Further Studies of the Action of a Partially Purified Bacteriocin against a Murine Fibrosarcoma

R. P. Hill and H. Farkas-Himsley

ABSTRACT

We have reported previously that a partially purified bacteriocin (PPB) from Escherichia coli HSC,, is toxic to KHT cells growing in vitro as micrometastases but apparently has no activity against a tumor growing i.m. We report here experiments to investigate possible reasons for this difference. The PPB was shown to become less effective against micrometastases initiated by i.v. injection of KHT cells, as the time between cell injection and PPB treatment increased. The kinetics of the loss of efficacy did not, however, correlate exactly with the growth kinetics of the nodules as assessed by survival following radiation treatment at different times after cell injection. This suggests the possibility of a diffusion limitation although it was found that s.c. injections of PPB were nearly as effective against micrometastases as i.p. injections. We also demonstrated that the lifetime of the majority of the toxic activity of PPB in vivo was relatively short (<1 day) and that the majority of its effect was not caused by stimulating macrophages to act against the tumor cells. The PPB was found to be cytotoxic to KHT cells in vitro but the effect was reduced at high cell density (~10⁶ cells/ml). The PPB did not induce an immune reaction against itself in C3H mice nor was it toxic to either bone marrow stem cells or jejunal crypt cells at doses which were effective against KHT micrometastases. We conclude that PPB may have potential as a cytotoxic agent to act against circulating tumor cells or very small deposits of tumor cells but is limited in its efficacy against larger tumor masses probably because of diffusional and/or cell density effects.

INTRODUCTION

We have demonstrated previously that a PPB obtained from Escherichia coli HSC,, has significant activity against malignant mammalian cells in vitro (1–4) and in vivo (5–7). Single doses of PPB were found to be able to prevent the growth of murine KHT fibrosarcoma cells in the lungs of mice when injected i.p. 24 h after KHT sarcoma cells had been injected i.v. (6). In these studies, we also observed that multiple doses (up to 4 given at 24-h intervals) of PPB could significantly reduce and sometimes eliminate the formation of metastases arising from KHT tumors growing locally in the leg when given early during the growth of the tumor. In contrast, similar treatments had no detectable effect on the growth of the local tumor, even though the treatment was initiated at a time soon after inoculation of the tumor cells into the leg, when the tumor was not yet palpable. We suggested that these results might be explained if the PPB was most capable of acting against cells in the circulation or only against small aggregates of the tumor cells, perhaps because it could not penetrate the tumor mass.

In the present paper, we describe experiments which further characterize the action of PPB in vivo against KHT cells. We show that the PPB is cytotoxic to KHT cells in vitro and that the majority of its action in vivo apparently does not involve stimulating macrophages to act against the tumor cells. However, delaying treatment of tumor nodules growing in the lung results in a loss of efficacy which suggests a diffusion limitation or a cell density effect. We also show no adverse effect on the colony-forming ability of proliferating normal murine bone marrow cells or of jejunal crypt cells at doses of PPB which are effective against the lung nodules.

MATERIALS AND METHODS

Tumor and Animals. Tumors were initiated by an i.m. injection of 2 × 10⁶ KHT sarcoma cells, on day 0, into the left hind leg of male C3H/HeJ mice (The Jackson Laboratory, Bar Harbor, ME). The animals, 8–12 weeks of age, were kept at the Ontario Cancer Institute animal colony with 5 mice/cage and were sustained on mouse/rat chow pellets and water ad libitum.

Tumor size was measured by testing the ability of the tumor-bearing legs to pass through holes of decreasing diameter in a plastic rod. The diameter of the leg thus determined was converted into a tumor weight using a previously obtained calibration curve (Hill, but see, e.g., Ref. 11). Growth of lung nodules in the mice was initiated by injecting appropriate numbers of KHT cells admixed with 2 × 10⁶ plastic microspheres (3M Co., St. Paul, MN) i.v. into groups of 5–7 mice. The mice were killed 18–21 days later, the lungs were removed and fixed in Bouin's solution, and the number of tumor nodules was counted with a dissecting microscope.

Preparation of PPB. PPB was prepared and titrated as described previously (8). Briefly, E. coli HSC,0 cells were treated with mitomycin C (0.5 μg/ml) during exponential growth and were then pelleted by centrifugation. The cells were disrupted in a French press and cell debris was removed by centrifugation. The supernatant was precipitated with ammonium sulfate, dialyzed against water, placed on a DEAE-Sephadex column, and washed with NaCl of gradient molarity to produce the PPB. Protein content was determined quantitatively by the method of Lowry et al. (9) and by the Bio-Rad method (10). PPB appears to be quite stable in solution in neutral buffer and can be stored frozen for months or lyophilized for years without losing activity.

Radiation or Drug Treatment. Tumors growing in the legs or the thoracic cavity of the mice were irradiated locally using a double-headed 100 kVp X-ray unit at a dose rate of approximately 1150 cGy/min (11). In both cases, the mice were appropriately positioned within a circular field with a diameter of 2.2 cm defined with lead collimators. The output of the machine was measured using ferrous sulfate dosimetry. Mice were given whole-body irradiation at a dose rate of about 65 cGy/min by using a double-headed Cs 137 γ-ray unit (12). Cyclophosphamide (Procytox; Horner, Montreal, Canada) was rehydrated according to the manufacturer’s instructions, diluted with saline, and injected i.p. into the mice at the indicated dose in a volume of 0.01 ml/g. Silica particles (0.5–10 μm; Sigma Chemical Co., St. Louis, MO) were suspended in saline and injected both i.p. and i.v. into C3H/HeJ mice at the times and doses indicated. Carrageenan (type V, c-carrageenan; Sigma) was

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3 The abbreviations used are: PPB, partially purified bacteriocin; Con A, concanavalin A; CV, cyclophosphamide.

Results in Fig. 1, left, show that the survival of the lung nodules following irradiation at 2 and 24 h were similar but when irradiation was delayed until 72 h, the survival values were higher, indicating that proliferation of the cells had occurred. Fig. 1, right, indicates the actual number of lung nodules observed in mice irradiated with 7 or 9 Gy at various times after cell injection. Since these doses are in the exponential region of the survival curve, any increase in surviving nodule number indicates an increase in the numbers of cells at risk in each nodule (effectively an increase in multiplicity). There is actually a small decrease in the number of surviving metastases between 2 and 24 h followed by a continuous increase at later times, which indicates that the cells destined to form nodules are proliferating. Similar results are seen following both radiation doses and in an experiment in which cells alone (no microspheres) were injected. In this latter case, the fraction of cells which form nodules is reduced by a factor of about 10 as expected from previous studies (13). The reason for the initial decrease in nodule number between 2 and 24 h is unclear. It is possible that early after injection the cells are partially hypoxic and therefore more resistant to the radiation treatment, as a result of interference with blood supply. Alternatively, it may be that induction of radiation damage to the lung (capillaries) early after cell injection allows a larger fraction of the cells to extravasate and escape destruction in the vascular system.

Treatment of KHT Cells with PPB in Vitro. A line of KHT cells (KHT-C cells) specially adapted to grow both in culture and in vivo were used for these studies. The cells grow in monolayer in «-minimal (KHT-C cells) specially adapted to grow both in culture and in vivo

Kinetics of Metastatic Nodule Formation in Lungs. Mice (C3H/HeJ) were injected i.v. with 10^7 or 10^8 KHT cells (+2 x 10^9 microspheres). The thoracic cavity of mice was then irradiated with 100 kVp X-rays (11) at various times after injection of the cells. The mice were killed 18 days after the irradiation and the lung nodules were counted. The surviving fraction was calculated relative to the number of nodules in untreated mice.

Combination of PPB with Radiation or Cyclophosphamide: Effect on Solid Tumors. Our previous experiments had demonstrated that up to 4 doses of PPB (10.4 μg/mouse) given one
per day were ineffective in treating KHT fibrosarcomas growing locally in the leg of C3H mice, even though the treatment was initiated before the tumors were palpable (6). We, therefore, examined whether PPB would be more effective against a tumor growing locally if combined with either radiation or CY treatment which can induce a delay in tumor growth (18, 19). Results of one such experiment are shown in Fig. 2 as a series of tumor growth curves. A second experiment gave similar results. Tumors were initiated as described in “Materials and Methods” and groups of 7 mice were treated with either 15 Gy of X-rays or 100 mg/kg CY on day 4 (at a tumor weight of 0.25 g); treatments which induced a significant growth delay. As seen from Fig. 2, giving four daily doses of PPB (10 μg/mouse) on days 5, 6, 7, and 8 had no significant extra effect on the regression or regrowth of the local tumors. In contrast, examination of the number of detectable metastases present in the lung at the time the mice were killed (when their leg tumors reached a size >1.5 g) indicated a significant effect of the PPB treatment in the mice given 15 Gy X-rays locally to the leg tumor (see Table 1). These results are similar to those reported previously (6) and again demonstrate the difference in response of locally growing KHT tumors and their metastases to treatment with PPB. The CY treatment significantly reduced the number of lung metastases, but this reduction was largely abrogated by the addition of PPB treatment. We currently have no explanation for this apparently anomalous result.

**Treatment of Micrometastases Growing in the Lung.** The efficacy of PPB in the treatment of metastases of different sizes was examined by giving mice injections of PPB at different times after KHT cells had been injected i.v. into the mice. The KHT cells arrest in the lungs of the mice and grow to form nodules with kinetics as described in Fig. 1. The results of an experiment to examine the response of these nodules to treatment with PPB at various times after the cell injection is shown in Fig. 3. Another experiment gave similar results. Three different PPB doses were used and in all cases, there was a rapid loss of efficacy of the PPB treatment as it was delayed beyond 1 day. However, 40 μg PPB/mouse were more effective than 10 or 4 × 10 μg/mouse. The loss of efficacy presumably reflects the inability of PPB to gain access to and kill all the cells in the individual nodules as they grow larger. The slopes of the steeply rising sections of the curves in Fig. 3 (days 1–3) are equivalent to an increase in the surviving fraction of nodules by a factor of 30–40 over a period of 2 days. Such an increase cannot be explained by cell proliferation, since the results in Fig. 1 suggest only a small increase in cell number over the first 2 days after cell injection. Furthermore, the total increase in nodule number demonstrated in Fig. 1 from 24 to 96 h after cell injection is equivalent to a factor of only about 10, which is consistent with a doubling time for the cells in the nodules of about 24 h, similar to that reported previously (20). These findings suggest that other factors, apart from cell number in the individual nodules, or their proliferative state, may be partly responsible for loss of efficacy of the PPB treatment.

One possibility is that the active moiety of the PPB treatment is limited in its ability to penetrate through tissue. Indeed, we

**Table 1 Lung metastases arising in mice with KHT tumors growing i.m.**

Radiation and cyclophosphamide (CY) were given on day 4. PPB treatment (10 μg/mouse) was given on days 5, 6, 7, and 8. The growth of the i.m. tumors in these mice is shown in Fig. 1. All mice were killed when the i.m. tumors reached >1.5 g. Statistical testing using the Mann-Whitney U test; Group 2 versus Group 4, P < 0.05; Group 3 versus Group 5, P < 0.05; Group 1 versus Group 3, P < 0.01.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Mice with metastases</th>
<th>Median (range)</th>
<th>Day on which mice killed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Control</td>
<td>5/5</td>
<td>27 (10–35)</td>
<td>14</td>
</tr>
<tr>
<td>2. 15 Gy</td>
<td>7/7</td>
<td>32 (6–150+)</td>
<td>22–25</td>
</tr>
<tr>
<td>3. 100 mg/kg CY</td>
<td>4/7</td>
<td>2 (0–6)</td>
<td>25–29</td>
</tr>
<tr>
<td>4. 15 Gy + PPB</td>
<td>5/7</td>
<td>4 (0–24)</td>
<td>20–27</td>
</tr>
<tr>
<td>5. CY + PPB</td>
<td>7/7</td>
<td>15 (1–22)</td>
<td>25–29</td>
</tr>
</tbody>
</table>

![Fig. 2. Growth curves for KHT tumors growing i.m. in hind legs of C3H mice. Mean tumor weight is plotted as a function of time after injection of 2 × 10^7 KHT cells for groups of mice (7/group) given treatments as indicated. Standard errors for the control group are similar in size to the points. They are omitted from the treatment groups for clarity. Local irradiation (15 Gy) or systemic CY (100 mg/kg injected i.p.) were given on day 4. Daily injections of PPB (10 μg/mouse) were given i.p. on days 5, 6, 7, and 8.](image)

![Fig. 3. Surviving fraction of lung nodules plotted as a function of the time of PPB treatment after the injection of the KHT cells. Treatment with PPB was given by i.p. injection at the dosage and times indicated. The multiple dose treatment (4 × 10 μg/mouse) was given daily starting on the day shown. Each point is the mean value ±SE (bars) of the lung nodules in a group of 5–7 mice; where the points overlap, bars have been omitted for clarity.](image)
have found that PPB treatment is somewhat less effective when given s.c. at day 1 to treat KHT cells growing in the lung rather than i.p. (see Table 2). This suggests that the active moiety is probably limited in diffusing through tissue and gaining access to the circulation. However, the fact that 40 µg PPB/mouse is more effective than 10 µg/mouse over the whole time range suggests that the vessel wall does not provide an absolute barrier.

It is possible, however, that PPB kills the KHT cells in vivo not only by direct cytotoxic action but rather also by stimulating natural immune mechanisms to kill the tumor cells. It may be these mechanisms which are limited once the cells enter the interstitial space. We have examined this possibility in a number of different ways.

In Vitro Cytotoxicity of PPB. Initially, we confirmed that the KHT cells were sensitive to PPB treatment in vitro. The cells were exposed (at 2 × 10⁴ cells/ml) to various concentrations of PPB for 4 h in suspension prior to washing and plating in normal growth medium for colony formation or they were plated into growth medium containing various concentrations of PPB and incubated for 10 days for colony formation. The results shown in Fig. 4 indicate a high degree of sensitivity, particularly for prolonged exposure. Thus, it is clear that PPB can act directly against the cells in vitro. A further study was undertaken in which the effect of high cell density (up to 2 × 10⁶ cells/ml) on the activity of PPB was examined for in vitro exposures of 4 h. The results are shown in Fig. 5 and indicate that there is a small reduction in the efficacy of PPB treatment at higher cell densities. In solid tumors, the cell density is far higher; thus the efficacy of the PPB may be further reduced.

Lifetime of Cytotoxic Action of PPB in Vivo. The large difference in the cytotoxicity of PPB when the cells were exposed for 4 h or continuously during colony growth prompted us to examine the lifetime of exposure in vivo. We opted to do this using a biological assay since the active moiety in the PPB is unknown. We injected PPB i.p. 10 µg/mouse at various times prior to or after the injection of KHT cells i.v. As previously, the reduction in the number of lung nodules which formed in the mice 18 days later was taken as an indication of the cytotoxic activity of the PPB. The results shown in Fig. 6 indicate that most of the efficacy of the PPB treatment is lost within 1 day but that there may be a small amount of residual activity which lasts longer.

Stimulation of the Immune System. We examined the effect of PPB on the immune system by examining first whether C3H mice would mount an immune response against the material itself. Groups of mice were given 1 or 2 priming doses of PPB (10 µg/mouse) at 2-week intervals, then 2 weeks after the last injection, KHT cells were injected i.v. and a further dose of PPB (10 µg/mouse) was given 24 h later. The results in Table 3 indicate that pretreatment with PPB neither affected the number of lung nodules which formed in the mice nor influenced the efficacy of the final PPB treatment given 24 h after the cell injection. This suggests that PPB treatment does not stimulate the immune system of the mice.

We next studied more directly whether PPB treatment activated macrophages in the mice to kill KHT cells. Preliminary studies had indicated that abrogation of macrophage (or NK cell) activity in otherwise untreated mice had no effect on lung nodule formation by KHT cells. Consequently, in the present experiments, we treated the mice with carrageenan or silica to

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Table 2 Activity of PPB against micrometastases induced by i.v. injection of KHT cells

<table>
<thead>
<tr>
<th>No. of cells injected</th>
<th>PPB dose* (µg/mouse)</th>
<th>% of survival of lung nodules after PPB treatment given</th>
</tr>
</thead>
<tbody>
<tr>
<td>10⁵</td>
<td>0.4</td>
<td>59% (±5) i.p.</td>
</tr>
<tr>
<td>2.5 × 10⁴</td>
<td>1.0</td>
<td>43% (±5) s.c.</td>
</tr>
<tr>
<td>10⁵</td>
<td>4.0</td>
<td>13.8% (±5) i.p.</td>
</tr>
<tr>
<td>2.5 × 10⁴</td>
<td>10.0</td>
<td>5.9% (±5) s.c.</td>
</tr>
<tr>
<td>10⁴</td>
<td>40.0</td>
<td>&lt;3%</td>
</tr>
</tbody>
</table>

* PPB treatment given 1 day after injection of KHT cells i.v.

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S. D. Young and R. P. Hill, unpublished observations.
reduce macrophage activity (21) and then injected KHT cells and treated with PPB 24 h later. In two experiments, shown in Table 4, the silica treatments had no significant effect on lung nodule formation but there appeared to be a small reduction in the cytotoxic action of PPB. The carrageenan treatment had some toxicity for the tumor cells (and for the mice when combined with PPB) and again appeared to give a small reduction in the effect of the PPB on the KHT cells.

### DISCUSSION

We have demonstrated that a partially purified bacteriocin preparation produced by *E. coli* HSC10 (PPB) is capable, when injected i.p., of reducing the formation of lung metastases (Table 1) from KHT tumors growing i.m. but has no action against the local tumor (Fig. 2). This is despite the fact that the PPB treatment was combined with other effective cytotoxic treatments and initiated early during the growth of the local tumor. We have also found that the PPB treatments rapidly lose their ability to act against the lung metastases as treatment is delayed (for a few days) after the seeding (i.v. injection) of the metastases (Fig. 3).

In investigating possible reasons for these observations we demonstrated that: (a) PPB is directly cytotoxic to KHT cells in vitro and this activity is more pronounced with increased exposure time (Fig. 4). However, the activity of the PPB is reduced if the cells are treated at high cell density (>10⁶ cells/
whole-body doses of radiation between 10 and 12 Gy. Delaying PPB treatment of lung after seeding is observed following both i.p. or s.c. injection of femoral bone marrow cells and 3 days later, the mice were injected with various doses of PPB.

Table 5 Effect of PPB on proliferating normal bone marrow cells

In Experiment 1, mice were given i.p. injections of various doses of PPB; 21 h later femoral bone marrow was harvested and the cells were injected i.v. into groups of 5 recipient mice which had received 9 Gy whole-body irradiation. In Experiment 2, whole-body irradiated (9 Gy) mice were given i.v. injections of KHT cell suspensions into the lungs of mice by i.v. injection, there is a continuous loss of efficacy (Fig. 3).

Table 6 Activity of PPB against murine jejunal crypt cells

Mice were given i.v. injections of various doses of PPB and immediately given whole-body doses of radiation between 10 and 12 Gy. Regardless of the reason for lethal toxicity of the PPB, it is clear from the present experiments that doses about 10 times less than the 50% lethal dose are quite effective in preventing the growth of micrometastases from the KHT sarcoma, although they are ineffective against larger tumor masses, probably because of diffusion limitations and cell density effects. Our current studies are aimed at identifying the active molecules in vivo that are responsible for the lethal toxicity to the animals.

Efficacy of treatment with 40 μg PPB/mouse relative to that with 10 μg PPB/mouse seen in Fig. 3 also suggests that the PPB is able to diffuse to the micrometastases in the lung.

The results in Fig. 6, which indicate that most of the cytotoxic activity of PPB is lost within 1 day of administration to mice, suggest that giving treatments at daily intervals, as was done for the results in Fig. 2, may not be optimum. Furthermore, in vitro results (Fig. 4; Ref. 24) demonstrate that extending exposure time to PPB results in greater cell killing. Thus, giving injections at 6- or 12-h intervals might have resulted in greater activity against the tumor cells in vivo. Our previous studies (6) demonstrated that the 50% lethal dose for C3H mice is in the region of 80–100 μg/mouse when given as a single dose and that the animals usually die within 1 week of the treatment. We have not examined whether larger doses would be tolerated if given over a period of time, nor have we identified the cause of death of the mice, even though the organs of mice which died following high dose treatment were examined histopathologically. In this context, it is interesting that we could observe no effect of doses of PPB up to 40 μg/mouse on either normal bone marrow or jejunal crypt cells. Furthermore, in vitro PPB showed no cytotoxicity to proliferating normal human lymphocytes at doses which were cytotoxic to KHT cells. This suggests that the lethal toxicity is not associated with proliferating cell populations in the animal.

Regardless of the reason for lethal toxicity of the PPB it is clear from the present experiments that doses about 10 times less than the 50% lethal dose are quite effective in preventing the growth of micrometastases from the KHT sarcoma, although they are ineffective against larger tumor masses, probably because of diffusion limitations and cell density effects. Our current studies are aimed at identifying the active molecules in the PPB to determine whether they have increased activity against the tumor and whether it is possible to separate the moieties responsible for the cytotoxicity to the tumor cells from those responsible for the lethal toxicity to the animals.

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REFERENCES


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