SHORT ARTICLES

INHIBITION OF DIFFERENT SEROTYPES OF *LISTERIA MONOCYTOGENES* BY ENTEROCINS IN SOLID AND LIQUID MEDIA

J. KRÄMER

*Institute of Medical Microbiology and Immunology, University of Bonn,
Germany*

THE production of bacteriocins (enterocins) is a very common property of enterococci (Sherwood *et al.*, 1949; Stark, 1960; Brock, Peacher and Pierson, 1963; Brandis, Brandis and Van De Loo, 1965; Pleceaš, 1970; Tzannetis, Leonardopoulos and Papavassiliou, 1970; Brandis and Šmarda, 1971; Bottone, Allerhand and Pisano, 1974). Two bacteriocins, enterocin E1A and E1B, produced by *Streptococcus faecium* strain E1 and studied in detail (Krämer and Brandis, 1975*a* and *b*) acted not only against certain strains of enterococci and *Streptococcus salivarius* but also inhibited strains of *Clostridium perfringens* and *Clostridium septicum* (Krämer and Schallehn, 1974) and strains of *Listeria monocytogenes* in solid media (Brandis and Brandis, 1962; Krämer and Brandis, 1975*a*). This paper describes the production of enterocins by several strains of different species of enterococci in solid and liquid media and the action of these inhibitory substances on different serotypes of *L. monocytogenes*.

MATERIALS AND METHODS

Cultures. All strains of bacteria tested were isolated from human sources. The organisms were grown in tryptose phosphate broth (TPB, Difco). Strains of enterococci were classified as *Streptococcus faecium*, *Streptococcus faecalis*, *Streptococcus faecalis* v. *zymogenes* or *Streptococcus faecalis* v. *liquefaciens* (Heeschen, Tolle and Zeidler, 1967; Esper, 1970). Isolates of *L. monocytogenes* were identified by biochemical methods and further subdivided into the serotypes 1, 2, 3, 4a, 4b, 4c, 4d and 4e.

Demonstration of enterocin activity

Stab plate method. A modification of the method of Fredericq (1948) was used. Cultures of enterococci were stabbed into TPB agar plates, incubated for 18 h, and after exposure to chloroform vapour were overlaid with 3.0 ml of soft TPB agar containing approximately 10^7 cells of the indicator strain per ml. Inhibition zones around the stab culture after incubation for 18 h at 37°C indicated enterocin sensitivity.

Spot test. Single drops of purified enterocin E1A (see below) or of TPB culture supernatant fluids, the latter filtered through Millipore membranes (Millipore, London) of 0.22 µm porosity, or heated at 80°C for 15 min., were spotted on to TPB agar containing 10^7 cells of the indicator strain per ml. The plates were examined for inhibition of growth after incubation for 18 h at 37°C.

Enterocin titre. Serial dilutions of supernatant fluids of cultures of enterococci were incubated with equal volumes of exponential-phase broth cultures of the indicator strain containing 10^4 bacteria per ml. The reciprocal of the highest dilution that completely inhibited growth after incubation for 8 h at 37°C was recorded as the titre of the sample.

Production and purification of enterocin E1A. Enterocin E1A, found without induction in the supernatant fluid of cultures of *S. faecium* strain E1, was purified 400-fold by consecutive ammonium sulphate fractionation, gel filtration on Sephadex G-75 and chromatography on DEAE-Cellulose (Krämer and Brandis, 1975*a*).

Received 5 Aug. 1975; revised version accepted 10 Sept. 1976.

TABLE I
Demonstration of enterocins in solid and liquid cultures

Enterocin-producing streptococcus	Number of strains tested	Number of strains showing inhibitory activity in		
		solid medium	supernate of fluid culture	
			Heated	Membrane-filtered
<i>S. faecium</i>	7	7	5	3
<i>S. faecalis</i>	4	4	0	1
<i>S. faecalis</i> v. <i>zymogenes</i>	4	4	0	4
<i>S. faecalis</i> v. <i>liquefaciens</i>	6	6	0	4

TABLE II
Inhibition of *Listeria monocytogenes* by enterococcal cultures in solid media

Donor streptococcal strain	Number of sensitive <i>L. monocytogenes</i> strains of the stated serotype (and number of strains tested)								
	1 (22)	2 (2)	3 (1)	4a (1)	4b (22)	4c (1)	4d (1)	4e (1)	
<i>S. faecium</i> strain no. E1	21	1	1	1	21	0	1	1	
3	20	1	1	1	20	0	0	1	
25	12	1	1	1	8	0	0	0	
38	21	2	1	1	22	0	1	1	
44	20	2	1	1	20	0	1	0	
59	21	2	1	1	20	0	0	0	
77	20	2	1	1	20	0	0	0	
<i>S. faecalis</i> strains no. 8, 61, 68, 98	0	0	0	0	0	0	0	0	
<i>S. faecalis</i> v. <i>zymogenes</i> strain no. 24	2	0	0	0	0	0	0	0	
78	0	0	0	0	0	0	0	0	
81	0	0	0	0	0	0	0	0	
85	1	1	0	0	0	0	0	0	
<i>S. faecalis</i> v. <i>liquefaciens</i> strains no. 4, 5, 12, 66, 75	0	0	0	0	0	0	0	0	

Lethal effect of enterocin E1A. Indicator cells from exponential phase cultures in TPB were centrifuged, washed and resuspended in fresh medium. Purified enterocin E1A or, as a control, 0.06 M-phosphate buffer, were added and the mixtures incubated aerobically at 37°C. The number of surviving viable cells was determined at intervals thereafter by subculturing diluted 0.1-ml samples on to TPB agar.

RESULTS

Demonstration of soluble enterocins in liquid cultures of enterococci

Twenty-one of the strains of different species of group D streptococci found by Brandis *et al.* (1965) to produce at least one enterocin when grown on solid media were examined for their production of soluble enterocins in liquid cultures. The strains tested are listed in table II. Enterocins were detected in the supernatant fluid of cultures of 14 of them (table I). The

TABLE III

Susceptibility of Listeria monocytogenes serotypes to soluble enterocins of Streptococcus faecium

Enterocin-producing <i>S. faecium</i> (strain no.)	Titre of enterocin inhibitory action against <i>L. monocytogenes</i> of serotype							
	1	2	3	4a	4b	4c	4d	4e
E1	320	40	640	320	40	0	160	0
3	20	10	40	40	10	0	10	0
25	0	0	0	0	0	0	0	0
38	160	40	80	320	10	0	80	0
44	80	0	10	40	0	0	10	0
59	320	80	320	10	40	0	320	0
77	10	10	10	40	10	0	20	0

soluble enterocins of *S. faecium* were heat stable but significantly adsorbed to membrane filters whereas the enterocins produced by *S. faecalis*, *S. faecalis* v. *zymogenes* and *S. faecalis* v. *liquefaciens* were completely inactivated by heating for 15 min. at 80°C.

Inhibition of Listeria monocytogenes in solid media

When the susceptibility to enterocins of *L. monocytogenes* was tested by the stab plate method, the zones of inhibition produced were of different sizes depending on the enterocinogenic strain and the strain of *L. monocytogenes* under test. As shown in table II enterocin activity was species-specific. All the strains tested except *L. monocytogenes* serotype 4c were susceptible to the enterocins of three or more strains of *S. faecium*. However, four only of 51 strains of *L. monocytogenes* were inhibited by cultures of other species of enterococci.

Inhibition of Listeria monocytogenes in liquid media

There was a noticeable variation in the sensitivity of different serotypes of *L. monocytogenes* to the action of enterocins from different strains of *S. faecium* (table III). Two serotypes (4c and 4e) were completely resistant; 4 serotypes (1, 3, 4a and 4d) were inhibited by all but one of the soluble enterocins tested and two serotypes (2 and 4b) were of intermediate sensitivity.

Killing effect on sensitive cells

S. faecium strains E1, 38 and 59 caused the most marked inhibition of the greatest number of strains of *L. monocytogenes*. It has been shown (Krämer and Brandis, 1975a and b) that the inhibitory substance produced by *S. faecium* strain E1 can be separated into two distinct bacteriocins—enterocin E1A and E1B. The figure shows the lethal action of purified enterocin E1A at different concentrations on sensitive cells of *L. monocytogenes* serotype 1, as demonstrated by loss of colony forming units. Purified enterocin E1A with a titre of 150 killed over 99% of the indicator cells in 10 min. at 37°C. The optical densities of the cell suspensions with and without enterocin E1A did not show any appreciable difference during incubation for 1 h.

DISCUSSION

Bacteriocins are defined as antibacterial substances produced by various species of bacteria which are active usually against closely related micro-organisms; however, some activity on species outside the bacteriocinogenic group is common, especially for bacteriocins produced by Gram-positive bacteria (Hamon and Peron, 1963). Apparently, most bacteriocins produced by *S. faecium* and by a few strains of other enterococci are of the type active also against some unrelated genera. Thus, most of the 51 strains of *L. monocytogenes*

FIGURE—The effect of different concentrations of enterocin E1A on the viability of cells of *Listeria monocytogenes* strain LB3 in suspension. ○—○ = control, 0.06M phosphate buffer; ▲—▲ = A, purified enterocin at a titre of 100; △—△ = B, at a titre of 500; and ●—● = C, at a titre of 1500.

tested were found to be sensitive to at least one bacteriocin produced by different strains of enterococci. The inhibition of *L. monocytogenes* could be demonstrated as easily on agar medium as in liquid cultures. There were, however, great differences in the spectrum of activity of the bacteriocins according to the species of the producer strain. The bacteriocins of *S. faecium* had the widest range and inhibited 50 strains of *L. monocytogenes*, whilst relatively feeble activity against *L. monocytogenes* was shown by bacteriocins of *S. faecalis*, *S. faecalis* v. *zymogenes* and *S. faecalis* v. *liquefaciens*. Activity of *S. faecium* bacteriocins has been demonstrated also against different strains of enterococci (Krämer and Brandis, 1975a) and against strains of *C. septicum* (Krämer and Schallehn, 1974), and other workers have also demonstrated a broad spectrum of activity for bacteriocins from *S. faecium* (Pleceaş, Bogdan and Vereanu, 1971), *S. faecalis* v. *zymogenes* (Brock *et al.*, 1963; Bottone *et al.*, 1974) and other enterococci (Pohunek, 1961).

Brandis *et al.* (1965) demonstrated that bacteriocins of *S. faecium* were inactivated by the action of trypsin in solid media, whereas bacteriocins of *S. faecalis*, *S. faecalis* v. *zymogenes* and *S. faecalis* v. *liquefaciens* were resistant to trypsin. Furthermore, the present study shows that these bacteriocins vary not only in their spectrum of activity but also in their response

to membrane filtration and to heat; the bacteriocins produced by strains of *S. faecium* differ clearly in these two respects from those produced by other enterococci.

The action of purified *S. faecium* enterocin E1A on *L. monocytogenes* appeared to be primarily bactericidal but despite a rapid decrease in the number of viable cells of *L. monocytogenes* after the addition of enterocin E1A there was no striking reduction in optical density of the cell suspension. These results suggest that the lethal effect was not accompanied by significant cell lysis as demonstrated for other enterocins which are both strongly haemolytic and strongly bacteriolytic (Davie and Brock, 1966; Jackson, 1971). Bactericidal action with no reduction in optical density has also been observed when enterocin-sensitive cells of *S. faecium* were used instead of *L. monocytogenes* (Krämer and Brandis, 1975b) and after the addition of a bacteriocin of *S. faecalis* v. *zymogenes* to a sensitive strain of *C. perfringens* (Bottone *et al.*, 1974).

SUMMARY

Twenty-one enterocinogenic strains of enterococci were examined for their ability to inhibit 51 strains of *Listeria monocytogenes* belonging to eight different serotypes; 50 strains of *L. monocytogenes* were uniformly inhibited on solid media and in broth cultures by strains of *Streptococcus faecium*. However, only four strains of *L. monocytogenes* were inhibited by strains of *Streptococcus faecalis*, *S. faecalis* v. *zymogenes* or *S. faecalis* v. *liquefaciens*. Enterocin E1A prepared from the supernatant fluid of *S. faecium* strain E1 rapidly killed sensitive cells of *L. monocytogenes* but did not lyse them.

I wish to thank Mrs Christel Reichertz for her excellent technical assistance.

REFERENCES

- BOTTONE, E., ALLERHAND, J. AND PISANO, M. A. 1974. Effects of a bacteriocin produced by *Streptococcus faecalis* var. *zymogenes* (E-1) on susceptible microorganisms. *Antonie van Leeuwenhoek*, **40**, 385.
- BRANDIS, H. AND BRANDIS, U. 1962. Über einen bacteriocinartigen Stoff aus Enterokokken. *Pathologia Microbiol.*, **25**, 632.
- BRANDIS, H., BRANDIS, U. AND VAN DE LOO, W. 1965. Bacteriocinähnliche Stoffe von Enterokokken. *Zentbl. Bakt. ParasitKde*, I. Abt. Orig., **196**, 331.
- BRANDIS, H. AND ŠMARDÁ, J. 1971. Bacteriocine und bacteriocinähnliche Substanzen, *Jena*, p. 350.
- BROCK, T. D., PEACHER, B. AND PIERSON, D. 1963. Survey of the bacteriocines of enterococci. *J. Bact.*, **86**, 702.
- DAVIE, J. M. AND BROCK, T. D. 1966. Action of streptolysin S, the group D hemolysin, and phospholipase C on whole cells and spheroplasts. *J. Bact.*, **91**, 595.
- ESPER, S. F. 1970. Untersuchungen über die biochemischen und serologischen Reaktionen von Streptokokken der Gruppe D, sowie Isolierung enterokokken-spezifischer Bakteriophagen und Anwendung dieser Phagen zur Charakterisierung von Enterokokkentypen. Dissertation, *Bonn*.
- FREDERICQ, P. 1948. Actions antibiotiques réciproques chez les Enterobacteriaceae. *Revue belge Path. Méd. exp.*, **19**, Suppl. 4, 1.
- HAMON, Y. AND PERON, Y. 1963. Quelques remarques sur les bactériocines produites par les microbes gram-positifs. *C. r. hebdomadaire Séances Acad. Sci.*, **257**, 1191.
- HEESCHEN, W., TOLLE, A. AND ZEIDLER, H. 1967. Zur Klassifizierung der Gattung Streptococcus. *Zentbl. Bakt. ParasitKde*, I. Abt. Orig., **205**, 250.
- JACKSON, R. W. 1971. Bacteriolysis and inhibition of gram-positive bacteria by components of *Streptococcus zymogenes* lysis. *J. Bact.*, **105**, 156.
- KRÄMER, J. AND BRANDIS, H. 1975a. Purification and characterization of two bacteriocins from *Streptococcus faecium*. *J. gen. Microbiol.*, **88**, 93.
- KRÄMER, J. AND BRANDIS, H. 1975b. Mode of action of two *Streptococcus faecium* bacteriocins. *Antimicrob. Agents Chemother.*, **7**, 117.

- KRÄMER, J. AND SCHALLEHN, G. 1974. Enterocinwirkung auf *Clostridium perfringens* und *Clostridium septicum*. *Zentbl. Bakt. ParasitKde*, I. Abt. Orig., **226**, 105.
- PLECEAȘ, P. 1970. Bactériocines des streptocoques groupe D. I. Leur incidence. *Archs roum. Path. exp. Microbiol.*, **29**, 229.
- PLECEAȘ, P., BOGDAN, C. AND VEREANU, A. 1971. The bacteriocins of group D streptococci. II. Their action. *Archs roum. Path. exp. Microbiol.*, **30**, 351.
- POHUNEK, M. 1961. Streptococci antagonizing the vaginal lactobacillus. *J. Hyg. Epidem. Microbiol. Immun.*, **5**, 267.
- SHERWOOD, N. P., RUSSELL, B. E., JAY, A. R. AND BOWMAN, K. 1949. Studies on streptococci. III. New antibiotic substances produced by beta hemolytic streptococci. *J. infect. Dis.*, **84**, 88.
- STARK, J. M. 1960. Antibiotic activity of haemolytic enterococci. *Lancet*, **I**, 733.
- TZANNETIS, S., LEONARDOPOULOS, J. AND PAPAVASSILIOU, J. 1970. Enterocinogeny and lysogeny in enterococci. *J. appl. Bact.*, **33**, 358.