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EFFECTS OF SOME FACTORS INFLUENCING THE ADHERENCE OF MASTITIS PATHOGENS TO EPITHELIAL CELLS OF THE BOVINE MAMMARY GLAND

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Adherence was studied tests in vitro, by mixing a bacterial suspension (10^8/ml) with a suspension of epithelial cells (10^4-10^5/ml). The bacterial suspension (10^8/ml) was pretreated with either the β-toxin of Staphylococus aureus, or blood serum from healthy cows, or milk from healthy cows, or blood serum from mastitis affected cows, or milk from mastitis affected cows. The effect of mixing a Streptococcus agalactiae suspension (10^8/ml) with a suspension of epithelial cells (10^4-10^5/ml) pretreated with lipoteichoic acid was also examined.

Adherence was estimated on the basis of the relative number of epithelial cells with adhered bacteria, the number of bacteria adhering to 100 epithelial cells and the adherence index.

The adherence index of Staph. aureus was in the range from 7 to 84, Str. agalactiae 5-80, Micrococcus spp. 27-62 and Str. uberis 16-42. The number of epithelial cells with adhered Staph aureus was in the range from 16 to 85, Str. agalactie 32-81, Micrococcus spp. 27-62. Str. uberis 68-82. The number of bacteria adhering to 100 epithelial cells ranged as follows: for Staph. aureus from 100-781, Str.agalactiae 198-578, Micrococcus spp. 90-457 and Str. uberis 128-331. The adherence of Str. agalactiae and Staph. aureus was higher than the adherence of Micrococcus spp. and Str. uberis ($P<0,01$).

Lipotechoic acid, the β-toxin of Staph. aureus, blood serum from healthy and mastitis affected cows significantly ($P<0,01$) inhibited the adherence of Str. agalactiae to epithelial cells of the bovine mammary gland. The β-toxin of Staph. aureus, blood serum, milk of healthy cows and milk of mastitis affected cows significantly ($P<0,01$) inhibited the adherence of Staph. aureus to epithelial cells of the bovine mammary gland.

Key words: adherence, epithelial cells, Str. agalactiae, Staph. aureus, Str. uberis, Micrococcus spp., LTA, β-toxin, blood serum, milk.

INTRODUCTION

It has become evident that most natural infections are initiated by the attachment of microorganisms to mucosal surfaces (Beachey, 1981). Not only must the organisms attach, but they must also be able to multiply at a sufficiently rapid rate to replenish renewed mucosal surfaces as old mucosal cells are removed by desquamation. In this way the successful organism establishes itself in stable colonies on the mucosal surface (Beachey, 1986). Adherence of bacteria to bovine mamary gland epithelium has been shown to be the first step in the pathogenesis of bovine mastitis (Frost, 1975, Frost et al. 1977). To be successful in infection, the mastitis pathogen has to transverse the teat canal, resist the specific and nonspecific defence mechanisms, and withstand the washing-out effect at milking (Nelson et al. 1991). Many bacteria possess surface structures that bind to specific macromolecules on host cells in a lock-and-key fashion analogous to the combination of an enzyme with its substrate, or antibody with its antigen (Beachey, 1986). The adherence of different srotypes and phagotypes of Group B streptococci to human vaginal epithelial cells is mediated by lipoteichoic acid (Teti et al., 1985). Adherence was inhibited by milk from a quarter infected with Staph aureus and by anti-staphylococcal antiserum (Wanasinghe, 1981). The adhesive properties of the organisms associated with sporadic mastitis were generally much poorer than Staph. aureus and Str. agalactiae (Frost et al., 1977., Wanasinghe, 1981).

The aim of the present paper was to evaluate the ability of the mastitis pathogens to adhere to epithelial cells of the bovine mammary gland and to study the effects of lipoteichoic acid, β-toxin of Staph. aureus, blood serum and milk of healthy cows and milk from Str. agalactiae mastitis affected cows on the adherence of Str. agalactiae and Staph. aureus to epithelial cells of the bovine mammary gland.

MATERIALS AND METHODS

Epithelial cells. The udder was removed intact from freshly slaughtered clinically normal lactating cows. It was washed out and cleaned and the required quarter was dissected out. The epithelial cells were collected and used within 3h of slaughter of the cows. The epithelial lining of the ductular system from the streak canal to the ductioles was exposed by cutting with a pair of sharp-pointed scissors beginning from the teat orifice. Excess milk was rinsed off with 0,01 M phosphate-buffered saline (PBS). Major lactiferous ducts were gently brushed with a fine pipette brush, and the cells removed in this way were gently suspended in PBS. The cells were then washed three times in PBS by centrifugation at 1300 r.p.m. for 15 min. After the final wash, the cells were resuspended to a concentration of approximately 10^4-10^5 cells per ml in PBS.

Preparation of the bacterial suspension. Freshly isolated strains of Str. agalactiae, Str. uberis, Staph. aureus and Micrococcus spp. from milk of cows affected with subclinical mastitis were grown in Todd-Hewitt broth for 18h at 37^0C.

The bacteria were then centrifuged at 3000 r.p.m. for 20min, washed three times in PBS and resuspended to acontent of 10^8 per ml.

Adherence of bacteria to epithelial cells. Aliquots of 0,5 ml each of the epithelial cell suspension and culture were mixed in test tubes and shaken at 37^0C for 30 min. to allow contact between the bacteria and cells. The mixtures were then washed five times in PBS in this manner to remove the unattached bacteria from the epithelial cell suspension. Smears were made on glass slides by spreading 0,01ml of the cell suspension over approximately 1 cm^2.

Staining and microscopic examination of smears. The smears were air-dried and fixed by gentle heating and in ethanol for 10 min. The fixed slides were stained for 15 min. with azure II (30mg) and eosin (20mg) in 100 ml. distilled water. The stained smears were aid-dried and examined by oil immersion microscopy. The numbers of bacteria adhering to 100 epithelial cells and the numbers of epithelial cells which had bacteria attached were recorded. Only those bacteria which were either on the cells or directly associated with the cells and only bacteria attached to undamaged cells which were single or together in clumps of 2 or 3 cells were counted.

Pretreatment of bacteria with PBS, β-toxin, LTA, blood serum or milk. To study the effect of PBS, β-toxin from Staph. aureus, and milk from healthy and infected cows on the adherence of Staph. aureus, the bacteria were pretreated with these substances. The pretreatment was done by suspending the washed bacteria in the respective solutions of the substances for 1 h at 37^0C. After pretreatment, the bacteria were washed and resuspended in PBS (pH 7.4) prior to being used in the system.

Since all factors could not be examined in one experiment, variables were grouped into several experiments. An untreated control was included in each experiment.

Pretreatment of epithelial cells with lipotechoic acid. The effect of lipotechoic acid (LTA) on the adherence of Str. agalactiae was examined. Pretreatment was done by suspending the washed epithelial cells in solutions of LTA for 1h at 37^0C. After pretreatment, the epithelial cells were washed again and resuspended in PBS (pH 7.4) prior to use in the system. An untreated control was included in the experiment.

Adherence index. The number of pretreated bacteria adhering to epithelial cells was represented as the percentage of adhering bacteria in relation to the bacteria adhering in the untreated control.

RESULTS

The results for adherence of mastitis pathogens to epithelial cells of the bovine mammary gland ore shown in Table 1.

Table 1. Adherence of different bacterial strains recovered from cases of mastitis to epithelial cells of the bovine mammary gland

Species	Strain	No of cells[a] with adhering bacteria per 100 cells	No adhering[a] bacteria per 100 cells	Adherence Index
Staph. aureus (control)	1	85	781	100
	2	88	661	84
	3	81	541	69
	4	80	493	63
	5	80	467	59
Str. agalactiae	1	81	541	68
	2	65	283	36
	3	60	295	37
Str. uberis	1	82	128	16
	2	68	331	42
Micrococcus spp	1	62	413	52
	2	58	457	58
	3	46	186	24
	4	33	104	13
	5	27	90	12

a Mean values of three determinations

The highest adherence index was exhibited by Staph. aureus (59-84), followed by Str. agalactiae (36-69). A lower adherence index was noted for Micrococcus spp. (12-58) and Str. uberis (16-42). Significant differences were not found when the adherence index was compared for Str. agalactiae and Staph. aureus. However, when Str. agalactiae and Staph. aureus were compared to Micrococcus spp. and Str. uberis the difference was significant. Since Str. agalactiae and Staph. aureus are most frequently demonstrated as mastitis pathogens, the adherence of these organisms was of primary interest to the present authors. These organisms were recovered from the udders of cows orginating from small farms with 5-6 cows (Experiment 1) and farms with 150-200 cows (Experiment 2) as shown in Table 2.

High adherence levels were noted for Staph. aureus and Str. agalactiae in both experiments. The highest variations in the adherence index for Staph. aureus (7-80) were recorded in organisms recovered in Experiment 1. In Experiment 2 the organisms were recovered from a farm where mastitis pathogens were attributed equally to Str. agalactiae and Staph. aureus. A higher adherence index to epithelial cells of the bovine mammary gland was noted for Str. agalactiae (34-72) compared to Staph.aureus (13-54).

Since it was demonstrated that the most frequent mastitis pathogens were Staph. aureus and Str. agalactiae, the effect of various factors upon the adherence of these organisms to epithelial cells of the bovine mammary gland was examined. The factors were PBS, β-toxin, blood serum and milk from healthy and mastitis affected cows and the results are shownin Table 3.

Table 2. Adherence to bovine mammary gland epithelial cells of Staph. aureus and Str. agalactiae recovered from mastitis cases originating in different herds

Experiment	Strain	No of cells[a] with adhering bacteria per 100 cells	No adhering[a] bacteria per 100 cells	Adherance Index
1	Staph. aureus 1/1 (control)	76	573	100
	Staph. aureus 1/2	31	104	18
	Staph. aureus 1/3	43	271	34
	Staph. aureus 1/4	42	271	47
	Staph. aureus 1/5	45	111	19
	Staph. aureus 1/6	67	380	66
	Staph. aureus 1/7	30	110	19
	Staph. aureus 1/8	16	41	7
	Staph. aureus 1/9	57	463	80
	Staph. aureus 1/10	33	66	11
	Staph. aureus 1/11	73	368	64
2	Staph. aureus 2/1 (control)	55	757	100
	Staph. aureus 2/2	35	297	39
	Staph. aureus 2/3	46	411	54
	Staph. aureus 2/4	22	100	13
	Staph. aureus 2/5	30	189	24
	Str. agalactiae 2/1 (control)	51	566	100
	Str. agalactiae 2/2	40	236	41
	Str. agalactiae 2/3	38	311	54
	Str. agalactiae 2/4	37	198	34
	Str. agalactiae 2/5	35	409	72

[a] Mean values of three determinations

Table 3. Effects of pretreatment with PBS, β-toxin, blood serum and milk of the cow on the adherence of Staph. aureus to epithelial cells of the bovine mammary gland.

Treatment	No of cells with[a] adhering bacteria per 100 cells	No of bacteria[a] adhering to 100 cells	Adherence Index
Control	65	557	
PBS	64	446	80
β - toxin	56	232	42
Control	63	573	
milk healthy cow	36	133	23
milk infected cow	26	68	12
blod serum - healthy cow	47	290	50
blod serum - infected cow	47	261	45

[a] Mean values of six determinations

Milk from infected cows exhibited the strongest effect on the adherence of Staph. aureus to epithelial cells of the bovine mammary gland. The adherence index in such cases amounted to 12. The β-toxin of Staph. aureus, milk and blood serum originating from infected and healthy cows significantly suppressed the

adherence of Staph. aureus to epithelial cells of the bovine mammary gland ($P<0,01$). The adherence index for Staph. aureus previously treated with milk from healthy cows, β-toxin, blood serum from infected and healthy cows amounted to 23, 42, 45 and 50 respectively. The effects of PBS, β-toxin, LTA and blood serum of a healthy cow on the adherence of Str. agalactiae to epithelial cells of the bovine mammary gland are shown in Table 4.

Table 4. Effects of pretreatment with PBS, β-toxin on the adherence of Str. agalactiae to epithelial cells of the bovine mammary gland

Treatment	No of cells with[a] adhering bacteria per 100 cells	No of bacteria[a] adhering to 100 cells	Adherence Index
Control	39	182	
PBS	47	253	140
β - toxin	18	54	30
LTA	26	88	48
Control	61	282	
Blod serum - healthy cow	41	231	34
Blod serum - infectrd cow	37	147	21

[a] Mean values of six determinations

It was faund that the blood serum of a cow affected with mastitis suppressed to the highest level the adherence of Str. agalactiae, the adherence index being 21. β-toxin from Staph. aureus, LTA and blood serum of a healthy cow significantly suppressed the adherence of Str. agalactiae to epithelial cells of the bovine mammary gland ($P<0,01$). The adherence index for Str. agalactiae previously treated with β-toxin and the blood serum of a healthy cow amounted to 30 and 34, and the adherence index for Str. agalactiae treated with LTA amounted to 48.

DISCUSSION

The most frequent mastitis pathogens are Str.agalactiae, Staph. aureus, other streptococci and coliform organisms (Harmon, 1994). For the farm from which the organisms were recovered for use in the in vitro adherence test, mastitis pathogens were Str. agalactiae (34,78%), Staph. aureus (13,04%), Micrococcus spp. (13,04%), Str. uberis (8,69%) and Corynobacterium bovis (8,69%). The highest adherence index was established for Staph. aureus and Str. agalactiae. These results are in accordance with the findings of Frost (1977) and Wanasinghe (1981) suggesting that the highest adherence index could be correlated with the most frequent mastitis pathogens. In order to demonstrate whether the herd size was responsible for the predominance of a particular mastitis pathogen, the organisms recovered from small farms were compared to those from big farms.

According to the results shown in Table 2, the adherence of Stapah. aureus was higher in organisms recovered from bovine udders from small farms where no cows affected with agalactiae Str. were present, compared to the organisms recovered from bovine udders from a big farm were both the former and the latter organisms were recorded.

The suppression of adherence of Staph. aureus and Str. agalactiae pretreated with blood sera of cows affected with mastitis was attributed to the binding of antibodies from the blood serum to antigens on bacterial cell surfaces. The suppression of adherence of Str. agalactiae to epithelial cells, pretreated with LTA, according to Mamo et al. (1986), Teti et al. (1985), Beachy (1981, 1986) and Simpson et al. (1987) was correlated with binding of LTA to epithelial cells which blocked the receptors at the epithelial cell surfaces thus preventing the adherence of Str. agalactiae. Moreover, it was found that the adherence index of Str. agalactiae and Staph. aureus for epithelial cells was significantly decreased after these organisms were pretreated with β-toxin. This phenomenon was attributed to the role of β-toxin in the lysis of bacterial walls. They are the site of adherence of toxins and β-toxin is the most frequent hemolysin produced by staphylococci of bovine origin.

The present authors suggest that further studies of bacterial adherence to epithelial cells should be carried out in order to understand better the pathogenesis of mastitis and the pathogenicity of studied organisms for the bovine udder. They also point out that studies on the factors affecting the adherence of mastitis pathogen to epithelial cells of the bovine mammary gland should enable more successful control of mastitis.

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UTICAJ NEKIH FAKTORA NA ADHERENCIJU UZROČNIKA MASTITISA NA EPITELNE ĆELIJE MLEČNE ŽLEZDE KRAVA

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SADRŽAJ

In vitro test adherencije je izvođen mešanjem suspenzije bakterija (10^8/ml) i suspenzije epitelnih ćelija (10^5/ml). Uticaj različitih faktora na adherenciju je ispitivan in vitro testom mešanjem suspenzije bakterija (10^8/ml) predhodno tretiranih sa β-toksinom Staph. aureus, krvnim serumom zdrave krave, mlekom zdrave krave, krvnim serumom krave obolele od mastitisa ili mlekom krave obolele od mastitisa. Takođe je mešanjem suspenzije Str. agalactiae (10^8/ml) i suspenzije epitelnih ćelija (10^4-10^5/ml) predhodno tretiranih sa LTA ispitivan i uticaj LTA na adherenciju.

Adherencija je određivana na osnovu broja epitelnih ćelija sa adheriranim bakterijama, brojem bakterija adheriranih na 100 epitelnih ćelija i indeksa adherencije.

Index adherencije za Staph. aureus se kretao od 7-84, Str. agalactiae 5-80, Micrococcus spp. 27-62 i Str. uberis 16-42. Broj epitelnih ćelija sa adheriranim bakterijama se kretao od 16-85 za Staph. aureus, od 32-81 za Str. agalactiae, od 27-62 za Micrococcus spp. i za Str. uberis od 68-82. Broj bakterija na 100 epitelnih ćelija se kretao za Staph. aureus od 100-781, za Str. agalactiae od 198-578, za Micrococcus spp. od 90-457 i za Str. agalactiae od 128-331. Adherencija Str.agalactiae i Staph.aureus je bila veća nego adherencija Micrococcus spp. i Str. uberis ($P<0,01$). Lipotejkonska kiselina, β-toksin Staph. aureus, krvni serum zdrave krave i krave obolele od mastitisa su značajno inhibirali adherenciju Str. agalactiae za epitelne ćelije mlečne žlezde krava ($P<0,01$). β- toksin Staph. aureus, krvni serum, mleko zdrave krave i krave obolele od mastitisa su značajno inhibirali adherenciju Staph. aureus za epitelne ćelije mlečne žlezde krava ($P<0,01$).