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Activation of Intestinal Mucosal Immunity in Tumor-bearing Mice by Lactoferrin

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We have previously demonstrated that oral administration of bovine lactoferrin (bLF) markedly increases CD4$^+$ and CD8$^+$ T cells and NK (asialoGM1$^+$) cells in the blood of tumor-bearing mice and enhances anti-metastatic activity. In this paper, we document that oral administration of bLF and bLF-hydrolysate (bLFH) is associated with strong increases in CD4$^+$ and CD8$^+$ T, as well as asialoGM1$^+$ cells in lymphoid tissues and lamina propria of the small intestine in mice, especially in tumor-bearing animals in which Co26Lu cells were implanted subcutaneously. Moreover, IgM$^+$ and IgA$^+$ B cells in lamina propria of the small intestine were also significantly increased by bLF and bLFH. Bovine apo-transferrin (bTF) did not exhibit such activity. In the colon, only CD8$^+$ cells were significantly increased by treatment with bLF, while asialoGM1$^+$ cells were significantly decreased. bLF and bLFH induced cytokines to activate T, B and asialoGM1$^+$ cells. Administration of bLF and bLFH, but not bTF, increased production of interleukin-18 (IL-18), interferon-gamma (IFN-γ) and caspase-1 in the mucosa of the small intestine. Particularly high levels of IL-18 were found in the epithelial cells of the small intestine. Moreover, administration of bLF and bLFH, but not bTF, induced IFN-γ presenting cells in the small intestine. Caspase-1, which processes proIL-18 to mature IL-18, was also induced in the epithelial cells of the small intestine following treatment with bLF and bLFH, but not with bTF. These results suggest that enhanced production of IL-18 and IFN-γ and caspase-1 induction by treatment with bLF may be important for elevation of intestinal mucosal immunity.

Key words: Lactoferrin — Small intestine — Interleukin-18 — Interferon-gamma — Caspase-1

Lung metastatic colony formation in mice subcutaneously implanted with highly metastatic colon carcinoma (Co26Lu) cells has been found to be significantly decreased by oral administration of bovine lactoferrin (bLF),[1)] in line with the observed inhibition of tumor growth and lung metastatic colony formation upon intraperitoneal or subcutaneous administration of human LF[2)] and bLF.[3)] In addition, oral administration of bLF strongly inhibits colon carcinoma development after azoxymethane treatment in rats.[4)] Possible mechanisms underlying the antimetastatic and anticarcinogenesis activity of bLF are postulated to be enhanced NK activity[1,5)] and anti-angiogenesis.[3)] Tumor cells in the blood released from primary lesions may be killed by T and NK cells, so that lung metastatic colony formation is decreased. We confirmed that CD4$^+$ and CD8$^+$ T cells and asialoGM1$^+$ (NK) cells after oral administration of bLF were markedly increased in the white blood cell fraction.[1)] CD8$^+$ T and asialoGM1$^+$ cells showed marked cytotoxicity against Co26Lu cells *in vitro*. bLF is also reported to stimulate the phagocytic activity of neutrophils.[6)] For such an immunological phenomenon, cytokine production may be important. The intestinal mucosa is the first line in host defense and is exposed to a great number of antigens from foods and bacteria. Orally administered bLF may also interact with the epithelial cells and lymphocytes in gut mucosa and stimulatory activity on mucosal immunity, with enhanced IgA and IgG secretion, has been reported.[7)] Recently we found a pro-inflammatory cytokine, interleukin-18 (IL-18), to be elevated in epithelial tissues of the small intestine following treatment with bLF (unpublished results). IL-18 is produced by various cells such as macrophages, keratinocytes and intestinal epithelial cells,[8)] and acts on various types of cells to influence strongly their expression of various genes, including interferon-gamma (*IFN-γ*) gene. IL-18 also enhances Th1-type T and asialoGM1$^+$ cell responses and generates CD8$^+$ T cells.[9)] In this study, we determined T, B and NK cells in the small intestine and detected IL-18 and IFN-γ by immunohistochemical methods. Moreover, caspase-1, which is related to the activation of proIL-18, was also detected immunohistochemically.

MATERIALS AND METHODS

bLF, bLF-hydrolysate (bLFH) and bovine apo-transferrin (apo-bTF) bLF (purity 98%) and bLFH, generated by acid-pepsin hydrolysis,[10)] were purchased from Mori-

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naga Milk Industry Co., Ltd. (Zama, Kanagawa). bTF was purchased from Sigma Chemical Co. (St. Louis, MO).

Mice BALB/c mice, 5-week-old males, were obtained from Charles River Japan (Atsugi). The animals were allowed free access to CE-2 pellet diets (CLEA Japan, Tokyo) and tap water, and were maintained in plastic cages on woodchip bedding under specific pathogen-free conditions with a controlled temperature (24±2°C) and a 12 h light-dark cycle. The experiments were performed when the mice were 6 weeks old.

Murine colon carcinoma 26Lu (Co26Lu) implantation The highly metastatic Co26Lu line[11] (1×10^5 cells per mouse) was implanted subcutaneously into the right thighs of the mice. bLF, bLFH and bTF were orally administered daily at 300 mg/kg from day 11 for 3 days to tumor-bearing or non-tumor-bearing mice (5 mice/group).

Tissue sections and staining Twenty-four hour after oral administration of bLF at 300 mg/kg for 3 days, mice were anesthetized and killed, and the small intestine was removed. Tissues were fixed in acetone at 4°C, embedded in paraffin, serially sectioned at 3.5 μm, and stained with anti-CD4 mAb (rat anti-mouse L3T4, GK1.5, Southern Biotechnology Association, Inc., Birmingham, AL), CD8 mAb (rat anti-mouse Lyt-2, 53-6.7, Southern Biotechnology Association, Inc.), rabbit anti-mouse caspase-1 p10 (Santa Cruz Biotechnology Inc., Santa Cruz, CA) and rat anti-mouse IFN-γ (RMMG-1, PBL Biomedical Lab., New Brunswick, NJ). Immunoreactions were detected using biotinylated rat or rabbit IgG and a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). Goat anti-mouse IL-18 antibody and anti-goat IgG-FITC were purchased from Santa Cruz Biotechnology Inc. Goat anti-mouse IgM (μ-chain specific)- and goat anti-mouse IgA (α-chain specific)-FITC conjugates were purchased from Sigma Co.

Results were expressed as the ratio of the total number of cells (%) in the lymphoid tissues or the mean of the number of positive cells in lamina propria per 10 light microscope fields (×200).

RESULTS

Influence of orally administered bLF, bLFH and bTF on T, B and asialoGM1$^+$ cells in the small and large intestines of mouse bLF and bLFH administration has both prophylactic and therapeutic effects against lung metastasis.[1] In this study, we investigated whether orally administered bLF or bLFH has immune-modulating functions in lymphoid tissues of the small intestine and colon of normal or tumor-bearing mice. Oral administration of bLF significantly increased the number of CD4$^+$ (Fig. 1), CD8$^+$ (Fig. 2), and asialoGM1$^+$ cells (Fig. 3) in the lymphoid tissues (A) and lamina propria (B) of the small intestine, but bTF did not. In lymphoid tissues of the colon, CD8$^+$ cells were also significantly increased by treatment with bLF (Fig. 4B). In contrast, asialoGM1$^+$ cells were markedly decreased (Fig. 4C). IgM$^+$ (Fig. 5A) and IgA$^+$ (Fig. 5B) cells were also significantly increased in the small intestine, especially in tumor-bearing mice.

Induction of IL-18 and IFN-γ positive cells by treatment with bLF, bLFH and bTF IL-18 is constitutively expressed in intestinal epithelium.[8] When bLF was orally administered to mice, high levels of IL-18 were found in the intestinal epithelium (Fig. 6, A and B). IL-18 is reported to activate T and NK cells,[12, 13] which produce IFN-γ.[14] Fig. 6C and D show that IFN-γ positive cells were markedly increased in the bLF, but not the bTF treat-

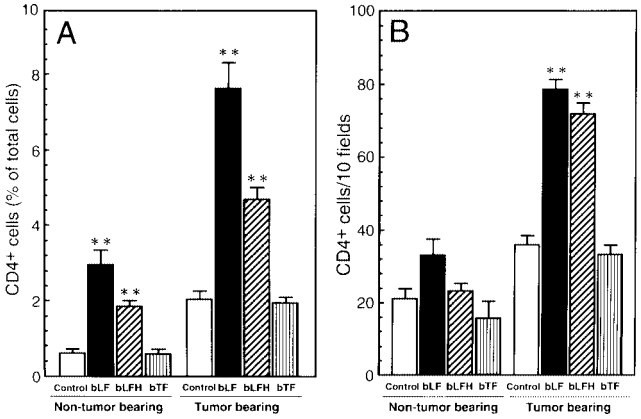

Fig. 1. Effects of oral administration of bLF, bLFH and bTF on numbers of CD4$^+$ cells in lymphoid tissues (A) and lamina propria (B) of small intestine of non-tumor-bearing and tumor-bearing mice (×200). Mean±SE (n=5), ** P<0.01.

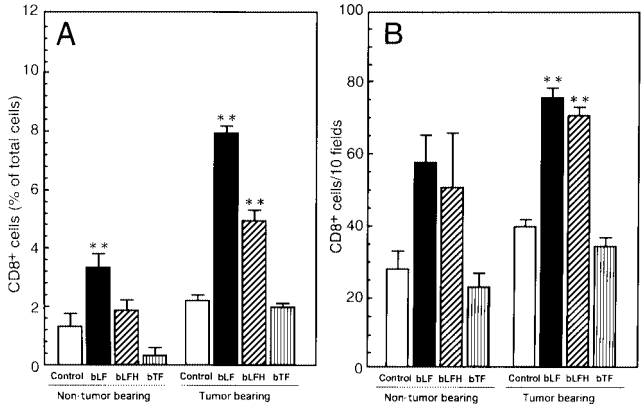

Fig. 2. Effects of oral administration of bLF, bLFH and bTF on numbers of CD8$^+$ cells in lymphoid tissues (A) and lamina propria (B) of small intestine of non-tumor-bearing and tumor-bearing mice (×200). Mean±SE (n=5), ** P<0.01.

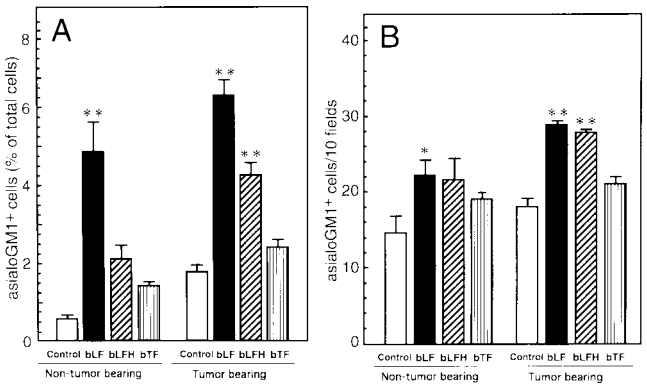

Fig. 3. Effects of oral administration of bLF, bLFH and bTF on numbers of asialoGM1$^+$ cells in lymphoid tissues (A) and lamina propria (B) of small intestine of non-tumor-bearing and tumor-bearing mice (×200). Mean±SE (n=5), ** P<0.01.

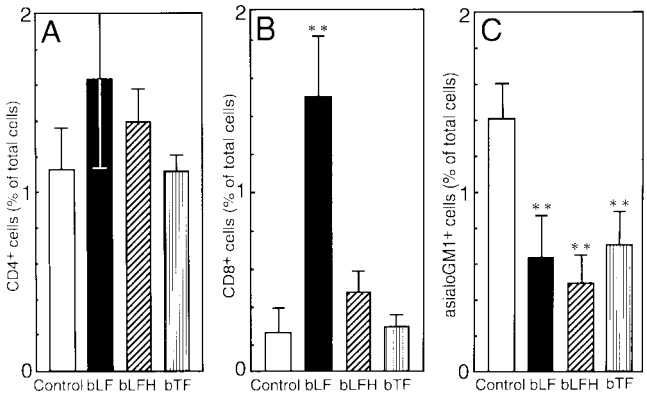

Fig. 4. Effects of oral administration of bLF, bLFH and bTF on numbers of CD4$^+$ (A), CD8$^+$ (B) and asialoGM1$^+$ (C) cells in lymphoid tissues of colon of non-tumor-bearing mice (×200). Mean±SE (n=5), ** P<0.01.

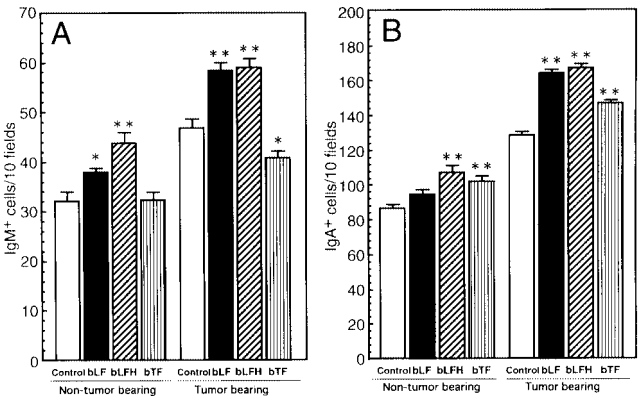

Fig. 5. Effects of oral administration of bLF, bLFH and bTF on numbers of IgM$^+$ (A) and IgA$^+$ (B) cells in lamina propria of small intestine of non-tumor-bearing and tumor-bearing mice (×200). Mean±SE (n=5), * P<0.05, ** P<0.01.

Table I. IFN-γ$^+$ Cells Following Treatment with bLF, bLFH and bTF in the Lamina Propria of the Small Intestine

Treatment	No. of IFN-γ$^+$ cells
Non-tumor-bearing mice	
Untreated control	23.8±0.85
bLF	65.1±1.93[a)]
bLFH	79.1±2.82[a)]
bTF	22.6±1.06
Tumor-bearing mice	
Untreated control	51.3±1.00
bLF	91.4±1.89[a)]
bLFH	100.7±2.22[a)]
bTF	52.0±0.83

The IFN-γ$^+$ cells were determined by an immunostaining test using mouse IFN-γ monoclonal antibody 24 h after 3 days of treatments with bLF, bLFH and bTF at 300 mg/kg/day. The values are arithmetic means±SE (n=5) of the number of cells in 10 fields (×100) expressing the marker.
a) P<0.01 compared with the untreated control value (Dunnett's modification of Student's t test).

ment group (Table I). bLF and bLFH treatment increased IFN-γ-positive cells 1.8 and 2.0-fold compared with untreated control tumor-bearing mice, respectively.

Production of caspase-1 following treatment with bLF, bLFH and bTF Murine IL-18 is synthesized as a proIL-18 (24 kD) and cleaved by IL-1β converting enzyme (ICE, caspase-1) to the active form (18 kD).[15] As shown in Fig. 6E and F, caspase-1 was also found to be elevated after bLF, but not bTF treatment.

DISCUSSION

The intestinal mucosa, as the first line in host defense, is exposed to a great number of antigens. Induction of a mucosal immune response is not simple, owing to the development of oral tolerance, but under some conditions, bacteria and foods can activate this immune system. The gut associated lymphoid tissue is thus a well developed immune network that is involved in protection of the host against pathogens. Lactoferrin and several of its peptides generated by gastric pepsin (LFH) may bind to epithelial cells of the small intestine or interact with M cells of Peyer's patches and induce a mucosal immune response. We have previously demonstrated that orally administered

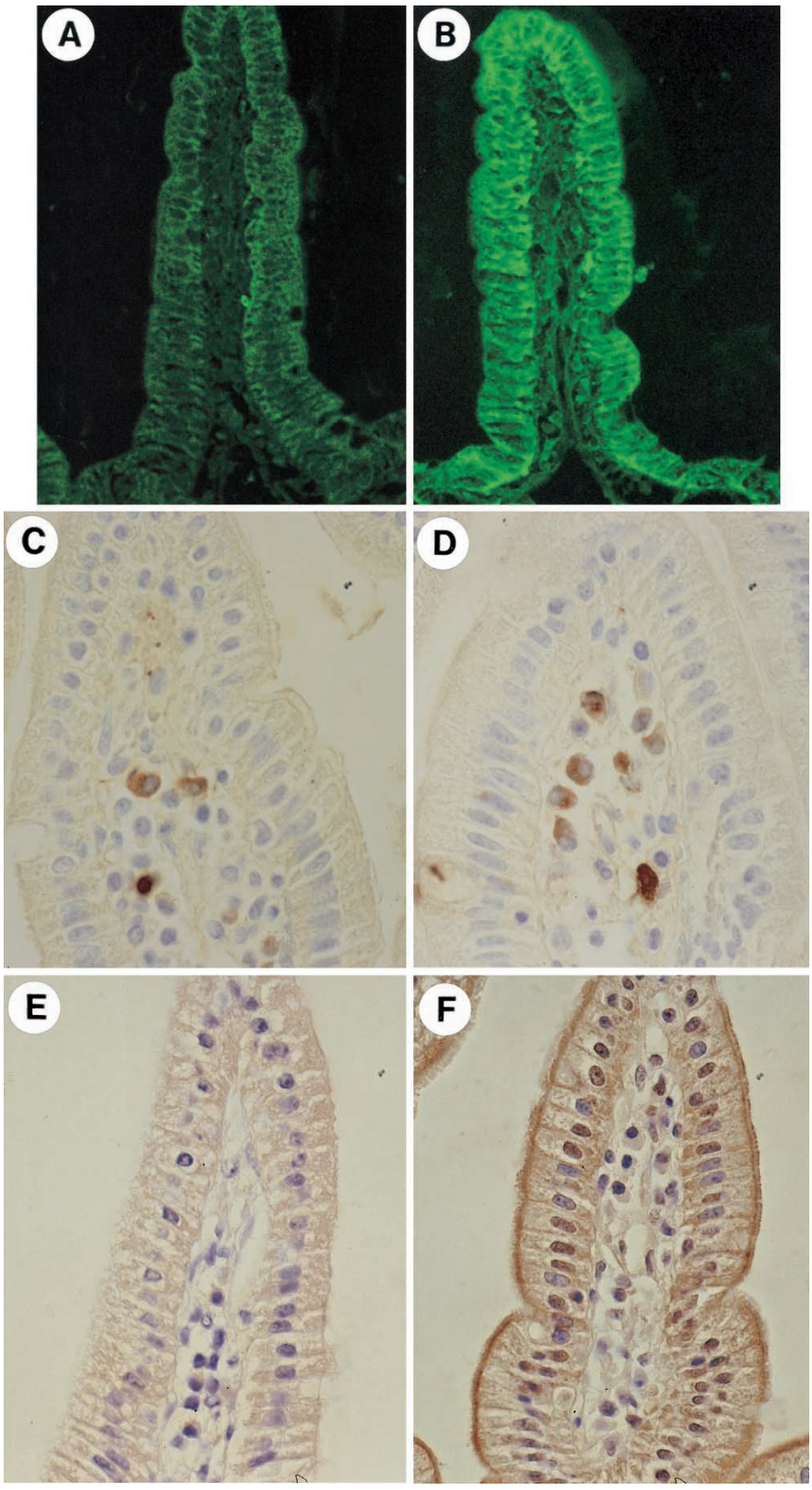

Fig. 6. Immunohistochemical staining of IL-18 (A, B, original magnification, ×200), IFN-γ (C, D, original magnification, ×400) and caspase-1 (E, F, original magnification, ×400) in small intestine of untreated (A, C, E) and bLF-treated tumor-bearing mice (B, D, F).

bLF markedly increases $CD4^+$ and $CD8^+$ T cells and asialoGM1$^+$ cells in the blood and inhibits lung metastatic colony formation.[1)]

In this study, $CD4^+$ and $CD8^+$ T cells, IgM^+ and IgA^+ B cells and asialoGM1$^+$ cells in the small intestine were markedly increased by bLF treatment, especially in tumor-bearing mice. However, $CD4^+$ and $CD8^+$ T cells were not increased by bLF treatment in athymic nude mice (data not shown). In our previous report,[1)] we did not find any intact bLF in serum after oral administration of bLF, suggesting that bLF itself may directly affect the immuno-system in the gastrointestinal tract. Though bLF and bLFH may induce an antigen-specific proliferative response of T cells though M cells,[16)] activation of T and NK cells could be related to cytokines released from cells in the mucosa of the small intestine. The constitutive *IL-18* gene expression present in epithelial cells of the small intestine, as well as whole blood cells and spleen cells, was here found to be elevated by bLF. Intestinal epithelial cells may be the main producers of IL-18 under physiological conditions and this may have an important role in the induction of mucosal immunity.[8)] Moreover, caspase-1 also appeared to be markedly induced by treatment with bLF and bLFH in epithelial cells. Along with $CD4^+$ T cells, IL-18 plays an important role in the generation of $CD8^+$ T cells.[9)] The many IFN-γ^+ cells observed in the lamina propria of the small intestine after treatment with bLF or bLFH might thus be $CD8^+$ T cells and asialoGM1$^+$ cells.

Anti-metastasis[1)] and anti-carcinogenesis[4, 5, 17, 18)] by bLF or bLFH might be due to enhanced cellular immunity mediated by IL-18 production. The increase in the T, B and asialoGM1$^+$ cells in the small intestine suggests enhancement of their migration and/or generation by bLF or bLFH. Thus, bLF may activate gut mucosal immunity by stimulating production of IL-18.

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REFERENCES

1) Iigo, M., Kuhara, T., Ushida, Y., Sekine, K., Moore, M. A. and Tsuda, H. Inhibitory effects of bovine lactoferrin on colon carcinoma 26 lung metastasis in mice. *Clin. Exp. Metastasis*, **17**, 35–40 (1999).
2) Bezault, J., Bhimani, R., Wiprovnick, J. and Furmanski, P. Human lactoferrin inhibits growth of solid tumors and development of experimental metastases in mice. *Cancer Res.*, **54**, 2310–2312 (1994).
3) Yoo, Y.-C., Watanabe, S., Watanabe, R., Hata, K., Shimazaki, K. and Azuma, I. Bovine lactoferrin and lactoferricin, a peptide derived from bovine lactoferrin, inhibit tumor metastasis in mice. *Jpn. J. Cancer Res.*, **88**, 184–190 (1997).
4) Sekine, K., Watanabe, E., Nakamura, J., Takasuka, N., Kim, D. J., Asamoto, M., Krutovskikh, V., Baba-Toriyama, H., Ota, T., Moore, M. A., Masuda, M., Sugimoto, H., Nishino, H., Kakizoe, T. and Tsuda, H. Inhibition of azoxymethane-initiated colon tumor by bovine lactoferrin administration in F344 rats. *Jpn. J. Cancer Res.*, **88**, 523–526 (1997).
5) Sekine, K., Ushida, Y., Kuhara, T., Iigo, M., Baba-Toriyama, H., Moore, M. A., Murakoshi, M., Satomi, Y., Nishino, H., Kakizoe, T. and Tsuda, H. Inhibition of initiation and early stage development of aberrant crypt foci and enhanced natural killer activity in male rats administered bovine lactoferrin concomitantly with azoxymethane. *Cancer Lett.*, **121**, 211–216 (1997).
6) Miyauchi, H., Hashimoto, S., Nakajima, M., Shinoda, I., Fukuwatari, Y. and Hasawa, H. Bovine lactoferrin stimulates the phagocytic activity of human neutrophils: identification of its active domain. *Cell. Immunol.*, **187**, 34–37 (1998).
7) Debbabi, H., Dubarry, M., Rautureau, F. and Tome, D. Bovine lactoferrin induces both mucosal and systemic immune response in mice. *J. Dairy Res.*, **65**, 283–293 (1998).
8) Takeuchi, M., Nishizaki, Y., Sano, O., Ohta, T., Ikeda, M. and Kurimoto, M. Immunohistochemical and immuno-electron-microscopic detection of interferon-gamma-inducing factor ("interleukin-18") in mouse intestinal epithelial cells. *Cell Tissue Res.*, **289**, 499–503 (1997).
9) Okamoto, I., Kohno, K., Tanimoto, T., Ikegami, H. and Kurimoto, M. Development of $CD8^+$ effector T cells is differentially regulated by IL-18 and IL-12. *J. Immunol.*, **162**, 3202–3211 (1999).
10) Tomita, M., Bellamy, W., Takase, M., Yamauchi, K., Wakabayashi, H. and Kawase, K. Potent antibacterial peptides generated by pepsin digestion of bovine lactoferrin. *J. Dairy Sci.*, **74**, 4137–4142 (1992).
11) Iigo, M., Tsuda, H. and Moriyama, M. Enhanced therapeutic effects of anti-tumour agents against growth and metastasis of colon carcinoma 26 when given in combination with interferon and interleukin-2. *Clin. Exp. Metastasis*, **12**, 368–374 (1994).
12) Kohyama, M., Saijyo, K., Hayasida, M., Yasugi, T., Kurimoto, M. and Ohno, T. Direct activation of human

$CD8^+$ cytotoxic T lymphocytes by interleukin-18. *Jpn. J. Cancer Res.*, **89**, 1041–1046 (1998).
13) Tomura, M., Zhou, X. Y., Maruo, S., Ahn, H. J., Hamaoka, T., Okamura, H., Nakanishi, K., Tanimoto, T., Kurimoto, M. and Fujiwara, H. A critical role for IL-18 in the proliferation and activation of $NK1.1^+$ CD3-cells. *J. Immunol.*, **160**, 4738–4746 (1998).
14) Micallef, M. J., Ohtsuki, T., Kohno, K., Tanabe, F., Ushio, S., Namba, M., Tanimoto, T., Torigoe, K., Fujii, M., Ikeda, M., Fukuda, S. and Kurimoto, M. Interferon-gamma-inducing factor enhances T helper 1 cytokine production by stimulated human T cells: synergism with interleukin-12 for interferon-gamma production. *Eur. J. Immunol.*, **26**, 1647–1651 (1996).
15) Ghayur, T., Banerjee, S., Hugunin, M., Butler, D., Herzog, L., Carter, A., Quintal, L., Sekut, L., Talanian, R., Paskind, M., Wong, W., Kamen, R., Tracey, D. and Allen, H. Caspase-1 processes IFN-gamma-inducing factor and regulates LPS-induced IFN-gamma production. *Nature*, **386**, 619–623 (1997).
16) Neutra, M. and Krachenbuhl, J. M cells as a pathway for antigen uptake and processing. *In* "Essentials of Mucosal Immunology," ed. M. Kagnoff and H. Kiyono, pp. 29–36 (1996). Academic Press, Inc., San Diego, CA.
17) Ushida, Y., Sekine, K., Kuhara, T., Takasuka, N., Iigo, M., Maeda, M. and Tsuda, H. Possible chemopreventive effects of bovine lactoferrin on esophagus and lung carcinogenesis in the rat. *Jpn. J. Cancer Res.*, **90**, 262–267 (1999).
18) Tanaka, T., Kawabata, K., Kohno, H., Honjo, S., Murakami, M., Ota, T. and Tsuda, H. Chemopreventive effect of bovine lactoferrin on 4-nitroquinoline 1-oxide-induced tongue carcinogenesis in male F344 rats. *Jpn. J. Cancer Res.*, **91**, 25–33 (2000).