The virulence of *Enterobacter cloacae* and *Serratia marcescens* in experimental bladder infection in diabetic mice

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Summary. Diabetic mice were significantly more susceptible than normal mice to bladder infection with *Enterobacter cloacae* or *Serratia marcescens* but not to intraperitoneal infection. Normal urine inhibited the growth of *E. cloacae* and *S. marcescens*, whereas urine from diabetic mice permitted multiplication. The addition of urea to urine from diabetic mice restored its antibacterial properties for *E. cloacae* and *S. marcescens*. We consider that the decreased urea content of the urine of diabetic mice was responsible for their increased susceptibility to bacterial infection of the bladder.

Introduction

Patients with diabetes mellitus have an increased susceptibility to infectious diseases. In such patients, the three most common sites of infection are the lung, skin and urinary tract (Forland *et al.*, 1977). Furthermore, studies in rabbits, rats and mice with chemically induced diabetes have sometimes, though not always (Cruickshank, 1954), shown increased susceptibility to various bacterial and fungal infections (Raffel *et al.*, 1981; Edwards and Fuselier, 1983; Obana *et al.*, 1987).

The present investigation was undertaken to determine the effects of alloxan-induced diabetes on experimental bladder infections in mice due to *Enterobacter cloacae* and *Serratia marcescens*, bacterial species frequently isolated from immunocompromised patients, and to compare the antimicrobial properties of urine obtained from normal and diabetic mice.

Materials and methods

Bacteria

Seven strains each of *E. cloacae* and *S. marcescens* were used. *E. cloacae* strain 113 and *S. marcescens* strain K-3 were isolated from the urine of patients with urinary tract infections. The source of the other strains was unknown. Cultures were maintained on Nutrient Agar slopes (Nissui Pharmaceutical Co., Ltd) at room temperature.

Animals

Std-ddY female mice weighing 20–22 g were obtained from the Shizuoka Agricultural Cooperative Association for Laboratory Animals.

Induction of diabetes

The method of producing diabetes has been described in detail elsewhere (Obana *et al.*, 1985b). Four days after the intraperitoneal injection of alloxan monohydrate (Nakarai Chemicals, Ltd) at a dose of 250 mg/kg body weight, urine from each animal was examined for glucose by means of N-Multistic[®]SG (Miles-Sankyo Co., Ltd). Animals with glycosuria (glucose > 10.0 mg/ml) were used for the study.

Intraperitoneal infection

The test bacteria were cultured in Nutrient Broth (Nissui Pharmaceutical Co., Ltd) at 37°C for 18 h. Serial 10-fold dilutions of this culture were mixed with an equal volume of 6% hog gastric mucin (Nakarai Chemicals, Ltd); each dilution was used to inoculate 10 mice intraperitoneally (dose 0.5 ml). Control mice received similar inocula in which mucin was replaced by sterile diluent. The final mortality was recorded 3 days after inoculation.

Bladder infection

Normal and diabetic mice whose water supply had been restricted for a period of 3 h were given bladder infections with *E. cloacae* strain 113 or *S. marcescens* strain K-3 by urethral inoculation, in a dose volume of

0.05 ml (see Obana *et al.*, 1985a). The mice were killed 24 h after inoculation and their bladders were removed aseptically. Each bladder with its contents was homogenised in sterile physiological saline, and dilutions were plated to obtain colony counts.

Collection of urine and determination of its antimicrobial activity

Groups of 10 mice each were forced to void urine by gentle compression of the bladder through the external abdominal wall. In each group the urine samples were pooled and sterilised by filtration through a 0.45- μ m filter. Sterilised urine samples not used in experiments on the same day were stored at -20°C . The urea nitrogen content of pooled urine from groups of normal and diabetic mice was measured by the urease-indophenol method (Okuda *et al.*, 1981). Growth of the bacterial strains in the urine samples was then studied. Nutrient broth was seeded from a stock culture and incubated at 37°C for 18 h. For each test $c. 10^6$ organisms, contained in 0.1 ml of an appropriate saline dilution of broth culture, were inoculated into 0.9 ml of urine preheated to 37°C . Immediately, and again after incubation for 2 and 4 h at 37°C , a sample was removed from the tube to determine the viable count.

Effect of urea on the growth of bacteria

The effect of different concentrations of urea on the growth of *E. cloacae* and *S. marcescens* was examined in nutrient broth adjusted to pH 6.5. Nutrient broth seeded with stock culture was incubated at 37°C for 18 h and then diluted in nutrient broth, pH 6.5, to a concentration of $c. 10^5$ organisms/ml. The diluted culture was transferred in 4.5-ml volumes to test tubes, which were then placed in a water bath (37°C) before the addition of 0.5-

ml volumes of medium containing 10 times the required final concentration of urea. Immediately, and again after incubation for 2 and 4 h at 37°C , a sample was removed from each tube to determine the viable count.

Results

Intraperitoneal infection

Normal and diabetic mice did not differ significantly in their susceptibility to intraperitoneal infection with *E. cloacae* strain 113 or *S. marcescens* strain K-3 (table I).

Bladder infection

Table II shows that diabetic mice were strikingly more susceptible than normal mice to experimental infection of the bladder with *E. cloacae* strain 113 and *S. marcescens* strain K-3.

Antibacterial activity of urine and urea

Table III shows that seven strains each of *E. cloacae* and *S. marcescens* invariably gave higher viable counts after incubation in the urine of diabetic mice (pH 6.2; urea nitrogen 14.9 mg/ml) than in normal urine (pH 6.5; urea nitrogen 37 mg/ml). Table IV shows that the antibacterial properties of normal urine could be conferred on urine from diabetic mice by the addition of urea. Similar results were obtained by the addition of urea to nutrient broth (table V). Furthermore, the addition of glucose 20 mg/ml to normal urine to simulate

Table I. Virulence of *Enterobacter cloacae* 113 and *Serratia marcescens* K-3 for normal and diabetic mice by the intraperitoneal route

Organism	Dose of viable organisms ($\log_{10}$)/mouse	Mortality (%) produced by intraperitoneal infection			
		with mucin, in groups of 10		without mucin, in groups of 10	
		normal mice	diabetic mice	normal mice	diabetic mice
<i>E. cloacae</i> 113	7.54	100	100	0	10
	6.54	100	80	0	0
	5.54	60	50	0	0
	4.54	0	0	ND	ND
<i>S. marcescens</i> K-3	7.70	70	80	0	0
	6.70	20	10	0	0
	5.70	0	0	ND	ND
	4.70	0	0	ND	ND

ND = not done.

Table II. Bacterial counts in the bladders of normal and diabetic mice inoculated urethrally with *E. cloacae* 113 and *S. marcescens* K-3

Organism	Dose of viable organisms (log ₁₀)/mouse	Counts (log ₁₀)* of bacteria, 24 h after infection, in the bladders of		
		normal mice	diabetic mice	p value†
<i>E. cloacae</i> 113	5.70	4.00 (0.32)	7.27 (0.47)	<0.01
	4.70	3.00 (0.42)	6.67 (0.31)	<0.01
	3.70	1.07 (0.94)	5.67 (1.30)	<0.01
	2.70	1.25 (1.26)	4.47 (2.23)	≤0.05
<i>S. marcescens</i> K-3	5.60	3.27 (0.89)	6.73 (0.50)	<0.01
	4.60	2.79 (1.21)	6.43 (0.25)	<0.01
	3.60	2.08 (1.16)	5.24 (1.56)	<0.05
	2.60	0	3.95 (1.77)	

*Each value represents the mean (SD) of 5 mice.

†Student's *t* test.

glycosuria did not appear to enhance multiplication of the bacterial strains tested (table IV).

Discussion

Clinicians have noted differences in the response of diabetic patients to opportunist infections, but there are few relevant laboratory studies. The present study showed that alloxan-induced diabetic mice were strikingly more susceptible than normal mice to bladder infection with *E. cloacae* or *S.*

marcescens but not to intraperitoneal infection. This suggests that, for these two organisms, the virulence factors necessary for bladder infection differ from those for intraperitoneal infection.

The growth of bacteria in mouse urine is probably an essential preliminary to bladder infection. It has commonly been stated that urine is an excellent culture medium for the bacteria that cause urinary tract infection (Cox and Hinman, 1961), but Davis and Hain (1918) demonstrated that dog urine was bactericidal for gram-negative bacteria. Our studies

Table III. Growth of *E. cloacae* and *S. marcescens* in urine from normal and diabetic mice

Organism	Strain	Bacterial counts (log ₁₀ /ml) in urine before incubation	Bacterial counts (log ₁₀ /ml) in the urine of			
			normal mice, after incubation for		diabetic mice, after incubation for	
			2 h	4 h	2 h	4 h
<i>E. cloacae</i>	106	5.70	6.24	6.74	7.00	8.36
	108	5.83	6.30	6.57	7.24	8.40
	110	5.72	6.20	6.52	6.89	8.48
	112	5.52	5.52	5.75	6.60	7.40
	113	5.74	6.24	6.72	7.00	8.42
	114	5.74	6.00	6.32	7.00	8.18
	718	5.86	6.20	6.71	7.24	8.51
<i>S. marcescens</i>	K-3	5.52	5.67	5.67	6.00	7.17
	K-4	5.18	5.00	4.49	6.00	6.51
	K-16	5.18	5.00	4.54	5.89	6.44
	T-3	5.18	5.20	5.44	6.24	7.23
	T-8	5.48	5.30	5.23	6.00	6.74
	T-11	5.62	5.62	5.45	6.20	7.28
	T-55	5.79	5.90	5.90	6.30	7.70

Table IV. Growth of *E. cloacae* 113 and *S. marcescens* K-3 in urine from normal mice, and in that from diabetic mice with and without the addition of urea

Urine	Urea added	Bacterial counts (log ₁₀ /ml) after the stated periods of incubation in urine seeded with			
		<i>E. cloacae</i> 113*		<i>S. marcescens</i> K-3†	
		2 h	4 h	2 h	4 h
From diabetic mice	None	7.10	8.41	5.84	6.93
	25 mg/ml	6.50	7.39	5.15	5.34
	50 mg/ml	5.70	5.63	4.89	4.32
Normal	None	6.15	6.71	5.20	5.34
Normal, with added glucose (20 mg/ml)	None	6.20	6.87	5.30	5.54

Bacterial counts (log₁₀/ml) before incubation = *5.63 and †5.08.

Table V. Growth of *E. cloacae* 113 and *S. marcescens* K-3 in nutrient broth, with and without the addition of different concentration of urea

Urea concentration (mg/ml)	Bacterial counts (log ₁₀ /ml) after the stated periods of incubation in broth seeded with			
	<i>E. cloacae</i> 113*		<i>S. marcescens</i> K-3†	
	2 h	4 h	2 h	4 h
0	6.30	7.80	6.24	7.82
10	6.25	7.73	6.15	7.39
30	6.00	7.37	5.80	6.57
50	5.70	6.26	5.00	4.85

Bacterial counts (log₁₀/ml) before incubation = *5.26 and †5.41.

have shown that normal mouse urine possessed bacteriostatic properties for *S. marcescens* and *E. cloacae* but that urine from diabetic mice supported bacterial growth. The addition of glucose 20 mg/ml to normal urine to simulate the glycosuria of diabetes did not affect bacterial growth appreciably. Moreover, normal urine and urine from diabetic mice differed little in pH. It seemed possible that the difference between the antibacterial properties of the two types of urine might be related to urea

nitrogen content. Kaye (1968) reported that the antibacterial activity of urine was related to urea concentration, and to a lesser degree to osmolality and ammonia concentration. Neter and Clark (1944) demonstrated that urea added to urine in concentrations ranging from 3.1 to 6.3 mg/ml greatly increased antibacterial activity, and Hadas *et al.* (1977) reported similar findings. Jackson and Griebel (1957) found, however, that the addition of urea to urine in a concentration of only 0.5 mg/ml did not inhibit multiplication of bacteria. In the present experiments the addition of urea to urine from diabetic mice rendered it partly bacteriostatic or bactericidal to *E. cloacae* and *S. marcescens*. The intensity of the effect increased with the concentration of urea. The decreased antimicrobial activity of urine in diabetic mice may result from the dilution of inhibitory substances such as urea by diuresis; it is well known, however, that the decrease of urea nitrogen content in human diabetic patients with ketoacidosis is due to the increase of ammonia concentration.

Possibly the susceptibility of diabetic mice to bladder infection is due to a combination of low antibacterial activity of the urine, a depression of the hosts' defences, and to a recently demonstrated (unpublished) increase in the adhesive capacity of bladder epithelial cells, but further studies are needed.

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