Selective Localization and Growth of *Bifidobacterium bifidum* in Mouse Tumors following Intravenous Administration¹

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**ABSTRACT**

A strain of domestic bacteria, *Bifidobacterium bifidum* (Lac B), which is nonpathogenic and anaerobic, selectively localized and proliferated in several types of mouse tumors following i.v. administration. None of the same bacilli could be detected in the tissues of healthy organs such as the liver, spleen, kidney, lung, blood, bone marrow, and muscle 48 or 96 hr after i.v. administration into tumor-bearing mice.

Proliferation of Lac B in the tumor was artificially stimulated by i.p. administration to DDD-H-2* mice of a synthesized disaccharide, lactulose (4-O-β-D-galactopyranosyl-D-fructofuranose), a sugar which is not metabolized by mammalian tissue cells.

Lac B, which survives and proliferates selectively in the tumor following i.v. administration into the tumor-bearing host, should aid in diagnosis and selective therapy for cancer.

**INTRODUCTION**

Regardless of the type of prescribed therapy, it is of cardinal importance in treating cancer to distinguish neoplastic from healthy tissue. However, the basic criterion for a clear distinction is yet to be established. Chemotherapy has the advantage of being able to attack cancer cells scattered in undetectable sites. It also has a disadvantage, in that it is capable of attacking rapidly growing normal cells such as those of gastrointestinal epithelia or of bone marrow. Although studies on the possible affinity of certain microorganisms to cancer have been done (24, 27—29), a practical approach has been awaited. In our investigations on cancer, we concluded that a new approach toward distinguishing between cancer cells and nonmalignant cells was required.

Focal centers of necrosis with accompanying zones of low oxygen tension are frequently seen in cancer lesions. When anaerobic bacteria are given i.v. to the tumor-bearing host, the bacteria will proliferate freely in those zones of low oxygen tension (24, 29); there is no proliferation in healthy tissues rich in oxygen, as supplied by an adequate blood flow. Thus, the presence of anaerobic bacteria in tumor tissues may be a clue which would aid in distinguishing tumor and normal tissues, a marker which could be used effectively in tumor diagnosis or treatment. For such purposes, the anaerobic bacterium must be nonpathogenic.

Lac B² is anaerobic and is one of the domestic, nonpatho-

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² The abbreviations used are: Lac B, Lactobacillus bifidus, a synonym of the new nomenclature, *Bifidobacterium bifidum*; PBS, phosphate-buffered saline (2.7 mM KCl, 8.1 mM NaH₂PO₄, 1.5 mM KH₂PO₄, and 0.8% NaCl, pH 7.4); FITC, fluorescein isothiocyanate-conjugated.

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**Lactulose Solution.** Lactulose (4-O-β-D-galactopyranosyl-D-fructofuranose) was also kindly provided by Nikken Chemicals Co., Ltd., and used as a 20% water solution after sterilization by passing through a 0.45-μm Millipore filter (Millipore Co., Bedford, Mass.).

**Treatment of Mice with Lactulose.** Preliminary experiments revealed that the number of bacilli per g of tumor tissues of Ehrlich solid tumor-bearing mice given in a single i.v. administration of Lac B 7 days before, followed by daily i.p. administrations of 1 ml 20% lactulose solution, was about 1000-fold more than that of the mice given daily i.p. administrations of 1 ml PBS.

Thus, throughout the present study, the bacterium suspension was given i.v. to the mice only once on Day 0, and thereafter 1 ml of 20% lactulose was given i.p. daily starting from Day 0 to the day of sacrifice. In the control groups, the same volume of PBS instead of lactulose was given by the same way and for the same period of time.

**Preparation of Tissue Homogenate.** Both the tumor-bearing and non-tumor-bearing mice were sacrificed by cervical dislocation. Organs and tissues were excised and minced thoroughly with scissors, and a sample was weighed and placed in a Potter-Elvehjem homogenizer to prepare a 10% homogenate with cold PBS.

The above procedure was carried out under aseptic conditions at 0°. As samples of nonmalignant tissues, the muscles (pectoral, deltoid, biceps, and triceps brachi of both sides; gluteal and thigh muscles of the non-tumor-bearing side); both lungs; blood; and bone marrow of bilateral humerus, radius, and ulna and of femur, tibia, and fibula of the non-tumor-bearing side were used. The whole tumor, which grew at the right thigh, was enucleated and weighed after removal of the bony material. Then the tumor tissue was minced thoroughly, and a sample was processed as described above. Since bone marrow could be neither obtained in an adequate amount nor prepared easily from bony material, the above-mentioned bones were mixed together and weighed, crushed with scissors, and then homogenized with cold PBS.

Blood was obtained aseptically from the femoral vessels of non-tumor-bearing thigh. An aliquot (200 μl) of blood was pipetted quickly into a test tube containing 50 μl of heparin solution (1000 units/ml). The heparin in this blood-heparin mixture was preliminarily confirmed to have no effect on the growth of Lac B in vitro.

**Culture Medium.** CTJ medium utilized in this study was kindly provided by Nikken Chemicals Co., Ltd. The composition of the medium was as follows: pancreatin-hydrolyzed casein, 5 g (as casein); sodium acetate anhydrous, 25 g; K2HPO4, 2.5 g; lactose, 35 g; ammonium acetate, 2 g; adenine-HCl, guanine-HCl, uracil, and xanthine, 10 mg each; DL-tryptophan, DL-alanine, and L-cystine, 200 mg each; L-asparagine, 100 mg; DL-alanine, and L-cystine, 200 mg each; L-asparagine, 100 mg; fumaric acid, 500 mg; sodium ethylxaloacetate, 500 mg; thiamine-HCl and riboflavin, 200 μg each; calcium pantothenate, 400 μg; pyridoxine-HCl, 1200 μg; nicotinic acid, 600 μg; p-aaminobenzoic acid and folic acid, 10 μg each; biotin, 4 μg; cyanocobalamin, 5 μg; Salts B (10 g of MgSO4·7H2O, 0.5 g of FeSO4·7H2O, 0.5 g of NaCl, and 0.335 g of MnSO4·H2O in 250 ml of H2O), 5 ml; Tween 80, 1 ml; agar, 10 g. pH was adjusted to 6.4 by adding 10% NaOH, and the whole volume was adjusted to 1000 ml with distilled water. The medium was autoclaved at 15 pounds for 10 min, then cooled rapidly, and stored in a refrigerator.

**Culture Conditions.** Refrigerated solidified culture medium (CTJ medium without vitamin C) was melted thoroughly in boiling water, and vitamin C (1 mg/ml, final concentration; Takeda Chemical Industries, Ltd., Osaka, Japan) was added to the medium when the temperature dropped below 55°. Thereafter, the medium was kept in a 48° water bath ready for use. Also, P-3 Petri dishes, prewarmed at 48° in an incubator, were placed on a thermocontrolled table-type heater prewarmed and kept at 48° during the procedure of pouring and mixing the medium and homogenates. The medium was poured into the dishes, 4 ml/dish. Usually, appropriately diluted tissue homogenate, 50 μl/dish, was inoculated into 4 dishes per sample and thoroughly mixed with the medium using a glass rod spreader. After the agar medium was solidified at room temperature, all the dishes were placed in a completely airtight desiccator. The air in the desiccator was replaced with 0.5 atm of CO2, and incubation was carried out at 37° under these anaerobic conditions for a given period. On Day 3 of culture, colonies grown in one of the 4 dishes were morphologically and bacteriologically identified; those in the remaining 3 dishes were counted.

Under this culture condition, the number of colonies ranging from 40 to 400 per plate (P-3 Petri dish) closely corresponds to the number of Lac B in the materials.

**Test of the Time Course Distribution of i.v.-administered Lac B in Various Organs or Tissues.** Forty tumor-bearing mice and 8 non-tumor-bearing mice were given 5 × 105 viable bacilli i.v. and lactulose solution i.p. as described above. At 12, 24, 48, 96, and 168 hr (7 days) after injection of the bacilli, 8 tumor-bearing mice each were sacrificed. Another group of 8 non-tumor-bearing mice was sacrificed at 168 hr to observe the bacilli in normal tissues of the non-tumor-bearing mice at the same time when bacilli were not detectable in normal tissues of the tumor-bearing mice.

**Confirmation of the Growth-promoting Effect of Lactulose on Intratumoral Lac B.** Fifty tumor-bearing mice were given 1 ml of 20% lactulose solution i.p. daily for the first week after receiving Lac B. At the beginning of the second week, the mice were divided into 2 groups: Group A, 20 mice; and Group B plus C, 30 mice. To the mice in Group A, lactulose administration was continued for 2 more weeks. At the end of the second and third week, 10 mice each were sacrificed for the determination of intratumoral Lac B. For the remaining 30 mice (Group B plus C), lactulose administration was suspended during the second week. At the end of the second week, 10 mice were sacrificed for the determination of the intratumoral Lac B. The remaining 20 mice were then divided into 2 groups of 10 each (Groups B and C). To the mice in Group B, lactulose was again given i.p. daily during the third week. The mice in Group C were not given lactulose injection during the third week. At the end of the third week, all the mice of Groups B and C were sacrificed and examined for intratumoral Lac B in the same way that the mice in Group A were examined.

**Assessment of Possible Proliferation of i.v.-administered Lac B in the Traumatized or Inflammatory Lesion.** To determine whether the i.v.-administered Lac B would be detectable in the traumatized or inflammatory lesions, hematomas were made artificially by injecting 1 ml of blood obtained aseptically...
from the donor mice into crushed thigh muscle lesions of 8 recipient mice. Four days later, Lac B (5 × 10^6 viable organisms/mouse) was given i.v. Lactulose treatment was also given as in the other experiments for 7 days. On Day 7 after Lac B injection, the hemotoma tissues were harvested and examined for Lac B.

Test on the Effect of i.v.-administered Lac B on the Life Span of Tumor-bearing Mice. To determine the effect of i.v.-injected Lac B on the survival of Ehrlich solid tumor-bearing mice, 3 experiments were repeated. Female 6- to 7-week-old DDD mice were inoculated with Ehrlich ascites tumor cells in the right thigh muscle. On Day 28 (Experiment a), 33 (Experiment b), or 26 (Experiment c) of tumor growth, 2 × 10^7 viable Lac B bacilli were given i.v. only once; 20% lactulose solution, 1 ml/mouse, was given i.p. once a day for 7 days (Experiment a) or for 21 days (Experiments b and c), the first injection being given on Day 0 immediately after the Lac B injection.

As controls, one group of tumor-bearing mice was not treated; another group was given lactulose solution (1 ml/mouse) alone i.p. for the period corresponding to that of each test group. The mice were inspected daily, and their body weight and tumor size were recorded once a week.

Immunofluorescence Studies. Anti-Lac B rabbit serum (titer, 1:256) was a gift from Ohmiya Research Institute, Nikken Chemicals Co., Ltd. (Ohmiya, Japan). For indirect immunofluorescence tests, tumor slices were frozen in dry ice:99.6% ethanol at −78°, and 5-μm-thick sections were prepared in a cryostat (Lipshaw Manufacturing Co., Detroit, Mich.) at −18°. The sections were dried and fixed in 95% ethanol at −20° for 20 min and then dried again. After a rinsing with a cold staining buffer (2.9 mM NaH₂PO₄, 9.0 mM Na₂HPO₄, and 0.8% NaCl, pH 7.2), the sections were covered with 30-fold diluted anti-Lac B rabbit serum and incubated in a moist chamber at 37° for 1 hr. After incubation, the sections were rinsed thoroughly with the buffer and stained with goat FITC-anti-rabbit γ-globulin (Boehringer Institute, Marburg, West Germany) at a 1-mg/ml concentration of γ-globulin (fluorescein:protein molar ratio, 3.2) at room temperature for 20 min. After staining, the sections were again thoroughly washed with the buffer and mounted in a buffered glycerol (pH 7.2). The preparations were examined under a Leitz Ortholux microscope. As control, some sections were stained with goat FITC-anti-rabbit γ-globulin without preincubation with anti-Lac B serum.

Histology. Tumors of mice given Lac B i.v. 7 days before were fixed in 10% formalin solution and embedded in paraffin. A Gram stain was applied to 5-μm-thick sections, as reported by Brown and Hopps (6).

RESULTS

Distribution of Lac B in Various Organ Tissues. Chart 1 shows the number of Lac B organisms per g of various tissues at various time intervals after i.v. administration of 5 × 10^6 viable bacilli into tumor-bearing mice. The number of Lac B in the tumor continued to increase for 1 week (1-68 hr).

Contrastingly, the number of Lac B organisms in nonmalignant tissues, such as the liver, spleen, kidney, lung, blood, bone marrow, and muscle from the tumor-bearing mice, began to decrease immediately after Lac B injection. The bacilli completely disappeared in 24 to 96 hr. Disappearance of Lac B from normal tissues of non-tumor-bearing mice was also confirmed at 168 hr.

Growth-promoting Effect of Lactulose on Intratumoral Lac B. This experiment was performed to confirm the growth-promoting effect of lactulose on intratumoral Lac B. Since all the tumor-bearing mice given injections of Lac B were a part of the
same material used in the preceding experiment, the results obtained (Chart 2) represent a continuation insofar as the number of the intratumoral Lac B is concerned. Consequently, in Chart 2, the data within 7 days after i.v. administration of

Lac B are those of the preceding experiment. The number of Lac B in the tumor decreased after the interruption of lactulose administration (Group B plus C on Day 14 and Group C on Day 21), as compared with Group A in which daily lactulose injections were given for 14 or 21 days ($p < 0.05$, t test) without interruption. However, the number of Lac B organisms in the tumors of Group B began to increase again with resumption of lactulose administration ($p < 0.05$, t test).

**Detectability of Lac B in the Traumatized or Inflammatory Lesion.** This experiment was done to determine whether the i.v.-administered Lac B would be detectable in a traumatized or inflammatory lesion at the time when the injected Lac B was detectable in tumor but not in any normal tissues. On Day 7 after injection of Lac B ($5 \times 10^6$ viable organisms/mouse), this bacterium was not detectable in any hematoma-containing crushed tissue of the thigh muscles, induced 4 days before Lac B injection.

**Effect of Lac B on the Life Span of Tumor-bearing Mice.** As shown in Chart 3, 3 repeated experiments showed neither significant prolongation nor shortening of the life span of tumor-bearing mice after a single i.v. administration of Lac B followed by a daily i.p. administration of lactulose. There was no significant difference in body weight or tumor size among the 3 groups.

**Detectability of Intratumoral Lac B In Relation to Tumor Size.** Detectability of Lac B in the Ehrlich solid tumor following a single administration of $5 \times 10^6$ bacilli i.v. and with a daily i.p. lactulose administration into the tumor-bearing mice was examined on Day 7 in relation to the tumor size. As shown in Table 1, the detectability of Lac B was 90.6% (58 of 64) when the tumor diameters were more than 15 mm. A decrease to 64.3% (9 of 14) occurred when the tumor diameters were less than 15 mm. However, when $1.2 \times 10^8$ bacilli were administered, the detectability increased to 100% (10 of 10) even when the tumor diameters were less than 15 mm. Bacilli were
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not detected in the normal tissues even with this higher dose of Lac B.

Detectability of Lac B in Other Kinds of Tumor. Detectability of intratumoral Lac B in other kinds of tumor was also tested as described above on Day 7, following a single administration of 5 \times 10^6 bacilli i.v. and daily lactulose administration. The numbers in parentheses, number of cases in which presence of intratumoral Lac B was positive/number of cases tested.

<table>
<thead>
<tr>
<th>Tumor diameter (mm)</th>
<th>Inoculum of bacilli (\times 10^6/mouse)</th>
<th>Detectability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;15</td>
<td>120</td>
<td>100 (10/10)</td>
</tr>
<tr>
<td>&gt;15</td>
<td>5</td>
<td>64.3 (9/14)</td>
</tr>
<tr>
<td>15–25</td>
<td>5</td>
<td>90.6 (58/64)</td>
</tr>
</tbody>
</table>

* Geometric mean of tumor diameters measured in 3 dimensions.
* Numbers in parentheses, number of cases in which presence of intratumoral Lac B was positive/number of cases tested.

DISCUSSION

Coinciding with the embryonic achievements in bacteriology as a natural science, use of microorganisms or their products as possible therapeutic means against cancers began in the latter part of the last century. The first report of this kind concerning human tumors was made by Busch (7) in 1868. On the basis of his own clinical experience, he attempted to induce erysipelas infection in a 19-year-old woman with inoperable sarcoma; apparently, he obtained encouraging results. Since then, many varieties of microorganisms and their products have been tested to determine whether or not they are effective therapeutic agents. The history of these attempts has been well reviewed by Reilly (34).

Penicillin-attenuated Streptococcus hemolyticus (OK-432) (23, 31) and Bacillus Calmette-Guérin (16, 25, 33, 37) are reportedly effective as nonspecific immunostimulators in cancer therapy. There are also antineoplastic fungal products or antibiotics, e.g., actinomycin D, mitomycin C, bleomycin. However, these are not universally effective against all forms of cancer. Furthermore, their usefulness is limited because they do not strictly distinguish cancer cells from normal cells and have serious side effects.

Little attention was paid to the selective affinity per se of microorganisms to a tumor tissue until Malmgren and Flanigan (24) reported the selective germination of the vegetative form of Clostridium tetani in the locus of a mouse tumor after i.v. administration of its spores. They established that all tumor-bearing mice died of tetanus intoxication within a few days, while all mice without tumor remained healthy. On the other hand, nonpathogenic and anaerobic Clostridium butyricum is reportedly oncolytic when given i.v. to tumor-bearing mice (29).

The oncolytic action in this case apparently depended on the action of H_2O_2 (21) and kinase (5, 27, 28) produced by the bacteria. A clinical application of the bacteria has apparently not been reported. The above-mentioned workers attempted to treat tumor using bacteria but did not utilize the bacteria for specific diagnostic detection of cancer.

Recently, the distribution of killed Corynebacterium parvum labeled with ^125I (10) or with FITC (36) was studied after its i.v. administration into tumor-bearing mice. A similar study using ^99mTc-labeled C. parvum was done by other researchers (4). In these investigations, the distribution of C. parvum was determined indirectly by assaying the radioactivity emanating from the isotopes which had been conjugated to the bacteria. However, the radiological method can merely show the presence and localization of the labeled bacteria in animals. The viability and rate of proliferation of the bacteria in vivo cannot be assessed by this method. We examined organ distribution and proliferation of Lac B following i.v. administration into tumor-bearing mice directly by quantitative assay of the living microorganisms in various organs and the tumors.

Bifidobacterium bifidum (Lactobacillus bifidus) was first described by Tissier (38) as constituting almost the entire flora of the stools of breast-fed infants. The nature of this microorganism was extensively studied by György et al. (3, 15, 17, 18, 30, 39, 40). The nonpathogenicity and importance of Lac B in maintaining the health of infants is now generally acknowledged. In Japan, Lac B has been widely prescribed clinically as one of the most popular drugs for the control of intestinal flora.

When we gave Lac B to tumor-bearing mice i.v., the distribution was first seen over the whole body, but after 48 to 96 hr it was detectable only in the tumor tissue (Chart 1). The fact that the bacilli are not only detected but can also proliferate in the tumor tissue implies that this tissue possesses an environment suitable for the growth of this bacterium. The suitability may be ascribed to either an anaerobic or hypoxic environment. We found in cultures that the tumor homogenate per se revealed no growth-promoting effect on Lac B (data not shown).

It is interesting that the growth of Lac B in the tumor tissue can be remarkably stimulated artificially by systemic administration of lactulose to the tumor-bearing host (Chart 2). This synthesized sugar, lactulose (19, 32), is not found in nature but can be prepared from lactose by alkaline isomerization (26). Since the sugar cannot be metabolized by humans, rats, and pigs (8, 12, 14, 35), it is assumed that lactulose in vivo stimulates the growth of Lac B in the tumor without otherwise affecting the mammalian tissues.

Here, a discussion on the utility of this nonpathogenic and
an aerobic bacterium, Lac B, in the field of cancer diagnosis and therapy may be useful. Our data suggest that Lac B can be used profitably as a tool for diagnosis and selective therapy of cancer. For example, if radioisotopically labeled antibody (1, 2, 22) against Lac B or antibody-anticancer drug complex (9, 11, 13, 20) is applied to the tumor-bearing and Lac B-treated host more than 96 hr after the administration of Lac B, the radioisotope or the drug complex would be selectively localized in the tumor.

Since the presence of Lac B was demonstrated even in tumors about 10 mm in diameter, detection of the locus of a tumor or of a metastatic focus may be diagnostically feasible with the aid of radiodiagnosis. In fact, colonies of Lac B were obtained from a rice grain-sized inguinal lymph node with metastasis of Ehrlich solid tumor of a mouse which had been given a single i.v. administration of Lac B 7 days before. A selective tumor damage by means of a radioisotopically labeled antibody to Lac B could also be envisioned.

Although the nonpathogenicity of Lac B is generally acknowledged and no significant harmful effect on tumor-bearing mice was observed in the present study (Chart 3), further tests should be done. In fact, a clinical case of bacteremia, which was caused by mixed infection with Eubacterium lentum and Bifidobacterium species as a result of a perforated colonic diverticulum with subsequent intraabdominal abscess formation and peritonitis, has been reported (41). However, the strain of Bifidobacterium was not specified in this report. Nonetheless, it should be kept in mind that even in the same species of bacterium there are many strains displaying differences in pathogenicity [for instance, Cl. tetani (24) and Cl. butyricum (27–29)]. In our tests using mice, hamsters, guinea pigs, and rabbits, no significant pathological changes in body weight, peripheral leukocyte counts, body temperature, the lie of fur, and life-span resulted from the i.v. administration of Lac B. In hopes that genetic engineering may provide an oncolytic capacity by introducing a suitable plasmid or some such related compound in Lac B, further investigations of the applicability of Lac B to cancer diagnosis and therapy are in progress.

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REFERENCES


Fig. 1. Fluorescence photomicrograph of Lac B in Ehrlich solid tumor tissue stained by FITC-antibody method. × 400.

Fig. 2. Photomicrograph of Lac B in Ehrlich solid tumor tissue stained by a modified Gram stain method. a, part of the border between necrotic and nonnecrotic Ehrlich tumor tissue; b, necrotic tissue of the tumor. × 400.

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