Bacterial Proteinaceous Products (Bacteriocins) as Cytotoxic Agents of Neoplasia

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SUMMARY

Several bacteriocins, bacterial proteinaceous antibiotics, are shown to markedly inhibit the division of various established (neoplastic) mammalian cell lines. The bacteriocins tested originated from Escherichia coli, Pseudomonas aeruginosa, Vibrio cholerae, and Vibrio eltor. Using exposure to the bacteriocin was observed prior to mitosis and reflected the effects on growth inhibition. Maximal sensitivity to the bacteriocin was observed prior to mitosis in the G2 phase of the cell cycle.

INTRODUCTION

The available evidence on the mode of action of vibriocin, a bacteriocin from Vibrio comma (3, 11, 12, 19-21, 24-26) and of other bacteriocins, such as colicins (1, 17, 23, 37), pyocins (22), and megacins (15), indicates that, upon the interaction of these bacteriocins with the membrane receptors of sensitive bacterial cells, a mechanism is triggered leading to inhibition of DNA synthesis and then to DNA degradation, accompanied by release of acid-soluble material. These events inhibit cell division and consequently prevent colony formation. The association of cell membranes and DNA replication in bacteria (13, 17, 18) or mammalian cells (4, 5, 30) is well documented. The greater the bacterial multiplication rate, the more sensitive are the cells to the bacteriocin (20). Tumor cells, in analogy with the bacterial culture in the exponential growth phase, have a fast rate of recycling and are probably selective targets for inhibition by bacteriocins, provided suitable membrane receptors are present.

The polypeptide antibiotics, neocarzinostatin and macromomycin, were shown to interact with cell membranes of both rapidly multiplying bacterial and mammalian sarcoma cells, leading to inhibition of DNA synthesis (16, 27). These antitumor agents do not penetrate the cell, and their lethal effect can be reversed by trypsin within a certain period of time after cell interaction (16, 27). They constitute thus a unique group among the known antitumor agents that interfere with cell replication, since they do not penetrate the target cells (16, 27). Also, vibriocin (21) and other bacteriocins (15, 32, 33, 37) do not penetrate the cell when exerting their lethal effect on bacteria and, similarly, this lethal action can be alleviated by trypsin. Indeed, in preliminary experiments, we found that vibriocin and a colicin had a marked cytotoxic effect on HeLa and mouse fibroblast L-cell cultures, inhibiting cell multiplication (8). These findings prompted us to investigate other bacteriocins, which are known to affect DNA synthesis in bacteria, for their selective growth inhibition of mammalian neoplastic cells and study their mode of action. A preliminary note has already been published (9).

It is reported here that not only the vibriocin tested earlier (8), but also a number of other bacteriocins such as other vibriocins from Vibrio cholerae and Vibrio eltor strains, colicins from enteropathogenic Escherichia coli, and pyocins from Pseudomonas aeruginosa were highly effective in inhibiting neoplastic L-cell division; that the incorporation of DNA precursor into DNA was inhibited and that such inhibition is concentration dependent; that a certain exposure time is required for the inhibition to become expressed; and that the rate of inhibition was dependent on the stage of the cell cycle, affecting mainly the immediate premitotic G2 stage.

MATERIALS AND METHODS

Preparation of Bacteriocins

The bacteriocins were prepared and titrated as previously described for vibriocin by Jayawardene and Farkas-Himsley (20). Briefly, the cultures were induced with mitomycin C (Sigma Chemical Co., St. Louis, Mo.), incubated and then resuspended in SL2. Upon further incubation, a cell lysate in SL containing the bacteriocin was obtained. The cell debris was then removed by centrifugation and the bacteriocin-containing supernatant was concentrated by filtration with a PM 10 membrane (Amicon Corp., Lexington, Mass.) and sterilized with the use of Millipore filters (0.45-μm pore size). The LU were estimated by survival ratio (36) and by growth inhibition measured by turbidity increase of a sensitive bacterial indicator culture (46). The effectiveness of the bacteriocins varied widely. The available evidence on the mode of action of vibriocin, a bacteriocin from Vibrio comma (3, 11, 12, 19-21, 24-26) and of other bacteriocins, such as colicins (1, 17, 23, 37), pyocins (22), and megacins (15), indicates that, upon the interaction of these bacteriocins with the membrane receptors of sensitive bacterial cells, a mechanism is triggered leading to inhibition of DNA synthesis and then to DNA degradation, accompanied by release of acid-soluble material. These events inhibit cell division and consequently prevent colony formation. The association of cell membranes and DNA replication in bacteria (13, 17, 18) or mammalian cells (4, 5, 30) is well documented. The greater the bacterial multiplication rate, the more sensitive are the cells to the bacteriocin (20). Tumor cells, in analogy with the bacterial culture in the exponential growth phase, have a fast rate of recycling and are probably selective targets for inhibition by bacteriocins, provided suitable membrane receptors are present.

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The abbreviations used are: SL, 0.5% NaCl solution at pH 7.8; LU, lethal units; UVAM, UV-absorbing material; NHS, normal horse serum; 1066 NHS, Medium 1066 supplemented with 10% (v/v) undialyzed normal horse serum; NCS, newborn calf serum; TCA, trichloroacetic acid; CI, cytotoxic index; GIU, growth-inhibitory unit; Cl50, a 50% inhibition in growth of the target L cells; MOI, multiplicity of interaction; MI, mitotic index.
preparation was also studied by DNA degradation and release of UVAM (10, 20, 21, 24).

Cell Lines

L6OT mouse fibroblast cells, a subline of Earle’s L-cells, adapted to grow in the absence of exogenous thymidine but capable of using an exogenous source when supplied (42), were routinely grown in Eagle’s minimum essential medium containing Earle’s balanced salt solution and 5% newborn calf serum. The cells were incubated in roller tubes at 37° in a rotator (New Brunswick Scientific Co., New Brunswick, N. J.) at 47 rpm.

Nonsynchronized (Random) Cells

These were obtained from the exponential growth phase, grown for 21 to 24 hr, then centrifuged (1085 × g, 10 min at 4°), and resuspended in twice-concentrated Medium 1066 and 20% NHS (34) at 8 × 10^6 cells/ml.

Partially Synchronized Cells

These were obtained as described by Xeros (47) and Tooze (44).

Fully Synchronized Cells

These were obtained by the double-blocking technique, using excess thymidine (44). Thus, a 24-hr culture at the exponential phase was centrifuged and resuspended at the cell density of 5 × 10^6 cells/ml in Eagle’s minimum essential medium containing Earle’s balanced salt solution and 5% newborn calf serum, supplemented with 2 mM thymidine and incubated over 13 hr, a period equivalent to G2 plus M plus G1 (40). The block was released by washing and resuspending the cells in prewarmed Medium 1066 (34) supplemented with 10% (v/v) undialyzed NHS (Woodlyn Farms, Guelph, Ontario, Canada) (1066 NHS) and incubated for 9 hr, an excess of the duration of S. Then a 2nd block, as described for the 1st, was imposed for another 8 hr. During this period, the entire population was expected to accumulate at a point immediately prior to S. The culture was then released from the 2nd block by washing and resuspension to a cell density of 4 × 10^6/ml in 1066 NHS and grown on the rotator. These cells were exposed to the bacteriocin at various times after removal of the 2nd block, while they progress in synchrony through the cell cycle.

Determination of the Cytotoxic Effect

Counts in a Hemacytometer and Electronic Coulter Counter. L6OT cells at the exponential phase were centrifuged and the cells were resuspended in twice concentrated 1066 NHS. Equal aliquots of the different bacteriocins, each at various dilutions in SL (test), or SL alone, or heat-inactivated bacteriocins, or noninduced culture supernatants (controls), were added to a constant number of 8 × 10^4 cells/ml and incubated in the rotator at 47 rpm for another 24 hr. The cytotoxic effect was determined by the final cell count, with a hemacytometer or an electronic cell counter (Coulter Electronics, Hialeah, Fla.). Membrane damage was tested by the trypan blue exclusion test (2).

Colony Counts. One ml of the cell suspension was taken from each roller tube and serially diluted so that the final concentration of cells was approximately 2 to 3 × 10^3/plate, the dilution being carried out in Medium 1066 supplemented with 5% NHS and 7% newborn calf serum. Five-ml portions of this suspension were then plated into each of 3 60-× 15-mm plastic tissue culture dishes (Falcon Plastics, Los Angeles, Calif.). These dishes were then incubated for 7 days at 37° in a humidified atmosphere containing 5% CO2, at which time the resulting colonies were stained with methylene blue and counted. A cell was considered to have retained colony-forming ability i.e., be viable, if it gave rise to a colony consisting of 64 cells or more. The untreated cells had a plating efficiency of about 65%.

Measurement of DNA Synthesis. L6OT cell suspensions in the exponential phase (24 hr), for a random cell culture or following treatment as required for synchrony (44, 47), were washed twice by centrifugation (1085 × g, 10 min at 4°) and resuspended to a concentration of 8 × 10^5 cells/ml thymidine-free Medium 1066 (42), supplemented with 10% NHS. Simultaneously, 0.5 µCi/ml, 2 µCi/mmol, of [methyl-3H]thymidine (New England Nuclear, Boston, Mass.) and bacteriocin at the appropriate dilution (test) or SL (control), were added to the cell suspension and incubated in the rotator, as described under “Cell Lines.” To determine [3H]thymidine incorporation into DNA, 3 ml culture suspensions were sampled at various time intervals, filtered (Millipore filters, 0.45-µm pore size), washed twice with 10 ml cold phosphate-buffered saline (pH 7.4), twice with 10 ml cold 5% TCA, and twice with 10 ml cold 95% ethyl alcohol, and then thoroughly dried. Alternatively, the cells were treated with the bacteriocin for a set time, while suspended in complete Medium 1066 in absence of [3H]thymidine. After the bacteriocin was removed by centrifugation, the cells were resuspended in the thymidine-less Medium 1066 and pulse labeled for 30 min with [3H]thymidine at 6 µCi/ml. The cells were then immersed in an ice bath and six 1-ml samples were removed, filtered, and washed, as described above. The label in the acid-insoluble material was then determined in a Mark 1 liquid scintillation spectrometer (Nuclear Chicago Corp., Des Plaines, Ill.) by use of an organic scintillation fluid consisting of Spectrafluor PPO-PPOP (Amersham/Searle, Arlington Heights, Ill.). The incorporation percentage, taking the number of cpm in the control cells as 100%, was calculated, using an average of all the samples.

Effect of Duration of Exposure of L-Cells to Pyocin.

To determine the effect of exposure time to the bacteriocin, the nonsynchronized or synchronized cells were treated with the bacteriocin as described above under “Determination of the Cytotoxic Effect.” At the required time, the cell-bacteriocin mixture was diluted with prewarmed medium below the effective concentration of the bacteriocin. The effectiveness of the dilution was tested in a control experiment where bacteriocin alone, at an equal dilution, had lost its inhibitory power. The test and control cells (0.1 ml) were added to MicroTest II plates (No. 3040;
The Effect of Bacteriocins on Cell Division. Some characteristics of the bacteriocins are given in Table 1 (footnote), describing the bacterial producer and indicator cultures and their origin, the potency of the bacteriocins in terms of bacterial LU/ml; their effectiveness on the release of UVAM from sensitive bacterial indicator cells, and their cytotoxic effect on L60T mouse fibroblast cells in suspension expressed as the CI, derived as described in "Materials and Methods" (mean ± S.E.). The data were obtained by cell counts after 24 hr of interaction with the bacteriocin. To arrive at the CI, we compared the results based on (a) the final number of cells following interaction with bacteriocin and (b) the final number less the membrane-damaged cells [dye exclusion method (2)]. Very little difference was found (approximately 1%), indicating that the main effect was on cell division. However, extreme concentrations of bacteriocins (>C150), affected the cell membranes, thus affecting cell morphology. A concentration-dependent and statistically significant effect (p < 0.05 by Student's t test) was found for all the bacteriocins at about 1 to 2 x 10^9 LU/ml. It can also be seen in Table 1 that bacterial lysates devoid of bacteriocin activity, either by heat inactivation (boiling for 10 min) or by use of supernatants from noninduced cultures, did not exert a cytotoxic effect. Furthermore, the GIU is given for all the bacteriocins tested, being that concentration of bacteriocin in terms of bacterial LU/ml producing a Cl50. This value was obtained when the cell number, after an exposure for 24 hr to the various bacteriocins, was plotted as a function of bacteriocin concentration, as shown in Chart 1 for 3 bacteriocins, a colicin, pyocin, and vibriocin.

The MOI, also given in Table 1, was derived as described in "Materials and Methods," and shows that about 6 to 10 x 10^8 bacterial LU are required for the inhibition of an L-cell.

The concentration-dependent activity of pyocin 1-4 was again noted in studies with [3H]thymidine incorporation into TCA-precipitable material (Chart 1). It can be seen that the same concentration that inhibited cell proliferation also inhibited DNA synthesis. The observed inhibition could either be due to a decrease in the rate of DNA synthesis of the individual cells in the culture or be a result of a decreased cell number in the treated suspension in which the individual surviving cells may still synthesize DNA at the rate of the cells in the control suspension.

The Effect of Extreme Concentration of Pyocin 1-4 on L60T Cells. Since at high concentrations of bacteriocin, with CI above 50, fewer cells remained than were present at the start, it was clear that the bacteriocin was not merely inhibiting cell proliferation but rather was disrupting or eliminating cells from the system. It was therefore of interest to evaluate the effect of extreme concentrations of bacteriocin on L60T cells. Thus, increasing concentrations of pyocin 1-4 were added to a nonsynchronized population of L60T, and the cell number was monitored in a Coulter counter (Chart 2). The control cells doubled in about 28 hr (the delay was due to growth interruption during sampling). When the bacteriocin at approximately 1 GIU was added, the cells remained at the initial number throughout the test period. Upon increasing the concentration of pyocin to 5 x 10^9 LU/ml (about 3 GIU), the cells were only slightly reduced below the initial number. This effect became more pronounced with increasing concentrations of pyocin and, as can be seen, in the presence of pyocin at 5 x 10^10 LU/ml (about 31 GIU), only 30% of the initial number of cells could be accounted for. [The effect on cell morphology can be seen in Fig. 1, where 1 to 3 GIU's are shown not to affect adversely cell shape but, rather, their number (Fig. 1b) as compared to the controls (Fig. 1a). However, the adverse effect of high concentrations of pyocin (13 GIU) can readily be seen in the disrupted cell debris or shrunken cells (Fig. 1c).]

Effect of Duration of Exposure of L-Cells to Pyocin. After examining the toxicity of the pyocin preparation for L-cells, it was of interest to determine the minimal required exposure time to pyocin at concentrations that inhibited division and caused a detectable growth inhibition. A synchronous L-cell culture was obtained by the double-block method, using high concentrations of thymidine to induce a feedback mechanism (44), and then treated as described in "Materials and Methods." The synchronous burst of the cell culture is shown in Chart 3, inset. The effect of the duration of exposure to pyocin of L-cells in the various growth phases is summarized in Table 2. It can be seen that cell-bacteriocin interaction for 1.5 hr, at various phases of the cell cycle, was not effective in inhibiting cell growth as indicated by a CI of less than 50 and a nonsignificant inhibition as expressed by the p value determined by Student's t test. However, for cells in the late G2 or early mitotic stage, at 11 hr after removal of the feedback inhibition, even at 1.5-hr contact, a CI of 62 was obtained with a statistically significant p value of 0.001. At 3 hr cell-bacteriocin interaction, inhibition of cell growth was noted also for cells in the

OCTOBER 1976

3563
Table 1

Cytotoxicity of various bacteriocins for mouse fibroblast L-cell suspensions

Cytotoxicity of the bacteriocins for nonsynchronous L6OT cells was evaluated by counts in the hemacytometer, after 24 hr of incubation, as described in "Materials and Methods." Data represent the mean of 4 separate counts of a typical experiment.

<table>
<thead>
<tr>
<th>Bacteriocin</th>
<th>Bacterial culture</th>
<th>Bacteriocin potency</th>
<th>Cytotoxic effect</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>LU/ml* (x 10⁶)</td>
<td>UVAM b</td>
</tr>
<tr>
<td>Vibriocin 41-S</td>
<td>Vibrio cholerae NIH41 (1p*)</td>
<td>1.0 +</td>
<td>85.4 ± 4.5b 0.001</td>
</tr>
<tr>
<td></td>
<td>Vibrio eltor HKI (1i)</td>
<td>0.5</td>
<td>52.2 ± 4.2 0.025</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.2</td>
<td>18.8 ± 5.8 NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.1</td>
<td>18.8 ± 4.7 NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.74</td>
<td>26.1 ± 4.6 NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.37</td>
<td>16.3 ± 5.9 NS</td>
</tr>
<tr>
<td>Vibriocin 506</td>
<td>V. eltor T-506 (2p)</td>
<td>7.4 +</td>
<td>186.7 ± 0.8 0.001</td>
</tr>
<tr>
<td></td>
<td>V. eltor K (2i)</td>
<td>3.7</td>
<td>54.4 ± 6.7 0.025</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.8</td>
<td>50.6 ± 4.3 0.025</td>
</tr>
<tr>
<td>Vibriocin 506 heat inactivated (100°, 10 min)</td>
<td>V. eltor T-506 (2p)</td>
<td>0</td>
<td>-15.8 ± 9.6 NS</td>
</tr>
<tr>
<td></td>
<td>V. eltor K (2i)</td>
<td>0.37</td>
<td>6.6 ± 7.3 NS</td>
</tr>
<tr>
<td>Pyocin P-1</td>
<td>Pseudomonas aeruginosa P1 (3p)</td>
<td>3.4 +</td>
<td>120.2 ± 2.7 0.001</td>
</tr>
<tr>
<td></td>
<td>P. aeruginosa I-1 (3i)</td>
<td>1.7</td>
<td>115.0 ± 3.9 0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.68</td>
<td>8.6 ± 4.4 NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.34</td>
<td>9.5 ± 7.9 NS</td>
</tr>
<tr>
<td>Pyocin I-4</td>
<td>P. aeruginosa 1-4 (4p)</td>
<td>10.0 +</td>
<td>113.8 ± 3.2 0.001</td>
</tr>
<tr>
<td></td>
<td>P. aeruginosa I-1 (3i)</td>
<td>5.0</td>
<td>82.4 ± 2.9 0.005</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.0</td>
<td>15.6 ± 6.4 NS</td>
</tr>
<tr>
<td>Colicin HSC 10</td>
<td>Escherichia coli HSC</td>
<td>4.0 +</td>
<td>63.6 ± 3.7 0.005</td>
</tr>
<tr>
<td></td>
<td>E. coli Y/10 (5i)</td>
<td>2.0</td>
<td>57.8 ± 2.3 0.01</td>
</tr>
<tr>
<td></td>
<td>10 (5p)</td>
<td>0.8</td>
<td>38.8 ± 6.3 NS</td>
</tr>
<tr>
<td>Colicin HSC 4</td>
<td>E. coli HSC 4 (6p)</td>
<td>2.2 +</td>
<td>63.6 ± 0.6 0.001</td>
</tr>
<tr>
<td></td>
<td>E. coli Y/10 (5i)</td>
<td>1.1</td>
<td>27.6 ± 3.2 NS</td>
</tr>
<tr>
<td></td>
<td>5 (6p)</td>
<td>0.44</td>
<td>27.6 ± 3.2 NS</td>
</tr>
<tr>
<td>Colicin HSC 4 heat inactivated (100°, 10 min)</td>
<td>E. coli HSC 4 (6p)</td>
<td>0</td>
<td>-64.4 ± 9.2 NS</td>
</tr>
<tr>
<td></td>
<td>E. coli Y/10 (5i)</td>
<td>0.22</td>
<td>-1.8 ± 3.7 NS</td>
</tr>
<tr>
<td>Supernatant HSC 4 noninduced</td>
<td>E. coli HSC 4 (6p)</td>
<td>0</td>
<td>-24.2 ± 7.3 NS</td>
</tr>
<tr>
<td></td>
<td>E. coli Y/10 (5i)</td>
<td>0</td>
<td>-8.8 ± 7.5 NS</td>
</tr>
</tbody>
</table>

* Determined by survival ratio (36) and by turbidity increase inhibition (46).

b UVAM leakage was determined by absorbance (260 nm) of filtrates from indicator strains exposed to a bacteriocin for 30 min at 37° (test) or at 0° (control) (10, 20, 24).

c Derived as described in "Materials and Methods."

p Value by Student's t test.

As described in "Materials and Methods."

d Derived as described in "Materials and Methods."

1p, origin: Burrows, University of Chicago, Chicago, Ill., through Feeley, Division of Biological Standards, NIH, Bethesda, Md.; 1i, origin: Basu, University of California at Los Angeles, Los Angeles, Calif.; 2p, origin: Weissman, Center for Disease Control, Department of Health, Education and Welfare, Atlanta, Ga.; 2i, origin: Central Laboratories, Ontario Ministry of Health, Toronto, Ontario, Canada. Isolated from a case of cholera in Kingston, Ontario, 1974; 3p, origin: Gillis and Govan (14); 3i, origin: Gillis and Govan (14); 4p, origin: Gillis and Govan (14); 4p, origin: Fleming, Hospital for Sick Children, Toronto, Ontario, Canada; 5i, origin: Fuerst, Ontario Cancer Institute, Toronto, Ontario, Canada; 6p, origin: as for 5p.

Mean ± S.E.

NS, not significant.

early G₂ phase, at 10 hr after the release of the thymidine block. Exposure of the cells to pyocin for 24 hr had a greater effect on inhibiting cell growth than exposure for 3 or 4 hr (4 hr not shown), and cells from all phases of the cell cycle were significantly affected. Nevertheless, an increased sensitivity of cells at late G₂ or early mitotic phase was still clearly evident. These results may mean that, during 24 hr of exposure to the inhibitor, such cells that at first escape inhibition may continue to progress through the cell cycle and reach a sensitive stage, which would contribute to the additional sensitivity observed, compared with 3 hr of exposure.

When a nonsynchronized culture is exposed for 3 to 4 hr to pyocin, the inhibitory effect on cell multiplication can be seen clearly (not shown).

Sensitivity of a Specific Phase in the Cell Cycle, to Pyocin I-4. These results of increased sensitivity at a specific phase of the cell cycle were then confirmed by means of 2 other approaches: (a) in estimating the rate of [³H]thymidine incorporation into DNA and (b) in evaluating

3564  CANCER RESEARCH VOL. 36
Synchronized cells were obtained by the double-blocking technique, as described in "Materials and Methods." As a function of time, aliquots of the cell culture were mixed with pyocin 1-4 at m = 3 GIU's (test) or with SL (control) for 1.5, 3, 4, or 24 hr. The cells were washed free of pyocin and growth inhibition evaluated by cell count in the hemacytometer, as described in "Materials and Methods.

Stage of cell cycle

<table>
<thead>
<tr>
<th>Exposure time (hr)</th>
<th>S 4 hr</th>
<th>End of S-G 2</th>
<th>G 2</th>
<th>Late G 2-M</th>
<th>M 12 hr</th>
<th>G 1 13 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cl p*</td>
<td>Cl p</td>
<td>Cl p</td>
<td>Cl p</td>
<td>Cl p</td>
<td>Cl p</td>
</tr>
<tr>
<td>1.5</td>
<td>2 NS</td>
<td>24 NS</td>
<td>26 NS</td>
<td>14 NS</td>
<td>62 0.001</td>
<td>2 NS</td>
</tr>
<tr>
<td>3</td>
<td>± 4 6</td>
<td>± 4.9</td>
<td>± 5.2</td>
<td>± 9.7</td>
<td>± 3.3</td>
<td>± 0.9</td>
</tr>
<tr>
<td></td>
<td>± 6.7</td>
<td>± 5.3</td>
<td>± 2.2</td>
<td>± 3.9</td>
<td>± 1.2</td>
<td>± 8.0</td>
</tr>
<tr>
<td>24</td>
<td>94 &lt;0.001</td>
<td>114 &lt;0.001</td>
<td>114 &lt;0.001</td>
<td>132 &lt;0.001</td>
<td>146 &lt;0.001</td>
<td>74 &lt;0.001</td>
</tr>
<tr>
<td></td>
<td>± 2.5</td>
<td>± 3.1</td>
<td>± 2.2</td>
<td>± 3.8</td>
<td>± 4.5</td>
<td>± 1.9</td>
</tr>
</tbody>
</table>

* Determined by Student's t test; statistically significant at 0.05.
* Mean ± S.E.
* NS, not significant.

Table 2

Effect of duration of exposure of L-cells to pyocin

Synchronized cells were obtained by the double-blocking technique, as described in "Materials and Methods." As a function of time, aliquots of the cell culture were mixed with pyocin 1-4 at m = 3 GIU's (test) or with SL (control) for 1.5, 3, 4, or 24 hr. The cells were washed free of pyocin and growth inhibition evaluated by cell count in the hemacytometer, as described in "Materials and Methods.

Chart 2: Concentration effect of pyocin on L-cells. Nonsynchronous L6OT cells were treated with pyocin 1-4 at 1 × 10^9 to 10^10 LU/ml (0.6 GIU to 82 GIU), as described in "Materials and Methods." The cell number was monitored in the Coulter counter and was normalized for each concentration of bacteriocin, relative to the number of cells at 0 hr. The increase or decrease in the number of cells at each time was then related to the average number of cells during 8 hr, at which time the fluctuation in the number of cells from the normal was less than 1%. However, for high concentrations of bacteriocin (GIU of about 31) the average of 6 hr was taken as the period without change in the original cell number. The S.E. (SEM) was adjusted for this change of scale.

Fig. 1. Cytotoxic effect of pyocin from P. aeruginosa on L6OT cells after 24 hr of interaction. The cytotoxic effect was determined by counts in MicroTest II plates as described under "Effect of Duration of Exposure." a, control SL; b, pyocin 1-4 at >1 GIU; c, pyocin 1-4 at 13 GIU. × 170.
shown to exist for various bacteriocins tested here, such as various mammalian cell lines to a bacteriocin may vary, as the effect of a bacteriocin on cell division is not surprising. The face plays a major part in controlling the division of bacteria. The uniqueness of the bacteriocins lies in their interaction with the cell surface without penetrating the target cells, yet easily obtainable in the laboratory, have a cytotoxic effect on cells from human origin. The inhibitory effect on neoplastic mammalian cell lines of murine origin. The results presented indicate that bacteriocins, bacterial proteinaceous products that are widespread in nature and easily obtainable in the laboratory, have a cytotoxic effect on neoplastic mammalian cell lines of murine origin. The effect on cells from human origin was reported earlier (8, 9). It is also shown here that cell sensitivity to pyocin depends on their stage in the cell cycle. Thus, the sensitivity increases transiently, being amplified during the late G2 or early M phase (Table 2; Chart 3). Such increased sensitivity during G2 phase could occur on account of some specific bacteriocin-sensitive event(s) related to that phase which may prevent the affected cell from eventually entering the S phase. Alternatively, such cells may enter the S phase, but lose their ability to synthesize DNA due to an earlier interference such as with thymidine kinase activity. Indeed, interference with thymidine phosphorylation may lead to the rate-limiting step in the incorporation of thymidine into DNA (35), as was found for hamster cells in culture (39). Conversely, an exposure to an adequate concentration of the inhibitor, for the period required by the cells to pass through the cell cycle (approximately 24 hr), will assure that even such cells that at first escape inhibition and continue to progress through the cell cycle will eventually reach the sensitive phase and be inhibited (Table 2). It seems likely that the higher the MI of a culture, the greater the opportunity for the sensitive surface to interact with the bacteriocin. Consequently, as was shown with the adenocarcinoma cells (9), fast-multiplying tumor cells could provide a selective target and be more susceptible than "normal" cells. However, the possibility of specific surface receptors in tumor cells with greater affinity for bacteriocins than those present in normal cells cannot be excluded.

DISCUSSION

The results presented indicate that bacteriocins, bacterial proteinaceous products that are widespread in nature and easily obtainable in the laboratory, have a cytotoxic effect on neoplastic mammalian cell lines of murine origin. The effect on cells from human origin was reported earlier (8, 9). It is also shown here that cell sensitivity to pyocin depends on their stage in the cell cycle. Thus, the sensitivity increases transiently, being amplified during the late G2 or early M phase (Table 2; Chart 3). Such increased sensitivity during G2 phase could occur on account of some specific bacteriocin-sensitive event(s) related to that phase which may prevent the affected cell from eventually entering the S phase. Alternatively, such cells may enter the S phase, but lose their ability to synthesize DNA due to an earlier interference such as with thymidine kinase activity. Indeed, interference with thymidine phosphorylation may lead to the rate-limiting step in the incorporation of thymidine into DNA (35), as was found for hamster cells in culture (39). Conversely, an exposure to an adequate concentration of the inhibitor, for the period required by the cells to pass through the cell cycle (approximately 24 hr), will assure that even such cells that at first escape inhibition and continue to progress through the cell cycle will eventually reach the sensitive phase and be inhibited (Table 2). It seems likely that the higher the MI of a culture, the greater the opportunity for the sensitive surface to interact with the bacteriocin. Consequently, as was shown with the adenocarcinoma cells (9), fast-multiplying tumor cells could provide a selective target and be more susceptible than "normal" cells. However, the possibility of specific surface receptors in tumor cells with greater affinity for bacteriocins than those present in normal cells cannot be excluded.

Experiments in parallel with those described here, using a colicin and a vibriocin preparation, have already been carried out. These preparations were as effective as the preparation of pyocin described here. Preliminary in vivo tests on C3H mice implanted with KHT fibrosarcoma tumor cells (28) and Fischer 344 rats implanted with CA cells (obtained from a carcinoma of the bladder) have reacted favorably to crude preparations of pyocine 1-4, in that the toxicity was rather low at multiple GIU's administered over a lengthy period, whereas the inhibition of tumor growth or even regression relative to controls was statistically significant. Details of these results are at present being prepared for publication.

In view of the described cytotoxic activity of the bacteriocins and their widespread occurrence (3), it may well be justifiable to speculate that the observations of sponta-
neous tumor regression following bacterial infection (7, 31, 45) are related not only to an immune response, but rather also to the presence of bacteriocins. Whichever is the case, it is of importance to stress that neoplastic cells are sensitive to bacterial products that are widespread in nature and readily obtainable at low cost in the laboratory.

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REFERENCES

Bacterial Proteinaceous Products (Bacteriocins) as Cytotoxic Agents of Neoplasia

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