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Study of Several Factors Affecting the Agglutinating Activity of K99-Positive *Escherichia coli* Strains

C. M. Ferreirós and M. T. Criado

Departamento de Microbiología
Facultad de Farmacia y Medicina
Universidad de Santiago de Compostela
Santiago de Compostela (Spain)

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The effect of several factors on *Escherichia coli* K99-plasmid associated agglutination has been studied. The results obtained indicate that *Escherichia coli* 637 ($K99^+$) mediated red blood cell agglutination is unspecific although the agglutination titres for several erythrocyte species are significantly different.

The agglutination is highly stable (at least with sheep red blood cells) to changes in temperature (from 4°C to 37°C), to changes in pH (from 5 to 9) and to the presence or absence of several metallic cations.

Treatment of sheep erythrocytes with certain proteolytic enzymes (papain and trypsin) results in a increment in their agglutinability. Also, the extraction of galactose after the removal of sialic acid residues from the oligosaccharides present on the erythrocyte membranes results in a great increment in their agglutinability.

On the other hand, only thyroglobulin, mucin, fetuin, and the oligosaccharides extracted from the last two glycoproteins are able to inhibit K99-plasmid mediated sheep red blood cell agglutination.

At the moment, it is well known that certain bacterial surface proteins can act as binding bridges between pathogenic microorganisms and sugar residues present on the surface of a great variety of animal cells. The bacterial binding molecules are frequently identified with *pili* and related to lectins (sugar binding proteins).

In 1975, ORSKOV *et al.* (15) identified a bacterial surface adhesive antigen with proteic nature and resembling *pili* in enterotoxigenic *Escherichia coli* associated with calf and lamb diarrhoea. Initially named Kco, its actual designation is K99. $K99^+$ *E. coli* were recently found in many cases of diarrhoea in piglets. K99 antigen and its associated adhesin are encoded by a thermosensitive, transmissible plasmid.

We have recently purified the molecule responsible for the agglutinating properties associated to the antigen K99 antigen (6). In this paper we study several factors

affecting or modifying the agglutinating activity of K99-positive *E. coli* strains.

Materials and Methods

Escherichia coli 637 strain ($K99^+$), kindly donated by E. Gonzalez and J. Blanco (Departamento de Microbiología, Universidad de Santiago, Spain) was used throughout this study. All chemicals and enzymes were from Sigma and culture media were obtained from Oxoid Ltd. (England). Sheep red blood cells were supplied by Flow General (USA); human blood was obtained from the Hospital General de Galicia (Santiago, Spain), whereas that of the other animal species was obtained from the Municipal Slaughterhouse.

The strain *E. coli* 637 was cultured in tubes with 5 ml of MINCA medium (9) inoculating 10^4 bacteria/ml from a 18 h old preinoculum. Culture conditions were always 37°C for 24 h. After cultivation the bacteria were centrifuged at 8.000 per *g* for 15 min at 4°C, washed once in phosphate-buffered saline (PBS) and suspended at 10^{12} cells/ml in PBS.

Erythrocytes for the agglutination assays were prepared from whole blood by centrifugation over Ficoll-Hypaque (1). After washing several times in PBS, the erythrocytes were suspended to 1 % (v/v) in the same buffer. Agglutination assays were made in Microtiter plates by mixing 50 µl of serial twofold dilutions of the bacterial suspension with 50 µl of the erythrocyte suspensions. Agglutination was allowed to occur at room temperature (except when indicated) and titres were read two hours later.

The influence of metallic ions on the agglutination was assessed by adding 10 µl of $CaCl_2$, $MgCl_2$, $MnCl_2$ or $FeCl_3$ (0.1 M, pH 7.2) or 2 % EDTA to 100 µl of the bacterial suspensions before doing the serial dilutions and mixing with the erythrocytes.

To test the effect of the temperature on the agglutination reaction, the plates were allowed to stand at 4°C, 20°C, 30°C or 37°C for two hours after mixing the cell suspensions.

The effect of pH was assessed by mixing 1 ml aliquots of both bacterial and erythrocyte suspensions (in 0.14 M NaCl) with 50 µl of the following buffers before doing the agglutination experiment: a) Glycine-HCl 0.2 M, pH 3; b) Acetate buffer 0.2 M, pH 4 or 5; c) Phosphate buffer 0.2 M, pH 6, 7 or 8; d) Tris-HCl 0.2 M, pH 9; e) Carbonate buffer 0.2 M, pH 10.

Formaldehyde-treated erythrocytes were obtained according to HERBERT (10). Treatment with papain was performed as described by GILBOA-GARBER (8) whereas treatment with trypsin, pronase and glycosidases was done according to SALIT and GOTSCHLICH (16). Metaperiodate treatment was done as described by OFEK *et al.* (14).

Inhibition of bacterial hemagglutination was measured as described in a previous paper (7).

Results

The agglutination titres obtained with erythrocytes tested indicate that the agglutination mediated by the K99-positive strain *E. coli* 637 is unspecific but with

Table I. Escherichia coli *637 mediated agglutination of several erythrocytes species.* The bacteria were cultured for 18 h at 37° C in MINCA medium and suspended in PBS (10^{12} cells/ml). Serial dilutions of the suspension were mixed (vol/vol) with 1 % erythrocytes in PBS in Microtiter plates.

Erythrocytes	Titre
Sheep	256
Guinea-pig	64
Calf	4
Human (A group)	2048
Human (0 group)	2048

Table II. *Effect of several factors (pH, temperature and ions) on SRC agglutinability.* Temperature effect was assessed by allowing the bacteria-erythrocyte mixtures to interact at different temperatures for 2 h. The influence of pH was measured by suspending the cells in different buffers (see text). The effect of ions was tested by including them in the PBS.

Factor		Titre
Temperature	4° C	256
	20° C	256
	30° C	256
	37° C	256
pH	3	(lysis)
	4	(lysis)
	5	256
	6-8	512
	9	256
	control	256
Ion	Ca	256
	Mg	256
	Mn	256
	Fe	256
	EDTA	256
	control	256

significant variations in the titres with the different erythrocytes (table I). These titres were from 4 with calf erythrocytes to 2048 with human ones. For the subsequent experiments sheep erythrocytes (SRC) were used throughout (titres 256).

SRC titres remain unchanged when the plates are allowed to stand at temperatures between 4°C and 37°C or when varying the pH of the agglutination buffer (table II). Also, SRC agglutination is not affected by the presence of several ions or EDTA. Variations in agglutination titres with pH are without signification.

Table III. Escherichia coli *637 mediated agglutination of SRC treated with proteolytic enzymes and other agents.*
Erythrocyte treatments were made as previously described (8, 16) and agglutination was done as above.

Treatment	Titre
None	256
Papain	2048
Trypsin	1024
Pronase	256
Metaperiodate	256
Formaldehyde	0

Table IV. Escherichia coli *637 mediated agglutination of SRC treated with various glycosidases.*
The erythrocytes were treated as described by SALIT and GOTSCHLICH (16) and the agglutinations were made by mixing equal volumes of the bacterial suspension (10^{13} cells/ml) and 1 % erythrocytes in PBS.

Treatment	Titre
N	256
N + G	512
N + M	2048
N + Gl	512
N + G + M	512
N + G + Gl	2048
N + G + M + Gl	2048
N + G + Gl + M	2048

N: neuraminidase; G: galactosidase; M: α-mannosidase; Gl: N-acetyl-glucosaminidase.

Table III shows the titres obtained after treatment of SRC with proteolytic enzymes and other chemicals. Papain and trypsin produce an increment in the SRC agglutinability. Metaperiodate does not affect the agglutination, whereas formaldehyde-treated SRC are not agglutinated.

Results obtained when the SRC are treated with different glycosidases, individually or sequentially, show that the extraction of galactose after removal of sialic acid produces an eigh-fold increment in agglutinability (table IV). Posterior extraction of mannose and or N-acetyl-glucosamine does not affect the agglutinability of sialic acid-galactose deprived SRC.

None of the simple sugars tested was able to inhibit the *E. coli* 637-mediated SRC agglutination. Only the glycoproteins thyroglobulin, mucin and fetuin or the oligosaccharides obtained from the

Table V. Escherichia coli *637 mediated SRC agglutination inhibition by sugars and glycoconjugates.*
Bacterial suspensions were mixed with serial dilutions of the potential inhibitors for 2 h before the addition of the SRC.

Inhibitor	Titre	Inhibitor	Titre
NANA	—	Melibiose	—
Galactose	—	Lactose	—
N-acetyl-glucosamine	—	Sorbose	—
Mannose	—	Dulcitol	—
L-Fucose	—	p-NP-Galactose	—
Glucose	—	Glucose-6-P	—
Xylose	—	Glucuronic acid	—
Arabinose	—	p-NP-Glucuronic acid	—
α-methyl-mannoside	—	Polygalacturonic acid	—
Rhamnose	—	Thyroglobulin	64
N-acetyl-galactosamine	—	Fetuin	2
Trehalose	—	Mucin	8
Fructose	—	Gangliosides (type III)	—
Raffinose	—	Fetuin oligosaccharides	+
Mucin oligosaccharides	+		

last ones were able to produce inhibition (table V).

Discussion

The most frequently used method to assess bacteria-mediated agglutination consists in the mixing of bacterial and erythrocyte suspensions on a microscopic slide (3, 4). This method is only qualitative and provides very little information about the agglutination. The use of serial dilutions of the bacterial suspension in Microtiter plates permits to give the titre values, which are an indication of the degree of specificity of the bacteria to the erythrocytes.

The K99 associated adhesin shows higher agglutination titres (is therefore more specific) with human than with calf erythrocytes (512-fold higher titres). This higher specificity can be due to the presence of higher numbers of receptors for the adhesin, because the formation of binding bridges between the erythrocytes is easier. This hypothesis considers the binding sites in the receptors of all erythrocyte types to be identical. Alternatively, a second hypothesis considers the binding sites to be different in each type of erythrocyte and with different affinity to the adhesin.

Those cells with higher affinity can be agglutinated in the presence of lower adhesin concentration because the bridges between the cells are stronger than in the erythrocytes with lower affinity.

Lastly, and on the basis of the membrane model proposed by LOOR (11) it can be postulated that the receptors implicated in the erythrocytes agglutination could be located in different membrane domains in different erythrocytes. This implicates differences in receptor mobility and in the formation of zones with high density of receptors which determine the density of intercellular binding bridges and therefore the agglutinability. It is possible that more than one of the postulated hypotheses determine the erythrocyte agglutinability.

SRC-agglutination produced by the K99 antigen associated adhesin in the strain *E. coli* 637 appears to be stable within a pH range from 5 to 9 and a tem-

perature range from 4°C to 37°C. Our results on temperature stability do not agree with those of BURROWS *et al.* (2) who found that the K99 associated agglutination decreases or even disappears when te incubation is done at 37°C. The reason for this difference could be in the probable existence of several types of K99 plasmids (13) which could encode for different adhesins.

For many active proteins (especially enzymes and lectins) to be functional the presence of certain metallic ions was found to be essential. Apparently, those ions help to maintain the stability of proteins or their active sites. In our case, none of the ions tested had any effect on the agglutinating ability of the K99-positive *E. coli* strain.

The results obtained in agglutination experiments carried out with SRC treated with proteolytic enzymes indicate that the adhesin receptor is not a peripheral membrane protein (the enzyme treatment is weak and does not allow the extraction of structural membrane proteins).

The increment in SRC agglutinability after treatment with papain or trypsin suggests that the adhesin receptors might be masked by peripheral proteins so that after their removal, the receptors can be more accessible for the adhesin. On the other hand, the enzymes could be supposed to act inside the membrane on the microtubules and microfilaments, rendering the membrane components free to move and form patches or clusters which increase the SRC agglutinability. This hypothesis agrees with the lack of agglutinability observed in formaldehyde-fixed SRC.

FARIS *et al.* (5) suggested that the most probable K99 receptor seems to be a glycoconjugate similar to GM-2 gangliosides, in which the sialic acid residues play a main role (their extraction from human erythrocytes renders them non-agglutinable). Nevertheless, they were unable to demonstrate the same effect with sheep erythrocytes. They further showed that human red cell agglutination mediated by K99 is inhibited by GM-2 (but not by GM-3) gangliosides. On the other hand, MORRIS *et al.* (12) found that N-acetylglucosamine inhibits the SRC agglutination by purified K99, suggesting that this sugar is a component of the K99 molecule which is recognized by a certain structure on the red cell surface.

Our results on sugar inhibition do not allow us to confirm these data since we only found inhibition by thyroglobulin, fetuin and mucin. GM-3 gangliosides did not inhibit the agglutination, results which agree with the data presented by FARIS *et al.* (5). The results on the agglutination of glycosidase-treated SRC suggest that, contrary to the results from these authors, neuraminic acid is not implicated in K99-mediated SRC agglutination (it seems that neuraminic acid extraction produces an increment in the agglutinability). Subsequent treatment with galactosidase, but not with mannosidase or glucosaminidase, produces a marked increase in SRC agglutinability. This can only be explained by postulating that both neuraminic acid and galactose, located in terminal positions in the oligosaccharide chains, interfere with the access of the K99-associated adhesin to its binding site in the receptor.

The non alteration of the SRC agglutinability after treatment with mannosidase, glucosaminidase or both (after removal of neuraminic acid and galactose) indicates that both mannose and N-acetyl-glucosamine are not implicated in the adhesin binding-site, at least when located in positions immediately vicinal to the terminal neuraminic acid and galactose residues.

Our results do not disagree with the receptor proposed by FARIS *et al.* (5) although the unalteration of the SRC agglutinability after treatment with sodium metaperiodate, a reagent that cleaves the bonds between vicinal carbon atoms with

free hydroxyl groups, characteristics of sugar molecules (14) should be borne in mind.

Resumen

Se estudia el efecto de diversos factores en la aglutinación asociada al plásmido K99 de *Escherichia coli*. Los resultados obtenidos indican que la aglutinación de glóbulos rojos asociada a la cepa *E. coli* 637 ($K99^+$) es inespecífica aunque los títulos son significativamente diferentes según las especies de eritrocitos.

En eritrocitos de carnero, la aglutinación es altamente estable a los cambios de temperatura en un rango entre 4°C y 37°C, a los cambios de pH (entre 5 y 9), y a la presencia o ausencia de distintos iones metálicos.

El tratamiento de los glóbulos rojos de carnero con ciertos enzimas proteolíticos (papaína y tripsina) da lugar a un incremento de su aglutinabilidad. Además, la extracción de galactosa, después de extraer los resíduos de ácido siálico de los oligosacáridos de la membrana de los eritrocitos origina un fuerte incremento de la aglutinabilidad de los mismos.

Solamente la tiroglobulina, mucina, fetuína y los oligosacáridos extraídos de estas dos últimas glicoproteínas son capaces de inhibir la aglutinación de los glóbulos rojos de carnero mediada por el plásmido K99.

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