Effects of Microcin B17 on Microcin B17-immune Cells

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When microcin B17-immune cells are treated with microcin B17 they show many of the physiological effects displayed by microcin B17-sensitive cells treated in the same way. DNA replication stops immediately and several SOS functions are subsequently induced. In sensitive cells these effects are irreversible and lead to cell death, whereas in immune cells they are reversible and there is no loss of viability. This is an unusual mechanism of immunity because it does not prevent the primary action of the microcin. The implications of this mechanism concerning the mode of action of microcin B17 and the induction of the SOS system are discussed.

INTRODUCTION

Microcin B17 is a low-molecular-weight antibiotic peptide produced by *Escherichia coli* strains carrying the 70 kb plasmid pMccB17, previously referred to as pRYC17 (Baquero *et al.*, 1978; Hernández-Chico *et al.*, 1982). This plasmid also encodes immunity to microcin B17, thus ensuring that producer strains are protected against the killing effect of the anti-bacterial agent.

Microcin B17 kills sensitive *E. coli* K12 cells. Its primary effect is the inhibition of DNA synthesis. DNA degradation follows and expression of the SOS repair system is induced. This induction depends on the bacterial products RecA and RecBC and requires the presence of an active chromosome replication fork in the treated cells (Herrero & Moreno, 1986). Whatever the target of the microcin, the antibiotic seems to inactivate, irreversibly, the active replication complexes of sensitive cells.

As with colicins (Konisky, 1982), microcin immunity is a general property of microcinogenic cells (Baquero & Moreno, 1984). Immunity is specific for each bacteriocin (colicin or microcin) and it protects against both endogenous (that synthesized by the cells) and exogenous microcin. The molecular and physiological basis for immunity to colicins has been extensively studied for several colicins (Jakes, 1982; Konisky, 1982). Briefly, immunity to a colicin is mediated by a protein specific for the colicin and is encoded by the plasmid that encodes the cognate colicin. For colicins with cytoplasmic targets (E2, E3 and DF13), the immunity protein interacts with the colicins, masking their catalytic site (Konisky, 1982). It is thought that channel-forming colicins (E1, Ia and Ib) interact with their immunity protein in the cytoplasmic membrane (Weaver *et al.*, 1981). In both cases, these interactions prevent the action of colicins on their targets.

In contrast, the results presented here indicate that immunity to microcin does not prevent this antibiotic from exerting its primary effect. Indeed, microcin B17 has similar physiological effects in sensitive and immune cells but immune cells are not killed. This represents a novel

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mechanism of immunity and the implications concerning the mode of action of microcin B17 and induction of the SOS repair system are discussed.

METHODS

Bacteria and plasmids. The strains of *E. coli* used are listed in Table 1. Plasmid pColE2-P9 determines synthesis of colicin E2 and immunity to this colicin (Mock & Pugsley, 1982). Plasmids pMM833 (Mcc⁺ Imm⁺) and pMM827 (Mcc⁻ Imm⁺) are two derivatives of the low copy number wild-type plasmid pMccB17 obtained by insertion of Tn5 (San Millán *et al.*, 1985*b*). Multicopy plasmids pMM102 (Mcc⁺ Imm⁺), pMM120 (Mcc⁻ Imm⁺), pMM201 (pMM102::Tn5, Mcc⁻ Imm⁺) have been described by San Millán *et al.* (1985*a*). pMccB17 derivatives were introduced into strains by conjugation, while pMM102 derivatives were introduced by transformation.

Media and experimental methods. These were all as described in the accompanying paper (Herrero & Moreno, 1986). Microcin extracts and non-microcin control extracts were prepared from RYC893 and RYC895, respectively, and were assayed on strain BM21. β -Galactosidase activity was measured, and units were calculated, as described by Miller (1972).

RESULTS

Microcin B17 inhibits DNA replication in microcin B17-immune cells

During studies on the production of and immunity to microcin B17 we observed that the growth of immune cells was inhibited in the presence of high concentrations of exogenous microcin, but that cell death did not follow. These observations led us to study in detail the behaviour of immune cells in the presence of the antibiotic. To evaluate the effects of exogenous microcin, experiments were done with cells carrying plasmids encoding the immunity function but not microcin synthesis, thus eliminating possible effects of endogenous microcin. We examined cell growth, cell viability, DNA metabolism and expression of the SOS repair system, phenomena known to be affected in sensitive cells treated with microcin B17 (Herrero & Moreno, 1986).

Table 1. *E. coli* strains used

Strain	Genetic characters	Reference or source*
BM21	GyrA ⁻ (λ^+)	Hernández-Chico <i>et al.</i> (1982)
MC4100	<i>araD139</i> Δ <i>lacU169</i> RpsL ⁻ RelA ⁻ ThiA ⁻	Casadaban (1976)
RYC511	As MC4100, pColE2-P9	Hernández-Chico <i>et al.</i> (1982)
RYC384	As MC4100, pColE2-P9, pMM827	
pop3351	As MC4100, Δ <i>malB1</i>	Hernández-Chico <i>et al.</i> (1982)
RYC388	As pop3351, pMM827	
RYC389	As pop3351, pMM201	
RYC893	As pop3351, pMM102	} San Millán <i>et al.</i> (1985 <i>a</i>)
RYC895	As pop3351, pMM120	
HfrH	GalE ⁻	J. Beckwith
RYC379	As HfrH, pMM827	
GC4415	Thr ⁻ Leu ⁻ His ⁻ PyrD ⁻ <i>trp</i> ::Muc ⁺ Δ <i>lac</i> MalB ⁻ GalK ⁻ RpsL ⁻ <i>srl-300</i> ::Tn10 <i>sfiA</i> ::Mud1(Ap ^R <i>lac</i>)	Huisman & D'Ari (1981)
RYC354	As GC4415, Δ (<i>sbmA-phoA</i>)14	
RYC355	As GC4415, pMM827	
RYC356	As GC4415, pMM833	
RYC357	As GC4415, pMM201	
RYC359	As RYC354, pMM833	
RYC361	As pop3351, ThyA ⁻	Herrero & Moreno (1986)
RYC394	As RYC361, pMM827	
RYC382	Thr ⁻ Leu ⁻ <i>his-4</i> ArgE ⁻ Δ <i>lacU169</i> <i>recA441</i> <i>sfiA11</i> <i>umuC</i> ::Mud(Ap ^R <i>lac</i>) <i>malE</i> ::Tn5	Herrero & Moreno (1986)
RYC383	As RYC382, pMM827	
GC2375	Thr ⁻ Leu ⁻ Pro ⁻ <i>his-4</i> <i>argE3</i> Thi ⁻ Lac ⁻ Gal ⁻ RpsL ⁻ <i>sup-37</i> [λ (<i>recA</i> :: <i>lac</i>)clind]	Casaregola <i>et al.</i> (1982)
RYC392	As GC2375, pMM827	
GC2421	As GC2375, <i>recB21</i>	Casaregola <i>et al.</i> (1982)
RYC393	As GC2421, pMM827	

* Strains for which no reference or source is given are described in this paper. Address: J. Beckwith, Harvard Medical School, Boston, USA.

Fig. 1

Fig. 2

Fig. 1. Effect of microcin B17 on the growth of immune cells. A culture of GC2375(pMM827), grown exponentially in M63 glucose medium, was split into two subcultures. At time zero, one subculture received microcin (200 AU ml⁻¹) and the other an equivalent volume of a non-microcin control extract. Incubation was continued and at various times the cell mass was determined spectrophotometrically and the number of c.f.u. was determined by plating on LB medium. ○, Control; ●, microcin B17; —, absorbance; ----, c.f.u. ml⁻¹.

Fig. 2. Inhibition of the rate of DNA synthesis in microcin B17-immune cells in the presence of microcin B17. Bacterial cultures were grown exponentially in M63 glucose. When cultures reached an OD₆₀₀ of 0.5 (time zero) each received 200 AU microcin B17 ml⁻¹ and incubation was continued for 1 h. At various times, 0.3 ml samples were removed and pulse-labelled with [*methyl*-³H]thymidine for 3 min, in the presence of 2-deoxyadenosine (200 µg ml⁻¹). The samples were precipitated in 5% (w/v) TCA, filtered and radioactivity on the filters was counted. The rates of incorporation are expressed as a percentage of that of the untreated control: the absolute values in the control were 50000–100000 c.p.m. △, pop3351; ○, pop3351(pMM827); ●, pop3351(pMM201).

When exponentially growing microcin-sensitive cells were treated with microcin B17 (200 AU ml⁻¹) the bacterial mass continued to increase normally for about 1 h. However, cells immediately lost viability upon antibiotic treatment. Under the same experimental conditions, microcin B17 only slightly affected the growth of immune cells, as measured by the increase in the optical density of the culture, and the number of c.f.u. remained constant for 2–3 h (Fig. 1). Microscopic observation of immune cells 2 h after addition of the antibiotic indicated that, as with sensitive cells, most had formed filaments. But, whereas cell filaments from sensitive strains were not viable, those from immune strains gave colonies when plated on microcin-free medium.

We have previously shown that the primary effect of microcin B17 on sensitive cells is to inhibit DNA synthesis. This also occurred with immune cells. In fact, the reduction in the rate of synthesis was the same in sensitive pop3351 cells and in immune pop3351(pMM827) cells when they were treated with microcin B17 at 200 AU ml⁻¹ (Fig. 2). Less inhibition of DNA synthesis was observed when the immune bacteria harboured the multicopy plasmid pMM201, indicating that the extent of inhibition depends on the copy number of the immunity genes.

Microcin B17 induces expression of the SOS system in immune cells

Agents or culture conditions that damage DNA or interfere with DNA replication induce the SOS response (Little & Mount, 1982). This response involves the inhibition of cell division and an increased capacity for DNA repair and mutagenesis, and may lead to colicin induction and derepression of a number of prophages. After treatment with an inducing agent, a signal is generated which reversibly activates the RecA protein, which in its active form can cleave the LexA protein, and some phage repressors. The inactivation of the LexA protein allows increased

Table 2. *Induction of SOS gene expression in sensitive and immune cells in the presence of microcin B17*

Cultures growing exponentially in supplemented M63 medium received microcin B17 at time zero. β -Galactosidase activity (Miller units) was assayed at the indicated times. The values are means of two independent experiments, the results of which agreed to within 95%.

Gene fusion	Strain	Microcin (AU ml ⁻¹)	β -Galactosidase activity (Miller units)			
			0 min	45 min	90 min	135 min
<i>recA::lacZ</i>	GC2375	150	150	600	1120	1390
		30	–	260	700	1000
	RYC392	150	150	400	970	1250
		30	–	180	320	440
<i>umuC::lacZ</i>	RYC382	150	7	30	50	–
		30	–	25	30	–
	RYC383	150	2	7	17	27
		30	–	3	7	12
<i>sfiA::lacZ</i>	GC4415	150	26	360	1070	900
	RYC355	150	28	240	1030	870

–, Not measured.

expression of many genes (SOS genes) normally repressed by this protein in uninduced cells. Using operon fusions of Mud1(Ap^R *lac*) and SOS genes (*recA*, *sfiA*, *umuC*) we have shown that microcin B17 induced expression of β -galactosidase in immune cells as well as in microcin-sensitive cells (Table 2). The extent of the induction depended on the microcin concentration. The immunity gene copy number also affected the level of β -galactosidase activity. For example, after 90 min treatment with 200 AU microcin B17 ml⁻¹ strain RYC355 (single-copy of immunity gene) had 1500 Miller units of β -galactosidase, while RYC357 (multi-copy immunity) had 500 units.

Colicin production is repressed in colicinogenic strains by the LexA repressor (Pugsley, 1984a). As expected from our findings, microcin B17 derepressed the production of colicin E2 in microcin-immune strains. We found the same increase in the level of colicin E2 (10⁴-fold) when we incubated MC4100(pColE2-P9) or MC4100(pColE2-E9, pMM827) for 100 min in the presence of 150 AU microcin ml⁻¹.

As in sensitive cells, the microcin B17-induced SOS expression in immune cells also required the RecBC product. No induction of *recA-lacZ* expressions as observed in GC2421 and RYC393 strains. All of the above results indicated that, when microcin B17 was present at a sufficiently high concentration, the extent of its inhibition of DNA synthesis and induction of SOS functions were practically indistinguishable between sensitive and immune cells. However, whereas sensitive cells were killed, immune cells remained viable.

Differences in the response to microcin B17 in sensitive and immune cells

First, all of the effects due to the microcin were reversible in immune cells, but not in sensitive cells. For example, immune cells eventually recovered their ability to divide (see Fig. 1). Specifically, when microcin was removed from a culture of pop3351(pMM827) treated for 1 h with microcin (under conditions like those described in Fig. 2), 1 h incubation in microcin-free fresh medium was sufficient to recover almost completely the ability to synthesize DNA. The rate of DNA synthesis reached 70% of the value of the untreated control, whereas DNA synthesis in sensitive cells remained at an undetectable level (<1%). The reversibility of microcin B17 action is also illustrated in Fig. 3, where it is apparent that for full induction of *sfiA* in immune cells, continuous presence of the microcin was required. In contrast, in microcin-sensitive bacteria, short treatments with the same amount of microcin were more efficient for *sfiA* induction than long treatments (see Fig. 3). The results obtained with immune strains were similar to those found with nalidixic acid, a reversible inhibitor of DNA gyrase (Sugino *et al.*,

Fig. 3

Fig. 4

Fig. 3. Induction of *sfiA* expression with microcin B17 in sensitive and immune cells. Cultures of GC4415 and GC4415(pMM827) grown exponentially in supplemented M63 medium were harvested, washed and resuspended in fresh medium. Microcin B17 (200 AU ml⁻¹) was added to both cultures. After 5 min incubation, half of each culture was washed and resuspended in antibiotic-free medium. The remainder of each culture was maintained in the presence of microcin. At various times the β -galactosidase activity in each culture was determined. GC4415: continuous treatment (○); 5 min treatment (●). GC4415(pMM827): continuous treatment (△); 5 min treatment (▲). Dashed line: GC4415 and GC4415(pMM827) treated with non-microcin control extract.

Fig. 4. Microcin B17-induced DNA degradation. Exponentially growing cultures of RYC361 and RYC361(pMM827), prelabelled with [*methyl*-³H]thymidine, were washed and then treated with microcin B17 (200 AU ml⁻¹). Duplicate samples of 0.2 ml were taken at various times. TCA-soluble counts are shown for treated (●) and untreated (○) sensitive cells, and treated (▲) and untreated (△) immune cells.

1977); the continuous presence of this antibiotic was required to obtain an efficient *sfiA* induction (Herrero & Moreno, 1986).

Secondly, microcin B17 is not mutagenic in immune cells. Mutagenesis by UV and many chemicals is an inducible SOS function (Witkin, 1976). The enhancement of the spontaneous mutation frequency requires the products of the *umuC* and *umuD* genes, which form an operon repressed by the LexA product (Bagg *et al.*, 1981; Shinagawa *et al.*, 1983; Walker, 1984). In microcin-sensitive bacteria *umuC* induction was accompanied by an increase in the mutation rate of the *lac* and *gal* operons which was dose-dependent (Herrero & Moreno, 1986). In contrast, treatment of strain HfrH GalE⁻ harbouring pMM827, in conditions in which *umuC* was efficiently expressed (1 h with 200 AU ml⁻¹), failed to increase the mutation rate in these operons.

Thirdly, no significant DNA degradation was observed in immune cells, whereas the DNA of sensitive cells was extensively degraded in the presence of antibiotic (Fig. 4). Thus, immunity does not prevent the inhibition of DNA replication but does protect DNA from the degradation which follows inhibition of DNA replication.

The sfiA-lacZ fusion is expressed constitutively at low level in cells producing microcin B17

Exogenous microcin induces the SOS system in sensitive and immune cells. Hence, the expression of this system in cells producing microcin was also investigated. This was done by examining the expression of the *sfiA-lacZ* fusion in microcin B17 producers and non-producers. Strain GC4415(pMM827) (Mcc⁻ Imm⁺) had 25 units of β -galactosidase throughout exponential growth, a value indistinguishable from that of the plasmid-less strain. In contrast, GC4415(pMM833) (Mcc⁺ Imm⁺) had 50 units of β -galactosidase. Growth and viability of these

strains were not significantly different. The twofold increase in *sfhA-lacZ* expression was abolished when an *shmA* mutation (which confers resistance to microcin B17, M. Laviña, A. Pugsley & F. Moreno, unpublished) was introduced into the producing strain (RYC359).

DISCUSSION

The results presented here and in the accompanying paper (Herrero & Moreno, 1986) show that both sensitive and immune *E. coli* cells stop synthesizing DNA and induce several SOS functions when treated with microcin B17. For sensitive cells these effects were irreversible. In immune cells, these effects were reversible: treated bacteria were not killed, and eventually recovered the ability to synthesize DNA and resumed normal growth. Since an active replication fork is required for microcin induction of SOS functions and since inhibition of DNA synthesis after microcin treatment is immediate, we concluded that the primary effect of microcin B17 is to block the DNA elongation process (Herrero & Moreno, 1986). No significant differences in the inhibition of DNA synthesis and in the induction of several SOS functions in sensitive and immune cells was detected. Therefore, the immunity mechanism does not affect the primary action of microcin, but rather must act after this event to block the later events which lead to bacterial death. Cell death is most likely to be due to the DNA degradation that follows inhibition of DNA synthesis. Massive release of nucleotides from chromosomal DNA of sensitive bacteria was observed 30 min after treatment with microcin, whereas no DNA degradation was detected in immune cells.

In immune cells the extent and duration of the inhibition of DNA synthesis and the efficiency of the microcin-induced SOS response depended on the copy number of the immunity gene(s). Cells recovered from the microcin treatment more quickly when more copies of the immunity genes were present. Indeed the extent of the observed effects and the time taken to recover depended on the relative ratio of microcin to immunity product(s). How does immunity prevent DNA degradation and how does it make the block in DNA replication reversible? All our results are consistent with the notion that immunity neutralizes or removes microcin from the replication fork after it has inhibited DNA synthesis, but before DNA degradation starts. This implies that the interaction between the microcin and the replication machinery does not produce structural damage to the DNA but only blocks DNA elongation. In sensitive cells the block would be irreversible and would somehow initiate DNA degradation and cell death. In immune cells the block would be reversed by removal of the microcin from its site of action by the immunity protein(s).

The level of *sfhA* expression was twofold higher in microcin-producing strains than in strains carrying a *Mcc⁻Imm⁺* plasmid. This increase in the basal level of *sfhA* expression was not observed in cells mutated in the *shmA* locus. These mutants are completely resistant to exogenous microcin. It follows that SOS expression above the basal level in cells producing microcin is due to microcin produced, excreted and then internalized by the cells. A corollary of this is that within microcin-producing cells the synthesis of the antibiotic and the molecule(s) conferring immunity are balanced to avoid the inhibiting effects of the antibiotic.

Colicinogenic bacteria are immune to the lethal effect of the colicin that they produce (Konisky, 1982). However, the immunity to cognate colicin is not absolute and genetically immune cells are sensitive to very high concentrations of colicin (Frederick, 1958; Mock & Pugsley, 1982; Pugsley, 1984*b*). This phenomenon, known as immunity breakdown, has been analysed in detail only with colicin Ib. In this case, the biochemical effects which occurred during the immunity breakdown were similar to the effects found in sensitive cells exposed to low concentrations of colicin Ib (Levisohn *et al.*, 1968). But, in contrast to what occurred with microcin, colicin Ib immune cells were killed by high colicin concentrations. This difference is probably a consequence of the different mode of action of these molecules. Inhibition of DNA synthesis does not have to be lethal, provided the block is eventually removed. However, the formation of ion-permeable channels in the membrane, the mode of action of colicin Ia and Ib (Konisky, 1982), will be lethal if immunity is not sufficient to prevent channel formation or function. The mechanism of immunity to microcin B17 seems to be a novel one in the sense that

it does not neutralize the antibiotic agent before it reaches its site of action, but prevents the subsequent events that lead to cell death.

Microcin B17 is mutagenic in sensitive cells and this activity is *recA*-dependent (Herrero & Moreno, 1986). The *recA* and *umuC* gene products are required for SOS-induced mutagenesis (Walker *et al.*, 1982). These genes were efficiently induced in microcin-treated immune cells. Yet, microcin failed to increase the mutation frequency in RecA⁺ UmuC⁺ immune strains. This failure may be related to the lack of DNA degradation and killing in these strains and suggests that mutations in sensitive cells may be generated during the processing of pre-mutagenic lesions directly or indirectly caused by microcin. Most likely, this damage would be targeted around the replication forks blocked by the antibiotic.

The presence of wild-type alleles of *recB* and *recC* is required for SOS induction by treatments which block DNA elongation (Gudas & Pardee, 1976; Oishi & Smith, 1978; Oishi *et al.*, 1978). The RecBC product is also required for microcin to induce the SOS system in immune cells, as well as in sensitive cells. However, it has to be pointed out that our results with microcin B17 immune cells are not consistent with hypotheses which propose that the endonuclease activity of the RecBC protein is responsible for generating the signal which switches on the SOS response (Irbe & Oishi, 1980; Oishi & Smith, 1978; Oishi *et al.*, 1978). All the SOS genes and functions we tested were highly expressed, in spite of the fact that no DNA degradation could be detected in these cells. Similar results were obtained by Bockrath & Hanawalt (1980) who investigated the UV induction of SOS in *uvrB recB* mutants. It appears that, at least in these cases, another activity (not nuclease) of the RecBC product is required for RecA activation. On the basis of *in vitro* results, single-stranded DNA has been proposed to be part of the inducing signal *in vivo* (Roberts & Devoret, 1983; Roberts *et al.*, 1982). If RecA activation occurs when RecA protein binds to single-stranded DNA in the vicinity of the stalled replication fork, the role of the RecBC protein could be to facilitate this binding. Indeed, besides the nuclease activities of the RecBC protein, an unwinding and rewinding activity of this enzyme has been described (Taylor & Smith, 1980). This could be the activity required for SOS induction in the presence of microcin.

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