NOTE *Public Health*

Susceptibilities against Bovine Lactoferrin with Microorganisms Isolated from Mastitic Milk

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ABSTRACT. Antibacterial effects of bovine lactoferrin were studied *in vitro* against microorganisms isolated from mastitic milk in Tokachi area, Hokkaido, Japan. Microorganisms isolated were *Eschelichia coli* (11 isolates), *Klebsiella pneumoniae* (5 isolates), enterococci (8 isolates), *Staphylococcus aureus* (10 isolates), coagulase negative staphylococci (CNS, 13 isolates), streptococci (11 isolates), *Prototheca zopfii* (7 isolates) and yeast-like fungi (9 isolates). Lactoferrin has been known as a multifunctional protein and its antimicrobial effect is one of the most essential function of it. In order to compare their susceptibilities against lactoferrin, the minimal inhibitory concentration values were estimated by a microplate assay method using 96-well microplate, which involved measuring the optical density of the cultures. *Prototheca zopfii* was highly sensitive to bovine lactoferrin and complete inhibition of this microorganism was observed even at the low concentration of 7 μg/ml. On the other hand, *E. coli* and enterococci showed resistance against lactoferrin action and staphylococci showed strain-dependent resistance.

KEY WORDS: antimicrobial activity, lactoferrin, mastitic milk.

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Lactoferrin, an iron-binding glycoprotein of the transferrin family, is present in most biological fluids including milk, saliva, tears, mucous secretions and blood. Lactoferrin consists of a single polypeptide chain having a molecular mass of about 80 kDa and it possesses two iron ion-binding sites per molecule [1]. Bacteriostatic and bactericidal activities of lactoferrin against Gram positive and Gram negative bacteria, some yeasts, fungi [2, 5, 6], parasite [29] and virus [18] have been reported. Moreover, several physiological functions of lactoferrin including modulation of the inflammatory response, activation of the immune system and control of myelopoiesis have been reported as reviewed in many articles [4, 23, 30]. The mechanism of the antimicrobial activity of lactoferrin has been understood to involve its iron ion chelating properties, making this essential factor inaccessible to invading pathogens [6, 7], and to involve their membrane binding properties, causing the release of lipopolysaccharides from Gram negative bacteria [5]. It is known that the lactoferrin content is high not only in colostrum [12, 20, 27] but in the milk of non-lactating period [12, 26] and in mastitic milk [10, 24, 25]. The latter suggests the contribution of lactoferrin on the mammary gland defence system. One of the authors has reported that the decreased levels of lactoferrin in peracute *Escherichia coli* mastitis may be associated with the progress of inflammation in the early phase of mastitis [13]. The aim of this study is to test the resistance or susceptibility of the microorganisms isolated from mastitic milk to lactoferrin *in vitro*. The lactoferrin anti-bacterial spectrum against strains isolated from mastitic milk would be expected to give us basic and useful information for the protection and treatment of mastitis.

The strains of microorganisms tested in this study were isolated from mastitic milk from dairy farms located in the Tokachi area, Hokkaido Japan. The microorganisms isolated from mastitic milk included Gram positive and Gram negative bacteria and other species as shown in Table 1. They are *Eschelichia coli* (11 isolates), *Klebsiella pneumo-*

Table 1. The minimal inhibitory concentration (MIC) values of 74 isolates obtained from mastitic milk, as determined by the microplate method

species	MIC values	Number
Escherichia coli	> 50 mg/ml	11 isolates
Klebsiella pneumoniae	0.88 mg/ml	5 isolates
Staphylococcus aureus	0.22 mg/ml	6 isolates
	> 50 mg/ml	4 isolates
Staphylococcus capitis	> 50 mg/ml	1 isolate
Staphylococcus epidermidis	> 50 mg/ml	1 isolate
Staphylococcus equorum	> 50 mg/ml	1 isolate
Staphylococcus haemolyticus	1.75 mg/ml	1 isolate
Staphylocuccus schleiferi	3.5 mg/ml	1 isolate
Staphylococcus simulans	> 50 mg/ml	5 isolates
Staphylococcus xylosus	> 50 mg/ml	3 isolates
Streptococcus dysgalactiae	> 50 mg/ml	2 isolates
Streptococcus equinus	> 50 mg/ml	1 isolate
Streptococcus uberis	0.055 mg/ml	8 isolates
Enterococcus faecalis	> 50 mg/ml	1 isolate
Enterococcus faecium	> 50 mg/ml	6 isolates
Enterococcus raffinosus	> 50 mg/ml	1 isolate
Prototheca zopfii	0.007 mg/ml	7 isolates
Yeast-like fungi	1.75 mg/ml	9 isolates

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niae (5 isolates), enterococci (8 isolates), *Staphylococcus aureus* (10 isolates), coagulase negative staphylococci (CNS, 13 isolates), streptococci (11 isolates), *Prototheca zopfii* (7 isolates) and yeast-like fungi (9 isolates) as identified according to the method of the National Mastitis Council [17].

The microorganisms isolated were cultured three times in heart infusion broth (for staphylococci, streptococci, enterococci and *Klebsiella*), Mueller-Hinton broth (for yeast-like fungi) and bacto-tryptone glucose broth (TG broth, for *Prototheca*). The growth assays were carried out at 37°C (for staphylococci, streptococci, enterococci, *Klebsiella* and yeast-like fungi), or 27°C (for *Prototheca*) for 24 hr. Since the growth rate of each microorganism was different, viable cell counts of the stock culture of each microorganism were determined in preliminary studies using standard plate count. Referring to the results of these preliminary studies, the stock culture of each microorganism was adjusted to 1.0 ($\pm$ 0.5) $\times$ 10^5 cfu/2 μl. Antimicrobial activity was estimated turbidimetrically by the microplate assay method developed in the author's laboratory [14], using 96-well microplate and a microplate reader. Fifty μl of each sterilized broth was added to the wells of the microplate followed by addition of filter-sterilized lactoferrin at a concentration in the range of 1 μg/m*l* to 50 mg/m*l*. Then, 2 μl of the adjusted stock suspension of the microorganism was added and the mixture was incubated at the most suitable temperature. As a negative control, sterilized distilled water was substituted for the lactoferrin solution. The optical density at 600 nm of the culture was measured using a microplate reader. Before the measurement, the microplate was shaken gently with a microplate mixer for 30 sec to disperse the microbial cells homogeneously. Bovine lactoferrin used in these experiments was obtained from Morinaga Milk Industry Co., Ltd. (Nutritional Science Laboratory, Zama, Japan). The iron ion saturation degree of lactoferrin was 35–39% as determined using Fe-assay kit (Wako Pure Chemicals, Ltd., Osaka). Other reagents used were analytical grade.

By the microplate assay, MIC values of 74 strains were compared to see the lactoferrin effects on the growth of each microorganisms (Table 1). The growth tests to obtain MIC values were performed with strains which showed no growth with the lactoferrin concentration of 50 mg/m*l*. The growth of all isolates of *E. coli*, 4 of 10 isolates in *S. aureus*, most of CNS and all isolates of enterococci were not inhibited in the presence of bovine lactoferrin. On the other hand, 8 of 11 isolates of streptococci were susceptible against lactoferrin and they were *S. uberis*. Other isolates, *S. equines* and *S. dysgalactiae* showed resistance against lactoferrin. Five isolates of *K. pneumoniae* and all of yeast-like fungi showed moderate susceptibilities against lactoferrin. The most sensitive bacteria against lactoferrin was *P. zopfii* and their MIC values were 0.007 mg/m*l*.

It was reported that an experimentally induced *E. coli* infection of a bovine mammary gland resulted in a 30-fold increase in lactoferrin concentration in the mammary secretion by 90 hr postinoculation and the concentration of lactoferrin in mammary gland may be 7 to 8 mg/m*l* [11]. On the other hand, lactoferrin concentration in normal milk is reported to be 0.02 to 0.35 mg/m*l* [20]. This may mean that mastitis referable to certain pathogenic bacteria may induce self-therapy in some degrees. Furthermore, *E. coli* mastitis is characterized by the occurrence of acute necrosis because of its endotoxin production [22]. On the other hand, it has been reported that lactoferrin did not affect the growth of 3 strains of *E. coli* isolated from bovine mastitic milk [21]. In our experiments, a relatively high MIC value of more than 50 mg/m*l* was obtained in the case of the *E. coli* isolate, but lactoferrin is never found at such a high concentration level in milk, colostrum, involuted or mastitic milk. A relation between virulence and resistance of bacteria to lactoferrin was suggested [19], but we did not confirm such relation. From our results, it is clear that lactoferrin susceptibilities are species-dependent, however, it is very hard to explain the difference of bacterial susceptibilities against lactoferrin. Antimicrobial mechanism of lactoferrin may be different with Gram positive and Gram negative bacteria because of the different cell surface structure. However, both Gram positive and Gram negative bacteria included bacteria which showed both lactoferrin-susceptibility and lactoferrin-resistance. One of the antimicrobial effects of lactoferrin is bacteriostatic and lactoferrin scavenges iron ions from the bacterial environments. Another effect is bactericidal effect inducing the liberation of lipopolysaccharide from bacterial surface. It seems that such difference of antimicrobial mechanisms may result the wide range of MIC values. Adding to these differences, protease secreted by bacteria may hydrolyse lactoferrin into small peptides. In some cases, bacteria can intake such peptides as nutrients for their growth but it cannot be denied the production of a strong antimicrobial peptide, lactoferricin [3]. Moreover, it is known that certain microorganisms have lactoferrin-binding proteins or lactoferrin-receptor on their surface [6, 9]. And *S. aureus* is one of bacteria possessing lactoferrin-receptor [15, 16]. Therefore, there may be a possibility that the resistance of some *S. aureus* isolates to lactoferrin is due to the presence of lactoferrin receptor.

Bovine lactoferrin showed relatively potent antimicrobial activity against yeast-like fungi. It has been known that treatment of infected cows with antimicrobial agents has no effect on mastitis caused by fungi or yeast. And *Prototheca* infection of the mammary gland produces a serious mastitic condition [8]. These microorganisms show resistance to most antimicrobial agents used in mastitis treatment and it is very difficult to cure the cows infected. The results of our experiments indicate that *P. zopfii* shows high sensitivity to lactoferrin treatment although the mechanism has not been resolved. From the preliminary study using lactoferrin hydrolysate or EDTA, it seems that iron ion is not concerned with the growth inhibition of *P. zopfii*. The study using recombinant lactoferrin showed the effectiveness of lactoferrin to suppress the growth of *P. zopfii* [28], too. These results lead us to speculate that lactoferrin might have potentials for treatment of such kinds of bovine mastitis.

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ロファージで発現し，病巣周辺のアストロサイトの一部でも陽性であった．これらの結果はOPN蛋白の発現が脊髄損傷後は主に活性化したミクログリア／マクロファージで増殖することを示し，OPNが損傷初期のおそらく組織改築に導く細胞増殖に関与することを示唆する．

薬　理　学：

ハムスターを用いた酢酸誘発大腸炎に対する好中球エラスターゼ阻害剤(ONO-6818)の作用——廣田　泰[1)]・鈴木美紀恵[1)]・山口康二郎[1)]・藤田常夫[1)]・勝部伸夫[1)]([1)]小野薬品工業(株)福井安全性研究所) **1223-1228**

好中球から放出される好中球エラスターゼは，炎症における組織破壊や機能不全に関与する因子として知られる．今回，我々は経口吸収性を有する好中球エラスターゼ阻害剤であるONO-6818のハムスター酢酸誘発大腸炎に対する作用を検討した．大腸炎を惹起した動物では潰瘍面積，大腸の出血量，大腸組織ミエロペルオキシダーゼ活性(MPO活性)，大腸腔内の好中球エラスターゼ活性(NE活性)が上昇した．ONO-6818は経口投与あるいは皮下投与のどちらにおいても潰瘍面積，大腸出血量およびNE活性を有意に抑制した．しかし，MPO活性の有意な抑制は認められなかった．以上から，ハムスターの酢酸誘発大腸炎では，その病態の進展に好中球エラスターゼが深く関与し，好中球エラスターゼの阻害によりその病態が改善されることが明らかとなった．

生　理　学：

ラットの制限給餌下での予知反応における視床室傍核の関与(短報)——中原桂子[1)]・福井健人[1)]・村上　昇[1)]([1)]宮崎大学農学部獣医学科家畜生理) **1297-1300**

給餌時間を毎日定刻の2時間のみに制限すると，ラットは餌の来る時刻を予知し，予知行動を示すようになる．給餌時刻前後の脳内cFosの発現部位を調べた結果，視床室傍核に発現の差を認めた．そこで，この部位を電気破壊すると，予知行動のみ消失した．また，視床室傍核を破壊したラットでは，血中コルチコステロンの制限給餌直前のピークが形成されなかった．以上の結果は，視床室傍核が制限給餌で発動する予知行動に関与していることを示唆する．

公衆衛生学：

乳房炎乳由来微生物のラクトフェリンに対する感受性(短報)——李　乃元[1)]・河合一洋[2)]・中村一郎[1)]・田仲哲也[1)]・玖村朗人[1)]・島崎敬一[1)]([1)]北海道大学大学院農学研究科生物資源生産学専攻酪農科学研究室，[2)]十勝NOSAI清水家畜診療所) **1267-1269**

臨床型乳房炎乳から*E. coli*(11株)，*K. pneumoniae*(5株)，enterococci(8株)，*S. aureus*(10株)，CNS(13株)，streptococci(11株)，*P. zopfii*(7株)，yeast-like fungi(9株)を分離し，ラクトフェリンに対する感受性を比較した．その結果，*P. zopfii*がラクトフェリンによって最も増殖抑制を受け易かった．一方，*E. coli*やenterococciはラクトフェリンの影響を受けにくく，また*S. aureus*は菌株によって感受性の有無が分かれた．

臨床繁殖学：

黒毛和種牛の3つの発情周期における主席卵胞と黄体のエストロジェンレセプターERαの発現——AMROZI[1)]・上村俊一[1,2)]・安藤貴明[1)]・浜名克己[1,2)]([1)]山口大学大学院連合獣医学研究科，[2)]鹿児島大学農学部獣医学科家畜臨床繁殖学教室) **1183-1188**

黒毛和種牛の3つの発情周期における主席卵胞と黄体のエストロジェンレセプター(ERα)の発現を免疫組織学的に検査した．卵巣動態を1日2回超音波診断法により観察し，発情周期の7日(1群，n＝3)，10日(2群，n＝3)，18日(3群，n＝3)に卵巣を摘出した．細胞核におけるERαの発現を免疫組織学的に検査し，染色度合いを光学顕微鏡で観察した．主席卵胞(DF)の染色度合いは内卵胞膜(TI)が卵胞膜側顆粒細胞(mGC)や卵胞腔側顆粒細胞(aGC)，外卵胞膜(TE)より高い傾向にあった．3群のERα発現は，mGCで1群より低く，TEで2群より低い傾向にあった．黄体細胞のERα発現は黄体間質細胞より高く，細胞ごとでは2群の黄体細胞が3群より高かった．今回の結果，1) ERα