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Development of Method for Early Diagnosis of Mastitis in Dairy Cows by Chemiluminescence

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Abstract

The chemiluminescence (CL) assay measures the ability of a phagocyte to produce light. Milk CL assay can detect an early stage of mastitis precisely because phagocytic leukocytes, such as neutrophils, accumulate in milk as a protective initial reaction after bacterial invasion. Based on the emission of CL by phagocytic leukocytes in milk, we developed a method to diagnose mastitis at an early stage.

Discipline: Animal health
Additional key words: phagocytic leukocytes, milk chemiluminescence, GM-CSF, *Staphylococcus aureus*, mastitis diagnosing apparatus

Introduction

Mastitis which is a major disease of dairy cattle in terms of cost and loss of production is an important problem worldwide[6]. It is very difficult to treat animals with mastitis because the bacteria causing mastitis are anchored in the udder. Especially, *Staphylococcus aureus* (*S. aureus*) mastitis is known to be highly resistant to therapy with antibiotics, since *S. aureus* causes microabscesses or granulomas in the mammary gland tissues and becomes easily resistant to antibiotics[4]. Furthermore, one of the reasons for the lack of prevention of mastitis is that a simple and precise method of detecting the first stage or subclinical stage of mastitis has not been developed[6]. Therefore, it is impossible to implement effective treatment or countermeasures against mastitis. In this study, based on the fact that phagocytic leukocytes penetrate into milk just after the invasion of the bacteria in the udder, a method for the early diagnosis of mastitis in which the amount of chemiluminescence released from phagocytic leukocytes is used as an index was developed. Furthermore, by using the CL method mainly, the therapeutic effect of a cytokine, GM-CSF, on subclinical mastitis was examined[5,8,9].

Why dairy cows can be easily affected by mastitis?

Domestic fowls and animals like chickens, pigs, cattle are easily affected by various diseases because selective breeding is implemented in order to improve the production capacity of the animals. Dairy cattle are a typical example, and a high milk production became possible by breeding and improvement of the animals. However, due to this production system, dairy cows develop abnormally large udders. Furthermore, milk becomes a

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culture medium for bacteria since it is an ideal food in which nutrients such as sugars, proteins, fats, minerals and enzymes are combined.

When mastitis occurs due to the invasion of bacteria in the mammary gland, milk production stops, and cattle are often disposed of. The cost due to the damage is estimated at about ¥ 100 billion per year even in Japan where dairy farming is not as popular as in Europe and USA.

Why is it difficult to treat mastitis?

Mastitis occurs when the protective mechanisms of the host against bacterial infection are deficient. Therefore, prevention by the use of a vaccine is no longer possible[8]. Until now, treatment had been entirely carried out by the use of antibiotics. However, drug tolerance and drug residues, etc. have become a major problem. Since milk is an important food, antibiotics can not be used for the treatment of mastitis. Also in many cases, antibiotics are not effective, even when applied after isolation of cattle. The disease becomes chronic, and can not be treated by antibiotics when bacteria invade, proliferate and produce microabscesses or granulomas in the mammary gland. Among the causal agents of mastitis, the therapeutic rate of *S. aureus*-induced mastitis is very low due to the toxicity and persistence of the bacteria[4].

Conventional methods for the diagnosis of mastitis

Somatic cell counting (SCC), California Mastitis Test (CMT), enzymatic method and conductometry have been mainly used for the diagnosis of mastitis in dairy cattle (Fig. 1). SCC involves the count of populations of lactic gland epithelial cells, neutrophils, eosinophils, macrophages, plasmocytes, etc. in milk. The method enables to diagnose mastitis from a relatively early stage to the middle stage after bacterial infection. The drawback of SCC is that as mastitis progresses, numerous leukocytes adhere to the bacteria, producing bulb-like forms which make the measurement impossible. CMT, enzymatic method and conductometry enable to diagnose mastitis from the middle stage to the later stage after bacterial infection. However, the drawback of these methods is that the diagnosis is difficult until the reaction between the leukocyes and the bacteria has occurred, or after the increase of vascular permeability.

Principle of chemiluminescence

Molecules become unstable in a state of excitation by chemical reaction, and chemiluminescence (CL) is a phenomenon whereby light which is not accompanied by high temperature is emitted when the molecules revert from the excited state to a stable state. Light emission by *Cypridina hilgendorfi*, firefly and noctiluca or fertilization of sea urchin are examples of chemiluminescence. Recently, it has been shown that such a phenomenon occurs daily in the process of metabolic reactions in animals. Since the reaction is an oxidation reaction, a method of evaluating the degree of metabolic activity in organisms was developed, using chemiluminescence[1].

Diagnosis of mastitis by the application of the chemiluminescence method

Milk contains somatic cells such as lactic gland epithelial cells, neutrophils, eosinophils, lymphocytes, monocytes, plasmocytes, etc. These cells are virtually absent in milk when the animals are healthy, but when the udder is invaded by bacteria, leukocytes with a phagocytic bactericidal activity like neutrophils or eosinophils accumulate in the milk to interrupt bacterial proliferation. Among these, the neutrophils appear in large numbers after bacterial invasion[2]. The neutrophils kill bacteria mainly with active oxygen, and also emit a trace intensity of light (photons) in proportion to the amount of active oxygen released[1,3].

In the milk CL method, light of photons is detected, amplified and quantified. The phagocytic leukocytes with a bactericidal activity that accumulate in milk in response to bacterial invasion can be detected with a high sensitivity during a long period of time from the initial stage of bacterial invasion to the stage of severe mastitis (Fig. 1).

No apparatus had been developed hitherto for convenient and reliable diagnosis of mastitis based on the aforementioned CL activity measurement. In the current study, a method for the early diagnosis of mastitis whereby the CL emission of phagocytes in milk is used as an index was developed. The outline of the measuring method is shown in Fig. 2. The method has some advantages because CL diagnosis of mastitis enables to detect accurately the antimicrobial activity of phagocytes which accumulate in milk as a reaction to bacterial invasion. The advantages are as follows: 1) the invasion of pathogenic bacteria in the udder can be detected at a very early stage, 2) the measurement can be performed in milk which has a high viscosity, in colostrum which shows cohesive properties or in the case of severe mastitis, 3) the stage of mastitis can be objectively evaluated quantitatively, and 4) the measurement and operation are simple. An apparatus for the exclusive diagnosis of mastitis was manufactured (Fig. 3) by the Tokken Co., Ltd.

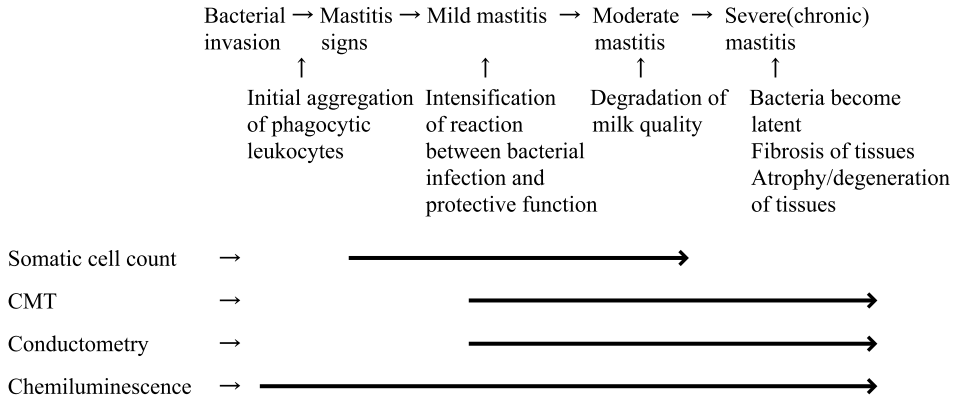

Fig. 1. Relationship between progression of mastitis and effectiveness of the major methods

Milk 50 μL
+
Cell-suspended liquid (5 mM Hepes Eagle's-MEM)
+ 450 μL
Luminol 10 μL
+
Opsonized zymosan
↓ 10 μL
↓ Incubation (37 ℃)
↓
Measurement of CL activity

Fig. 2. Method of measurement of milk chemiluminescence (CL) activity

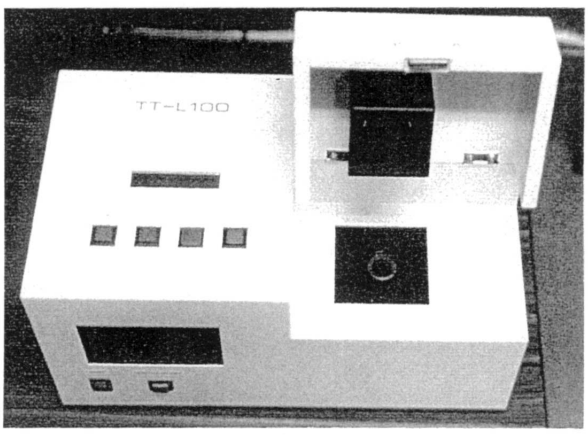

Fig. 3. Apparatus for the diagnosis of mastitis based on chemiluminescence method

Applications of the milk chemiluminescence method to the detection of mastitis in dairy cattle

1) Application of the milk chemiluminescence method to the forecasting of mastitis

Fig. 4 shows the relation between the progression of mastitis and milk CL activity when 35 dairy cows in a dairy farm were transferred from a rotary milking parlor to a parallel milking parlor. In the cows without mastitis, milk CL activity was almost zero throughout the experiment. The rise of the milk CL activity had already begun 1 week before the diagnosis of mastitis had been made by the conventional CMT method in cows with mastitis.

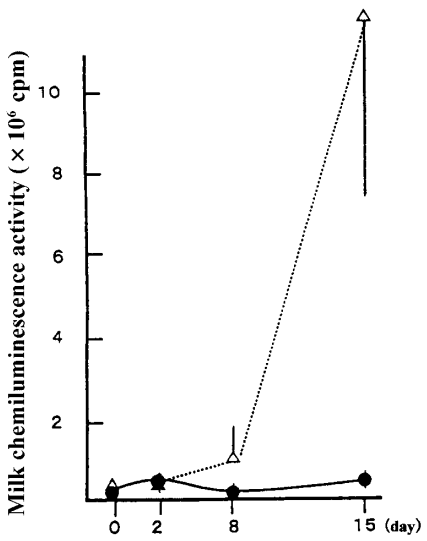

Fig. 4. Changes in milk chemiluminescence activity in cows with mastitis and healthy dairy cows

Diagnosis of mastitis was possible at 8 days after the change in the milking system using the chemiluminescence method, while at 15 days after the change using the conventional CMT method.
△ : Mean ± S.E. of 4 mastitis cows,
● : Mean ± S.E. of 31 healthy cows.

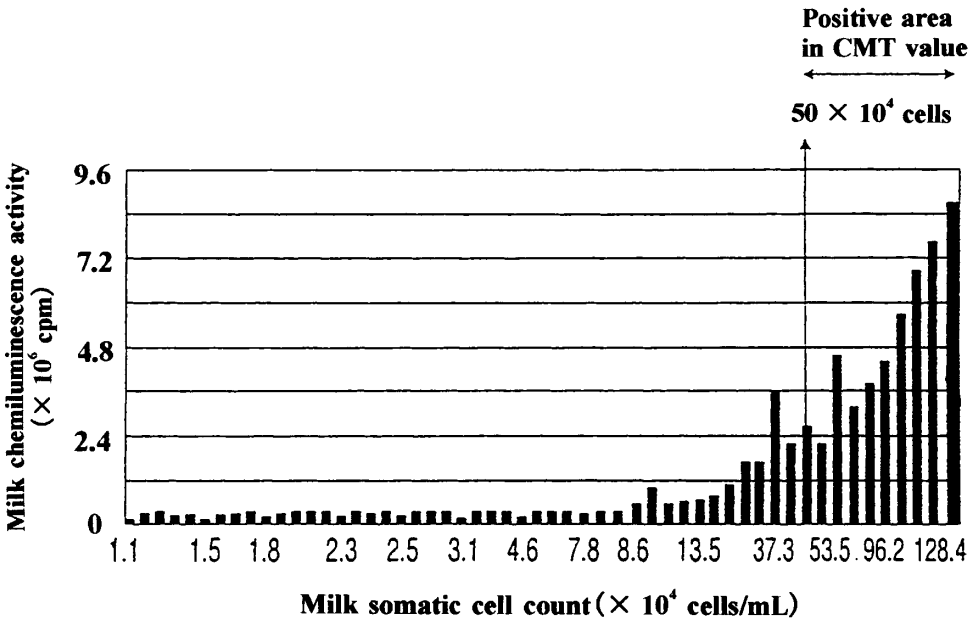

Fig. 5. Correlation between milk chemiluminescence activity, somatic cell count and CMT value

The results show that the milk CL method enabled to forecast the onset of mastitis earlier than the conventional diagnosis method[3,9].

2) Comparison of chemiluminescence method with conventional diagnosis method

Fig. 5 shows the results of the diagnosis of mastitis using milk sampled daily from 47 dairy cows in 2 dairy farms. The rise of the milk CL activity was well correlated with the increase in the milk somatic cell counts above 10×10^4 cells/mL. In the CMT method, the diagnosis of mastitis is possible when the somatic cell count reaches 50×10^4 cells/mL or more. However, the milk CL method enables to detect an increase in the somatic cell count of 10×10^4 cells/mL or more in milk. The results show that the use of the milk CL method enables to detect an initial bacterial infection of udders.

Cow No.	Total milk CL activity ×10⁶cpm	CMT
948	0.12	−
562	0.24	−
555	0.30	−
556	0.36	−
559	0.39	−
474	0.46	−
440	0.88	−
540	1.47	−
534	3.57	−

	Udder	Quarter milk CL activity ×10⁶cpm	CMT
	R.F.	0.12	−
	R.R.	0.10	−
	L.F.	0	−
*	L.R.	7.87	+
	R.F.	0.06	−
*	R.R.	6.15	+
	L.F.	0.11	−
	L.R.	0.09	−

S. aureus was separated from *-sealed quarter milk.

Fig. 6. Detection of subclinical mastitis in cows by the milk chemiluminescence method

3) Application of the milk chemiluminescence method to periodical examinations of mastitis

Fig. 6 shows the results of the milk CL activity in periodical examinations of mastitis at 2- week intervals in a dairy farm. For the diagnosis of mastitis, the CL method was compared with the CMT method by using total milk (mixed milk from 4 udders). It was found that the CL activity in total milk was high in 2 out of 9 head of cattle, though mastitis had not been detected in the 9 animals by the CMT method. It was found that 2 cows showed the first stage of mastitis, when the milk CL activity and CMT value were measured a second time by using "quarter milk" (milk from each udder). It was thus possible to detect mastitis at the subclinical stage, which was overlooked in the conventional method, by applying the milk CL method along with conventional periodical examinations.

Prospect for the control of mastitis

It is essential to diagnose mastitis at the initial stage of infection to initiate the treatment as early as possible because the treatment becomes very difficult after the bacteria are anchored in the mammary gland[4,6]. However, a method for early diagnosis that is both precise and

Table 1. Changes in CMT value after intra-udder injection of rBoGM-CSF in dairy cows with mastitis

Group	Cow No.	Days after GM-CSF injection							
		0	1	2	3	7	11	14	25
Chronic stage of mastitis	405	2+	4+	3+	4+	3+			
	479	4+	4+	4+	5+	5+	+		3+
	538	4+	4+	3+	4+	3+		2+	
	544	2+	4+	+	+	2+		2+	
Early stage of mastitis	429	+	4+	3+	+	–	–		–
	460	2+	4+	4+	4+	–	–		–
	970	±	2+	3+	3+	–		–	

convenient had not been developed hitherto. Indeed, the conventional method of diagnosis did not enable to detect mastitis at the initial or subclinical stage. On the other hand, the diagnosis of mastitis by the CL method can be conveniently and quickly performed to detect the degree of increase of the antibacterial activity of phagocytes which accumulate in the udder at the same time as the bacterial infection of the udder. By using the milk CL method, it is possible to detect the initial stage of mastitis since the method can detect even the bactericidal activity of a small number of phagocytes[6,9].

For the prevention and treatment of mastitis, attempts have been made to develop methods that enable to avoid the use of drugs like conventional antibiotics. Cytokine therapy[7,8,10] could become a promising method of treatment in future, as it is based on the activation of the host defense mechanisms. Furthermore, problems of drug tolerance and residues can be avoided, because cytokine is a biologically active substance naturally present in the animal. We participate in the Recombinant Cytokine Project which the National Institute of Animal Health is promoting and we are analyzing the effect of bovine GM-CSF of recombinant cytokine on udder affected with mastitis. In the experiment, the CMT value became negative 7 days after intra-udder injection of cytokine in an early case of mastitis (Table 1). As shown in Fig. 7(a), milk CL activity of the group with mastitis at an early stage markedly increased, with the maximum level recorded 6 h after the injection, followed by a rapid decrease to almost zero level 3 and 7 days after the cytokine injection. The increase in the CL activity corresponded to the inflow of phagocytes into the mammary cisterns and promotion of the bactericidal activity of the phagocytes[8]. As indicated in Fig. 7(b), milk SCC of the group with mastitis at an early stage markedly increased with a maximum level on day 1, followed by a rapid decline to the normal level (below 20×10^4 cells/mL) 7 days after the injection. Penetration of phagocytic leukocytes like neutrophils into the udder may have led to the

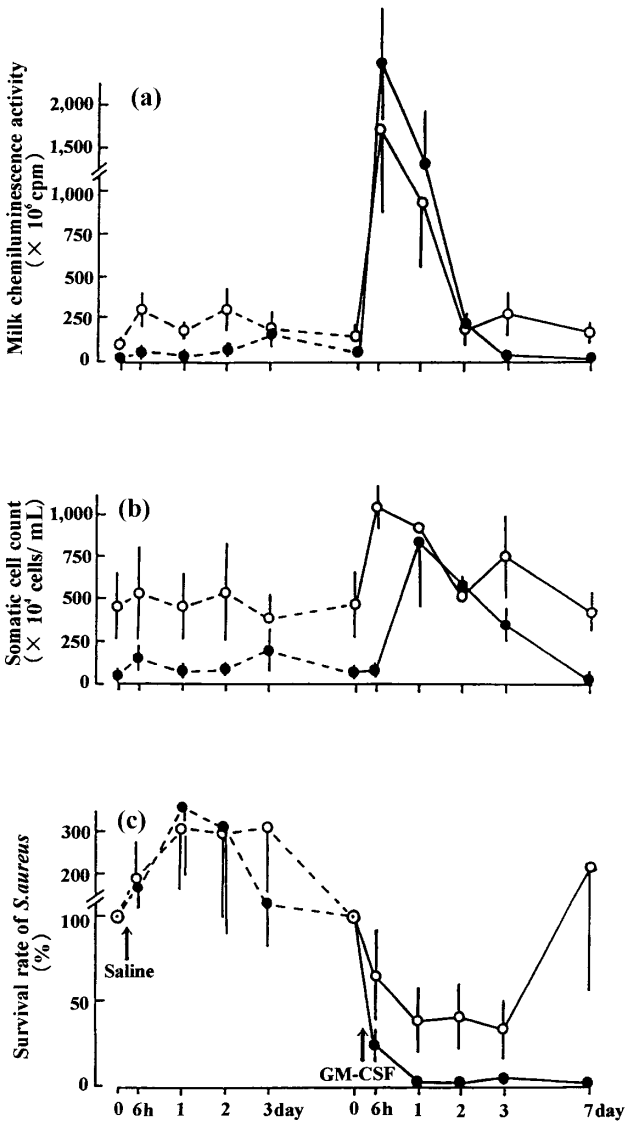

Fig. 7. Changes in milk CL activity, SCC and *S. aureus* survival rate following intra-udder injection of rBoGM-CSF in dairy cows with mastitis at early stage (● : n=3) and chronic stage (○ : n=4)

Each point and vertical bar represent the mean ± S.E. of 3 or 4 animals.

increase of milk SCC since GM-CSF activates the production and mobilization of neutrophils[10]. Furthermore, as indicated in Fig. 7(c), the survival rate of *S. aureus* declined drastically after the injection of cytokine. These results suggest that complete recovery is possible even in *S. aureus* mastitis which is most difficult to treat, if cytokine is applied at the initial stage of infection (Table 1 and Fig. 7)[9].

It is considered, as mentioned earlier, that the damage caused by mastitis amounts to ¥ 100 billion per year in Japan, with 80% due to the decrease in milk yield and quality. Therefore, it is important to diagnose mastitis at the initial and subclinical stages. In future, the use of the CL method for the early diagnosis of mastitis and the development of a method of administration of biologically active substances such as cytokine may enable to reduce the damage caused by mastitis.

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