Urol Res (1995) 22: 399–402

ORIGINAL PAPER

F. Busolo · L. Francescon · F. Aragona · F. Pagano

Solid-phase binding of clinical isolates of *Escherichia coli* expressing different piliation phenotypes

Effect of glycosaminoglycans

Received: 10 May 1994 / Accepted: 30 September 1994

Abstract Pili (or fimbriae) are frequently present on most *Escherichia coli* strains and they mediate binding to specific receptors. In the present work we used type 1 and P-fimbriated *E. coli* strains isolated from patients with urinary tract infections to study the antiadhesive effect of some synthetic commercial glucosaminoglycans (GAGs). Quantitative determinations of tritiated bacteria associated with specific receptor-activated resins indicated that displacement by GAGs was more effective in the presence of nonspecific binding. Glucuronilglucosaminoglycan sulfate (Glu-g) and esosaminoglycan sulfate (Eso-g) at a concentration of 10 mg/ml were the most active substances against P-fimbriated *E. coli*, while galactosaminoglucuronglycan sulfate (Gal-g) behaved as an antiadhesive agent on type 1 fimbriated *E. coli* also at a lower concentration. The possible use of GAGs for the prophylaxis or treatment of chronic urinary tract infections implies a previous characterization of the piliation phenotype of the clinical isolates.

Key words Glycosaminoglycans · Pili · Receptor Adherence

The interaction between microorganisms and mucosal surfaces is mediated by pili (or fimbriae). Bacterial adherence to cell surfaces is considered the first step in the pathogenesis of infection. Of the various types of fimbriae of *Escherichia coli*, the most common is type 1. The fimbriae are involved in bacterial colonization through binding to host epithelial cells via a mannose-sensitive receptor. A similar mechanism regulates colonization of the urinary tract by P-fimbriated bacteria. P-fimbriae are present on most pyelonephritogenic *E. coli* strains and mediate the specific binding to α-D-Gal-(1–4)-α-D-Gal on the epithelial cell surface. Electrochemical charge plays an important role in bacterial adherence together with the glycosaminoglycan (GAG) layer produced by bladder epithelium, which exerts an antiadherence effects [12, 16]. Many studies have been devoted to understanding the role of surface mucin in the bladder primary antibacterial defense. In this respect, the ability of hydrophilic sulfonate GAGs (heparin) and analogs to replace normal mucin in making a barrier of water between the bacterium and the transitional cells has also been considered [14]. Early studies showed that removal of the natural mucin from the bladder surface of rabbits was associated with an increased rate of infection [13]. Recently, Ruggieri et al. [15] introduced the use of an anion exchange resin to study inhibition of bacterial adherence in vitro. In the present investigation, we adopted the above system to explore the activity of commercial GAGs as potential antiadherence agents. Additionally we assessed the inhibitory activity of the GAGs by the use of receptor-activated resins.

F. Busolo (✉) · L. Francescon
Institute of Microbiology, Faculty of Medicine, Padua University, Via A. Gabelli 63, I-35121 Padova, Italy

F. Aragona · F. Pagano
Department of Urology, Faculty of Medicine, Padua University, I-35121 Padova, Italy

Materials and methods

Bacteria and culture media

Escherichia coli strains were obtained from patients with urinary tract infections, identified by routine diagnostic tests and cultured on colonization factor agar (CFA) as described by Evans et al. [2]. Type 1 fimbriated strains were cultured in CFA supplemented with 0.25% urea according to Ofek and Maayan [11]. Afimbriated, type 1 and P-fimbriated *E. coli* strains were radiolabeled by growing them in CF (colonization factor) broth containing 10 μCi/ml [^{3}H]uridine (specific activity = 28–30 Ci/mmol, Amersham) giving an efficiency of 1 cpm (counts per minute) = 1100 ± 40 CFU (colony-forming units).

Electron microscopy

Escherichia coli strains were fixed with glutaraldehyde, collected with 0.5% Formovar-coated grids (300 mesh) that were subsequently platinum coated in an evaporator. The specimen was then observed using an Hitachi H59 electron microscope.

Characterization of pili

Type 1 pilus was identified by a glass slide agglutination test as previously described [1]. Briefly, bacterial cells suspended in phosphate-buffered saline (PBS) (pH 7.2) to an optical density of 0.4 using a wavelength of 540 nm were mixed with an equal volume (20 µl) of 4% guinea pig erythrocyte suspension. Type 1 fimbriated *E. coli* gave a positive agglutination reaction within seconds. Such a reaction was inhibited when the bacterial suspension was prepared in PBS containing 1% D-mannose. To assess for P receptor binding specificity, Gal-gal-coated latex beads (PF agglutination test; Orion, Finland) were used.

Resins

Three types of resins were used: AG1-X2 (Biorad, Calif., USA), an activated commercial 2% agarose and an activated Affi-gel Hz. AG1-X2 (Biorad, Calif., USA), as employed by Ruggieri et al. [15], is a strong anion exchange resin with quaternary ammonium functional groups attached to a styrene lattice. This resin is usually used to separate peptides and its mechanism of binding bacteria is probably not fimbria specific. Receptor-activated agarose resins were used for experiments of binding inhibition in the presence of carbohydrate receptor analogs. An activated commercial 2% agarose (Sigma, USA) to which α-methyl-D-mannoside was covalently bound has been used to measure the ability of sulfated GAGs to inhibit bacterial specific binding. Affi-gel Hz resin, an agarose support that reacts with the aldehydes of oxidized carbohydrates to form stable, covalent bonds, was activated by a two-step procedure:

1. Periodate oxidation of the P-fimbriated *E. coli* (4-O-α-D-galactopyranosyl-D-galactopyranose, Sigma, USA) receptor.

2. Coupling of oxidized disaccharide. The procedure followed was the same suggested for antibody coupling to Affi-Gel Hz resin by Bio-Rad Laboratories (Richmond, Calif.).

Glycosaminoglycans (GAGs)

In the experiments of inhibition of fimbriated or afimbriated bacteria adhesion to the resins, four substances were used: galactosaminoglucuronglycan sulfate (Gal-g) (IBSA, Switzerland), glucosamino sulfate (Glu) (Rottapharm, Monza, Italy), esosaminoglycan sulfate sodium salt (Eso-g) (Parke-Davis, Milan, Italy) and glucuronilglucosaminoglycan sulfate (Glu-g) (NCSN, Rome, Italy). These GAGs have a long polysaccharide chain of repeating disaccharide units with highly negative charges due to the presence of sulfate or carboxyl groups on many of the sugar residues.

Effect of glycosaminoglycans on the adherence of various fimbriated strains

The method originally proposed by Ruggieri et al. [15] with minor modifications was used: 0.2 ml radiolabeled *E. coli* strains, corresponding to 8×10^6 CFU, was added to a plastic tube (13 × 100 mm, Falcon, Becton Dickinson, N.J., USA) containing 0.8 ml previously hydrated and washed AG1-X2 resin in 1 ml 50 mM TRIS buffer, pH 7. After mixing foir 10 min at room temperature and after incubation for 10 min at 37 °C, 5 ml TRIS buffer was added, making the final volume up to 7 ml. The tube content was then filtered through 10-mm pore size Teflon LCWPO2500 filters (Millipore, Bedford, Mass., USA), which allowed free bacteria to pass through, but retained the resin. After rinsing the tube with 5 ml TRIS buffer, the resin was scraped off the filter and transferred to vials containing 6 ml instafluor II scintillation fluid (Packard, Downers Groce, Ill., USA) that was vortexed for 15 s. Radioactivity was measured by a liquid scintillation spectrophotometer (LKB 1214). As a control, the radioactivity associated with 0.2 ml suspension of radiolabeled bacteria was considered as 100% in each experiment. The percentage of bacteria bound to the resin was determined in triplicate by measuring the ratio of the radioactivity associated with the resin versus the total radioactivity. The inhibition by GAGs of bacterial attachment was measured by the above-mentioned procedure, replacing 1 ml 50 mM TRIS buffer with 1 ml of the tested compound solution.

Statistics

Percentages obtained were compared and subjected to statistical analysis using the chi-square test and are reported as significant if $P < 0.05$.

Results

Electron microscopy studies and agglutination tests allowed us to identify wild fimbriated or afimbriated *E. coli* strains to test for the antiadherence effect of GAGs. *E. coli* strains recognized as "afimbriated" were characterized by the lack of external organelles microscopically comparable to fimbriae and by the negativity of the slide agglutination test. Due to the existence of afimbrial adhesins [9], all of these findings are inadequate to exclude the potential adhesiveness of bacteria. In our study at least two strains for each piliation phenotype were employed. Irrespective of the presence or the absence of fimbriae, the AG1-X2 resin showed the higher binding ability, with 42–45% of bacteria adhering to the resin (Table 1). This percentage was markedly reduced when receptor-activated resin was employed. Regardless as to how low it might be, the percentage of specific binding to different receptor-activated resins is such as to allow differentiation between afimbriated, type 1 fimbriated and P fimbriated bacteria. Indeed a 3.2% binding was observed for type 1 bacteria and a 6.3% binding for P-fimbriated bacteria; in both cases P was < 0.05. The mean number of CFUs used for each experiment was high enough (1 476 200 CFU = 1342 cpm × 1100 CFU/cpm) to give

Table 1 Adherence to the resins of bacteria with different piliation phenotypes. Values represent means of duplicate determinations of at least three separate experiments with two strains for each piliation phenotype

Type of resin	*Escherichia coli*		
	Afimbriated	Type 1	P-fimbriated
AG1-X2	42% (±5.6)	45% (±6)	44% (±3.4)
α-Methyl mannoside-activated resin	0.3% (±0.04)	3.2% (±0.7)	1% (±0.09)
α-Gal-(1,4)-β-Gal-activated resin	0.9% (±0.05)	1.9% (±0.1)	6.3% (±0.8)

Table 2 Inhibition by GAGs of bacterial adherence to AG1-X2 resin. Values represent means of duplicate determinations of three separate experiments with two strains for each piliation phenotype. Standard errors have been omitted but they were similar to those reported in Table 1 (*Glu-g*, glucuronilglucosaminoglycan sulfate, *Eso-g*, esosaminoglycan sulfate sodium salt, *Gal-g*, galactosaminoglucuronglycan sulfate, *Glu*, glucosamino sulfate)

GAGs	mg/ml	*Escherichia coli*		
		Afimbriated	Type 1	P-fimbriated
Glu-g	1	0	0	0
	5	40 (±6.2)	36 (±2.7)	23 (±2.2)
	10	89 (±9.6)	77 (±11)	88 (±7.7)
Eso-g	1	19 (±3.8)	6 (±1.2)	7 (±0.8)
	5	59 (±4.8)	42 (±5.9)	42 (±4.6)
	10	83 (±8.8)	78 (±9.3)	71 (±7.3)
Gal-g	1	15 (±4.8)	3 (±0.2)	4 (±0.4)
	5	34 (±3.9)	34 (±6.3)	28 (±3.8)
	10	53 (±8.5)	47 (±8.5)	67 (±5.3)
Glu	1	23 (±3.5)	28 (±2.7)	25 (±4.6)
	5	40 (±5.9)	29 (±3.8)	23 (±2.7)
	10	45 (±6.2)	33 (±3.8)	24 (±3.8)

Table 3 Inhibition by GAGs of bacterial adherence to α-methyl D-mannoside-activated and α-Gal-(1,4)-β-Gal-activated resins. Values represent means of duplicate experiments of three separate experiments with two strains for each piliation phenotype. Standard errors have been omitted but they were similar to those reported in Fig. 1 (*Glu-g*, glucuronilglucosaminoglycan sulfate, *Eso-g*, esosaminoglycan sulfate sodium salt, *Gal-g*, galactosaminoglucuronglycan sulfate, *Glu*, glucosamino sulfate)

GAGs	mg/ml	*Escherichia coli*	
		Type 1	P-fimbriated
Glu-g	1	3 (±0.3)	0
	5	4 (±0.7)	33 (±2.2)
	10	21 (±3.2)	81 (±7.7)
Eso-g	1	0	0
	5	15 (±2.9)	12 (±4.6)
	10	15 (±2.3)	84 (±7.3)
Gal-g	1	26 (±3.2)	0
	5	21 (±4.3)	10 (±3.8)
	10	25 (±8.5)	29 (±5.3)
Glu	1	3 (±0.7)	0
	5	5 (±0.7)	0
	10	16 (±3.8)	0
Specific competitors[a] (1 mg/ml)		46 (±4.3)	49 (±9.1)

[a] At variance with the above-reported data, these data express the detachment rate of previously linked bacteria by competitors (α-methyl mannoside and α-methyl D-galactoside for type 1 and P-fimbriated strains, respectively)

comparable results. When labeled bacteria were added to the resins in the presence of GAGs (Table 2), the higher antiadhesive effect was produced by Glu-g and Eso-g at the concentration of 10 mg/ml, compared with Gal-g and Glu. Inhibition by Glu-g and Eso-g ranged from 71% to 89%. However, this inhibition cannot be ascribed to specific competition at the receptor level, as demonstrated by the comparable results which were obtained with several *E. coli* strains differing for piliation phenotype. A net charge difference must account for the reduced effect of Gal-g and Glu, which are more electronegative than Glu-g and Eso-g. When *E. coli* type 1 fimbriated and P-fimbriated isolates were assayed for binding to their respective receptor-activated resins and activity of inhibitors was measured (Table 3), the greater competition was produced by Glu-g and Eso-g (at 10 mg/ml). The above effect was exclusively towards P-fimbriated microorganisms to bind to their specific activated resin. For type 1 fimbriated strains, the higher bacterial antiadhesive effect was obtained with Gal-g, which showed similar activity at the various concentrations tested. Glu was poorly active or inactive. With regard to the specificity of the inhibitory action, Gluc-g and Eso-g gave a 18% inhibition of adhesion (mean value) of type 1 isolates to α-methyl mannoside activated resin compared with a 37% inhibition of adhesion of P-fimbriated *E. coli* strains to the same resin (data not shown).

To determine whether receptor-activated resin was promoting a specific bacterial binding, we employed 1 mg/ml receptor analog solution (α-methyl mannoside or α-methyl D-galactoside as competitors) to detach the bacteria already bound. 46% of type 1 and 49% of P-fimbriated bacteria were rapidly detached by washing resins with analog solutions (Table 3), but not with TRIS buffer. This is further evidence that the antiadhesive effect of GAGs was not based upon interference with a specific binding.

Discussion

Bacterial adherence is frequently associated with the expression of fimbriae [6, 18]. Type 1 and P-fimbriae are involved in tissue colonization by binding to host epithelial cells [8]. Several findings would suggest that this binding is mediated by adhesins through the recognition of specific receptors containing α-mannose or α-Gal-(1,4)-α-Gal. Adhesins are minor protein components present in type 1 [4, 10] and in P-fimbriae [5], which are electrophoretically distinct from fimbrial subunits. Notwithstanding the questions over the role for type 1 fimbriae as virulence factor [17], there is enough evidence that P-fimbriated *E. coli* strains are frequently involved in the etiopathogenesis of pyelonephritis [7]. Since adhesin-receptor interaction represents an early step of infection, many studies have been devoted to the investigation of this phenomenon, mainly exploiting in vitro agglutination reactions.

An alternative possibility is based on the method of Ruggieri et al. [15], which measures bacterial binding to a solid phase. This method, which was originally meant to provide a tool for assaying the antiadhesive effect of natural mucin, has the advantage of allowing a quantitative evaluation of bacterial adhesiveness.

The aim of our study was to evaluate a number of GAGs as antiadhesive agents by taking advantage of

receptor-activated resins, the use of which has not been previously proposed. Employment of receptor-activated resins was designed to assay the specificity of bacterial adhesion and interference by GAGs. This approach has made it possible to demonstrate that binding to AG1 X2 is not a specific phenomenon. Indeed, (a) a much larger number of bacteria bind to AG1 X2 resin than the receptor-activated resin, (b) this binding is independent of the piliation phenotype and (c) competition experiments indicate lack of discrimination by GAGs between afimbriated, type 1 and P-fimbriated isolates when AG1 X2 resin is employed. Hence, the above resin cannot be adopted to study bacterial adherence or to infer the possible use of these clinical compounds as antiadhesive agents. It is worth recalling that, because of the net negative charge of the external cell surface, bacteria can only adhere to epithelial cell membrane through specific contacts in spite of electrostatic repulsion. In this context, the use of AG1 X2 resin that contains quaternary ammonium groups as the charged species can only probe for the occurrence of an electrostatic interaction, i.e., the more negative the bacterial cell surface the higher the binding to the solid phase. It is therefore obvious that the more negative the polyelectrolyte, the greater potential it has as an antiadherence agent [3]. In relation to the in vivo situation, it seems that the use of receptor-activated resin with reference to the piliation phenotype can give information both at the level of bacterial adhesion properties and of the possible use of selective inhibitors thereof. The results of the present investigation show that Glu-g and Eso-g compete at a higher level with binding of bacteria to their receptor-specific activated resin only in the case of P-fimbriated isolates, but not with type 1 isolates. This could account for a closer resemblance of Glu-g and Eso-g to the α-Gal-(1,4)-α-Gal receptor than α-methyl mannoside receptor or to a higher density of electronegativity charge of type 1 fimbriae.

As for susceptibility to the various GAGs tested, our experiments indicate a different response according to piliation phenotype, with type 1 fimbriated isolates being more sensitive to Gal-g with respect to P-fimbriated isolates. In conclusion, GAGs may inhibit bacterial adhesion not specifically by mucus barrier replacement. Additionally, under those conditions where the mucin layer is unaltered and bacterial adhesion is still taking place they could also act by mimicking the natural bacterial adhesin receptor. It is noteworthy in this respect that Glu-g and Eso-g, at the concentration of 10 mg/ml, are more active against P-fimbriated *E. coli*, while Gal-g is active against type 1 fimbriated bacteria even at a low concentration. These data suggest that piliation phenotype characterization of the clinical isolates must be performed if an effective prophylaxis or treatment with selective GAGs is to be pursued.

References

1. Conventi L, Errico G, Mastroprimiano S, D'Elia R, Busolo F (1989) Characterization of *Escherichia coli* adhesins in patients with symptomatic urinary tract infections. Genitourin Med 65:183
2. Evans DG, Silver RP, Evans Jr DJ, Chase DG, Gorbach SL (1975) Plasmid-controlled colonization factor associated with virulence in *Escherichia coli* enterotoxigenic for humans. Infect Immun 12:656
3. Hanno PM, Ruggieri MR, Levin RM (1984) Comparison of *Escherichia coli* adherence to the urinary bladder mucosa with the adherence to anion exchange resin. Surg Forum 35:637
4. Hanson MS, Brinton CC Jr (1988) Identification and characterization of *Escherichia coli* type-1 pilus tip adhesion protein. Nature 332:265
5. Hoschutzky H, Lottspeich F, Jann K (1989) Isolation and characterization of the α-galactosyl-1,4-α-galactosyl specific adhesin (P adhesin) from fimbriated *Escherichia coli*. Infect Immun 57:76
6. Hulygren SJ, Lindberg F, Magusson G, Kihlberg J, Tennet JM, Normark S (1989) The PapG adhesin of uropathogenic *Escherichia coli* contains separate regions for receptor binding and for the incorporation into the pilus. Proc Natl Acad Sci USA 86:4357
7. Kaack MB, Roberts JA, Baskin G, Patterson GM (1988) Maternal immunization with P-fimbriae for the prevention of neonatal pyelonephritis. Infect Immun 56:1
8. Klemm P, Orskov I, Orkov F (1982) F7 and type-1 like fimbriae from three *Escherichia coli* strains isolated from urinary tract infections: protein, chemical and immunological aspects. Infect Immun 36:462
9. Labigne-Roussel AF, Lark D, Schoonlik G, Falcow S (1984) Cloning and expression of an afimbrial adhesin (AFA-I) responsible for P blood group-independent, mannose-resistant hemaglutination from a pyelonephritic *Escherichia coli* strain. Infect Immun 46:251
10. Linberg F, Lund B, Normark J (1986) Gene products specifying adhesion of uropathogenic *Escherichia coli* are minor components of pili. Proc Soc Natl Acad Sci USA 83:1891
11. Ofek I, Mayan M (1986) Urinary factors which influence population shift in the expression of mannose specific lectin by *K. pneumoniae*. In: Proceeding of the IVth International Symposium on pyelonephritis. Goteborg, Sweden: Kompandie Trickeriet-Kallered, vol 63
12. Parsons CL (1982) Prevention of urinary tract infection by the exogenous glycosaminoglycan sodium pentosanpolysulfate. J Urol 127:167
13. Parsons CL, Shrom SH, Hanno PM, Mulholland SG (1978) Bladder surface mucin. Examination of possible mechanisms for its antibacterial effect. Invest Urol 16:196
14. Parsons CL, Pollen JJ, Anwar H, Stauffer C, Schmidt JD (1980) Antibacterial activity of bladder mucin duplicated in the rabbit bladder by exogenous glycosaminoglycan (sodium pentosanpolysulfate). Infect Immun 27:876
15. Ruggieri MR, Hanno PM, Levin RM (1985) Further characterization adherence to urinary bladder mucosa: comparison with adherence to anion exchange resin. J Urol 134:1019
16. Ruggieri MR, Levin RM, Hanno PM, Witkowski BA, Gill HS, Steinhardt GE (1988) Defective antiadherence activity of bladder extracts from patients with recurrent urinary tract infection J Urol 140:157
17. Salit IE, Vavougios J, Hofmann T (1983) Isolation and characterization of *Escherichia coli* pili from a diverse clinical source. Infect Immun 42:755
18. Svanborg-Eden C, Hanson LA, Jodal U, Lindberg H, Sohl-Akerhardt A (1976) Variable adherence to normal human urinary tract epithelial cells of *Escherichia coli* strains associated with various forms of urinary tract infection. Lancet II:490