Monoclonal Antibody against Bovine Lactoferricin® and Its Epitopic Site

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ABSTRACT. Bovine lactoferricin® (LFcin B) is a strong antimicrobial peptide derived from N-lobe of lactoferrin. To study the immunochemical and structural properties of LFcin B, monoclonal antibody (mAb) was prepared and the amino acid sequence concerning with the binding to mAb has been identified. Mice injected with LFcin B showed no production of antibody specific to this peptide, whereas those with LFcin B-KLH conjugate produced anti-LFcin B antibodies. None of the mAb reacted with bovine lactoferrin C-lobe, human lactoferrin or LFcin H. By the reactivity of the mAb against the peptides synthesized on cellulose membranes using SPOTs™ and against chemically modified derivatives of LFcin B, the antigenic determinant of LFcin B was identified to be the sequence of "QWR".—
KEY WORDS: lactoferricin, lactoferrin, monoclonal antibody.

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Lactoferrin is a multifunctional protein. It is known to exert bacteriostatic effects due to its ability to bind environmental iron [1]. Also, apo-lactoferrin has been shown to bind to microbial membranes and causes the direct destruction of microorganisms [7]. Other biological functions attributed to lactoferrin include roles in modulation of the inflammatory response, activation of the immune system, and control of myelopoiesis [5]. Recently, hydrolysates produced by gastric pepsin cleavage of human or bovine lactoferrin were found to contain a potent bactericidal peptide, named lactoferricin (LFcin) H and B, respectively [2]. The microbial killing effect of these peptides was 10 to 100 times stronger than that of undigested lactoferrin and LFcin B is about 9-fold more effective than LFcin H [2]. These active peptides were found to display broad-spectrum antibacterial properties, having effectiveness against Gram-positive and -negative bacteria, yeast and filamentous fungi [15]. LFcin B consists of a single peptide chain of 25 amino acid residues having the sequence FKCRRWQWRMKKLGAPSITCVRRAF [2], derived from the N-terminal region (17–41) of bovine lactoferrin. On the other hand, LFcin H is composed of two peptides linked by a disulfide bond and is comprised of 47 amino acid residues. LFcin B has been shown to have an affinity for cell membranes and may exert its lethal effect by disruption of essential membrane functions. It binds directly to lipopolysaccharide and disrupts the permeability barrier of the outer membrane of Gram-negative bacteria [16]. Also, LFcin B acts to disrupt the permeability properties of the cytoplasmic membrane, as suggested by the observation that it inhibits bacterial uptake of proline [3].

To clarify the structural relationship of the biological functions of LFcin, it seems that antibodies specific to LFcin B would be a useful tool. Therefore, the antigenicity of LFcin B was examined, and monoclonal antibody (mAb) specific to this peptide was prepared. Then the antigenic determinant recognized by the mAbs prepared in this study was identified by several methods.

Bovine lactoferrin, LFcin B, and chemically synthesized LFcin B were kindly supplied by the Nutritional Science Laboratory, Morinaga Milk Industry Co., Ltd. (Zama, Japan). Bovine lactoferrin C-lobe was prepared as reported previously [13].

The carrier protein coupling method [10] was used to enhance the antigenicity of the peptides. For this purpose, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide/HCl (Sigma Chem. Co., Ltd., St. Louis, MO) and keyhole limpet hemocyanin (KLH, Pierce Chem. Co., Rockford, IL) was used. To immunize mice with LFcin B or LFcin B-KLH conjugate, 100 μg/m*l* of the material was mixed with the same volume of Freund's complete adjuvant to form an emulsion. Eight week-old female BALB/c mice were injected intraperitoneally. On the days post primary inoculation, they were reinjected with the emulsified samples except in this instance Freund's incomplete adjuvant was used. Spleen cells isolated from the mice were fused with sp2/0-Ag-bearing mouse myeloma cells using polyethylene glycol (PEG 1500) [11]. Hybridoma cells were monoclonally selected twice by the limiting dilution culture method. Monoclonal antibody was obtained from the ascites fluid of BALB/c mice inoculated with the hybridoma cells.

Peptides were synthesized from Fmoc amino acid active esters on a pre-activated cellulose membrane [4] using SPOTs™ (Genosys Biotech. Inc., The Woodlands, TX). Detection of the mAb binding was carried out using horseradish peroxidase labelled secondary antibody. Functional groups of lysine, arginine and tryptophan residues of LFcin B were modified using succinic anhydride [9], 1,2-cyclohexanedione [12] and N-bromosuccinimide [14], respectively. The disulfide bond within LFcin B was reduced by treatment with dithiothreitol and then pyridylethylated (Pe-) with 4-vinylpyridine [6]. The Pe-LFcin B was separated from intact LFcin B by reverse phase (RP-) HPLC. For identification of Pe-LFcin B, partial N-

terminal amino acid sequences were determined using an Applied Biosystems Model 477A protein sequencer. Then, cyanogen bromide (CNBr) cleavage [8] of Pe-LFcin B was performed, and the fragments produced were separated by RP-HPLC. RP-HPLC was carried out using columns of TSKgel ODS-80T_S or ODS-120T_M (Tosoh Co., Ltd., Tokyo) at 40 °C. For elution, a mixture of eluents A (0.1% trifluoro acetic acid (TFA) in water) and B (0.1% TFA in 80% acetonitrile) was employed, using a linear gradient of A:B from 100:0 to 40:60 for 30 min at a flow rate of 1 ml/min.

None of the mice injected with LFcin B alone displayed detectable anti-LFcin B antibody production, even after the booster injection. Therefore, to produce mAbs specific to LFcin B, spleen cells were obtained from the mice inoculated LFcin B-KLH conjugate. After screening and cloning, four colonies were chosen for further study. One clone (5F12.1.2) showed reactivity to LFcin B at a concentration of 1 *ng*/m*l*. The other three clones showed reactivity to LFcin B at 100 *ng*/m*l*, but the reactivity was diminished at concentrations of 10 *ng*/m*l* or lower. Three clones showed high specificity to anti-mouse IgG1 antibody but the other clone (2F7.4.3) reacted with both anti-mouse IgG2b and IgM antibodies (Table 1). For further experiments, mAb produced by 5F12.1.2 cells was mainly used. These mAb showed reactivity against both chemically synthesized LFcin B, of which the sulfhydryl groups are acetamidomethylated, and natural LFcin B by ELISA. These antibodies showed a little reactivity against native state of bovine lactoferrin (Table 1), and they reacted with the denatured bovine lactoferrin (data not shown). None of them showed the reactivity with the C-lobe of bovine lactoferrin, human lactoferrin or LFcin H.

Fifty kinds of peptides corresponding to the LFcin B sequence were synthesized and their reactivity was studied (Fig. 1). Positive reactions were obtained with peptides 1, 2, 9, 10, 11, 18, 19, 20, 28, 29, 30, 31, 32, 39, 40, 41 and 42, and weak positive reactions were obtained with 21, 43 and 44. The common sequence found in each of the peptides recognized by the mAb is "QWR". The reactivity of the mAb against LFcin B derivatives having chemically modified lysine, tryptophan or arginine residues, as compared with intact LFcin B, was 96.5, 30.1 and 27.9%, respectively. The ratio of tryptophan residues modified was 70% estimated from the decrease in absorbance of the reaction mixture at 280 nm. The modified ratio of arginine and lysine residues has not been estimated. This observation supports the above result that the tryptophan and arginine residues of LFcin B are mainly involved in the epitope region recognized by the mAb. Therefore, we conclude that the sequence "QWR" is the antigenic determinant of LFcin B.

After the CNBr cleavage of the Pe-LFcin B fraction, the reaction mixture was separated into three fractions by RP-HPLC (Fig. 2). The last fraction (peak 3) eluted at the same position of the Pe-LFcin B having its disulfide bond cleaved and blocked. These three fractions were gathered, concentrated, and assayed for the reactivity against anti-LFcin B mAb. In the ELISA, the mAb did not show any reactivity against peaks 1 and 2, and showed only with the peak 3. This means that the epitope sequence is located very close to methionine residue, and the peptidyl

Table 1. Reactivity of mAbs to lactoferricin® B and lactoferrin and their subclass determination

Clones	lactoferricin B (*ng*/m*l*)			lactoferrin (*ng*/m*l*)	Subclass
	100	10	1	100	
5F12.1.2	0.55	0.42	0.25	<0.05	IgG1
2F7.4.2	0.64	0.08	0.01	<0.05	IgG1
2F7.4.3	0.48	0.04	0.00	<0.05	IgG2b, M
2F7.5.4	0.65	0.18	0.06	<0.05	IgG1

Reactivity of mAb is represented as the absorbance at 415 nm.

1 W**QWR**	14 MKKLG	26 KLGAPS	39 **KCRRWQWR**
2 **QWRM**	15 KKLGA	27 LGAPSI	40 **CRRWQWRM**
3 WRMK	16 KLGAP	28 **CRRWQWR**	41 **RRWQWRMK**
4 RMKK	17 LGAPS	29 **RRWQWRM**	42 **RWQWRMKK**
5 MKKL	18 **RRWQWR**	30 **RWQWRMK**	43 WQWRMKKL
6 KKLG	19 **RWQWRM**	31 **WQWRMKK**	44 QWRMKKLG
7 KLGA	20 **WQWRMK**	32 **QWRMKKL**	45 WRMKKLGA
8 LGAP	21 QWRMKK	33 WRMKKLG	46 RMKKLGAP
9 **RWQWR**	22 WRMKKL	34 RMKKLGA	47 MKKLGAPS
10 W**QWRM**	23 RMKKLG	35 MKKLGAP	48 KKLGAPSI
11 **QWRMK**	24 MKKLGA	36 KKLGAPS	49 KLGAPSIT
12 WRMKK	25 KKLGAP	37 KLGAPSI	50 LGAPSITC
13 RMKKL		38 LGAPSIT	

Fig. 1. Reactivity of anti-lactoferricin B mAb against synthetic peptides prepared by SPOTs™. Bold letters indicate that positive reactivity was observed (21, 43 and 44 were weakly positive). A common sequence in peptides reactive with the mAb is underlined. Antibodies obtained from clones 5F12.1.2 and 2F7.5.4 gave the same results.

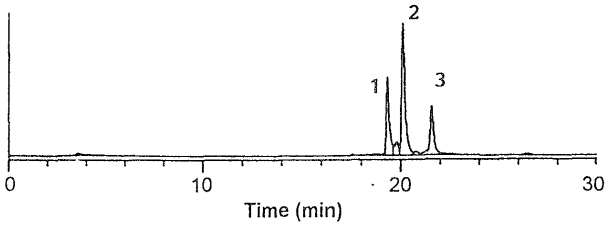

Fig. 2. Separation of the Pe-lactoferricin B (peak 3) and its fragments (peaks 1 and 2) after cyanogen bromide treatment. The column used was TSKgel ODS-120T (4.6 mm ID × 25 cm) and detection was at 210 nm.

homoserine lactone formed as a result of CNBr treatment [8] may hinder the binding of antibody. The antibody binding site of LFcin B is sequential or linear epitope, but the interaction of the bulky amino acid groups near this sequence would affect the antibody-peptide binding.

This "QWR" sequence could not been found in lactoferrin originated from human, pig, mouse and goat, transferrin originated from human, rabbit, pig, horse, rat, cockroach and African clawed frog, human melanotransferrin or ovotransferrin, all of which are members of the transferrin family proteins. These sequences were obtained from DNA Data Bank of Japan (Mishima). It has been reported that the sequence "RRWQWR" is the subregion essential for antimicrobial activity of LFcin B [15]. It is very interesting that the epitopic sequence "QWR" found in this study is located at the C-terminal half of this subregion sequence. The functional significance of this observation is now being explored in further experiments *in vivo* and *in vitro*.

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REFERENCES

1. Arnold, R. R., Cole, M. F., and McGhee, J. R. 1977. *Science* 197: 263–265.
2. Bellamy, W., Takase, M., Yamauchi, K., Wakabayashi, H., Kawase, K., and Tomita, M. 1992. *Biochim. Biophys. Acta* 1121: 130–136.
3. Bellamy, W. R., Wakabayashi, H., Takase, M., Kawase, K., Shimamura, S., and Tomita, M. 1993. *J. Appl. Bacteriol.* 75: 478–484.
4. Blankemeyer-Menge, B., Nimtz, M., and Frank, R. 1990. *Tetrahedron Lett.* 31: 1701–1704.
5. Brock, J. 1995. *Immunol. Today* 16: 417–419.
6. Cavins, J. F. and Friedman, M. 1970. *Anal. Biochem.* 35: 489–493.
7. Ellison, R. T. 1994. pp. 71-90. *In*: Lactoferrin: Structure and Function (Hutchens, T. W., Rumball, S. V., and Lönnerdal, B. eds.), Plenum Press, New York.
8. Gross, E. 1967. *Methods Enzymol.* 11. 238–255.
9. Klotz, I. M. 1967. *Methods Enzymol.* 11. 576–580.
10. Maloy, W. L., Coligan, J. E., and Paterson, Y. 1991. pp. 9.4.1–11. *In*: Current Protocols in Immunology (Coligan, J. E., Kruisbeek, A. M., Margulies, D. H., Shevach, E. M., and Strober, W. eds.), John Wiley and Sons, New York.
11. Oi, V. T. and Herzenberg, L. A. 1980. pp. 351–372. *In*: Selected Methods in Cellular Immunology (Mishell, B. B. and Shiigi, S. M. eds.), W. H. Freeman and Co., New York.
12. Patthy, L. and Smith, E. L. 1975. *J. Biol. Chem.* 250: 565–569.
13. Shimazaki, K., Tanaka, T., Kon, H., Oota, K., Kawaguchi, A., Maki, Y., Sato, T., Ueda, Y., Tomimura, T., and Shimamura, S. 1993. *J. Dairy Sci.* 76: 946–955.
14. Spande, T. F. and Witkop, B. 1967. *Methods Enzymol.* 11: 498–506.
15. Tomita, M., Takase, M., Bellamy, W., and Shimamura, S. 1994. *Acta Paediatrica Japonica* 36: 585–591.
16. Yamauchi, K., Tomita, M., Giehl, T. J., and Ellison, R. T. 1993. *Infect. Immun.* 61: 719–728.