Inhibition of Listeriolysin O-Induced Hemolysis by Bovine Lactoferrin

Kohji MORIISHI,^a Satoshi INOUE,^a Minako KOURA,^a and Fumio AMANO^{*,b}

Department of Veterinary Science,^a and Department of Biochemistry and Cell Biology,^b National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan. Received June 11, 1999; accepted July 23, 1999

Lactoferrin (LFR) plays an important role in the anti-microbial defense through iron binding, lipopolysaccharide binding and immunomodulation. In this study, we demonstrate that bovine LFR specifically inhibits the hemolytic activity of listeriolysin O (LLO) produced by *Listeria monocytogenes*. The hemolytic activity of LLO was completely inhibited in the presence of bovine LFR that was highly purified on two cation-exchange columns, whereas that of streptolysin O or perfringolysin O was not inhibited at all. A rabbit anti-LFR antibody canceled this inhibitory activity of bovine LFR. Although human transferrin exhibits 62% amino acid identity with bovine LFR, human apo-transferrin could not inhibit LLO-induced hemolysis. An increase in the concentration of FeCl₃ or the Fe³⁺-saturation of bovine LFR, however, slightly reduced its inhibition of the hemolysis. The inhibitory activity of bovine LFR was dependent on pH, since it was observed under neutral and alkali conditions, but not under acidic conditions. These results suggest that the inhibition of LLO-induced hemolysis by bovine LFR is influenced by pH and iron ions, both of which may lead to conformational changes of LFR.

Key words lactoferrin; *Listeria monocytogenes*; listeriolysin O; hemolysin; hemolysis

Listeria monocytogenes is a gram-positive, food-borne pathogen that causes the listeriosis involved in meningitis, encephalitis, and septicemia in domestic animals and humans.¹⁾ The intracellular growth of *L. monocytogenes* in these host cells is clearly correlated with the escape of the bacteria into the host cell cytoplasm.²⁾ A mutant with a transposon inserted into the *hlyA* gene failed to escape from phagosomes into the cytoplasm and thus became avirulent.³⁾ Expression of listeriolysin O (LLO) in *Bacillus subtilis* allowed the microorganism to escape into the cytoplasm of J774.1 macrophage-like cells.⁴⁾ These findings suggest that no factor except LLO is required for the escape into the host cytoplasm.

Lactoferrin (LFR) which comprises a single polypeptide chain with a molecular mass of 78 kDa, is an iron-binding protein that exhibits 62% amino acid identity with the serum iron-transporting protein, transferrin.⁵⁾ LFR is found in milk, tears and saliva, and is also a prominent component of the secondary granules of neutrophils.⁵⁾ Li *et al.*⁶⁾ reported that elevated levels of advanced glycation end products in tissues and serum of diabetic patients may inhibit endogenous anti-bacterial defense by binding to LFR and lysozyme, thereby increasing the susceptibility to bacterial infections of the diabetic population. Ballamy *et al.*⁷⁾ reported that the bactericidal effect on gram-positive and -negative bacteria is due to the N-terminal region of bovine LFR. These studies suggested that LFR plays an important role in non-specific anti-bacterial defense.

It is also known that LFR specifically binds to various microbial pathogens and that this binding seems to enhance its anti-microbial capacity. Wang *et al.*⁸⁾ reported that LFR secreted by neutrophils could inactivate lipopolysaccharide (LPS) to limit inflammation and prevent tissue damage through inhibition of priming against neutrophils. However, it was not known whether LFR could affect bacterial virulence factors other than LPS. In this paper, we demonstrate that bovine LFR specifically inhibits the hemolytic activity of LLO *in vitro*.

MATERIALS AND METHODS

Purification of LFR Bovine LFR of 95% purity was provided by Wako Chemicals (Tokyo, Japan), and was further purified by two cation-exchange column chromatographies to remove a little contamination. The starting preparation of LFR was dialyzed at 4 °C for 2 d against phosphate-buffered saline (PBS) containing 5 mM EDTA, and then at 4 °C overnight against 0.1 M phosphate buffer (pH 6.0). The resulting dialysate was applied to a 5 ml volume column of Econo-Pac[®] High CM Cartridge (Biorad, Hercules, CA) at the flow rate of 2 ml per min in 0.1 M phosphate buffer (pH 6.0). Bovine LFR was eluted as a single peak with a 0–0.5 M NaCl gradient. The eluted sample was collected as 2 ml fractions in glass tubes. Three fractions including the peak fraction were dialyzed against 1 l of 0.1 M phosphate buffer (pH 6.0) at 4 °C overnight. The dialysate was applied to a 1 ml volume column of Resource[™] S (Pharmacia Biotech, Uppsala, Sweden). The purified bovine LFR was eluted with a 0–1 M NaCl gradient.

Estimation of Hemolytic Activity and Inhibitory Activity toward Hemolysis Sheep erythrocytes (SRBC; Biotechnique Institute, Tokyo) were washed three times in PBS (pH 7.4) by centrifugation at 1000×*g* for 10 min, and then suspended in PBS (pH 7.4) containing 0.1 mg per ml bovine serum albumin (PBS-BSA). LLO was preincubated at 37 °C for the indicated times in 0.7 ml of PBS-BSA with or without LFR, and then added to 0.3 ml of 2.0×10⁸ erythrocytes per ml. The mixture was incubated at 37 °C for 30 min with rotation and then centrifuged at 10000×*g* for 10 s. The absorbance of the resulting supernatant was measured at 541 nm to detect the released hemoglobin. One hemolytic unit (HU) was taken as the amount of hemolysin needed to release one half of the hemoglobin from the erythrocytes, which was estimated graphically by plotting percentage lysis *versus* the amount of hemolysin on a log-probit graph. Although hemolytic activity of LLO was not stable and thus showed variation of the specific activity among the preparations, 1 HU usually corresponded to 100–200 ng protein when the hemolytic activity was assayed at pH 7.0. The inhi-

* To whom correspondence should be addressed.

bition of the hemolysis by bovine LFR was estimated as the difference between the hemolytic activity of 3 HU/ml hemolysin with and without bovine LFR. Sheep erythrocytes suspended in 0.012 M phosphate buffer (pH 5.8–7.9) containing 8 g/l NaCl, 0.2 g/l KCl and 0.1% bovine serum albumin were used for estimation of the pH dependency. Streptolysin O (SLO) and perfringolysin O (PLO) were generous gifts from Dr. Hiroko Sato (Department of Bacteriology, National Institute of Infectious Diseases). Bovine apo-LFR was prepared by dialysis at 4 °C for 2 d against 1 l of PBS containing 2 mM phenanthroline, and then at 4 °C overnight against 1 l of PBS. Iron-saturated LFR was prepared by the addition of 2 mM FeCl₃ to a 2 mg/ml solution of bovine LFR, followed by dialysis overnight against 1 l of PBS. Neutralization of the inhibitory effect of human LFR on LLO-induced hemolysis was performed by addition of anti-LFR antibody (Paesel & Lorei GmbH, Hanau, Germany) to the LFR followed by incubation with sheep erythrocytes and LLO.

Treatment of LFR with Proteases One hundred µg per ml of trypsin was mixed with a 10 fold higher concentration of soybean trypsin inhibitor (Sigma) in PBS, followed by incubation at 37 °C for 5 min. Bovine LFR was added at 1 mg/ml and the resultant mixture was used as an undigested control.

N-tosyl-L-phenylalanyl chloromethyl ketone (TPCK)-treated trypsin (Sigma) was finally added at 0.1 mg/ml to PBS containing 1 mg/ml LFR. The mixture was incubated at 37 °C for 2 h. The trypsinization was terminated by incubation at 37 °C for 5 min with a 10 fold higher concentration of soybean trypsin inhibitor. The resultant mixture was used as trypsinized LFR.

Pepsin was added at 0.1 mg/ml to 0.1 M glycine buffer (pH 3.0) containing 1 mg/ml LFR, followed by incubation at 37 °C for 2 h.⁷⁾ Proteinase K was added at 0.1 mg/ml to PBS containing 1 mg/ml LFR, followed by incubation at 37 °C for 2 h. The remaining activity of the protease was abolished by heat treatment at 90 °C for 5 min, respectively.

Construction of LLO Expression Vectors and Purification of Thioredoxin-Fused LLO A ThioFusion™ Expression System (Invitrogen, San Diego, CA), which allows a transformant to produce a thioredoxin-fusion protein, was used for the LLO expression.⁹⁾ Thioredoxin-fused LLO produced by *Escherichia coli* was purified on a rabbit anti-thioredoxin column as described previously.⁹⁾

Organisms *Listeria monocytogenes* strain LO28, and non-hemolytic mutant strains Bof415 and Bug337 were kindly donated by Dr. P. Cossart, Institut Pasteur, France. These strains were inoculated into 5 ml of brain heart infusion medium and then incubated at 37 °C overnight. The cultures were centrifuged at 2000×g for 10 min, and the resultant culture supernatants were kept at 4 °C until use.

RESULTS

Inhibition of LLO-Induced Hemolysis by Bovine LFR

L. monocytogenes mutant strain Bof415, derived from wild-type hemolytic strain LO28, becomes non-hemolytic on the insertion of a single copy of a transposon into the *hlyA* gene.²⁾ The codon, Trp-494, in the *hlyA* gene was mutated to Ala in mutant strain Bug337,³⁾ resulting in a non-hemolytic strain. Hemolytic activity was detected in the culture super-

Fig. 1. Expression of Hemolytic Activity in the Culture Supernatants of *L. monocytogenes* Strains and Inhibition of the Hemolytic Activity by Bovine LFR

Panel A: LLO and mutants of it were visualized with PhotBlot® Western Blot AP systems (Promega, Madison, WI) and an anti-LLO antibody in culture supernatants of *L. monocytogenes* wild-type strain LO28 (lane 1), and mutant strains Bug337 (lane 2) and Bof415 (lane 3). Panel B: The hemolytic activity in 0.05 ml of culture supernatant was estimated with or without 20 mg of bovine LFR. The results shown are representative of three independent experiments.

natant of LO28 but not in that of Bof415 or Bug337 (Fig. 1B), although the anti-LLO antibody reacted with a band corresponding to a molecular mass of 58 kDa for the culture supernatants of both LO28 and Bug337, and a band corresponding to a molecular mass of 42 kDa for the culture supernatant of Bof415 (Fig. 1A). Thus, the hemolytic activity in the culture supernatant of LO28 is due to the biological activity of LLO. The addition of bovine LFR inhibited the hemolytic activity in the culture supernatant of LO28 (Fig. 1B). The addition of bovine LFR also inhibited the hemolytic activity of recombinant LLO from *E. coli* (Fig. 2A). This inhibition was not due to the association of bovine LFR with LLO-fused thioredoxin, since thioredoxin by itself showed no inhibitory activity and did not influence the inhibitory activity of bovine LFR (Fig. 2B). A longer preincubation of thioredoxin with bovine LFR up to 30 min also had no effect (data not shown). Bovine LFR of 95% purity was further purified on Econo-Pac® High S and Resource™ S columns to confirm that this inhibition of hemolysis is caused by the bovine LFR molecule. A single peak was eluted with a gradient from 0.2–0.5 M NaCl on a column of Econo-Pac® High S (data not shown). The fractions around this peak were applied to a Resource™ S column with 1 ml of bed volume, following dialysis against 0.1 M phosphate buffer, pH 6.0. Three peaks were eluted between 0.5 and 0.7 M NaCl. Protein bands corresponding to the same molecular weight and the same inhibitory activity were detected around these peaks I, II, and III (data not shown). The purified bovine LFR in peak II gave a single band protein corresponding to a molecular mass of 75 kDa (Fig. 3A), which was detected with an anti-LFR antibody (Fig. 3B). The hemolytic activity of LLO was inhibited by the purified bovine LFR in peak II to the same extent as by the original preparation of bovine LFR (Fig. 3C). In addition, the anti-bovine LFR antibody abolished the inhibition of the hemolysis (Fig. 4). These results indicate that bovine LFR inhibits the hemolytic activity of LLO.

Figure 2A also shows that inhibition of the hemolysis was increased by preincubation of LLO with bovine LFR, suggesting that bovine LFR primarily interacts with LLO rather

Fig. 2. Inhibition of the Hemolytic Activity of Recombinant LLO in the Presence of LFR

Panel A: The hemolytic activity of 3 HU of thioredoxin-fused LLO was estimated with various concentrations of bovine LFR. LLO was preincubated at 37 °C for 5 min with bovine LFR (circles) and then mixed with sheep erythrocytes (SRBC). In the second case, LLO was preincubated at 37 °C for 5 min with SRBC (triangles) and then mixed with bovine LFR. In the third case, LLO was preincubated with bovine LFR and SRBC (squares) at 37 °C for 5 min. The hemolytic activity in each mixture was estimated by incubation at 37 °C for 30 min as described in the text. The results shown are representative of three independent experiments. Panel B: Thioredoxin (Thr) was purified from *E. coli* containing pTrxFus (Invitrogen) by chromatography on a Resource Q column. After 5 mg of bovine LFR had been incubated at 37 °C for 15 min with 30 mg of thioredoxin, 0.65 mg of LLO was added, followed by incubation at 37 °C for 5 min. Hemolytic activity was measured by addition of this mixture to SRBC as described in the text.

than with erythrocytes.

Effects of Bovine LFR on Hemolysis Induced by SLO and PLO LLO is one of the thiol-activated hemolysins, which include SLO, PLO, pneumolysin O and other hemolysins of gram-positive bacteria. LLO exhibits 40% and 43% amino acid identity with SLO and PLO, respectively. We attempted to determine whether or not bovine LFR could inhibit the hemolytic activities of thiol-activated hemolysins other than LLO. Figure 5 shows that bovine LFR could not inhibit the hemolytic activities of SLO and PLO even at concentrations above 10 μg/ml, whereas the hemolysis by 0.3 μg (5.2 pmol) of LLO was completely inhibited by more than 2 μg (26.1 pmol) of bovine LFR. These results suggest that the inhibition of LLO-induced hemolysis by bovine LFR is specific for LLO.

Protease Sensitivity of the Inhibitory Activity of Bovine LFR toward LLO-Induced Hemolysis LFR is a multifunctional molecule showing such activities as bactericidal effect, Fe³⁺ chelating activity, LPS binding and DNA binding. The treatment of bovine LFR with pepsin potentiated the bactericidal effect, since lactoferricin B, a fragment purified from a pepsin-treated LFR preparation, exhibits higher activity than that of the native molecule.⁷⁾ Figure 6 shows that the proteolysis of bovine LFR with pepsin, trypsin and proteinase K completely abolished the inhibition of the hemolysis. A trypsin inhibitor terminated tryptic digestion of LFR and reversed the trypsin-induced decrease in the inhibitory activity of bovine LFR.

pH Dependency of the Inhibitory Activity of Bovine LFR LLO is known to exhibit higher hemolytic activity at acidic pH than at neutral pH.¹⁰⁾ The amount of LLO for 50% hemolytic activity at pH 6.0 was about one-fourth that at pH 7.4 (Fig. 7A). Thus, inhibition of LLO-induced hemolysis by bovine LFR was measured in the pH range of 5.8 to 7.9. Figure 7B shows that bovine LFR did not inhibit the hemolytic activity of LLO under acidic conditions, whereas it completely inhibited this activity under neutral and alkaline conditions.

Effect of Iron-Binding on the Inhibition of LLO-Induced Hemolysis LFR is one of the iron binding proteins, like transferrin. Although human transferrin exhibits 62%

Fig. 3. Inhibition of LLO-Induced Hemolysis by Bovine LFR

Purified (lane 1) and crude (lane 2) bovine LFR were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then visualized by silver staining (panel A) or by PhotBlot[®] Western Blot AP systems using an anti-bovine LFR antibody (panel B). The inhibitory effects of purified bovine LFR (open) and crude (closed) LFR on the hemolysis of 3 HU of recombinant LLO were measured (panel C). The results shown are representative of three independent experiments.

Fig. 4. Effect of an Anti-Bovine LFR Antibody on the Inhibitory Activity of Bovine LFR

The suppressive effect of bovine LFR on hemolysis was measured after 5 µg of LFR had been incubated at 37 °C for 5 min with 10 or 100 µg of an anti-bovine LFR antibody. Then the mixture was incubated further with 3 HU of recombinant LLO at 37 °C for 5 min, and the hemolytic activity was assayed as described in the legend to Fig. 2A. The results shown are representative of three independent experiments.

Fig. 5. Specificity of the Inhibitory Activity of Bovine LFR

The hemolytic activity of SLO (squares), PLO (crosses), or LLO (circles) was assayed, and one hemolytic unit was calculated (panel A). The hemolytic activity of 3 HU hemolysin was also measured in the presence of various concentrations of bovine LFR (panel B). The results shown are representative of three independent experiments.

amino acid identity with LFR, the addition of human apo-transferrin to a final 0.37 mg/ml had no effect on the hemolytic activity induced by 300 ng/ml LLO. Inhibition of the hemolytic activity by LFR was slightly attenuated in the presence of 2 µM Fe³⁺. However, 20 µM Fe³⁺ could not greatly enhance this attenuation (Fig. 8A). Inhibition of the hemolytic activity by Fe³⁺-saturated LFR was slightly lower than that by apo-LFR (Fig. 8B). These results suggest that transferrin is not able to inhibit LLO-induced hemolysis, and that acquisition of Fe³⁺ by LFR leads to a moderate decrease in the inhibition of LLO-induced hemolysis by bovine LFR.

DISCUSSION

We have shown here that LLO-induced hemolysis was inhibited by bovine LFR in a dose-dependent manner. This inhibition of LLO-induced hemolysis is not due to contamination by a small amount of cholesterol and other compounds, since the extent of inhibition was not changed after purification of bovine LFR (Fig. 3); additionally, an anti-LFR antibody completely reversed the inhibition of hemolysis (Fig. 4). The inhibitory activity of bovine LFR seems to be specific toward LLO-induced hemolysis, because bovine LFR could not inhibit the hemolytic activity of SLO or PLO (Fig. 5).

E. coli LPS is known to bind to the N-terminal loop region comprising Arg-28 to Pro-34 of human LFR, which leads to impairment of neutrophil priming.¹¹⁾ Lactoferricin B, purified from a pepsin digest of bovine LFR, originates from the corresponding site comprising Phe-17 to Phe-41 in mature bovine LFR and is able to bind to [³H]LPS.^{7,12)} However, inhibition of the hemolysis by bovine LFR was completely abolished on treatment of LFR with pepsin. These findings suggest that the inhibition of LLO-induced hemolysis by bovine LFR is due to a region other than the bactericidal domain in the N-terminal region.

LFR is an iron-binding protein that exhibits 62% amino

Fig. 6. Effect of Protease-Treatment of Bovine LFR on Its Inhibition of Hemolysis

Panel A: Bovine LFR treated with pepsin (lane 2), trypsin (lane 3), or proteinase K (lane 5) was subjected to SDS-PAGE and then stained with Coomassie brilliant blue R-250. Molecular standard markers (lane M) and bovine LFR untreated (lane 1) or treated with a mixture of trypsin and trypsin inhibitor (lane 4) were also subjected to SDS-PAGE. Panel B: Inhibitory effect of bovine LFR on hemolysis was examined by incubation of LLO at 37 °C for 5 min with untreated or treated LFR. The results shown are representative of three independent experiments.

Fig. 7. pH Dependency of the Inhibitory Activity of Bovine LFR

The hemolytic activity of LLO was measured at pH 5.8 (open) and 7.4 (closed) to calculate one hemolytic unit (panel A). The hemolytic activity of 3 HU of LLO (150 to 600 ng) was measured at various pHs in the presence of a 65 fold higher molar amount of bovine LFR (10.6 to 53 μg). The results shown are representative of three independent experiments.

Fig. 8. Effect of Fe³⁺ on the Inhibitory Activity of Bovine LFR

The inhibition of LLO-induced hemolysis by bovine LFR was measured in the presence of various concentrations of iron ions (panel A). The hemolytic activity induced by 300 ng/ml LLO was also measured in the presence of various concentrations of iron-saturated (closed) or apo (open) bovine LFR (panel B). The results shown are representative of three independent experiments.

acid identity with transferrin. Human apo-transferrin, however, could not inhibit the hemolytic activity of LLO, despite the addition of a 1000-times higher amount of apo-transferrin. These results suggest that the inhibition of LLO-induced hemolysis by bovine LFR is not due to the transferrin-homologous region and is independent of iron chelation. The inhibitory activity slightly decreased on the addition of Fe^{3+} ions to the reaction mixture or on Fe^{3+} saturation of LFR (Fig. 8). The crystallographic structure of apo-human LFR showed a major conformational change compared to iron-loaded human LFR.¹³⁾ The conformational change of bovine LFR caused by iron binding might reduce its inhibitory effect on the hemolysis.

LLO disrupts the membranes of phagosomes, which are under acidic conditions, and thus allows bacteria to escape into the host cytoplasm. Similarly, the hemolytic activity of LLO was more potent under acidic conditions than under neutral or alkaline conditions¹¹⁾ (Fig. 7A). Figure 7 shows that bovine LFR inhibited the hemolytic activity of LLO under neutral and alkaline conditions, but not under acidic conditions even in the presence of excessive amounts of LFR to LLO. Thus, the escape of *L. monocytogenes* into the cytoplasm of host cells might not be prevented by the presence of LFR, since the intraphagosomal pH seems to be acidic.

A decrease in the number of host cells results in an increase in anti-listerial defense on exposure of *L. monocytogenes* to phagocytic cells. The apoptosis of dendritic cells¹⁴⁾ and hepatocytes¹⁵⁾ accompanied *L. monocytogenes* infection; apoptosis of the former was due to the lytic activity of LLO. The apoptosis of hepatocytes induced the release of neutrophil chemoattractants and thus eliminated infected cells rapidly, resulting in inhibition of the spread of *Listeria*.¹⁵⁾ Depletion of neutrophils, which secrete LFR from their secondary granules, enhanced the extent of the apoptosis.¹⁵⁾ LFR produced by neutrophils might inhibit the apoptosis of host cells through the inhibition of LLO-induced lytic activity. Unfortunately, it is not known whether LLO induces the apoptosis of hepatocytes or whether LFR inhibits the LLO-induced apoptosis. Further *in vivo* and *in vitro* studies are necessary to clarify of these points.

Acknowledgments This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Health and Welfare of Japan, as well as funds from the Japan Health Science Foundation, the Governmental Budget Related to Nuclear Research and Development for the National Research Organization, and funds from the Basic Research Core System for Facilitation of Science in Governmental Institutes from the Science and Technology Agency of Japan. We wish to thank Drs. Masashi Tatsumi and Takeshi Kurata for helpful discussions, and Drs. Hiroko Sato and Pascale Cossart for providing experimental materials.

REFERENCES

- 1) Sheehan B., Kocks C., Dramsi S., Gouin E., Klarsfeld A. D., Mengaud J., Cossart P., "Current Topics in Microbiology and Immunology," Vol. 192, ed. by Dangel J. L., Springer-Verlag, Berlin, Heidelberg, 1994, pp. 187—216.
- 2) Cossart P., Vicente M. F., Mengaud J., Bawuero F., Perez D. J. C., Berche P., *Infect. Immun.*, **57**, 3629—3636 (1989).
- 3) Michel K. A., Reich R., Favier P., Berche P., Cossart P., *Molecular Microbiol.*, **4**, 2167—2178 (1990).
- 4) Bielecki J., Philip Y., Patricia C., Portnoy D. A., *Nature* (London), **345**, 175—176 (1990).
- 5) Lonnerdal B., Iyer S., *Annu. Rev. Nutr.*, **15**, 93—110 (1995).
- 6) Li M. Y., Tan A. X., Vlassara H., *Nature Med.*, **1**, 1057—1061 (1995).
- 7) Ballamy W., Takase M., Wakabayashi H., Kawase K., Tomita M., *J. Appl. Bacteriol.*, **73**, 472—479 (1992).
- 8) Wang D., Pabst K. M., Aida Y., Pabst M. J., *J. Leukoc. Biol.*, **57**, 865—874 (1995).
- 9) Moriishi K., Koura M., Amano F., *FEMS Immunol. Med. Microbiol.*, **16**, 213—222 (1996).
- 10) Ellass-Rochard E., Roseanu A., Legrand D., Trif M., Salmon V., Motas C., Montreuil J., Spik G., *Biochem. J.*, **312**, 839—845 (1995).
- 11) Geoffroy C., Gaillard J. L., Alouf J., Berche P., *Infect. Immun.*, **55**, 1641—1646 (1987).
- 12) Yamauchi K., Tomita M., Giehl T. J., Ellison III R. T., *Infect. Immun.*, **61**, 719—728 (1993).
- 13) Anderson B. F., Baker M. H., Norris G. E., Rumball S. V., Baker E. N., *Nature* (London), **344**, 784—787 (1990).
- 14) Guzman C. A., Domann E., Rohde M., Bruder D., Darji A., Weiss S., Wehland J., Chakraborty T., Timmis K. N., *Mol. Microbiol.*, **20**, 119—126 (1996).
- 15) Rogers H. W., Callery M. P., Deck B., Unanue E. R., *J. Immunol.*, **156**, 679—684 (1996).