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Effect of a Bacteriocin Produced by *Mycobacterium smegmatis* on Growth of Cultured Tumor and Normal Cells

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ABSTRACT

Growth-inhibitory effects of a partially purified bacteriocin derived from *Mycobacterium smegmatis* ATCC 14468 on various animal cells transformed by tumor viruses, human malignant cells, and normal cells in the same species were studied. A growth-inhibitory effect of the bacteriocin on these cultured cells was determined by counting the residual cells. The bacteriocin inhibited virally transformed animal cells (mKS-A TU-7, 155-4 T2, and XC cells) and human malignant cells (AS-II and HGC-27 cells). The inhibitory effect increased with an increase in the bacteriocin activity. The bacteriocin sensitivities of transformed animal cells were relatively higher than were those of human malignant cells, while normal cells in the same species were practically insensitive to the bacteriocin. Differences in the degree of bacteriocin sensitivity were observed among tumor cell lines. Simian virus (SV) 40-transformed hamster cells (TSV-5 cells), which grow rapidly, were less sensitive to the bacteriocin. The cell membrane of SV40-transformed BALB/c mouse cells (mKS-A TU-7 cells) adsorbed the bacteriocin much more than did the cell membrane of nontransformed BALB/3T3 cells. The results seem to indicate that the inhibitory effect of bacteriocin 14468 on cultured mammalian cells probably depends on the binding sites for the bacteriocin which appear or increase by malignant transformation on cytoplasmic membrane.

INTRODUCTION

Bacteriocins, antibacterial substances of a proteinaceous nature for strains belonging to the same or closely related species, are produced by many species of bacteria (10, 17, 21). The cytoplasmic membrane of the cells is a primary target for the action of bacteriocin (2, 8, 9, 23).

Although bacteriocins interact only with sensitive strains among bacteria, they could be toxic to mammalian cells as these cells possess a number of receptors for various substances (15, 20). Farkas-Himsley and Cheung (4) reported that several bacteriocins, produced by gram-negative bacteria, such as colicin, vibriocin, and pyocin showed the toxic effect on established neoplastic L60T mouse cells. However, details on the comparative sensitivity of neoplastic and normal cells to bacteriocins have not been documented. Little is known about the toxic effects of bacteriocins produced by gram-positive bacteria. Recently, we found that HeLa-S3 cells were sensitive to a partially purified Bacteriocin 14468 originating from *Mycobacterium smegmatis* ATCC 14468 (19). This paper deals with the growth-inhibitory effects of Bacteriocin 14468 on tumor virus-transformed animal cells, human malignant cells, and normal cells in the same species.

MATERIALS AND METHODS

Cell Lines. The cell lines used are as follows. (*a*) mKS-A TU-7 cells, SV40-transformed BALB/c mouse tumor cells (13); (*b*) BALB/3T3 cells, established from BALB/c mouse; (*c*) 155-4 T2, herpes simplex virus 2-transformed hamster tumor cells (12); (*d*) TSV-5 cells, hamster tumor cells induced by SV40 (7); (*e*) BHK 21 cells, baby hamster kidney cells; (*f*) XC cells, rat tumor cells induced by Rous sarcoma virus; (*g*) NRK cells, normal rat kidney cells; (*h*) AS-II cells, human malignant cells of cancerous ascites derived from embryonal carcinoma of ovarium (11); (*i*) HGC-27 cells, human malignant cells from metastatic lymph node of gastric cancer (1); and (*j*) HEL cells, human embryo lung cells. All cells were maintained in MEM[2] (Wako Pure Chemicals Co., Tokyo, Japan) supplemented with 10% fetal bovine serum (MBA, Inc., Bethesda, Md.). For culture TSV-5 cells, MEM containing 3-fold of amino acids and vitamins was used.

Bacteriocin Preparations. The partial purification of Bacteriocin 14468 was described previously (19). Briefly, the bacteriocin in the cell-free extracts obtained by disintegrating *M. smegmatis* ATCC 14468 cells in a sonic oscillator was purified by means of chromatography on DEAE-cellulose, ammonium sulfate fractionation, gel filtration on Sephadex G-200, and chromatography on DEAE-Sephadex A-50.

Growth-inhibitory Effect of Bacteriocin. Cells cultured for 4 days were treated with 0.2% EDTA in MEM to detach them from glass bottles (5 × 15 cm), collected by centrifugation, and suspended in MEM containing 10% fetal bovine serum as growth medium. Five ml of cell suspensions (about 10^5 cells/ml) were cultured in Petri dishes (60 mm) or plastic tissue dishes (50 mm; Wako Pure Chemicals) at 37° for 24 hr in a humidified atmosphere containing 5% CO_2. After incubation, adherent cells were rinsed thoroughly with MEM and then exposed to 0.2 ml of the bacteriocin solution (64 to 256 AU/ml of MEM) at 37° for 3 hr. As controls, rinsed cells were treated with 0.2 ml of heat-inactivated bacteriocin solution (256 AU/ml) or MEM alone. After the bacteriocin solution was removed, the cells were rinsed with MEM and then incubated for up to 120 hr at 37° in 5 ml of the fresh growth medium. At various time intervals, the adherent cells were detached with 0.2% EDTA, collected by centrifugation, suspended in 0.85% NaCl solution, and counted with a hemocytometer.

Effect of Long Exposure of Cells to Bacteriocin. Twenty-four-hr old cells cultured in plastic tissue dishes (32 mm; Toyoshima Manufacturing Co., Tokyo, Japan) were incubated

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[2] The abbreviations used are: MEM, minimal essential medium; AU, arbitrary units.

in 1.0 ml of the growth medium with or without 64 AU/ml of bacteriocin at 37° for 48 hr. After incubation, adherent cells were harvested as mentioned above, and the percentage of inhibition was calculated from the number of adherent cells as follows:

$$\% \text{ inhibition} = 100 - \left(\frac{\text{the number of adherent cells in test}}{\text{the number of adherent cells in control}} \times 100\right)$$

Preparation of Cell Membrane and Assay of Bacteriocin Adsorption to the Cell Membrane. The cell membrane fractions of mKS-A TU-7 and BALB/3T3 cells were prepared according to the method of Emmelot *et al.* (3). The cell membrane-rich fraction in 10 mM Tris-HCl buffer (pH 7.5) was stored at −20°. Protein in the fraction was measured by the method of Lowry *et al.* (14). One-half ml of the bacteriocin solution (128 AU/ml) was incubated with an equal volume of the cell membrane preparations (0 to 3600 μg protein per ml) at 37° for 3 hr. After incubation, the mixture was centrifuged at 15,000 × *g* for 20 min at 4°, and an aliquot of supernatant was withdrawn and diluted 2-fold. The residual bacteriocin activity was titrated using indicator cells (*Mycobacterium diernhoferi* ATCC 19340) as described previously (19).

RESULTS

Effect of Bacteriocin on the Growth of Tumor Virus-transformed and Nontransformed Cells. Chart 1 shows the effect of Bacteriocin 14468 on the growth of SV40-transformed BALB/c mouse tumor cells (mKS-A TU-7 cells) and nontransformed BALB/3T3 mouse cells. In the case of mKS-A TU-7 cells, the number of adherent cells treated with 64 AU/ml of bacteriocin increased after 24 hr of incubation. However, the number no longer increased after 48 hr, and a logarithmic decrease occurred thereafter. At the activity of 128 AU/ml, the inhibitory effect was more conspicuous. In contrast, no significant inhibition of the bacteriocin (128 AU/ml) to BALB/3T3 cells was observed.

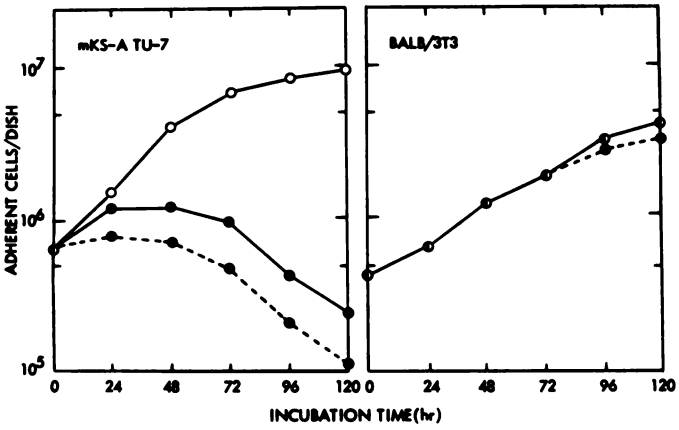

Chart 1. Inhibitory effect of Bacteriocin 14468 on growth of mKS-A TU-7 and BALB/3T3 mouse cells. Five ml of cell suspensions at a density of 10^5/ml of MEM supplemented with 10% fetal bovine serum were plated onto plastic tissue (50 mm) or Petri dishes (60 mm) and then incubated at 37° for 24 hr in a humidified atmosphere containing 5% CO_2. The cells were treated with or without 0.2 ml of native and heat-inactivated bacteriocin solution at 37° for 3 hr. The cells were rinsed and further incubated in 5 ml of the fresh growth medium at 37°. At indicated intervals, the residual adherent cells were counted with a hemocytometer. The values indicated were the mean values obtained from 3 or 5 dishes. In the following charts, the experimental methods are the same as mentioned above. ○, cells plus no or heat-inactivated bacteriocin; ●——●, cells plus 64 AU/ml, ●- - -●, cells plus 128 AU/ml. ◐, overlapped values of control (○) and experiment (●).

Chart 2 shows the effect of bacteriocin on virally transformed hamster tumor cells (155-4 T2 and TSV-5 cells) and nontransformed hamster cells (BHK 21 cells). In the case of 155-4 T2 cells, the increase in the number of cells treated with 64 AU/ml of bacteriocin stopped after 48 hr, and a decrease was seen thereafter. Inhibition of the cell growth was more effective when 128 AU/ml was used. In contrast to TSV-5 cells, significant inhibitory effects of the bacteriocin were not observed with BHK 21 cells. However, when TSV-5 cells at a density of about 10^4 cells/ml, preincubated for 24 hr at 37°, were treated with 128 AU/ml of bacteriocin, the number of adherent cells decreased gradually after 96 hr of incubation (data not shown).

Of the 2 rat cell lines shown in Chart 3, the growth of XC cells induced by Rous sarcoma virus was markedly inhibited by the bacteriocin (64 and 128 AU/ml) as in cases with mKS-A TU-7 and 155-4 T2 cells, while nontransformed NRK cells were resistant to the bacteriocin.

Effect of Bacteriocin on the Growth of Human Malignant and Normal Cells. Chart 4 shows the growth-inhibitory effect of the bacteriocin on human malignant cells (AS-II and HGC-

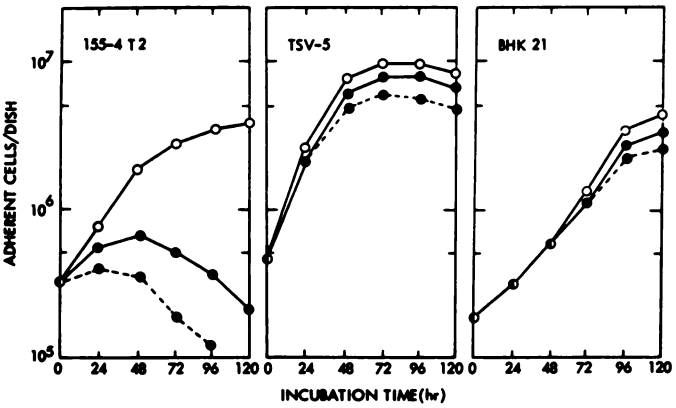

Chart 2. Inhibitory effect of Bacteriocin 14468 on growth of 155-4 T2, TSV-5, and BHK 21 cells. ○, cells plus no or heat-inactivated bacteriocin; ●——●, cells plus 64 AU/ml; ●- - -●, cells plus 128 AU/ml. ◐, the overlapped values of control (○) and experiment (●).

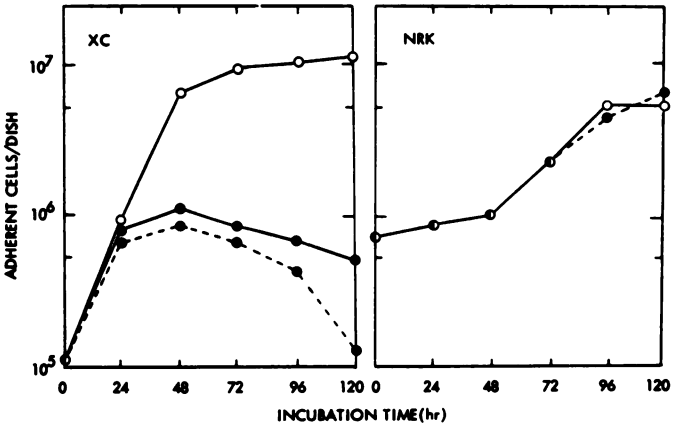

Chart 3. Inhibitory effect of Bacteriocin 14468 on growth of XC and NRK cells. ○, cells plus no or heat-inactivated bacteriocin; ●——●, cells + 64 AU/ml; ●- - -●, cells plus 128 AU/ml. ◐, overlapped values of control (○) and experiment (●).

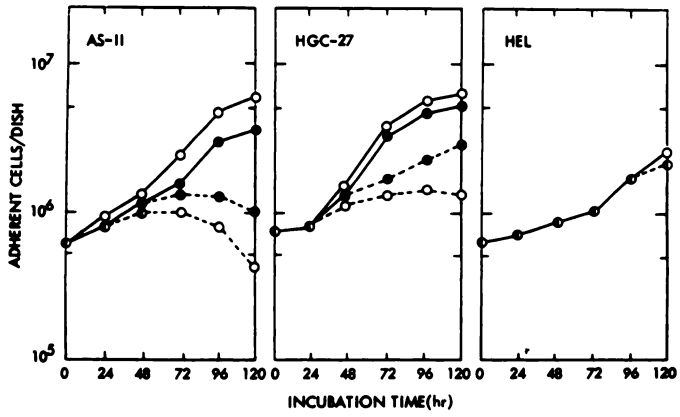

Chart 4. Inhibitory effect of Bacteriocin 14468 on growth of AS-II, HGC-27, and HEL cells. ○——○, cells plus no or heat-inactivated bacteriocin; ●——●, cells plus 64 AU/ml; ●- - -●, cells plus 128 AU/ml; ○- - -○, cells plus 256 AU/ml. ◐, overlapped values of control (○) and experiment (●).

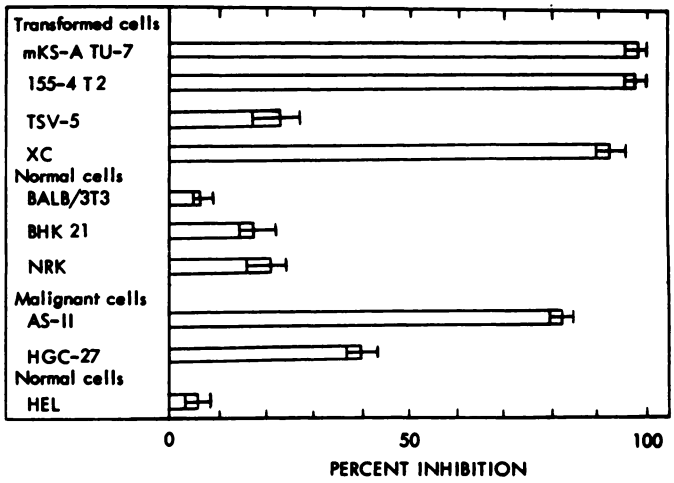

Chart 5. Inhibitory effect of continuous treatment with Bacteriocin 14468 on cells. Adherent cells, preincubated at 37° for 24 hr, were treated with or without 64 AU/ml of bacteriocin at 37° for 48 hr. After incubation, residual adherent cells were counted. Percentage of inhibition was calculated as follows:

$$\% \text{ inhibition} = 100 - \left(\frac{\text{No. of adherent cells in test}}{\text{No. of adherent cells in control}} \times 100\right)$$

Data were obtained from 5 culture dishes and S.D.'s are indicated.

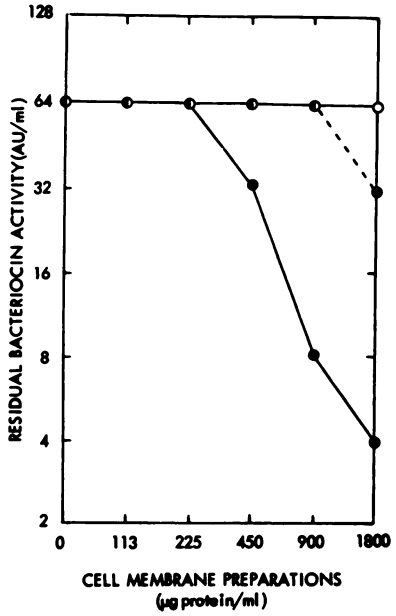

Chart 6. Adsorption of Bacteriocin 14468 to the cell membrane of mKS-A TU-7 and BALB/3T3 cells. One-half ml of the bacteriocin solution (128 AU/ml) was incubated with an equal volume of the cell membrane-rich preparations (0 to 3600 μg of protein per ml) at 37° for 3 hr. After incubation, the mixture was centrifuged, and an aliquot of the supernatant was withdrawn and diluted 2-fold. The residual bacteriocin activity was titrated as described previously (19). ○, controls; ●——●, cell membrane of mKS-A TU-7 cells plus bacteriocin; ●- - -●, cell membrane of BALB/3T3 cells plus bacteriocin. ◐, overlapped values of control (○) and experiment (●).

27 cells) and the resistancy of normal cells (HEL cells). In the case of AS-II cells, the number of adherent cells treated with bacteriocin (64 AU/ml) showed a depressed increase. When the cells were treated with 128 AU/ml, a decrease in the cell number was seen after 72 hr of incubation, and the degree of inhibition was more enhanced by the treatment at 256 AU/ml. On the other hand, the susceptibility of HGC-27 cells to the bacteriocin was considerably lower than that of AS-II cells. The growth curve of the cells treated with 64 AU/ml was similar to that seen in the control cells. However, the increase in adherent cells was slightly inhibited with 128 AU/ml and ceased after 96 hr of incubation with 256 AU/ml. In contrast, the growth of HEL cells was not inhibited by the treatment with 128 and 256 AU/ml.

Inhibitory Effect of Continuous Treatment with Bacteriocin on Cell Growth. Cells were incubated in the growth medium with or without 64 AU/ml of bacteriocin at 37° for 48 hr, and the percentage of inhibition was calculated from the number of adherent cells remaining in the control and the test. The results are shown in Chart 5. Among animal cell lines, the susceptibilities of transformed cells (mKS-A TU-7, 155-4 T2, and XC cells) to the bacteriocin were more conspicuous (except for TSV-5 cells) than those of normal cells (BALB/3T3, BHK 21, and NRK cells). Similar results were obtained in the human cell lines. However, the susceptibilities of human malignant cells (AS-II and HGC-27 cells) to the bacteriocin were lower than those of the transformed animal cells.

We have also found that DNA synthesis in mKS-A TU-7 cells treated with 64 AU/ml of bacteriocin was strongly inhibited after 6 hr of incubation, whereas DNA synthesis in BALB/3T3 cells was not.[3]

Adsorption of Bacteriocin to Cell Membrane of SV40-transformed and Nontransformed Mouse Cells. Chart 6 shows the bacteriocin-adsorbing capacity of the cell membrane of mKS-A TU-7 and BALB/3T3 cells. The bacteriocin activity that remained in the supernatant decreased with an increase in the amount of the cell membrane from mKS-A TU-7 cells. When the bacteriocin solution was incubated with up to 900 μg/ml of cell membrane from nontransformed BALB/3T3 cells, there was no decrease in the bacteriocin activity; however, a slight decrease was observed with the highest concentration of the cell membrane (1800 μg/ml).

[3] H. Saito and T. Watanabe, unpublished results.

DISCUSSION

The bactericidal action of a bacteriocin against sensitive bacteria is primarily defined by the specific receptors on the cell envelope (10, 17, 21). It has been demonstrated that bacteriocin molecules such as colicin E1 (2) and staphylococcin 1580 (23) pass through the cell wall, attach themselves to specific sites on the cytoplasmic membrane, and inhibit mac-

romolecular syntheses. In contrast, bacteriocin molecules such as colicin E3 (8) and pyocin R1 (9) first bind to specific receptors on the cell surface, penetrate the cell wall, reach specific loci in the cytoplasmic membrane, and finally inhibit macromolecular syntheses. Therefore, the cytoplasmic membrane seems to be an important biochemical target for the action of bacteriocins.

As a rule, bacteriocins are toxic only for sensitive bacteria belonging to the same or related species. However, they may have toxic effects on mammalian cells which possess a number of receptors for various substances such as viruses (15) and hormones (20) on the cell membrane. In fact, we recently found that a bacteriocin produced by *M. smegmatis* ATCC 14468 showed a toxicity to HeLa-S3 cells (19). Farkas-Himsley and Cheung (4) also reported that a growth of neoplastic L60T mouse cells was markedly inhibited by colicin, vibriocin, and pyocin, respectively. In the present study, we compared the toxicity of Bacteriocin 14468 to tumor and normal cells from the same species and found that the sensitivities of tumor virus-transformed animal cells except for TSV-5 cells and human malignant cells were much higher, in general, than were those of normal cells in the same species. Farkas-Himsley *et al.* (4, 5) speculated that the toxicity of bacteriocins to tumor cells is likely to be dependent on the phase of the cell cycle rather than existence of specific surface receptors with greater affinity for bacteriocins in tumor cells. In general, the level of the neutral glycopeptides and glycolipids in the cell membrane from virally transformed tumor cells is greatly increased compared to that of nontransformed parental normal cells (16, 18), and the changes of these components are common phenotypic markers of spontaneous tumor cells as well as virally transformed cells (6, 22). The increase in bacteriocin sensitivity of tumor cells may be due to the accumulation of glycopeptides and/or glycolipids in cell membranes of these cells which showed a greater affinity for the bacteriocin 14468 as compared with normal cells. Therefore, as the bacteriocin binds to these constituents, lethal events would ensue.

We found that the adsorption of Bacteriocin 14468 to the cell membrane of mKS-A TU-7 cells (SV40-transformed BALB/c mouse tumor cells) was more remarkable than that of bacteriocin to the cell membrane of BALB/3T3 mouse cells established from BALB/c mouse. Farkas-Himsley *et al.* (4, 5) reported that tumor cells showed high mitotic indices in accordance with their sensitivities to bacteriocins such as colicin, vibriocin, and pyocin, as compared with normal cells which showed relatively low mitotic indices and low sensitivities to these bacteriocins. However, one of our data reported here is incompatible with their proposal. Despite the fact that the cell division of TSV-5 cells is more rapid than that of other tumor cells, the cells were comparatively less sensitive to Bacteriocin 14468. Therefore, the differences in sensitivity of mammalian cells to the bacteriocin are dependent on the difference in their bacteriocin-binding capacities rather than that of mitotic indices of the cells. The accumulation of glycopeptides and/or glycolipids in the cell membranes of TSV-5 cells may not occur as in the case of other bacteriocin-sensitive tumor cells, or if it occurs, the degree of increase in constituents with specific affinity for the bacteriocin may be low.

Among tumor cells tested, the bacteriocin sensitivities of human malignant cells were relatively lower than those of tumor virus-transformed animal cells except for TSV-5 cells. Further studies are being done to determine if the phenomenon is due to the difference in species or in phenotypes of membranes of these cell lines. Studies on the nature of cell membrane components having bacteriocin-binding capacities from tumor and normal cells will be reported elsewhere.

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