BACTERIAL PATHOGENICITY

Role of type-1 fimbriae in the pathogenesis of chronic pyelonephritis in relation to reactive oxygen species

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The role of type-1 fimbriae in the pathogenesis of chronic pyelonephritis was studied for two *Escherichia coli* strains. Although both strains produced a similar total oxidative burst of chemiluminescence in macrophages from uninfected mice, the extracellular oxidative burst was greater with the non-fimbriate mutant *E. coli* BH-5 than its type-1 fimbriate parent *E. coli* 31-B. Moreover, macrophages from mice infected with the non-fimbriate mutant gave a much greater oxidative burst when stimulated with latex particles than that given by macrophages from mice infected with the type-1 fimbriate parent. These results correlated with the degree of renal inflammation and scarring as measured by malondialdehyde formation. Hence, the role of type-1 fimbriae in the pathogenesis of chronic UTI although documented does not appear to be significant.

Introduction

Urinary tract infections (UTI) range in severity from acute symptomatic pyelonephritis to chronic pyelonephritis and have the potential to lead to renal failure and death [1]. Re-infection with a different strain (80%) or relapse with the same organism (20%) may lead to recurrent or chronic UTI even after treatment [2]. Despite the high prevalence of the infection and years of study by many investigators, all the factors involved in the pathogenesis of chronic pyelonephritis have not yet been identified.

Escherichia coli strains that cause UTI usually belong to a restricted number of O- and K-antigen serogroups. They often possess special virulence properties such as resistance to serum bactericidal activity, production of haemolysin, and the capacity to adhere to epithelial cells from the urinary tract and the peri-urethral area [3]. However, many of these factors are absent in strains isolated from patients who develop chronic pyelonephritis or renal scars. The capacity of type-1 fimbriate bacteria to activate polymorphonuclear leucocytes (PMNL) *in vitro* and their capacity to cause scarring *in vivo* has been found to be related [4].

Renal scarring has been shown to be associated with increased PMNL infiltration [5]. It has also been observed that type-1 fimbriate strains of *E. coli* bind more readily to PMNL than non-fimbriate or P-fimbriate strains [6]. Strains of *E. coli* expressing type-1 fimbriae can cause significant release of lysosomal neutral protease from human neutrophils [7]. The present study was undertaken to confirm the role of type-1 fimbriae in the pathogenesis of chronic pyelonephritis in relation to reactive oxygen species (ROS) in an ascending, non-obstructive mouse model.

Materials and methods

Bacterial strains

Two strains of *E. coli* were used to examine the role of type-1 fimbriae in the pathogenesis of chronic pyelonephritis: *E. coli* 31-B, a uropathogenic, nalidixic acid-resistant strain derived from TN 675 that produced type-1 fimbriae and *E. coli* BH-5 a mutant of *E. coli* 31-B defective in its ability to produce type-1 fimbriae. Both the strains were kindly supplied by Takeda Chemical Industries Ltd, Japan.

Bacterial cultivation

The *E. coli* strains were grown in brain heart infusion (BHI) broth at 37°C for 18 h without agitation. The bacteria were harvested by centrifugation at 10 000 *g* for 10 min, washed in phosphate-buffered saline (PBS,

pH 7.2) and resuspended in PBS. To confirm the expression of type-1 fimbriae by *E. coli* 31-B, an agglutination test was performed with guinea-pig RBCs. Equal volumes (20 μ l) of bacterial suspension (5×10^9 cfu/ml of PBS) and guinea-pig erythrocytes (2% v/v) were mixed on a microscope slide at room temperature with gentle shaking. Agglutination was observed within 5 min. Agglutination was inhibited when the bacterial suspension was prepared in PBS containing D-mannose 1.5% w/v.

Animal model for experimental pyelonephritis

Female BALB/c mice 16–24 weeks old and weighing 20–30 g, from the Central Animal House, Panjab University, Chandigarh, India were fed on a standard antibiotic-free synthetic feed (JDB Agencies Pvt Ltd, India). Those without bacteriuria, as confirmed by urine culture, were selected for the study. Chronic pyelonephritis was induced by the method of Gupta *et al.* [8]. An inoculum of 0.1 ml of the cell suspension (3×10^9 cfu/ml) was injected slowly into the bladder of an anaesthetised mouse. To promote persistent chronic infection, mice were re-infected in the same way 2 weeks later. To establish renal scarring, i.e., an endpoint in chronic pyelonephritis, the process was repeated at 16 weeks after the first injection. The animals were killed by cervical dislocation in groups of six at 4, 8, 12, 16 and 20 weeks after the first infection. The kidneys were removed for biochemical investigations. Blood was cultured from all mice to exclude bacteraemia.

Isolation of peritoneal macrophages

Peritoneal macrophages were isolated from the mice of different groups by the method of Bjornson and Michael [9]. Mice were anaesthetised by ether and the peritoneal cavity was exposed without disrupting any blood vessels. Minimal essential medium (MEM) (8–10 ml) was injected into the peritoneal cavity and the abdomen was massaged for 1–2 min. The peritoneal lavage was removed to a sterile glass petri dish and placed for 1 h in a CO₂ 5% incubator at 30°C. After 1 h the supernate was removed, the thin layer of cells sticking to the glass was suspended in Hanks's balanced salts solution (HBSS) and the count was adjusted to 10^6 cells/ml. The purity of the cell population was established by esterase staining [10]: >95% of cells showed esterase activity and were confirmed as macrophages.

Chemiluminescence response

The chemiluminescence response was measured [11] in a Berthold Luminometer (Biolument LB 9500C). A polyethylene cuvette containing 1 ml of peritoneal macrophages suspended in HBSS (10^6 cells/ml) was allowed to equilibrate for 5 min at 37°C. One min after the addition of 20 μ l of luminol (5 mg/ml in 0.1 N

NaOH; Sigma) and mixing, the amount of chemiluminescence was recorded. To activate the system either latex (20 μ l; Difco; 0.81 μ m in diameter) or bacterial suspension (100 μ l, 10^8 cells/ml) was added and light emission was recorded after every 10-s interval. Two different reaction mixtures were used to quantify intracellularly and extracellularly generated chemiluminescence [12]: that for the measurement of extracellularly generated chemiluminescence contained 1 mM sodium azide and 4 U of horseradish peroxidase (HRP; Sigma) whereas that for the measurement of intracellularly generated chemiluminescence contained 2000 U of catalase (Sigma).

Malondialdehyde (MDA) estimation

MDA was estimated by the method of Ohkawa *et al.* [13]. Kidney tissue homogenate (0.2 ml) (10% w/v in KCl 1.15%) was added to 0.9 N H₂SO₄ 4 ml, 1 ml of thiobarbituric acid (TBA, 0.67% w/v in 0.1 N NaOH) solution (Romali, India) and 0.2 ml of SDS (8.1% w/v Sigma) solution. The contents were mixed thoroughly in covered tubes and kept at 90°C for 1 h. After cooling, 5 ml of n-butanol were added to each tube. The tubes were vortex mixed thoroughly for 1–2 min and centrifuged at 500 g for 10 min. The upper organic layer was removed and its absorbance at 532 nm was read immediately. Blank (0.2 ml of KCl 1.15%) and standard (1,2,3,3-tetraethoxypropane, diluted 1 in 10 000 with n-butanol; Sigma) were also run simultaneously. Protein was estimated by the method of Lowry *et al.* [14].

Results

The MDA levels (mean of six mice) in mouse renal tissue homogenate at different time intervals during the course of the disease are shown in Fig. 1. The MDA levels showed a steady increase with time in the

Fig. 1. Malondialdehyde in mouse renal tissue homogenates at different time intervals: ■, controls; ▨, mice infected with *E. coli* 31-B; □, mice infected with *E. coli* BH-5.

infected animals and these were greater in those infected with the non-fimbriate strain BH-5 than the fimbriate parent 31-B ($p < 0.05$).

E. coli 31-B and *E. coli* BH-5 did not differ significantly ($p > 0.05$) in the total luminol-dependent chemiluminescence (LDCL) response they caused in normal murine peritoneal macrophages (Table 1). On the other hand, *E. coli* BH-5 elicited a significantly higher ($p < 0.05$) extracellular LDCL than *E. coli* 31-B, although their intracellular LDCL responses were not significantly different ($p > 0.05$). Moreover, the peak of intracellular chemiluminescence was reached very late in comparison to the peak of extracellular chemiluminescence (CL) for both organisms.

Table 2 shows the results of the LDCL response (cpm $\times 10^4/10^6$ macrophages) of murine peritoneal macrophages isolated at different stages of infection. At all stages, the macrophages from both the *E. coli* 31-B and *E. coli* BH-5 infected groups showed significantly higher peak CL ($p < 0.001$) elicited by latex particles than those from the control group of uninfected animals. The time for attainment of the LDCL peak was shorter in the infected groups than the control. Moreover, it was greater and faster with the *E. coli* BH-5 infected group than the *E. coli* 31-B infected group during the progression of disease.

Discussion

The clinical significance of type-1 fimbriae in human urinary tract infections is not very clear, but studies

have suggested that the adherence of some uropathogenic *E. coli* strains to uroepithelial cells is mediated by the type-1 fimbriae [3]. Hence they may be important for initiation of infection. In addition, it has been reported that type-1 fimbriate *E. coli* strains bind more readily to PMNLs than non-fimbriate strains and are capable of initiating the respiratory burst of human PMNLs leading to the generation of toxic oxygen species [7]. Topley *et al.* [4] have shown a positive correlation between the capacity of type-1 fimbriate organisms to activate PMNLs *in vitro* and their capacity to cause scarring *in vivo*.

The extent of lipid peroxidation by the formation of MDA has been used as an index of damage mediated by oxygen free radicals [15]. Excessive production of MDA in chronic renal failure patients as a consequence of pyelonephritis has been observed [16]. The present study demonstrated an increase in the MDA levels in the infected groups at all the stages of infection. This supports the concept of peroxidative damage in pyelonephritis through production of ROS by inflammatory cells. This phenomenon was also observed following ischaemic damage [17]. Vascular occlusion leading to ischaemia was reported to be one of the mediators of tissue injury in pyelonephritis [18]. Tubulointerstitial injury has also been found to correlate directly with the amount of liquids, particularly of unsaturated fatty acids of membrane phospholipids [19].

Free radical generation by LDCL of mouse peritoneal macrophages isolated at different stages of infection was further evaluated. There was a signifi-

Table 1. Chemiluminescence (CL) response of macrophages from uninfected normal mice, elicited by *E. coli* strains 31-B and BH-5

<i>E. coli</i> strain	Mean (SD) cpm $\times 10^4/10^7$ macrophages – CL response		
	Total	Extracellular	Intracellular
Group I: 31-B	11.568 SD 1.434 (16)	2.334 SD 0.362 (20)	10.076 SD 1.224 (58)
Group II: BH-5	13.923 SD 2.004* (16)	3.384 SD 0.294† (18)	10.812 SD 1.318* (54)

(), time (min) at which the peak CL was observed.

* $p > 0.05$; † $p < 0.05$ (p-value for Student's *t* test between groups I and II).

Table 2. Effect of bacterial strain on peak LDCL of peritoneal macrophages isolated at different stages of infection as triggered by latex particles

Test group	Mean (SD) cpm $\times 10^4/10^6$ macrophages – LDCL response – at				
	4 weeks	8 weeks	12 weeks	16 weeks	20 weeks
Group I (uninfected control)	0.382 SD 0.0492† (140)	0.3972 SD 0.0468†§ (140)	0.4068 SD 0.0354†§ (140)	0.3588 SD 0.0414†§ (140)	0.3786 SD 0.0546†§ (140)
Group II <i>E. coli</i> 31-B	1.4886 SD 0.2154* (100)	1.3296 SD 0.1986*§ (40)	1.5006 SD 0.2079*§ (10)	1.5972 SD 0.1872*§ (0)	1.7610 SD 0.2892*§ (20)
Group III <i>E. coli</i> BH-5	3.0810 SD 0.4122† (90)	10.1028 SD 1.2872†** (30)	17.7828 SD 2.0873† (10)	23.5296 SD 1.9278† (0)	25.9080 SD 1.1538† (0)

(), Time (s) at which the peak LDCL response was observed.

p-values for Student's *t* test: (i) group I versus II, * $p < 0.001$; (ii) group II versus III, † $p < 0.001$; (iii) group I versus III, ‡ $p < 0.001$; (iv) compared to intermediate previous time interval of same group, § $p > 0.05$, || $p < 0.01$, ** $p < 0.001$.

cant progressive increase in ROS generation by peritoneal macrophages of infected groups with increasing post-infection time. As the amount of ROS produced increased significantly with increasing days of post-infection period and the time for attainment of the peak response decreased during the course of infection, there would be a gradual build up of phagocytic activity that reached its peak 5 months after infection. It was observed that phagocytosis during pyelonephritis was accompanied by lethal damage to both the phagocytes and surrounding tubules [20]. The present study found that the LDCL response was higher at all the stages of infection with the non-fimbriate mutant *E. coli* BH-5 than *E. coli* 31-B. The present observations support previous work, which showed that *E. coli* BH-5 was able to induce more histopathological damage to the kidney tissue and hence scarring [8] than *E. coli* 31-B. Moreover, the extracellular release of ROS was significantly higher ($p < 0.05$) with *E. coli* BH-5 than 31-B. The present data support the findings of Mundi *et al.* [12] that extracellular release of ROS from human phagocytes upon interaction with *E. coli* strains was the key to scarring, but contradicts other reports [4, 8] that type-1 fimbriae of *E. coli* play the most important role in this process and stimulate a unique pattern of degranulation by human PMNLs that may contribute significantly to their potential pathophysiological role in renal scarring.

Based on different parameters, it was observed that the changes were more pronounced in *E. coli* BH-5 than *E. coli* 31-B-infected animals. These results are in accord with May *et al.* [21], who reported that the type-1 fimbrial expression in *E. coli* decreases virulence in the rat peritonitis model. Iwahi and Imada [22] reported that the phagocytic resistance of *E. coli* BH-5 was not because of a decreased ability to attach to PMNLs but arose from an increased ability to resist the bactericidal process after bacterial attachment. If the bacteria can remain attached extracellularly and continue to trigger the release of oxidative metabolites and lysosomal enzymes without being ingested, they may potentiate the inflammatory process. Lock *et al.* [23] investigated the interaction between neutrophils and two different type-1 fimbriae-bearing *E. coli* strains and showed that although both adhered avidly to neutrophils, the strain with a rough LPS was ingested more effectively, whereas the other with a smooth LPS and K antigen resisted ingestion but induced a greater extracellular generation of ROS. *E. coli* BH-5, unable to express type-1 fimbriae, was more effective in the release of extracellular ROS than the fimbriate strain 31-B. Although the bacterial determinant of the extracellular release of oxidative metabolites in this strain (BH-5) remains to be identified, the present study establishes the key role of extracellularly generated ROS in chronic renal inflammation and scarring.

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