production [8] and enhance mucosal immunity [9], NK cell activity [10,11], LAK activity [11], neutrophil activity [12,13], and macrophage cytotoxicity [14,15]. Previously we found that the pro-inflammatory cytokine interleukin-18 (IL-18) was produced by epithelial cells of the small intestine following administration of bLF [9,16]. IL-18 enhances Th1 type T and NK cell responses and generates $CD8^+$T cells [17].

Bezault et al. [18] report a protective effect of human-LF against the growth of solid tumors and the development of metastases in mice. bLF also inhibits lung metastasis of B16 melanoma and colon 26 tumor cells in mice [19,20] and is protective against tongue, esophagus, intestinal, lung, and bladder carcinogenesis in rats [21–24]. bLF administration results in a significant increase of $CD4^+$ and $CD8^+$T cells and asialo GM_1^+ (NK) cells in the blood [16] and in the lymphoid tissues and lamina propria of the small intestine [9]: $CD8^+$T and asialo $GM1^+$ cells exhibit marked cytotoxicity against Co26Lu cells *in vitro* [16]. The observed increase in T and NK cells may be due to the increased levels of mature IL-18 produced by epithelial cells of the small intestine after treatment with bLF [16].

In Co26Lu bearing-SCID mice, which are deficient in T and B cells, bLF also showed significant inhibition of tumor growth (Fig. 1) and lung metastatic colony formation [7]. However, the number of lung metastatic colonies was markedly increased when these mice were treated with anti-asialo GM_1 antibodies to weaken their NK cell activity. Bezault et al. [18] also report that LF-mediated antitumor effects were lost upon elimination of NK cell activity by pretreatment of mice with anti-asialo GM_1 antibodies. These results demonstrate the importance of activated NK cells for LF-mediated inhibition of tumor growth and metastasis.

The immunomodulatory and antitumor activities of bLF suggest that it may be a promising chemopreventer of carcinogenesis [25,26]. In this study we investigated pathways by which ingested bLF can exert its effects.

2. Materials and methods

2.1. bLF, bLFH, bLFcin and bTF

Bovine lactoferrin (bLF) (purity: greater than 96% of total protein by HPLC; endotoxin: less than 5.0 pg/mg of bLF; iron content: 143 ± 19 ng/mg of bLF, $n = 3$), a pepsin-generated hydrolysate of bLF (bLFH: endotoxin, 101.3 ± 25.7 pg/mg of bLFH; iron content, 100 ± 0 ng/mg of bLFH, $n = 3$), and lactoferricin (bLFcin: endotoxin, 101.3 ± 25.7 pg/mg of bLFcin, $n = 3$) were obtained from Morinaga Milk Industry Co., Ltd. (Zama, Japan). Bovine transferrin (bTF) (purity: greater than 98% of total protein by agarose electrophoresis; endotoxin, 16.4 ± 2.8 pg/mg of bTF; iron content, 13 ± 3 ng/mg of bTF, $n = 3$) was purchased from Sigma Co. Ltd. (St. Louis, MO). Endotoxin (by the modified limulus test) and iron (by the 2-nitroso-5-[*N*-*n*-propyl-*N*-(3-sulfopropyl)amino]phenol test) content were determined by SRL Inc., Hachioji, Japan.

2.2. Cytokines and antibodies

Anti-asialo GM_1 Ab was purchased from Wako Pure Chemical Industries (Osaka, Japan; caspase-1/ICE inhibitor (Acyl-Tyr-Val-Ala-Asp-chloromethylketone) was purchased from Medical & Biological Laboratories Co., Ltd. (Nagoya, Japan); recombinant murine IFNγ was purchased from Intergen Company (Purchase, NY); recombinant murine IL-7 was purchased from PeproTech House (London, England); recombinant murine IL-18 was purchased from Medical & Biological Laboratories Co., Ltd.; recombinant IFNαA/D (2.04×10^7 IU/ml) was obtained from Nippon Roche Research Center, (Kamakura, Japan); anti-mouse IFNα antibody (clone F18, rat IgG_1) was purchased from HyCult Biotechnology b.v.; goat anti-murine IL-1β (M-20, goat IgG) was purchased from Santa Cruz Biotechnology, Inc.; anti-murine IL-7 polyclonal antibody was purchased from Pepro Tech EC Ltd.; anti-mouse IL-12 antibody (p40/p70) was purchased from PharMingen, BD Biosciences; rabbit anti-mouse IL-15 antibody was purchased from Torrey Pines Biolabs, Inc.

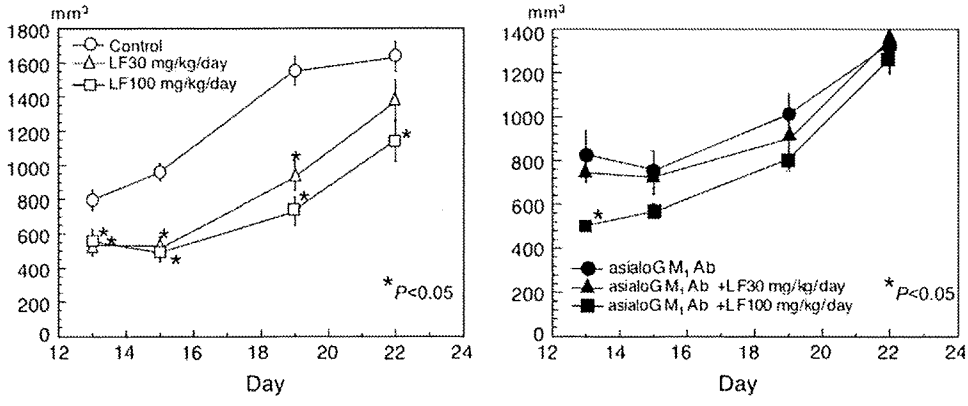

Fig. 1. Importance of activated NK cells for bLF-mediated inhibition of tumor growth (Co26Lu tumor-bearing SCID mice). 1×10^5 cells/mouse were subcutaneously implanted into the right thigh of SCID mice (Charles River Japan, female). bLF was administered at 30 or 300 mg/kg per day on days 5–9 and 12–16. Anti-asialo GM_1 Ab was injected (dilute 1/40, 0.2 ml/mouse i.v.) on days 5, 7, 12, and 14. The longest (a) and shortest (b) diameters of tumors at the Co26Lu cell injection site were measured twice a week using calipers, and the volume was calculated using the formula: $ab^2/2$ (mm^3). The experiment was terminated 22 days after implantation. The symbols represent the mean and standard error of tumor sizes ($n = 5–7$). *$p < 0.05$, Dunnett's multiple comparison *t*-test.

(Houston, TX); anti-rat IFNγ antibody (clone DB-1, mouse IgG_1) was purchased from BioLegend (San Diego, CA); and anti-TNFα (L-19) polyclonal antibody was purchased from Santa Cruz Biotechnology, Inc. Rabbit anti-human lactoferrin, specific for human lactoferrin, and rabbit anti-bovine lactoferrin antibodies, specific for bovine lactoferrin, were obtained from Morinaga Milk Industry Co. Ltd. (Zama, Japan). Anti-human IL-12/IL-23 p40 (clone: C8.3, mouse IgG_1) was purchased from BioLegend; anti-human IL-18 antibody (clone: 159-12B, rat IgG_{2a}:detects both pro-IL-18 and mature IL-18) and anti-human IFNα antibody (clone: #14, mouse IgG_1) were purchased from Medical & Biological Laboratories Co., Ltd.; anti-human IFNγ antibody (clone: MD-1, mouse IgG_1) was purchased from BioLegend (San Diego, CA).

2.3. Mice

BALB/c mice (wild type mice), 5-week-old male and female, were obtained from Charles River Japan (Atsugi, Japan). IFNγ knockout mice (mice with a targeted disruption of the IFNγ gene, BALB/c-Ifng^{tm1Ts}, 4 ~ 5-weeks-old female, GKO mice) [27] were purchased from the Jackson Laboratory, Bar Harbor, ME. The animals were allowed free access to CE-2 pellet diet (CLEA Japan, Tokyo, Japan) and water, and were maintained in plastic cages on woodchip bedding under specific pathogen-free conditions in our animal facility with a controlled temperature of 24 ± 2 °C, humidity 60 ± 10%, and a 12 h light–dark cycle. All experiments were initiated when the mice became 6 weeks-old, and performed according to "Guide for the Care and Use of Laboratory Animals" of the Animal Study Committee of National Cancer Center Research Institute, Tokyo, Japan.

2.4. Organ culture of the small intestine

BALB/c mice were anesthetized with diethyl ether and then killed. The jejunum was excised (2 cm) and then washed with physiological saline. The jejunum was turned inside out and cultured in RPMI-1640 culture medium at 37 °C for 30 min with bLF, bTF, or bLFcin as shown in Fig. 2. After the 30 min culture period, the tissue specimens were cut perpendicularly with scissors and opened onto glass plates. The entire surface (epithelium, lamina propria, submucosa) of the jejunum was harvested by scraping with a slide glass. The sample was then placed on an aluminum plate cooled by acetone-dry ice and pressed flat using another plate. These samples were stored at −80 °C until use.

2.5. Peritoneal macrophage culture

Mice were injected intraperitoneally with 4 ml of thioglycollate broth (3 g/100 ml, Eiken Chemical Co. Ltd., Tokyo). Four days after injection, peritoneal exudate macrophages were obtained: using a plastic 10 ml syringe with a 22-gauge needle, mice were injected intraperitoneally with saline and massaged and the saline solution containing peritoneal exudate macrophages was recovered. Macrophages were washed once with Hanks' balanced salt solution. The harvested cells were centrifuged at 200 × *g* for 10 min, resuspended in culture medium (RPMI-1640, 10% FBS), and plated into a 24-well tissue culture plate (2×10^6 cells/2 ml/well). After incubation for 2 h, non-adherent cells were removed by vigorous washing, and fresh culture medium (RPMI-1640, 5% FBS) was added to the monolayer.

2.6. Spleen cell culture

Mice were anaesthetized and killed and the spleen harvested. Spleens were minced in petri dishes containing 5 ml media and pressed through a 120-stainless-steel mesh. Cells obtained were washed with Hanks' balanced salt solution, centrifuged at 200 × *g* for 10 min, resuspended in culture medium (RPMI-1640, 10% FBS), and plated into a 24-well tissue culture plate (2×10^6 cells/2 ml/well).

2.7. Enzyme-linked immunosorbent assay (ELISA) — frozen organ culture samples

ELISA was used to determine the levels of various cytokines. Frozen tissues were homogenized with a Sonifier 450 (Branson Ultrasonics Corp., Daburg, CT) in lysis buffer (0.067 M phosphate buffer, pH 7.2, 1% Nonidet P-40, 0.5% sodium deoxycholic acid, 0.1% sodium dodecyl sulfate, and 10 μg/ml phenylmethylsulfonyl fluoride, 2.5 μg/ml leupeptin and 20 U/ml aprotinin), homogenates were clarified by centrifugation at 15,000 rpm for 10 min at 4 °C, and the supernatants were collected and stored at −80 °C until testing. ELISA kits for murine IL-1β were purchased from Genzyme Techne (Minneapolis, MN). Mature IL-18 levels were determined using a mouse IL-18 ELISA kit (minimum detection limit: 25.0 pg/ml) purchased from Medical & Biological Laboratories Co., Ltd. (Nagoya, Japan).

2.8. Enzyme-linked immunosorbent assay (ELISA) — peritoneal macrophage, RAW 264.7 cells, and spleen cell cultures

Peritoneal macrophages and spleen cells were harvested as described above. RAW 264.7 mouse macrophages were obtained from Riken (Tsukuba, Japan). Cells were plated into 24-well tissue culture plates (2×10^6 cells/2 ml/well). After incubation for 24 h in 300 μg/ml bLF, cells were harvested and lysed in 100 μl of chilled lysis buffer (0.067 M phosphate buffer, pH 7.2, 1% Nonidet P-40, 0.5% sodium deoxycholic acid, 0.1% sodium dodecyl sulfate, and 10 μg/ml phenylmethylsulfonyl fluoride, 2.5 μg/ml leupeptin and 20 U/ml aprotinin), and then clarified by centrifugation at 15,000 rpm for 10 min. The supernatants were collected and stored at −80 °C until testing. An ELISA kit for mature mIL-18 (minimum detection limit: 25.0 pg/ml) (Medical & Biological Laboratories Co., Ltd., Nagoya, Japan) was used to measure IL-18 levels.

2.9. Enzyme-linked immunosorbent assay (ELISA) — peritoneal macrophage cultures

Peritoneal macrophages were harvested as described above. Cells were plated into 24-well tissue culture plates

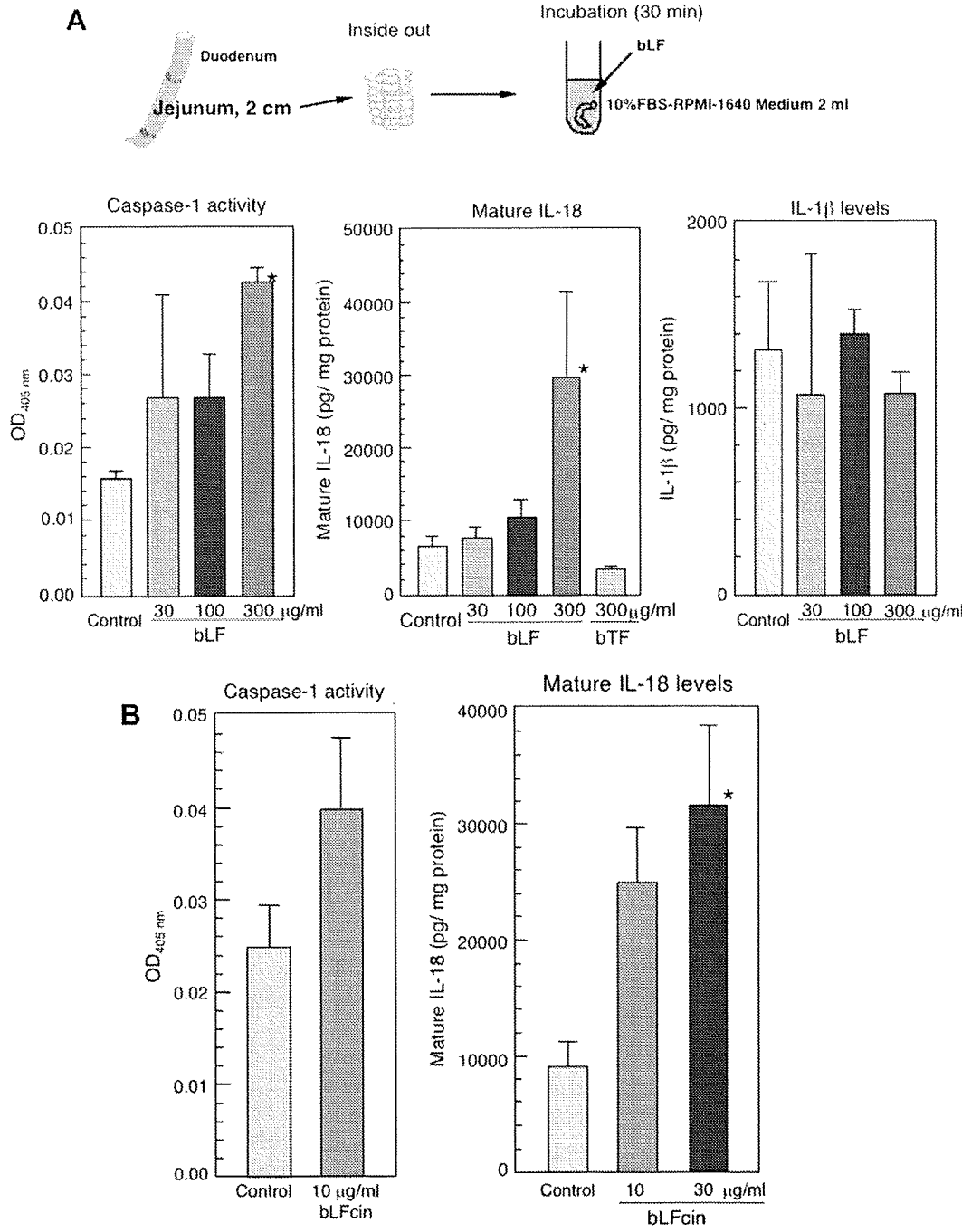

Fig. 2. Effects of bLF and bLFcin on caspase-1 activity and the level of mature IL-18 in the mucosa of organ cultured small intestine. Mouse jejunum in organ culture was incubated with lactoferrin (A) ($n = 7$) or lactoferricin (B) ($n = 3$) for 30 min, and caspase-1 activity and mature IL-18 or IL-1β levels were measured by ELISA. Error bars represent the standard error. *$p < 0.05$, Dunnett's multiple comparison t-test.

(2×10^6 cells/2 ml/well). After incubation for 24 h in bLF, rmIFNγ, anti-mIFNγ antibody, or caspase-1 inhibitor, cells were harvested and lysed in 100 μl of chilled lysis buffer (0.067 M phosphate buffer, pH 7.2, 1% Nonidet P-40, 0.5% sodium deoxycholic acid, 0.1% sodium dodecyl sulfate, and 10 μg/ml phenylmethylsulfonyl fluoride, 2.5 μg/ml leupeptin and 20 U/ml aprotinin), and then clarified by centrifugation at 15,000 rpm for 10 min. The supernatants were collected and stored at −80 °C until testing. An ELISA kit for mature mIL-18 (minimum detection limit: 25.0 pg/ml) (Medical & Biological Laboratories Co., Ltd., Nagoya, Japan) was used to measure IL-18 levels.

2.10. Caspase-1 activity

For assaying the activity of caspase-1, colorimetric protease assay kits were used (BioVision Research Products, Mountain View, CA). The samples were diluted to 200 μg protein in 50 μl Cell Lysis buffer and then 50 μl of Reaction buffer containing 10 mM DTT and 5 μl of 4 mM Tyr-Val-Ala-Asp-pNA

(chromophore *p*-nitroanilide) was added. The samples were incubated at 37 °C for 2 h. Caspase-1 activity levels were determined at 405 nm in a microtiter plate reader. In some experiments, Acyl-Tyr-Val-Ala-Asp-chloromethylketone (Bachem Biochemica GmbH, Heidelberg, Germany) at a concentration of 50 μM was added to inhibit caspase-1 activity; the inhibitor was applied 30 min before bLF addition.

2.11. Western blot analysis of cell cultures

Peritoneal macrophages were harvested as described above; Lewis Lung carcinoma cells and MCF7 human breast cancer epithelial cells were obtained from the National Cancer Center Research Institute (Tokyo, Japan); SW620 colon carcinoma cells were obtained from the American Type Culture Center (Rockville, MD); and SK-MEL 19 melanoma cells [28] were obtained from the Medical Research and Development Center, the Tokyo Metropolitan Institute of Medical Science (Tokyo, Japan). Cells were seeded in 24 well plates in RPMI-1640 medium with 10% FBS at a density of 1×10^6 cells/well and incubated for 24 h. The medium was then changed to serum-free medium containing various concentration of bLF. After a 24 h incubation, cells were washed twice with serum-free medium and lysed in 75 μl chilled lysis buffer containing 125 mM Tris–HCl (pH 6.8), 4.3% SDS, 30% glycerol, 10% 2-mercaptoethanol, 0.01% BPB, and protease inhibitor cocktail "Complete Mini" (Roche, Mannheim, Germany). Lysates were sonicated and clarified by centrifugation at 15,000 rpm for 45 min at 4 °C. Ten μg of each sample was separated by SDS-PAGE on 15/25% polyacrylamide gradient gels (Multigel II Mini, Daiichi Pure Chemicals Co. Ltd., Tokyo). Proteins were transferred to PVDF membrane (Immobilon-P, Millipore Corp., Bedford, MA) and detected by Western blot with appropriate antibodies. Immunoreactive bands were visualized with Amersham ECL Plus Western Blotting Detection System (GE Healthcare, Buckinghamshire, UK). Densitometric analysis of digitized blot images was carried out using NIH Image software.

2.12. Protein assay

Total protein content of the samples was assayed using Coomassie Dry Protein Assay Plates (Pierce, Rockford, IL) with bovine serum albumin as the protein standard (Fraction V, Sigma Chemical Co. St. Louis, MO).

2.13. Lung metastasis assay

Colon carcinoma 26 (obtained from the Cancer Institute of the Japanese Foundation for Cancer Research, Tokyo, Japan), are of BALB/c origin and have low metastatic potential. Metastatic tumor cells (Co26Lu) were obtained by sequential selection of tumor colonies in the lung: colon carcinoma 26 cells were subcutaneously implanted into the back of synergistic BALB/c mice and after tumor formation, colonies in the lung were recovered and cells from these lung metastases were subcutaneously implanted into the back of another mouse. After several rounds of sequential selection, highly metastatic Co26Lu cells were obtained.

Freshly excised Co26Lu cells from tumor-bearing mice were minced in Hanks' balanced salt solution (Life Technologies, Grand Island, NY) and strained through a 120-stainless-steel mesh. Viability was determined by trypan blue dye exclusion. Cells were diluted to the desired concentration and 1×10^5 cells/mouse were subcutaneously implanted into the right thigh of GKO and BALB/c mice ($n = 15$). bLF was administered at 30 or 300 mg/kg per day on days 3–7, 10–14, and 17–21 and the mice were killed on day 22. The lungs were excised and fixed in acetone and lung metastases were counted.

2.14. Treatment with bLF, bLFcin, and bTF and preparation of tissues

Normal BALB/c (WT) and GKO mice were used (five mice/group). bLF, bLFH, and bTF were dissolved in physiological saline (3 or 30 mg/ml) and administered at 30 mg/kg per day in consecutive treatments for 7 days or a single administration at 300 mg/kg. Three hours after the last oral administration of bLF, bLFH or bTF the mice were anesthetized with diethyl ether and killed. The small intestine, jejunum and ileum, was excised and washed with physiological saline. It was then cut perpendicularly with scissors and opened onto a glass plate. The entire surface (epithelium, lamina propria, submucosa) of the ileum was harvested by scraping with a slide glass. A portion of the harvested mucosa was used for RNA extraction; the remaining mucosa was placed on an aluminum plate cooled by acetone-dry ice and pressed flat using another plate. These samples were stored at −80 °C until use. The jejunum was fixed in acetone and used for immunohisochemistry.

2.15. Enzyme-linked immunosorbent assay (ELISA) – frozen tissue samples

ELISA was used to determine the levels of various cytokines. Frozen tissues were homogenized with a Sonifier 450 (Branson Ultrasonics Corp., Daburg, CT) in lysis buffer (0.067 M phosphate buffer, pH 7.2, 1% Nonidet P-40, 0.5% sodium deoxycholic acid, 0.1% sodium dodecyl sulfate, and 10 μg/ml phenylmethylsulfonyl fluoride, 2.5 μg/ml leupeptin and 20 U/ml aprotinin), homogenates were clarified by centrifugation at 15,000 rpm for 10 min at 4 °C, and the supernatants were collected and stored at −80 °C until testing. ELISA kits for murine IL-1β, IL-4, IL-6, IL-12, GM-CSF, and TNFα were purchased from Genzyme Techne (Minneapolis, MN). Mature IL-18 levels were determined using a mouse IL-18 ELISA kit (minimum detection limit: 25.0 pg/ml) purchased from Medical & Biological Laboratories Co., Ltd. (Nagoya, Japan).

2.16. PCR detection of IL-7, IL-15, IL-18, and caspase-1 mRNA expression

For investigation of IL-7, IL-15 IL-18, and caspase-1 mRNA levels, in the intestinal epithelium, bLF was administered orally to tumor-bearing mice on day 7 or days 1–7,

and the mice were killed under deep anesthesia with diethyl ether 3 h after the last oral administration and the small intestine was immediately removed. Total RNAs from mucosal tissues were isolated using ISOGEN (Nippon Gene, Toyama, Japan), followed by ethanol precipitation. Aliquots (5 μg) of total RNA were subjected to a DNase I digestion before reverse transcription (RT) to eliminate any remaining genomic DNA. The RT reaction was carried out for 1 h at 42 °C in a reaction mixture (20 μl) containing 50 ng of oligo(dT)12–18 primer, dNTP (0.5 mM each of dATP, dGTP, dCTP and dTTP), 200 units of Superscript™II (Life Technologies Inc., Gaithersburg, MD) and the first strand buffer, and terminated by incubation at 95 °C for 5 min.

PCR was performed in a reaction mixture (30 μl) containing cDNA, primers (10 pmol each), 0.2 mM dNTP and 1.5 units of Takara Ex Taq™ DNA polymerase (Takara Shuzo, Kyoto, Japan) in Ex Taq buffer. Primers (5′–3′) specific for murine IL-7, IL-15, IL-18, caspase-1 and β-actin sequences [29–32] were as follows:

IL-7, GCGGATCCATGTTCCATGTTTCTTTTAG and GCAAGCTTGTTTATATACTGCCCTTC, 483 bp;
IL-15, GCGGATCCAATGAAAATTTTGAAACC and GCGAATTCAGTCAGGACGTGTTGATG, 508 bp;
IL-18, GCGAATTCCAATGGCTGCCATGTCAG and GCAAGCTTACCTAACTTTGATGTAAG, 599 bp;
Caspase-1, GCCTGCAGATGGCTGACAAGATCCTGAGG and GCCTCGAGTTAATGTCCCGGGAAGAGGTAG, 1225 bp
β-actin, GCGGATCCATGGATGATGATATCGCCGC and GCAAGCTTCCTAGAAGCATTTGCGGTGG, 1144 bp.

Before the first cycle, a denaturation step was performed at 95 °C for 7 min, PCR was carried out using a PCR thermal cycler sp (Takara Shuzo, Kyoto, Japan) with a regimen of 95 °C for 1 min, 58 °C for 1 min, and 72 °C for 1 min (30 cycles), and a final extension period of 7 min at 72 °C. The products were analyzed by electrophoresis through 1.5% agarose gels and stained with ethidium bromide, and densitometric analysis of digitized gel images was carried out using NIH Image software.

2.17. Immunohistochemical studies

For investigation of the effect of bLF on immune cells, 3 h after the final oral administration of bLF (30 mg/kg per day for 7 days; four mice/group) mice were anesthetized and killed, and the jejunum was removed. Jejuna obtained from mice were fixed in acetone at 4 °C and embedded in paraffin. After cutting and deparaffination, sections were incubated in 1% skim milk (Yukijirushi, Sapporo, Japan) – PBS at room temperature for 1 h to block non-specific antigens.

$CD4^+$ and $CD8a^+$ cells were detected with rat-anti-mouse CD4 mAb and CD8a mAb (Japan BD Biosciences Pharmingen, Tokyo, Japan). After washing blocked sections three times, sections were incubated in PBS containing 10% hydrogen peroxide at room temperature for 30 min to remove endogenous peroxidase activity before antibody was added. Sections were then incubated with antibody in a humidified chamber at room temperature for 2 h. After washing, the sections were incubated with biotin-conjugated anti-rat IgG antibody (Vectastain Elite ABC kit, Vector Laboratories, Burlingme, CA) for 3 h. Labeled cells were visualized with 0.03% 3,3-diaminobezidine tetrahydrochroride solution containing 0.1% hydrogen peroxide for 5 min.

IgM^+ and IgA^+ cells were detected with goat anti-mouse IgM Ab-FITC and goat anti-mouse IgA Ab-FITC (Sigma). After washing blocked sections three times, sections were incubated with antibody in a humidified chamber at 4 °C for 2 h. Labeled cells were visualized by fluorescent microscopy.

IL-7, IFNα, and caspase-1 (p-20) expressing cells were detected with rat anti-mouse IL-7 Ab (PeproTech EC. Ltd. London, UK), rat anti-mouse IFNα mAb (HyCult Biotechnology b.v., Uden, The Netherlands), and rabbit anti-mouse caspase-1 (p-20) antibody (Santa Cruz Biotechnology), respectively. After washing blocked sections three times, sections were incubated in PBS containing 10% hydrogen peroxide at room temperature for 30 min to remove endogenous peroxidase activity before antibody was added. Sections were then incubated with antibody in a humidified chamber at room temperature for 2 h. After washing, the sections were incubated with biotin-conjugated anti-rat IgG antibody or biotin-conjugated anti-rabbit IgG antibody (Vectastain Elite ABC kit, Vector Laboratories, Burlingme, CA) for 3 h. Labeled cells were visualized with 0.03% 3,3-diaminobezidine tetrahydrochroride solution containing 0.1% hydrogen peroxide for 5 min.

Results were expressed as the mean of the number of positive cells in the lamina propria per 10 light microscope fields (×200).

2.18. Western blot analysis of frozen tissue samples

Frozen tissue samples (100 mg) were homogenized in 1 ml chilled lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitor cocktail "Complete Mini™" (Roche, Mannheim, Germany). Lysates were centrifuged at 600 rpm for 20 min at 4 °C to remove nuclei and cell debris, and then clarified by centrifuged at 15,000 rpm for 45 min at 4 °C. Twenty μg of protein from each sample were separated by SDS-PAGE on 10% acrylamide, 0.2% bis gels. Proteins were transferred to Immobilon™ transfer membranes (Millipore Corp., Bedford, MA) and detected by Western blot with appropriate antibodies. Immunoreactive bands were visualized with ECL Plus Western Blotting Detection System (GE Healthcare, Buckinghamshire, UK). Densitometric analysis of digitized blot images was carried out using NIH Image software.

2.19. Treatment of GKO mice with rmIL-7, rmIL-18 or rIFNαA/D and metastasis assay

Co26Lu cells (1×10^5 cells/mouse) were subcutaneously implanted into the right thigh of GKO mice. Recombinant

murine IL-7 (*Escherichia coli*, PeproTech House, London, England), recombinant murine IL-18 (Medical & Biological Laboratories Co., Ltd.), and recombinant IFNαA/D (2.04×10^7 IU/ml, Nippon Roche Research Center, Kamakura, Japan) were administered intraperitoneally (i.p.) to GKO mice at 1 and 0.1 μg/mouse, 0.02 μg/mouse and 1×10^5 IU/mouse, respectively, on days 10, 13, 17, and 20. Endotoxin levels of rmIL-7 and IL-18 were less than 0.1 ng/μg protein by the LAL method. The longest (a) and shortest (b) diameters of tumors at the Co26Lu cell injection site were measured twice a week using calipers, and the volume was calculated using the formula: $ab^2/2$ (mm^3). The experiment was terminated 22 days after implantation, and the mice were killed. The lungs were removed, rinsed with saline, and fixed in acetone, and the lung metastatic colonies counted.

3. Results

3.1. Caspase-1 activity and generation of mature interleukin-18 levels in the mucosa of the small intestine in organ culture by bLF

In C57BL/6 or BALB/c mice, oral treatment with bLF or bLFcin increases mature IL-18 levels in the mucosa of the small and large intestines [7]. However, bLF is digested into numerous peptides by pepsin in the stomach [33]. We employed an organ culture system using mouse small intestine to investigate the ability of intact bLF and an N-terminal peptide fragment of bLF (bLFcin) to affect the activity of caspase-1 and the generation of mature IL-18: caspase-1 cleaves the proform of IL-18 to generate the 18 kDa bioactive molecule [34–36].

In organ culture experiments, caspase-1 activity and mature IL-18 levels in the mucosa of the small intestine were significantly increased following addition of bLF (Fig. 2A). Exposure to bLFcin also resulted in increases in caspase-1 activity and mature IL-18 levels (Fig. 2B). These results were specific since exposure to bTF did not result in elevated levels of mature IL-18, and exposure to bLF did not affect IL-1β levels. These results are similar to those obtained from previous *in vivo* experiments in which treatment with bLF or bLFcin caused an increase in IL-18 levels and caspase-1 activity in the mucosa of the small intestine [9]. Therefore, both intact bLF and an N-terminal peptide fragment of bLF, bLFcin, stimulate caspase-1 activity and the generation of mature IL-18.

3.2. bLF or bLFcin induction of cytokine production by peritoneal macrophages

Next, we investigated caspase-1 and cytokine production by peritoneal macrophages treated with bLF or bLFcin *in vitro*. Peritoneal macrophages, the macrophage cell line RAW264.7, and spleen cells were treated with bLF, and caspase-1 activity and mature IL-18 levels were measured. Treatment with 300 μg/ml bLF enhanced caspase-1 activity in peritoneal macrophages and possibly to some extent in spleen cells, but not in RAW264.7 cells (Fig. 3 left panel). bLF markedly enhanced production of mature IL-18 in peritoneal macrophages but not in Raw264.7 or spleen cells (Fig. 3 right panel). bLF-mediated induction of mature IL-18 by peritoneal macrophages was dose dependent and bovine transferrin (bTF) had no effect (Fig. 4). Recombinant murine interferon-gamma (rmIFNγ) also induced mature IL-18 production by peritoneal macrophages (Fig. 4). Importantly, bLF-mediated induction of mature IL-18 was abrogated by co-incubation of the cells with caspase-1 inhibitor (Acyl-Tyr-Val-Ala-Asp-chloromethylketone, Ac-YVAD-CMK) (Fig. 5). bLF-mediated induction of mature IL-18 was also inhibited by co-incubation with anti-mIFNγ antibody (Fig. 5). Finally, IFNγ induced production of mature IL-18 (Fig. 5), and this induction was inhibited by co-incubation of the cells with anti-mIFNγ antibody (data not shown), confirming the validity of this assay.

In Western blot analysis of macrophage lysates after addition of bLF or bLFcin at 1000 or 30 μg/ml, respectively, bLF induced expression of IFNγ and IL-15, and bLFcin induced expression of IL-15 (Fig. 6). Treatment with bLF decreased expression of IFNα while treatment with bLFcin decreased expression of TNFα (Fig. 6).

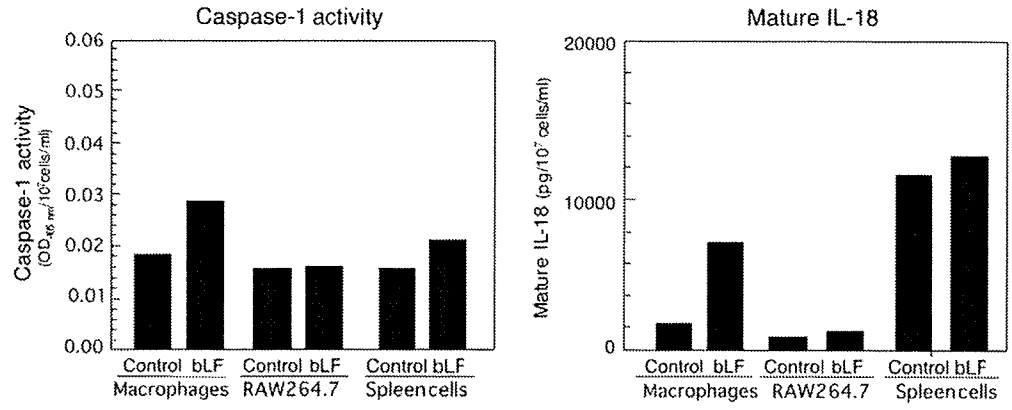

Fig. 3. Effects of bLF on caspase-1 activity and mature IL-18 production in peritoneal macrophages, RAW264.7 cells, and spleen (BALB/c mouse) cells. Cells were incubated for 24 h in bLF (300 μg/ml). Caspase-1 activity and mature IL-18 levels were determined by ELISA. Data are representative of two independent experiments.

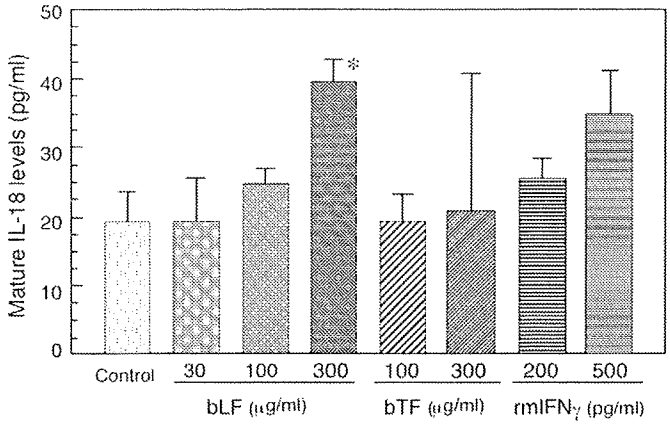

Fig. 4. Effect of bLF, bTF, and rmIFNγ on the production of mature IL-18 by macrophages. Macrophages were incubated for 24 h in bLF, bTF or rmIFNγ and mature IL-18 levels were measured by ELISA. Error bars represent the standard error ($n = 3$). *$p < 0.05$, Dunnett's multiple comparison t-test.

3.3. Cytokine induction in various tumor cell lines by bLF or bLFcin

bLF can induce caspase-1 activity and the generation of mature IL-18 in mouse intestinal tissue and macrophages and IFNγ and IL-15 expression in macrophages; bLFcin can induce caspase-1 activity and the generation of mature IL-18 in mouse intestinal tissue and IL-15 expression in macrophages. We next investigated the effect bLF or bLFcin had on cytokine expression in mouse and human tumor cell lines: Lewis lung carcinoma was the mouse tumor cell line used, and SW620 (colon), MCF-7 (breast), and SK-MEL-19 (melanoma) were the human cell lines used.

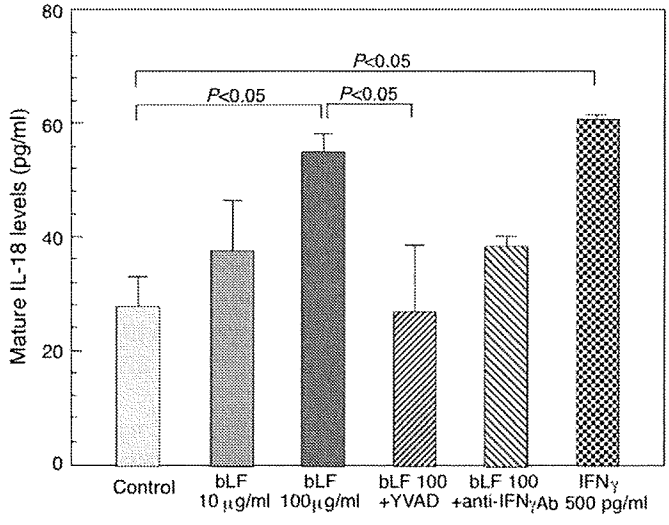

Fig. 5. Caspase-1 inhibitor (YVAD) and IFNγ antibodies block mature IL-18 production by macrophages treated with bLF. Peritoneal macrophages were treated with bLF, bLF + the caspase-1 inhibitor YVAD or bLF + IFNγ antibodies, and mature IL-18 levels were measured by ELISA. The error bars represent the standard error ($n = 3$), p values were calculated by Dunnett's multiple comparison t-test. YVAD = Acyl-Tyr-Val-Ala-Asp-chloromethylketone.

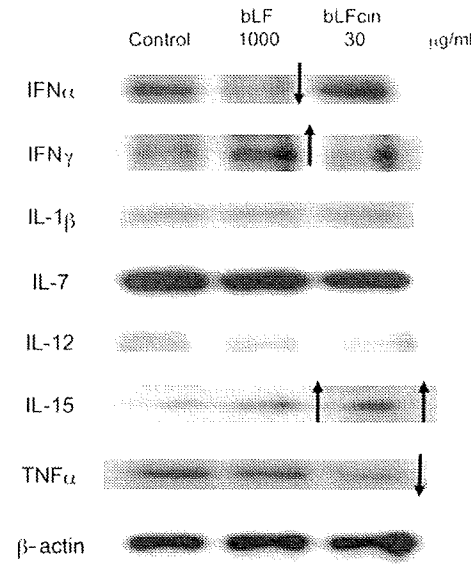

Fig. 6. Western blot analysis of cytokine production in peritoneal macrophages following addition of bLF or bLFcin (BALB/c mice). Macrophages were incubated for 24 h in bLF (1000 μg/ml) or bLFcin (30 μg/ml). Arrows indicate increases or decreases in expression compared to the control. Data are representative of two independent experiments.

Incubation of Lewis Lung carcinoma cells in bLFcin at 100 or 300 μg/ml for 48 h significantly decreased cell number, however, bLF at 1000 μg/ml or bLFcin at 30 μg/ml had no effect on cell number (Table 1). In Western blot analysis of Lewis lung carcinoma cells treated with bLF or bLFcin for 24 h, bLF induced increased expression of IFNγ (2-fold) and IL-1β (3-fold), and bLFcin (lower doses) induced increased expression of IL-1β (4-fold) (Fig. 7).

In Western blot analysis of human cells treated with bLF or bLFcin for 24 h, both bLF and bLFcin induced expression of human lactoferrin (hLF) (2-fold) and mature IL-18 (2 ~ 4-fold) by colon cancer SW620 cells (Fig. 8A); both bLF and bLFcin induced expression of hLF (7 ~ 29-fold), IFNα (4-fold), IL-12 (2 ~ 4-fold), and mature IL-18 (2 ~ 3-fold) by mammary cancer MCF-7 cells (Fig. 8B); and both bLF and bLFcin induced expression of IFNα (4 ~ 11-fold), IL-12 (2 ~ 5-fold) and mature IL-18 (3 ~ 4-fold) by melanoma SK-MEL19 cells (Fig. 8C). SK-MEL19 cells did not express hLF. In HT29 human colon cancer cells, hLF levels were unaffected by incubation in 1000 μg/ml bLF (87% of basal line), but were significantly decreased by incubation in 30 μg/ml bLFcin (46% of basal line) (data not shown).

Table 1
Effects of lactoferrin and lactoferricin on the growth of Lewis lung carcinoma cells 5% FCS in RPMI-1640 medium, 48 h incubation

	Concentration (μg/ml)	Growth (% of Control)
bLF	1000	96.2
bLFcin	30	100.0
	100	46.5
	300	13.2

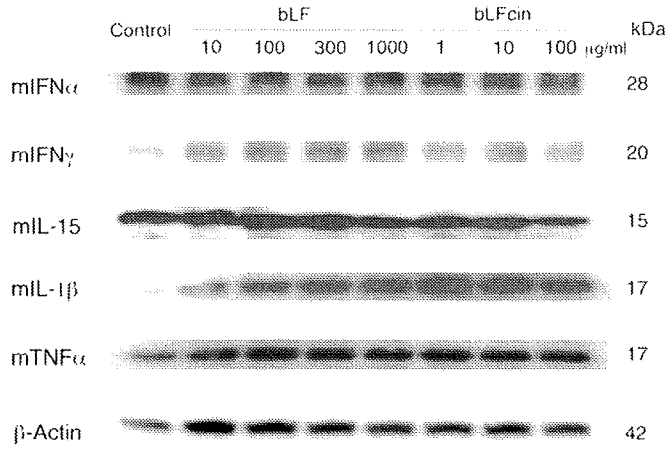

Fig. 7. Western blot analysis of cytokine production in Lewis Lung carcinoma cells following incubation with bLF or bLFcin for 24 h. Data are representative of two independent experiments.

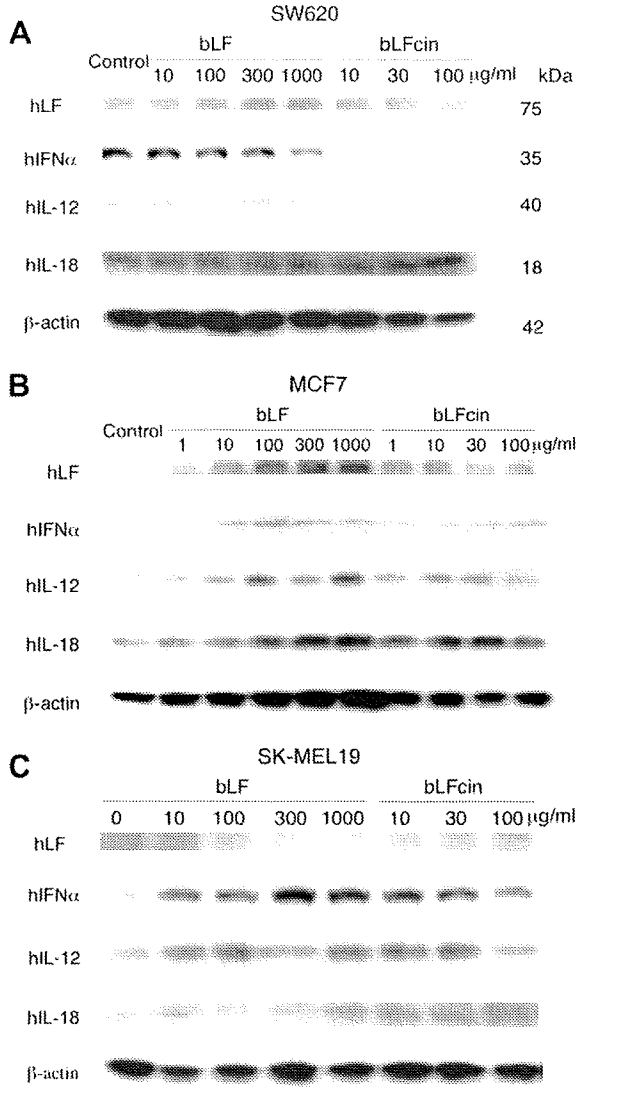

Fig. 8. Western blot analysis of cytokine production in SW620 (A) colon cancer cells, MCF7 (B) mammary carcinoma cells, and SK-MEL19 (C) melanoma cells following incubation with bLF or bLFcin for 24 h. Data are representative of three independent experiments.

In summary, exposure of cells to bLF or bLFcin tends to cause induction of a variety of cytokines. In addition, LF may activate an autocrine loop in some cells; notice the extremely high induction of hLF in the breast cancer cell line, MCF7. The specific effects of bLF and bLFcin, however, depend on the cell type.

3.4. Anti-metastatic activity following oral administration of bLF in GKO and BALB/c mice

We have shown that bLF and bLFcin can induce mouse small intestine to increase its production of mature IL-18. An important function of IL-18 is activation of T-cells and NK cells involved in cell mediated immune responses. Through its stimulation of immune cells, IL-18 has been shown to protect experimental animals against repeated challenges with tumor cells [37,38]. Therefore, we investigated the ability of bLF to inhibit lung metastasis by Co26Lu tumor cells. In addition, we used IFNγ KO (GKO) mice to assess the importance of IFNγ on the ability of bLF to inhibit lung metastasis of Co26Lu tumor cells.

The number of lung metastatic colonies in tumor-bearing GKO mice were markedly higher than in tumor-bearing BALB/c mice (Fig. 9), indicating that IFNγ inhibits the metastatic ability of Co26Lu cells. Oral treatment with bLF significantly inhibited lung metastatic colony formation in both GKO mice and BALB/c mice (Fig. 9), indicating that bLF can inhibit the metastatic ability of Co26Lu cells in the absence of IFNγ.

3.5. Cytokine levels in the small intestines in GKO and BALB/c mice following treatment with bLF

Since IFNγ is not required for bLF-mediated inhibition of Co26Lu metastasis to the lung, we compared bLF-mediated induction of cytokines in the small intestines of GKO and BALB/c mice. IL-1β, IL-6, and TNFα levels in the mucosa of the small intestine in both GKO and BALB/c mice were not markedly changed after treatment with bLF (Fig. 10). IL-12 levels showed a tendency to decrease in BALB/c mice. IL-4 and GM-CSF showed a tendency to increase in both GKO and BALB/c mice. Mature IL-18 levels in the small intestine of GKO mice were not affected by treatment with bLF, however, bLF did cause a significant increase in mature IL-18 in BALB/c mice (Fig. 11).

Expression of IL-7, IL-15, and IL-18 mRNA was examined in freshly isolated mucosal tissues of the small intestine of GKO and BALB/c mice following treatment with bLF at 30 or 300 mg/kg per day. IL-7 mRNA levels were significantly enhanced in GKO mice by administration of bLF (1.4- and 1.5-fold increase, $p < 0.05$ and $p < 0.01$, respectively), but bLF had no effect on IL-7 mRNA levels in BALB/c mice (0.9 ~ 1.3-fold increase $p > 0.05$); bLF had no significant effect on IL-15 mRNA levels in either GKO or BALB/c mice (Fig. 12); bLF had no significant effect on IL-18 mRNA levels

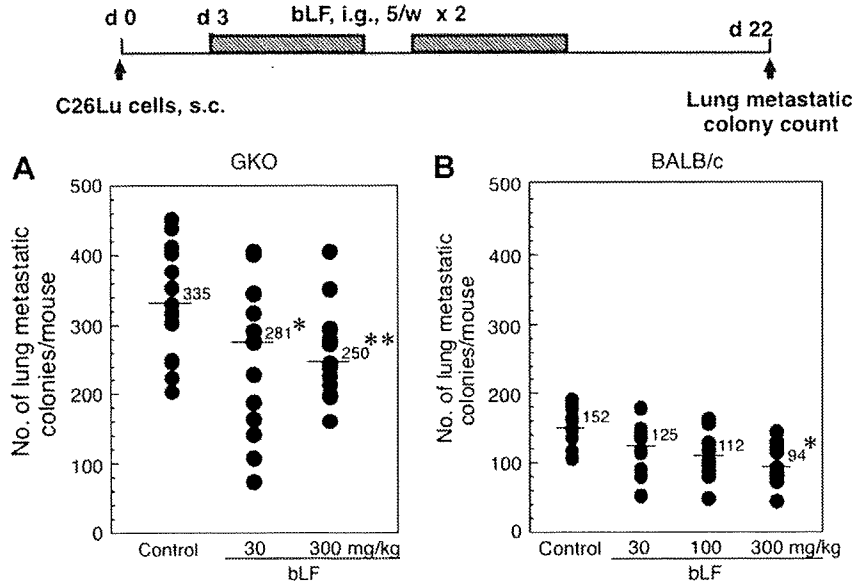

Fig. 9. Effects of oral administration of bLF on the number of lung metastatic colonies in Co26Lu-bearing GKO (A) and Co26Lu-bearing BALB/c (B) female mice. The numerical values represent the median of the number of lung metastatic colonies in 15 animals. $^{*}p < 0.05$, $^{**}p < 0.01$ vs Control, Mann–Whitney *U*-test.

in GKO mice (about 1.3-fold increase, $p > 0.05$), but bLF markedly elevated IL-18 mRNA levels in BALB/c mice (1.3 and 1.7-fold increase, $p > 0.05$ and $p < 0.01$, respectively) (Fig. 12).

Thus, bLF induced expression of IL-18 mRNA and generation of mature IL-18 in BALB/c mice. Since bLF had no effect on IL-18 expression in GKO mice, bLF induction of IL-18 is IFNγ dependent. In GKO mice, bLF induced expression of IL-7.

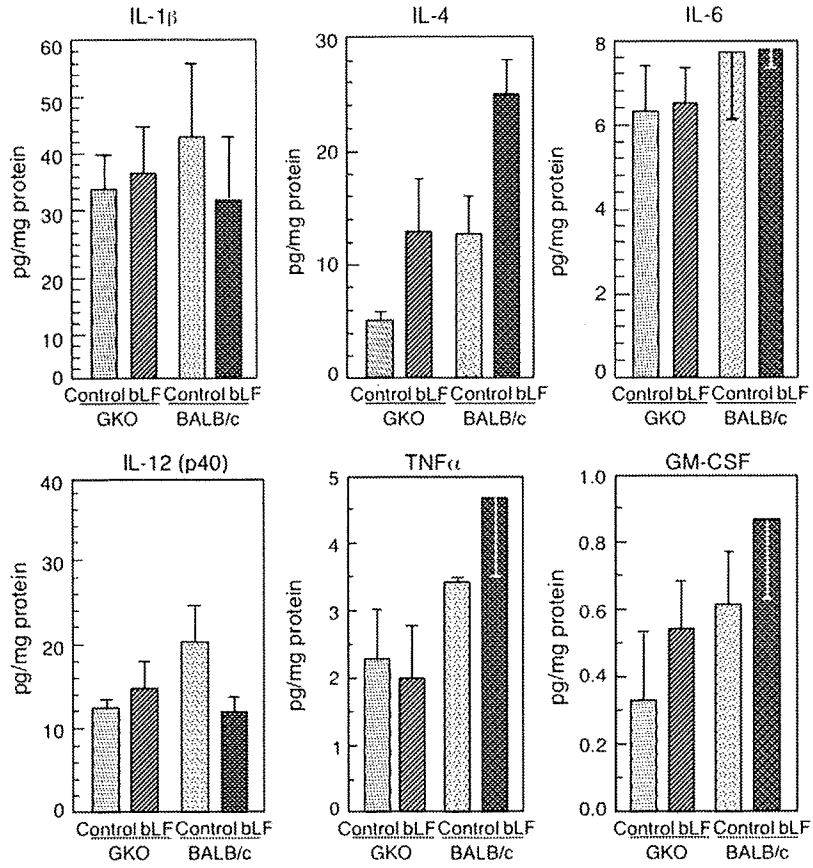

Fig. 10. Effect of bLF on various cytokine levels in the mucosa of the small intestines of GKO and BALB/c female mice. bLF was administered orally at 30 mg/kg per day for 7 days, and 3 h after the last administration the animals were anesthetized and killed, the jejunum removed, and cytokine levels measured. Error bars represent the standard error ($n = 5$).

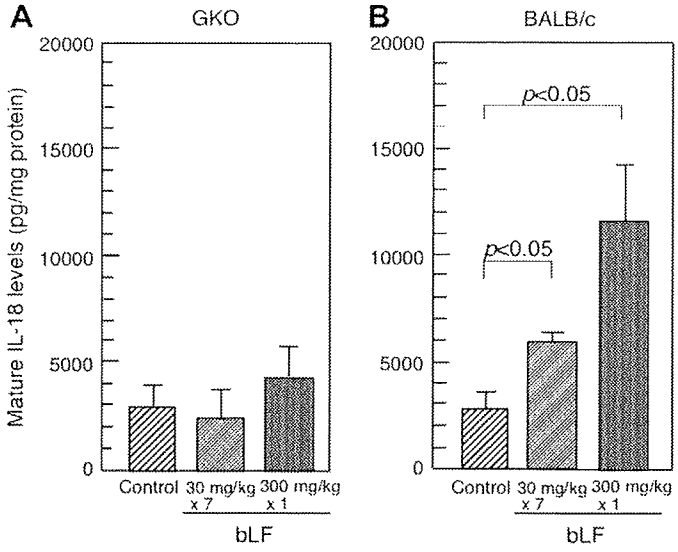

Fig. 11. Effect of bLF on mature IL-18 levels in the mucosa of the small intestine of GKO (A) and BALB/c (B) mice. bLF was administered orally at 30 mg/kg per day for 7 days or once at 300 mg/kg on day 7, and 3 h after last the administration the animals were anesthetized and killed, the jejunum removed, and mature IL-18 levels measured. Error bars represent the standard error ($n = 5$).

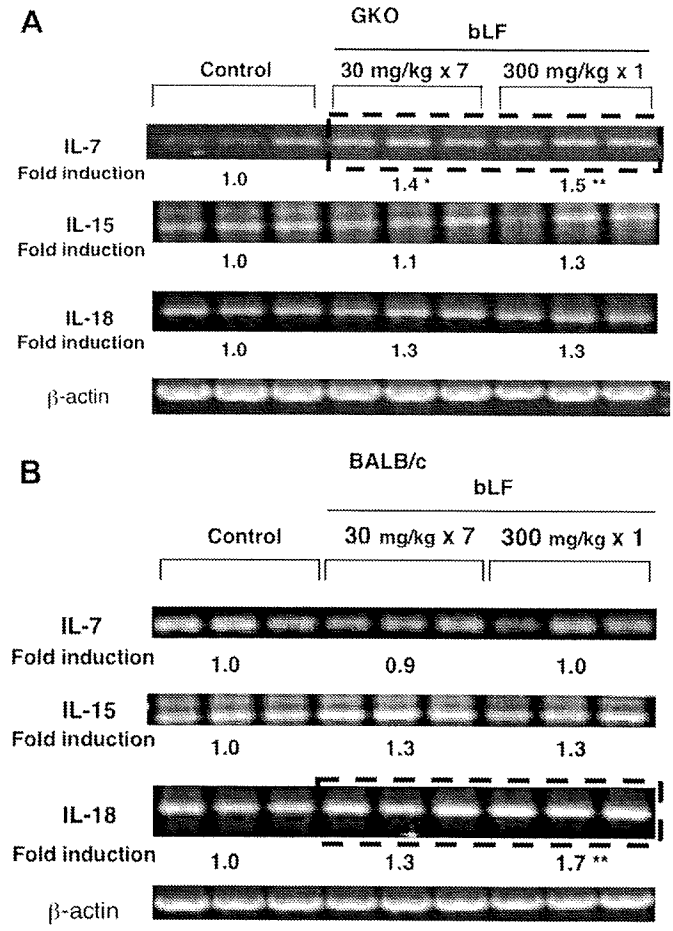

Fig. 12. Effect of bLF on the expression of IL-7, IL-15 and IL-18 mRNA in the mucosa of the small intestines of GKO (A) and BALB/c (B) female mice. bLF was administered orally at 30 mg/kg per day for 7 days or once at 300 mg/kg on day 7, and 3 h after the last administration of bLF the animals were anesthetized and killed and the jejunum removed. mRNA expression was detected by RT-PCR. Three mice were used for each condition. Expression of each transcript was normalized to β-actin and the mean expression values for each condition obtained. Numerical data represent the ratio of these values compared to the controls. Administration of bLF lead to an increase in IL-7 expression in GKO mice and to an increase in IL-18 expression in BALB/c mice. Data are representative of 2 independent experiments.

3.6. Analysis of caspase-1 expression and activity in the small intestine of GKO and BALB/c mice

Caspase-1 expression and activity in the small intestine of BALB/c mice is enhanced by treatment with bLF, and this effect is dependent on IFNγ. Therefore we investigated the role of IFNγ on bLF-mediated induction of caspase-1 expression and activity. In immunohistochemical and Western blot analysis, caspase-1 expression in epithelial cells of the small intestine was increased in both GKO and BALB/c mice after treatment with bLF (GKO: 1.6-fold; BALB/c: 1.9-fold). However, caspase-1 activity, as measured by generation of the cleaved-caspase-1 p-20 peptide, was markedly different between GKO and BALB/c mice (GKO: 0.8; BALB/c: 1.5-fold vs Control) (Fig. 13). Moreover, caspase-1 activity in the mucosa of the small intestine in GKO mice did not change following administration of bLF (120–123% of Control), but its activity in BALB/c mice was significantly enhanced (211–272% of Control, $p < 0.05$) (Table 2). Therefore, bLF induction of caspase-1 expression is not dependent on IFNγ, but generation of active caspase-1 is dependent on IFNγ.

3.7. Immunohistochemical analysis of various immune cells in the small intestine of GKO mice

bLF is thought to mediate its anti-metastasis activity by activation of immune cells in the intestine, and we have previously demonstrated that bLF increases the number of $CD4^+$ and $CD8^+$T and asialo GM_1^+ (NK) cells in BALB/c mice [9]. bLF treatment also inhibits Co26Lu metastasis to the lung in GKO mice, and this effect occurs in the absence of IFNγ, caspase-1, and IL-18. We therefore examined the effect of bLF on immune cells in the small intestine of GKO mice. $CD4^+$, IgM^+ (data not shown), and IgA^+ cells were significantly increased by treatment with bLF, but $CD8^+$ cells were not (Fig. 14).

$IL\text{-}7^+$ cells in the small intestine are mainly intraepithelial lymphocytes (IEL), fibroblasts, and vascular endothelial cells. bLF also caused a marked increase in the number of $IL\text{-}7^+$ intraepithelial lymphocytes in GKO mice (Fig. 15B). In contrast, bLF did not induce expression of IL-7 in the small intestine of BALB/c mice or an increase in $IL\text{-}7^+$ intraepithelial lymphocytes (data not shown).

$IFN\alpha^+$ cells exist in the lamina propria of the small intestine and the number of these cells was also increased by treatment with bLF in GKO mice, but not in BALB/c mice (Fig. 16). IFNα protein expression was increased (1.8-fold) as shown by Western blot analysis (data not shown).

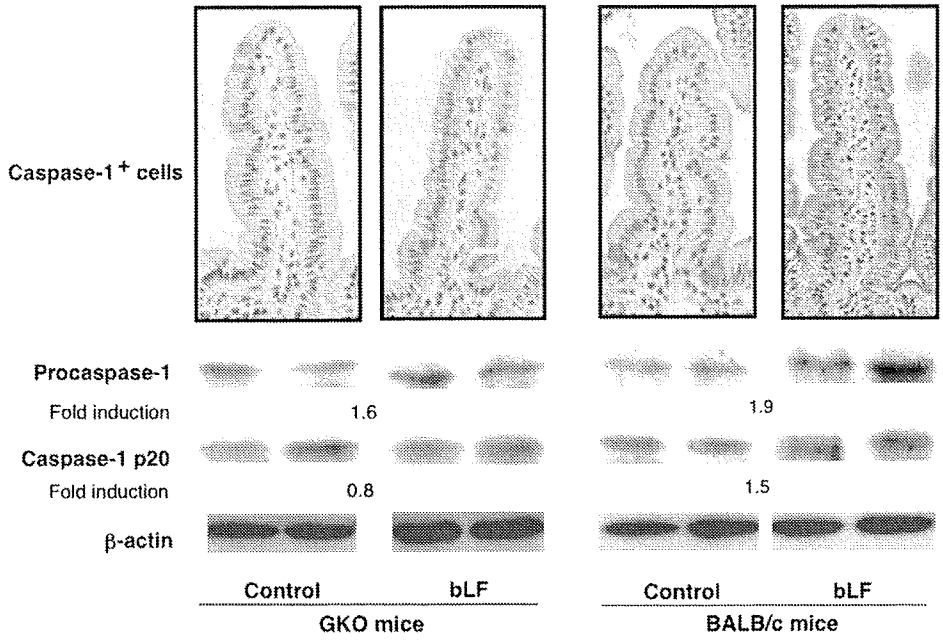

Fig. 13. Caspase-1$^+$ cells in the small intestine and Western blot analysis for procaspase-1 and active caspase-1 (caspase-1 p20). bLF was administered orally at 30 mg/kg per day for 7 days, and 3 h after the last administration of bLF the animals were anesthetized and killed, and the jejunum was removed. The results of two independent experiments are shown.

3.8. Anti-metastatic effects in tumor-bearing GKO mice by rmIL-18, rIFNαA/D, or rmIL-7 treatment

bLF inhibits Co26Lu metastasis to the lung in GKO mice in the absence of IFNγ, caspase-1, and IL-18. However, bLF induces IFNα and IL-7 in these mice. Therefore, we tested the effects of recombinant mature IL-18 (rmIL-18), recombinant IFNα (rIFNαA/D), and recombinant IL-7 (rIL-7) on Co26Lu metastasis to the lung in GKO mice. Intraperitoneal administration of IL-18 at 20 ng/mouse, IFNαA/D at 10^5 IU/mouse, or rmIL-7 at 0.1 μg/mouse caused significant retardation of tumor growth of subcutaneously implanted Co26Lu and also significant retardation of lung metastases (Table 3).

4. Discussion

LF is an important component of the innate immune system with potent antimicrobial activity and is found in a variety of exocrine secretions including gastrointestinal fluids. The small intestine has a well developed immune network that is involved in protection of the host from pathogens. For example, mucosa-associated bacteria (lactobacili and *E. coli*, etc.) in the small intestine can be potent stimulators of mononuclear cells of the intestinal lamina propria, inducing these cells to express IL-12 [39], which in turn activates intestinal immune cells and protects the small intestine against microbial infection. LF is part of the intestinal immune network [40–42]. It is an excellent marker of inflammatory bowel diseases such as ulcerative colitis and Crohn's disease [43], and fecal LF levels are also higher in patients with colorectal tumors than in healthy individuals [44]. Because ingested LF stimulates cytokine production [8] and enhances mucosal immunity [9], NK cell activity [10,11], LAK activity [11], neutrophil activity [12,13], and macrophage cytotoxicity [14,15], it is thought that orally ingested LF is also able to interact with epithelial cells and immune cells in the mucosa of the intestine.

Table 2
Effects of bLF on caspase-1 and -3 activities in the mucosa of the small intestine in GKO and BALB/c (WT) mice

Treatment (p.o.)	Caspase-1 activity	Caspase-3 activity (absorbance at 405 nm)
GKO mice ($n = 4$)		
Control (saline)	0.035 ± 0.002	0.214 ± 0.011
bLF (30 mg/kg × 7)	0.042 ± 0.011 (1.20)	
bLF (300 mg/kg × 1)	0.043 ± 0.012 (1.23)	0.221 ± 0.024 (1.03)
BALB/c (WT) mice ($n = 5$–7)		
Control (saline)	0.018 ± 0.003*	0.168 ± 0.014
bLF (30 mg/kg × 7)	0.049 ± 0.011* (2.72)	
bLF (300 mg/kg × 1)	0.038 ± 0.005 (2.11)	0.190 ± 0.026 (1.13)

Mucosal samples of the small intestine were diluted to 200 μg protein/50 μl. () = -fold. *$p < 0.05$.

There are several reports that administration of LF inhibits tumor growth and protects against carcinogenesis [18,45,46], reviewed in refs. [26,47,48]. In several rodent cancer models, it has been shown that this protective effect is associated with enhancement of the local intestinal mucosal immune response [9,16,47,49–52].

One pathway which appears to be an effector of LF is generation of mature IL-18 and consequent activation of immune cells. Administration of bLF induces expression of IL-18 in the small intestine [9,16,46]. IL-18 stimulates both Th1 and Th2 type T helper cells, CD8$^+$ effector T cells, NK cells, and LAK cells [10,11,17,53–56]; and up-regulation and/or enhanced activity of CD4$^+$ and CD8$^+$ effector T-lymphocytes and NK and LAK cells is also observed upon LF

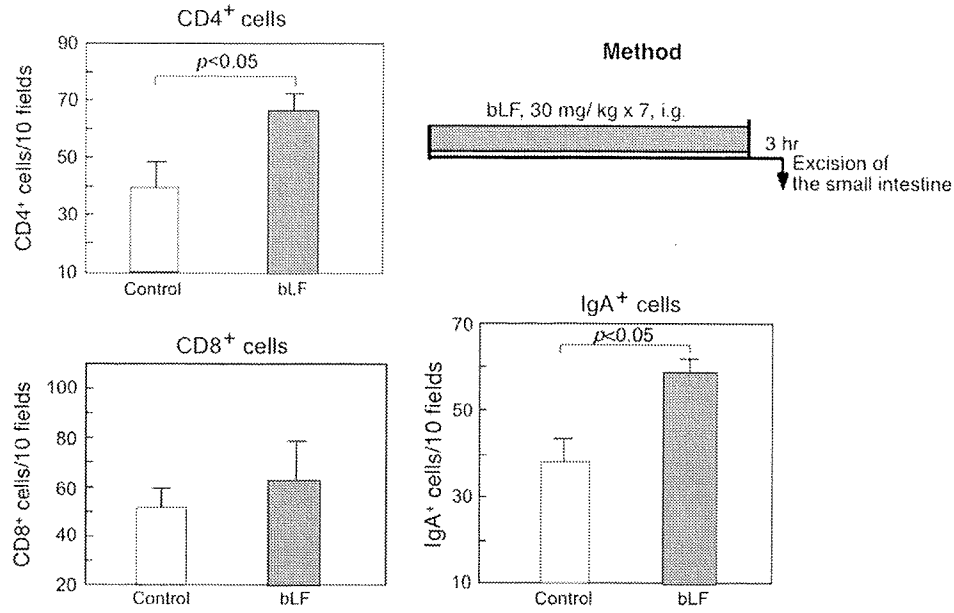

Fig. 14. Effects of bLF on the number of $CD4^+$, $CD8^+$, and IgA^+ cells in the lamina propria of the small intestine of GKO mice. bLF was administered orally at 30 mg/kg per day for 7 days, and 3 h after the last administration of bLF the animals were anesthetized and killed, and the jejunum was removed and the number of $CD4^+$, $CD8^+$, and IgA^+ cells counted. Error bars represent the standard error ($n = 5$).

administration [16,19,40,50,57]. LF also stimulates the induction and/or activation of B cells, macrophages, and neutrophils [54,58,59]. Wolf et al. report that hLF resulted in T cell-dependent tumor inhibition of head and neck squamous cell carcinoma *in vivo*, and that depletion of $CD3^+$ cells abolishes this effect [46]. Bezault et al. report that stimulation of NK cell activity is essential for LF-mediated prevention of metastasis in C57BL/6 mice: intraperitoneal administration of hLF to these mice inhibited the growth of solid tumors and prevented lung metastasis of murine melanoma cells, and this effect was lost upon deletion of NK cell function using antibody blocking experiments [18].

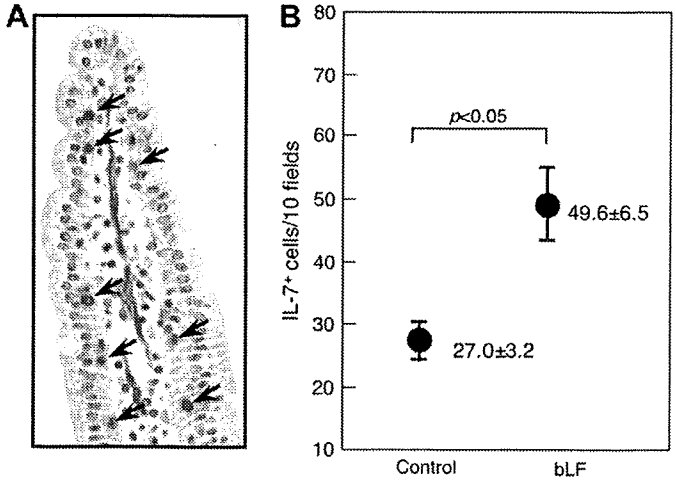

Fig. 15. Effects of bLF on the number of $IL\text{-}7^+$ cells in the small intestine of GKO mice. bLF was administered orally at 30 mg/kg per day for 7 days, and 3 h after the last administration of bLF the animals were anesthetized and killed, and the jejunum was removed. (A) $IL\text{-}7^+$ cells in the small intestine (arrows). (B) The number of $IL\text{-}7^+$ cells in the small intestine of GKO mice was significantly increased by treatment with bLF ($n = 5$).

In our study we confirmed the ability of bLF and digested bLF-peptides to induce expression of IL-18 and the generation of mature IL-18 in a variety of settings. Importantly, in IFNγ knockout (GKO) mice, bLF had no effect on IL-18 expression or maturation. Moreover, in settings in which bLF did induce generation of mature IL-18, maturation was abolished by caspase-1 inhibitors. Therefore, bLF-mediated induction of IL-18 expression is dependent upon IFNγ and bLF-mediated generation of mature IL-18 is dependent upon caspase-1.

Previously we showed that bLF can induce IFNγ in the intestine [50], and in this study we affirmed that bLF can induce expression of IFNγ by peritoneal macrophages and that both bLF and bLFcin can induce expression of IFNγ by Lewis Lung carcinoma cells. Moreover, bLF also induces expression

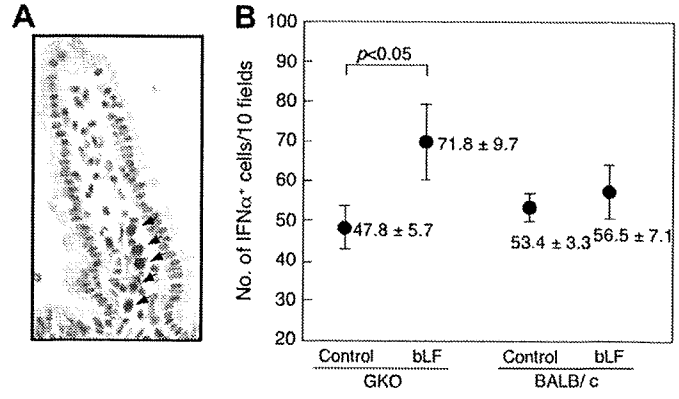

Fig. 16. Effects of bLF on the number of $IFN\alpha^+$ cells in the small intestine of GKO mice. bLF was administered orally at 30 mg/kg per day for 7 days, and 3 h after the last administration of bLF the animals were anesthetized and killed, and the jejunum was removed. (A) $IFN\alpha^+$ cells in the small intestine (arrows). (B) The number of $IFN\alpha^+$ cells in the small intestine of GKO mice was significantly increased by treatment with bLF ($n = 5$).

Table 3
Effect of IL-7, IL-18 and IFNαA/D on lung metastatic colony formation in Co26Lu-bearing GKO mice

Treatment (i.p.)	Dose (μg/mouse)	Tumor volume on day 20 (mm^3)	No. of metastatic colonies Median (range)	*p*-value
Control (saline)		1274 ± 254	233 (156–420)	
rmIL-7	0.1	1023 ± 68*	124 (53–165)	0.003
rmIL-7	1.0	1122 ± 172	143 (34–168)	0.007
rmIL-18	0.02	953 ± 55*	105 (51–169)	0.012
rIFNαA/D	1×10^5 IU	899 ± 59*	111 (91–168)	0.012

Cytokines were administered i.p. to Co26Lu-bearing GKO mice (5–7 mice/group) on days 10, 13, 17 and 20. *$p < 0.05$ vs Control.

of caspase-1 mRNA *in vivo*. However, while bLF administration results in generation of active caspase-1 in BALB/c mice, it has no effect on caspase-1 activity in GKO mice. Taken together, these results indicate that in BALB/c mice, bLF ingestion results in stimulation of IFNγ and caspase-1 expression; IFNγ in turn stimulates IL-18 expression and caspase-1 activity; and finally active caspase-1 cleaves pro-IL-18 to generate mature IL-18. The bLF stimulated generation of mature IL-18 subsequently acts on immune cells in the intestine which in turn effect the observed bLF anticarcinogenesis activity.

However, bLF also effects inhibition of tumor growth and metastasis in GKO mice, an animal model in which bLF administration does not result in induction of IFNγ expression or generation of caspase-1 activity or mature IL-18. This result is not inconsistent with some published reports. Kuhara et al. also report that bLF administration caused an increase in IFNγ and IL-18 and NK activity, however, bLF administration also caused increased NK activity in IL-18 KO mice [51]. In these mice, bLF induced increased expression of IFNα and IFNβ (type I IFNs) in Payer's patches and mesenteric lymph nodes, and blockade of induction of IFNα and IFNβ expression abolished bLF induction of NK activity. Using BALB/cByJ Jcl hypoglycemic mice, bLF administration does not result in elevated IFNγ or IL-18 expression [60]; however, bLF is host protective in this animal and does induce expression of IFNβ and IL-12. These cytokines are well characterized mediators of host defense against infections and cancer. bLF-mediated induction of IL-12 is also reported in other systems [61]. In the present study, we also show induction of type I IFNs and of interleukins other than IL-18 by bLF *in vitro* and in GKO mice *in vivo*. Therefore, administration of bLF can activate more than one effector pathway; which pathways are responsive to activation depend on the physiology of the gastrointestinal tract.

In the GKO mice, bLF did not induce IL-18 expression or maturation, but bLF did induce expression of IFNα and IL-7 and inhibited tumor growth and metastasis. In these mice, injection of recombinant murine IL-18 also inhibited tumor growth and metastasis. Thus, the IL-18 effector pathway is intact in GKO mice, but in the absence of IFNγ, bLF is unable to activate it. Importantly, injection of recombinant IFNαA/D or recombinant IL-7 also inhibited tumor growth and metastasis in these mice. Thus, in the absence of the IFNγ/IL-18 effector pathway, bLF is able to exert anticarcinogenesis activity by activation of a second effector pathway, an IFNα/IL-7 effector pathway.

Finally, it should be noted that bLF and bLFcin induced expression of IL-15 by peritoneal macrophages *in vitro*. That the induction of IL-15 was not observed when the entire intestinal mucosa was examined is not surprising since macrophages make up a very small proportion of this tissue. The specific role of IL-15, if any, in bLF-mediated immune modulation remains to be elucidated, however, IL-15 is a well known immune cell regulator [62–64] and could be a component of another bLF activated effector pathway.

5. Summary

Lactoferrin, originally isolated from milk, is an important component of the innate immune system. Several reports indicate that ingested lactoferrin can inhibit carcinogenesis. In this report we investigated effector pathways which link ingestion of lactoferrin to carcinogenesis. Our data support the concept that lactoferrin peptide fragments, especially lactoferricin, are able to stimulate cytokine production by cells in the mucosa of the small intestine. The subsequent activation of immune cells enhances immune surveillance, i.e., the ability of the immune system to recognize and remove malignant cells that arise during a person's lifetime, and lactoferrin's anticarcinogenesis effects are the result of this enhanced surveillance.

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A Medium-Term, Rapid Rat Bioassay Model for the Detection of Carcinogenic Potential of Chemicals

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ABSTRACT

The Ito Liver Model and the Ito Multi-organ Model are used in conjunction and constitute an efficient and rapid bioassay for the identification of both genotoxic and nongenotoxic carcinogenic chemicals. The Ito Liver Model is an 8-week bioassay system that uses the number and size of foci of hepatocytes positive for glutathione S-transferase placental form (GST-P) as the end-point marker. One hundred fifty-nine compounds were tested using the Ito Liver Model: 61 of 66 hepatocarcinogens tested positive, and 10 of 43 nonliver carcinogens were also positive. The false-positive detection of noncarcinogens was low; a single false-positive result was obtained from the 50 noncarcinogens tested. Since more than half of all known carcinogens are hepatocarcinogens in rodents, the initial 8-week bioassay is able to detect most carcinogens. The Ito Multi-organ Model is a 28-week bioassay system for the detection of carcinogens that were not identified by the Ito Liver Model. Results are evaluated by preneoplastic and neoplastic lesions in major organs. Forty-four compounds were tested using the Ito Multi-organ Model: 17 out of 17 liver carcinogens were positive, and 19 out of 22 (86%) nonliver carcinogens were positive. None of the 5 noncarcinogens tested positive.

Keywords: medium-term bioassay; carcinogens; liver GST-P; multi-organ.

INTRODUCTION

Identification and control of carcinogens in the environment are of prime importance to reduce cancer risk in humans. Long-term chronic administration assays for the detection of carcinogenicity and toxicity using rodents have been the standard for the evaluation of the carcinogenic potential of chemicals. The requirements call for testing in two rodent species, usually rats and mice, of each sex, at three dose levels (zero, low, middle, and high) of the test compound for 2 years.

Although this standard has long been used worldwide, 2-year carcinogenicity studies are too costly to test all the chemicals being introduced into the environment. Furthermore, there is political pressure to decrease the number of animals used for carcinogenicity testing because of animal welfare considerations (Ashby and Tennant 1991). A guideline proposed by the International Conference on Harmonization (ICH) recommends reducing long-term protocols by utilizing only one rodent species and replacing the second long-term rodent assay with an alternative bioassay (ICH Steering Committee 1997).

It is known that mutagenicity does not always correlate with carcinogenicity, and there are a variety of chemicals in use, typically represented by pesticides and herbicides, that are not mutagenic but are carcinogenic. A whole-body animal study is the only method to test the carcinogenic potential of a nongenotoxic chemical. Therefore, any alternative bioassay must be an *in vivo*, whole-body assay. Our laboratories have focused

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on the development of a rapid *in vivo* bioassay system able to detect both genotoxic and nongenotoxic carcinogens.

Since more than half of all known carcinogens are hepatocarcinogens in rodents, we initially focused on establishment of a medium-term liver bioassay system. We developed an 8-week rat liver bioassay, known as the Ito Liver Model, which is able to detect rat hepatocarcinogens with a high degree of accuracy (Shirai, Hirose, and Ito 1999). This protocol is cited in the sixth edition of *Casarett and Doull's TOXICOLOGY* as a potential alternative bioassay (Pitot and Dragan 2001). In addition, we developed a 28-week model, known as the as Ito Multi-organ Model, to detect carcinogens that are not identified by the Ito liver model.

Using the Ito Liver Model in conjunction with the Ito Multi-organ Model, most carcinogens can be identified after 8 weeks, and the remaining carcinogens can be identified after an additional 28 weeks. In the present review, the Ito Liver Model and the Ito Multi-organ Model are briefly described.

BACKGROUND

In 1976, Solt and Farber developed a protocol in which foci of liver cells expressing an altered repertoire of enzymes could be induced in rats within 4 weeks (Solt and Farber 1976). Their protocol was based on their observation that in rats treated with diethylnitrosamine (DEN) followed by the hepatocarcinogen 2-acetylaminofluorene (2-AAF), DEN-altered hepatocytes were able to respond to growth stimuli evoked by a two-thirds partial hepatectomy and form distinct foci. In contrast, normal hepatocytes were not able to respond to the growth stimuli because of the toxic effect of 2-AAF. This observation was described as a "selection process" by altered hepatocytes (Solt and Farber 1976). The application of this observation for carcinogen detection was examined by treating rats with test compounds to generate altered hepatocytes followed by feeding with 2-AAF and stimuli to induce hepatocyte proliferation, and the principle of the method was validated: treatment with representative carcinogens resulted in the formation of foci of altered hepatocytes (Tsuda, Lee, and Farber 1980).

In other studies, Peraino and associates reported a two-stage model in which hepatic tumor growth was enhanced by chemicals such as phenobarbital given after initiation with 2-AAF (Peraino et al. 1975, 1977, 1980). Their data suggested that a two-stage approach could be utilized for detection of carcinogenic responses to chemicals: either test compounds could given at the initiation stage followed by appropriate promoting agents or test compounds could be given during the promotion stage after initiation with DEN.

Based on the concepts presented above, we established an assay system to evaluate the hepatocarcinogenicity of chemicals for their promotion potential (Ito et al. 1996, 1997; Ito, Tamano, and Shirai 2003): we used the promotion potential of hepatocarcinogens because almost all carcinogens have a promotion effect when repeatedly administered (Peraino et al. 1975, 1977, 1980). The preneoplastic nature of altered hepatic foci and the usefulness of such lesions as indicators

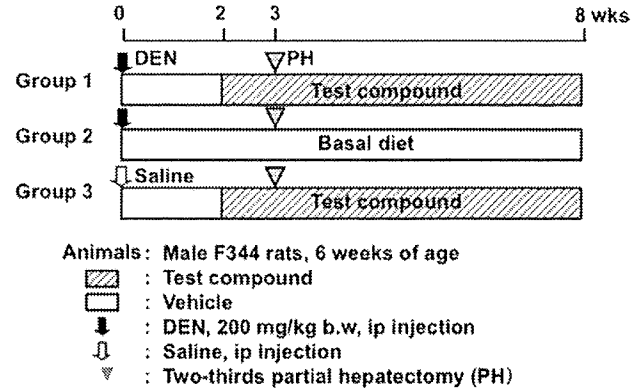

FIGURE 1.—Protocol of Ito Liver Model. Six-week-old male F344 rats are initially given a single intraperitoneal injection of diethylnitrosamine (200 mg/kg) to initiate liver carcinogenesis. Two weeks after initiation, the test compound is administered for 6 weeks. Animals are sacrificed at the end of week 8. All rats are subjected to two-thirds partial hepatectomy on week 3. The end-point marker is glutathione S-transferase placental form-positive (GST-P^+) liver cell foci. The numbers and sizes of GST-P^+ liver cell foci are analyzed using an image-analyzer and expressed as values per unit liver section (1 cm^2). When values, number, and/or area per unit area of GST-P^+ foci are significantly enhanced ($P < .05$) over the control value, a chemical is judged to possess carcinogenic potential for the liver.

of preneoplastic development are now well accepted (Bannasch 1986; Oesterle and Deml 1990; Tatematsu et al. 1977). The phenotypic characteristics of preneoplastic lesions in the liver have been extensively studied, and immunohistochemical staining for glutathione S-transferase placental form (GST-P) was found to be the best marker for visualization of lesions and their quantitative analysis (Ogiso et al. 1985; Tsuda et al. 2003, 1985).

ASSAY PROTOCOL AND RESULTS

Ito Liver Model

Figure 1 shows the protocol employed in Ito's laboratory as a medium-term liver bioassay model. Male F344 male rats, 5 weeks old, are divided into three groups consisting of 15-20 animals each. Group 1 is given a single intraperitoneal injection of DEN, 200 mg/kg b.w., dissolved in saline to initiate hepatocarcinogenesis. After 2 weeks, the rats receive a test compound mixed in the basal diet or drinking water or by repeated intraperitoneal, subcutaneous or intravenous injections. The rats are subjected to two-thirds partial hepatectomy (PH) at the end of week 3. Group 2 is given DEN and PH in the same manner as for group 1, but without administration of the test compound. Group 3 is injected with saline instead of DEN and then subjected to administration of the test compound and PH as in groups 1 and 2 (Figure 1). All animals are sacrificed at the end of week 8. The liver tissues, 3-4 slices from the cranial and caudal lobes of the right lateral lobe and caudal and/or cranial part of the caudal lobe, are excised and fixed in ice-cold acetone or 4% paraformaldehyde solution in phosphate buffer

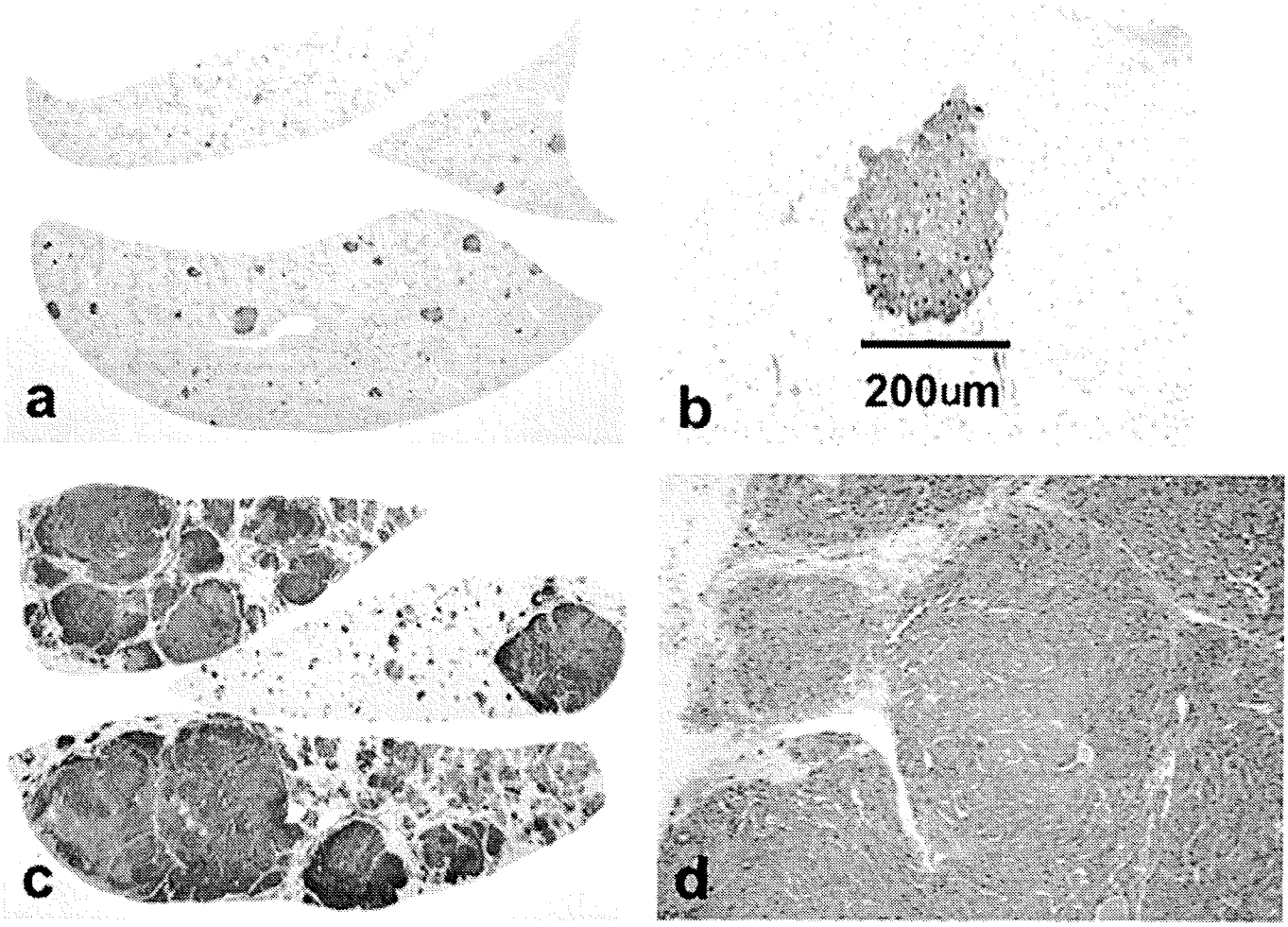

FIGURE 2.—GST-P-positive liver cell foci. Three to 4 slices from paraffin embedded liver (left) are immunostained with GST-P antibody. Lesions greater than 200μm (right) in diameter are included for counting. GST-P is consistently expressed from small foci to adenomas and hepatocellular carcinomas.

at pH. 7.4 for subsequent paraffin embedding and immunohistochemical demonstration of GST-P-positive foci. Numbers and areas of GST-P-positive foci more than 0.2 mm^2 in mean diameter are included for measuring by an image processor. The results are assessed by comparing the values between group 1 (DEN-test compounds) and group 2 (DEN alone). Group 3 serves to assay the potential of the test chemicals to induce GST-P-positive foci without prior DEN exposure. Statistical analysis of differences between means is carried out using Student's or Welch's *t*-tests after application of a preliminary *F*-test for equal variance, and scoring of carcinogenicity, promotion, or inhibition is made on the basis of differences in *P*-values between groups; positive = increase at $P < .05$ in either number or area of foci.

Until the protocol was finalized, the following were extensively investigated to maximize the predictive potential of the model (Hasegawa and Ito 1992; Ito et al. 1997, 1992; Shirai 1997; Shirai, Hirose, and Ito 1999):

1. use of PH as a tool for induction of hepatocyte proliferation,
2. the most suitable end-point marker enzyme,
3. whether results with GST-P-positive foci can predict carcinoma development in a dose dependent manner, and
4. specificity of the protocol for detection of carcinogens.

Since PH was introduced by Higgins and Anderson in 1931 (Higginson and Anderson 1931), it has been extensively employed for investigation of cell proliferation and regeneration. After two-thirds PH, the rodent liver recovers quickly and returns to near preoperative weight within 1 week with peak DNA synthesis at about 24 h; induction of hepatocyte growth factor appears to be one mechanism by which the liver recovers from PH (Matsumoto and Nakamura 1992). Use of hepatotoxins such as carbon tetrachloride (CCl_4) or D-galactosamine is

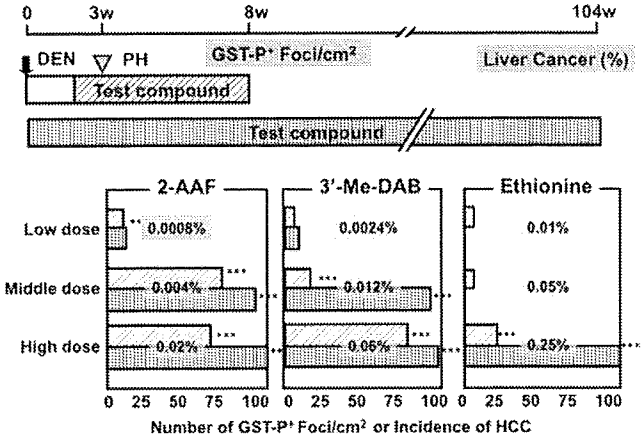

FIGURE 3.—Comparison of GST-P-positive foci and carcinoma. Results obtained from the Ito Liver Model and long-term 2-year studies are shown. Different doses of three representative hepatocarcinogens—2-acetylaminofluorene, 3'-methy-4-diaminoazobenzene, and ethionine—were administered. A clear correlation between GST-P-positive foci and the incidence of hepatocellular carcinomas can be seen. (a) A low magnification view of a slide from a rat treated with phenobarbital (0.05%, in the diet). (b) Smallest focus included for counting purposes. (c) A low-magnification view of a slide from a rat treated with 2-AAF (0.02%, in the diet). (d) Higher-magnification view of hepatocellular carcinoma: the carcinoma is clearly positive for GST-P.

TABLE 1.—Results for 159 Compounds in the Ito's Test

	No. of Positive Compounds/Examined (%)			
	Mutagenicity (Ames test)			
Test Compounds	Positive	Negative	Unknown	Total
Liver carcinogen	31/32(97)[a]	29/33(88)[b]	1/1(100)	61/66(92)
Non-liver carcinogen	7/26(27)	2/15(13)	1/2(50)	10/43(23)[d]
Not carcinogenic	0/6(0)	1/42(2)	0/2(0)	1/50(2)

[a] 4,4-Diaminodiphenylmethane gave negative results

[b] Four chemicals, Clofibrate, Di(2-ethylhexyl)adipate, Di(2-ethylhexyl)phthalate, Trichloroacetic acid, gave negative result.

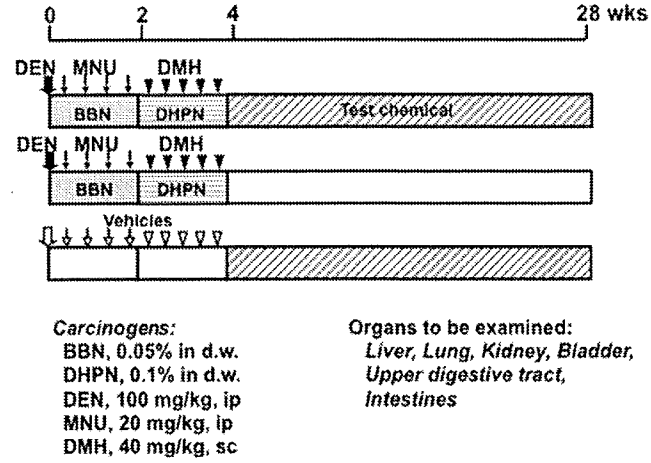

FIGURE 4.—Protocol of The Ito Multi-organ Model (DMBDD Model). Six-week-old F344 male rats are given i.p. injections of diethylnitrosamine (DEN, 100 mg/kg body wt.) and N-methylnitrosourea (MNU, 20 mg/kg body wt), s.c. injections of 1,2-dimethylhydrazine (DMH, 40 mg/kg body wt.), and 0.05% N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN) and 0.1% 2,2'-dihydroxy-di-n-propylnitrosamine (DHPN), both in the drinking water, for a total initiation period of 4 weeks (DMBDD treatment). The test compound is then administered for the following 24 weeks. The rats are sacrificed at the end of week 28. The liver, lung, thyroid, kidney, bladder, upper digestive tract (esophagus and forestomach), and intestines are examined for preneoplastic and neoplastic lesions and compared with the control rats.

an alternative method to induce liver cell proliferation. However, neither of these agents stimulates cell proliferation equivalent to PH in our system because induction of cells to enter S-phase of the cell cycle by these chemicals is sluggish (data not shown).

We have not yet elucidated the role of cell proliferation induced by PH at week 3 in the appearance of liver cell foci. It is possible that a majority of carcinogens are toxic to hepatocytes, causing retardation of the compensatory regenerative response to PH by noninitiated hepatocytes, allowing focal expansion of initiated hepatocytes. In this regard, it is known that initiated cells reduce phase I CYP enzyme expression and increase phase II enzyme expression (Liu et al. 2005; Tsuda et al. 1996), and this altered enzyme expression enables them to escape the effects of toxic compounds.

The expression of several different enzymes is altered in liver preneoplastic lesions (Ogawa et al. 1982; Tsuda et al. 1992). We compared the use of a variety of enzyme markers to visualize liver lesions (Tsuda et al. 2003, 1984). GST-P was found to be the most appropriate for practical use and is expressed continuously from the early lesion to the appearance of hepatocellular carcinoma (Kitahara et al. 1984; Tsuda et al. 1996, 2003).

Several studies have shown the validity of using GST-P-positive foci as a surrogate end-point in predicting carcinogenic potential (Ogawa et al. 1982; Tatematsu et al. 1985; Tsuda et al. 1984, 1988). One of these studies is shown in Figure 3. There was a clear correlation between GST-P-positive foci and incidence of hepatocellular carcinomas after administration of different doses of the well-known hepatocarcinogens 2-acetylaminofluorene, 3'-methy-4-diaminoazobenzene, and ethionine (Hagiwara et al. 1993).

A total of 159 compounds were examined using the Ito Liver Model. They are classified into three categories (Table 1): (1) hepatocarcinogens; (2) carcinogens targeting organs other than the liver (nonhepatocarcinogens); and (3) compounds negative for carcinogenicity in 2-year tests in rats and mice (noncarcinogens). The compounds can also be divided into three categories according to their reported mutagenicity: mutagenic compounds, nonmutagenic compounds, and compounds with unknown mutagenic potential. Comparisons of the results obtained using the Ito Liver Model and reported *Salmonella* mutagenicity and long-term carcinogenicity testing are summarized in Table 1. It is especially noteworthy that the Ito Liver Model identified

TABLE 2.—Results of 44 Compounds in the Medium-term Multi-organ Carcinogenesis Bioassay (DMD/DMBDD Model)

	Positive Compounds/Examined (%)			
	Mutagenicity (Ames test)			
Test compounds	Positive	Negative	Unknown	Total
Liver carcinogen	12/12(100)	5/5(100)	0/0(0)	17/17(100)
Non-liver carcinogen	10/11(91)[a]	8/10(80)[b]	1/1(100)	19/22(86)
Not carcinogenic	0/1(0)	0/4(0)	0/0(0)	0/5(0)

[a] One negative compound is Benzo[a]pyrene
[b] Two negative compounds are Sesamol and Daminozide

59 of 64 (92%) liver carcinogens, irrespective of their mutagenicity, leaving only 5 false-negatives; 30 out of 31 (97%) mutagenic and 29 out of 33 (88%) nonmutagenic hepatocarcinogens were identified. Three out of the 4 nonmutagenic carcinogens that gave false-negative results were carcinogenic peroxisome proliferators, known to suppress GST-P expression. It is noteworthy that the false-positive and false-negative rates are 2.1% and 3.1%, respectively. It was also noted that many chemicals positive in the Ito Liver Model were hepatotoxins (Ward et al. 1989). These results clearly demonstrate that this medium-term liver bioassay is excellent for detection of liver carcinogens (Ito, Tamano, and Shirai 2003; Shirai 1997).

A formula for the validity of carcinogen screening tests is described by Cooper, Saracci, and Cole (1979). This formula evaluates five categories: sensitivity, specificity, predictive value (positive predictivity), false-positive rate, and false-negative rate of the screening test. When the Ito Liver Model was evaluated, all five categories demonstrated excellent values (Shirai, Hirose, and Ito 1999). The Ito Liver Model was accepted as an alternative protocol to replace one of the 2-year chronic administration assays at the Fourth International Conference on Harmonization (ICH Steering Committee 1997).

Ito Multi-organ Model (DMBDD Model)

The Ito Multi-organ Model was developed for the detection of carcinogens not identified by the Ito Liver Model. F344 male rats are given i.p. injections of diethylnitrosamine (DEN, 100 mg/kg body wt.) and N-methylnitrosourea (MNU, 20 mg/kg body wt), s.c. injections of 1,2-dimethylhydrazine (DMH, 40 mg/kg body wt.), and 0.05% N-butyl-N-(4- hydroxybutyl)nitrosamine (BBN) and 0.1% 2,2'-dihydroxy-di-n-propylnitrosamine (DHPN), both in the drinking water, for a total initiation period of 4 weeks (DMBDD treatment) (Akagi et al. 1995; Ito et al. 1996). Then rats are given the test compound in the diet or drinking water or by injection for the following 24 weeks. The animals are sacrificed at the end of week 28. The organs targeted by the 5 different carcinogens—the liver, lung, thyroid, kidney, bladder, upper digestive tract (esophagus and forestomach), and intestines—are histologically examined for preneoplastic and neoplastic lesion development (Fukushima et al. 1991; Ito et al. 1996).

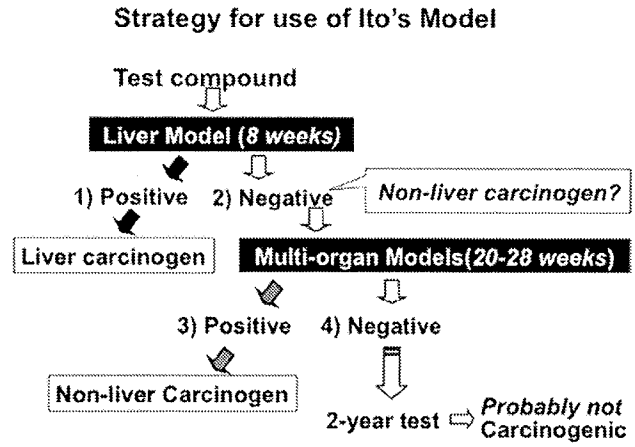

FIGURE 5.—Strategy for the Use of Ito's Model. (1) Positive compound in the liver model: is a liver carcinogen. (2) Negative compound in the liver model: test using the multi-organ model. (3) Positive compound in the multi-organ model: is a carcinogen. (4) Negative in both the liver and multi-organ models: is probably not carcinogenic.

A total of 44 compounds were examined using the Ito Multi-organ Model. A summary of the results is presented in Table 2. All 17 liver carcinogens tested positive, including peroxisome proliferators, and 19 of 22 nonliver carcinogens (86%) tested positive. The Ito Multi-organ Model was able to identify carcinogens irrespective of their mutagenicity; 22 of 23 mutagenic carcinogens and 13 of 15 nonmutagenic carcinogens were identified (Table 2).

The medium-term bioassay described here is a rapid, reliable, and practical tool for the prediction of the carcinogenic potential of chemicals. The strategy for the use of the Ito Model is presented in Figure 5.

1. Positive in liver model: carcinogen.
2. Negative in liver model: apply the multi-organ model.
3. Positive in the multi-organ model: carcinogen.
4. Negative in both the liver and multi-organ models: probably not carcinogenic.

The system is now internationally well recognized and recommended as an alternative carcinogenicity test.

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