Xu, F. (1997) Influences of light-dark shifting on the immune system, tumor growth and life span of rats, mice and fruit flies as well as on the counteraction of melatonin. *Biological Signals* **6**, 77–89.

Poeggeler, B., Reiter, R.J., Hardeland, R., Sewerynek, E., Melchiorri, D. and Barlowwalden, L.R. (1995) Melatonin, a mediator of electron transfer and repair reactions, acts synergistically with the chain breaking antioxidants ascorbate, trolox and glutathione. *Neuroendocrinological Letters* **17**, 87–92.

Reiter, R.J., Tang, L., Garcia, J.J. and Munoz-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., Gutnick, D. and Rosenberg, E. (1980) Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surface hydrophobicity. *FEMS Microbiology Letters* **9**, 29–33.

Stojiljkovic, I., Hwa, V., De Saint, M. *et al.* (1995) The *Neisseria meningitidis* haemoglobin receptor: its role in iron utilization and virulence. *Molecular Microbiology* **15**, 531–541.

Uberos, J., Molina, A., Liébana, J., Augustín, M.C. and Muñoz, A. (2000) The influence of different concentrations of melatonin on the cell-surface hydrophobic characteristics of *Neisseria meningitidis*. *Letters in Applied Microbiology* **31**, 294–298.