Effect of Pentazocine on the Cytotoxicity of Cortisone-Resistant Lymphocytes from Mouse Thymus

Matomo Nishio, Ikunobu Muramatsu and Shigeru Kigoshi

Department of Pharmacology, Fukui Medical School, Matsuoka, Fukui 910-11, Japan

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ABSTRACT — Pentazocine and its related compounds were examined for their effect on the cytotoxicity of cortisone-resistant lymphocytes (CR lymphocytes) against Ehrlich carcinoma cells. The following compounds were used: pentazocine, naloxone, levallorphan, eptazocine and morphine. CR lymphocytes were obtained from the thymus or spleens of mice injected i.p. with hydrocortisone acetate (125 mg/kg) 2 days before harvesting the lymphocytes. The mixture of tumor cells and CR lymphocytes was inoculated s.c. into mice after incubation in the presence or absence of 10 µM drugs. Five weeks after the inoculation, the percentage of mice developing a solid tumor among the recipients given the pentazocine-treated cell mixture of tumor cells and thymic CR lymphocytes was significantly smaller than the percentage in recipients given the cell mixture treated with or without other drugs (percent tumor takes: 21% and about 80%, respectively). Splenic CR lymphocytes did not show any cytotoxic effect, irrespective of the drug treatment. The pretreatment of CR lymphocytes or Ehrlich cells with 10 µM pentazocine did not affect the cytotoxicity of thymic and splenic CR lymphocytes. The proportion of the lymphocyte-conjugated tumor cells was significantly increased when the mixture of CR lymphocytes and tumor cells was incubated in the presence of pentazocine. The present results indicate that the cytotoxicity of thymic CR lymphocytes is enhanced by pentazocine possibly through the increase in the proportion of the lymphocyte-conjugated tumor cells but enhanced not by the other drugs.

Keywords: Pentazocine, Cortisone-resistant lymphocytes, Cytotoxicity, Ehrlich carcinoma cells, Thymus

It has been well-established that the treatment of mice with corticosteroids results in a rapid atrophy of the thymus and spleen and a pronounced decrease of lymphocytes in these lymphoid tissues (1). The lymphocytes remaining in the lymphoid tissues of mice after treatment with hydrocortisone are known as the cortisone-resistant lymphocytes (CR lymphocytes), which are abundant in T cells, and are cytotoxic to tumor cells or allogeneic cells (2–5). We have revealed that the cytotoxicity of CR lymphocytes is enhanced by the treatment of the donor mice for CR lymphocytes with group A streptococcus or Freund’s complete adjuvant (5).

Many studies have demonstrated that opioid peptides are involved in the immune systems, such as the production of antibody or interleukin-2, the proliferation of blood lymphocytes by lectins or the activity of natural killer cells (6, 7). In addition, there are several reports dealing with the effect of morphine and its analogues on the lymphocyte transformation by lectins, the rosette formation of T cells or the hemolysin production by lymphocytes (8, 9). However, little is known about the effect of morphine and its analogues on the cytotoxicity of T lymphocytes.

The present study was performed to examine the effect of morphine and its analogues on the cytotoxicity of CR lymphocytes against Ehrlich carcinoma cells. We found that among the morphine-related compounds, pentazocine enhanced the cytotoxicity of thymic lymphocytes with an increase in the proportion of CR lymphocyte-conjugated tumor cells.

MATERIALS AND METHODS

Drugs

The drugs used were as follows: pentazocine (15 mg/ml solution, Sankyo Co.), naloxone hydrochloride (0.2 mg/ml solution, Sankyo Co.), levallorphan tartrate (1 mg/ml solution, Takeda Chem. Indust.), eptazocine hydrochloride (15 mg/ml solution, Kaken Pharmaceut.
Preparing the cell suspension

The animals used were 7–8-week-old female mice of the ddY strain (Shizuoka Laboratory Animal Center). To obtain cortisone-resistant lymphocytes (CR lymphocytes), a total of 80 mice in a group was injected i.p. with 125 mg/kg of hydrocortisone acetate (Schering AG) 2 days before killing (3, 10). The suspension of CR lymphocytes was prepared from the thymus or spleen of the mice, using Hanks balanced salt solution (HBSS). The lymphocyte suspension contained 93–98% of lymphocytes and 2–6% of macrophages, and about 95% of the cells in the suspension were viable according to the trypan blue test.

Ehrlich carcinoma cells were obtained from mice at 10 days after i.p.-inoculation of the tumor cells, and they were suspended in HBSS (11). In the tumor cell suspension, 85–90% of cells were viable according to the trypan blue test.

Test for the direct cytotoxicity of the drugs

To examine the effect of drugs on Ehrlich carcinoma cells, the tumor cells were suspended in HBSS supplemented with 2% bovine albumin fraction V (Armour Pharmaceut. Co.) (albumin-HBSS), and then they were incubated at 37°C for 120 min with or without 10 μM drugs (2 X 10⁶ cells/ml) (11). After the incubation, the tumor cells were resuspended in HBSS and inoculated s.c. into the right flank of mice (10⁶ cells/mouse). The growth of solid tumors in the mice was observed for 5 weeks. In addition, the proportion of viable cells in the tumor cell suspension was examined after the incubation by the trypan blue test.

Examination of the cytotoxicity of CR lymphocytes

The effect of CR lymphocytes against Ehrlich carcinoma cells was examined by the Winn test, with a slight modification (5, 12). The CR lymphocytes suspended in albumin-HBSS (5 X 10⁶ lymphocytes/ml) were mixed with Ehrlich cells (tumor cells/lymphocytes: 1/10), and the cell mixture was incubated at 37°C for 30 to 120 min in the presence or absence of drugs. After incubation, the cell mixture was resuspended in HBSS and inoculated s.c. into the right flank of mice (10⁶ cells/mouse). The growth of the solid tumor in the mice was observed for 5 weeks. To evaluate the cytotoxicity of CR lymphocytes on the tumor cells, the percent of tumor takes was calculated for each group of recipient mice: Tumor takes (%) = (number of tumor-bearing mice/total inoculated mice) × 100.

RESULTS

Direct effect of drugs on the viability of tumor cells

Before testing the drug effect on the cytotoxicity of CR lymphocytes, pentazocine and its related compounds were examined for their direct effect on Ehrlich carcinoma cells. When the tumor growth was evaluated 5 weeks after the inoculation, no significant difference was observed in the growth of solid tumors among the groups of mice that were inoculated with Ehrlich cells treated with 10 μM of pentazocine, naloxone, levallorphan, eptazocine, morphine or no drug for 120 min before the inoculation (data not shown).

Effect of drugs on the cytotoxicity of CR lymphocytes

The effect of drugs on the cytotoxicity of CR lym-
phocytes was examined according to the modified Winn test described in the Methods section. Table 1 shows the number of tumor-bearing mice 5 weeks after the inoculation. When the mixture of Ehrlich cells and thymic CR lymphocytes was treated with 10 μM pentazocine for 120 min and then inoculated into mice, tumor takes were significantly smaller than those in the other groups of recipients (tumor takes: 21% and 79–83%, respectively, P < 0.01). The effect of pentazocine was dependent on the incubation time. No growth of the tumor was observed in a group of recipients given the cell mixture treated with 10 μM pentazocine for 180 min (Fig. 1A).

On the other hand, splenic CR lymphocytes did not show any cytotoxic effect, irrespective of the drug treatment. Tumor takes in groups of mice given the cell mixture treated with the drugs and without any drug for 120 min were 69–90% and 94%, respectively (Table 1 and Fig. 1B).

The concentration-dependence of the effect of pentazocine was examined. When the mixture of Ehrlich cells and thymic CR lymphocytes was treated for 120 min with 0.1–10 μM pentazocine before the inoculation, tumor takes were decreased with an increase in the drug concentration (Fig. 2). However, no significant enhancement of cytotoxicity was observed in the splenic CR lymphocytes treated with pentazocine. These results indicate that the enhancement of the cytotoxicity of CR lymphocytes on Ehrlich carcinoma cells is specific for the thymic CR lymphocytes and is produced by pentazocine in a concentration-dependent manner.

On the other hand, no enhancement was observed

Table 1. Effect of pentazocine and its related compounds on the cytotoxicity of CR lymphocytes against Ehrlich carcinoma cells

<table>
<thead>
<tr>
<th>Origin of CR lymphocytes</th>
<th>Tumor takes</th>
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<tbody>
<tr>
<td></td>
<td>Pentazocine</td>
</tr>
<tr>
<td>Thymus</td>
<td>9/44* (21%)</td>
</tr>
<tr>
<td>Spleen</td>
<td>24/35 (69%)</td>
</tr>
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</table>

The mixture of Ehrlich cells and cortisone-resistant (CR) lymphocytes was treated with 10 μM drugs and then inoculated into the mice. Results were expressed as [number of tumor-bearing mice 5 weeks after inoculation]/[total number of mice inoculated], which is then converted to a percentage. *Significantly different from the control values (P < 0.01 by the χ²-test).

Fig. 1. Effects of incubation time on the cytotoxicity of cortisone-resistant (CR) lymphocytes against Ehrlich carcinoma cells. The mixture of Ehrlich cells and the CR lymphocytes from the thymus (A) or spleen (B) was incubated for 60 to 180 min in the presence (●) or absence (○) of 10 μM pentazocine. The cell mixture was then inoculated s.c. into mice. The percentage of mice developing solid tumors 5 weeks after the inoculation was calculated as described in the footnote of Table 1 and plotted on the ordinate. Asterisks indicate statistically significant differences from the value in the absence of pentazocine (P < 0.01 by the χ²-test).
when either CR lymphocytes or tumor cells were pre-treated with pentazocine before they were mixed together. As shown in Tables 2 and 3, tumor takes were not significantly different between the untreated groups and the groups in which CR lymphocytes or tumor cells were treated with pentazocine, irrespective of the origin of the CR lymphocytes.

**Effect of drugs on the conjugation of tumor cells and CR lymphocytes**

In connection with the effect of pentazocine on the cytotoxicity of CR lymphocytes, the proportion of Ehrlich carcinoma cells conjugated with CR lymphocytes (proportion of the conjugated tumor cells) was examined in the mixture of these two cells after incubation for 15 to 90 min in the presence or absence of pentazocine (Fig. 3). When the mixture of Ehrlich cells and CR lymphocytes from the thymus or spleen was incubated with 10 μM pentazocine for 120 min and inoculated s.c. into mice thereafter. The percentage of mice developing tumors was evaluated at 5 weeks after the inoculation.

![Graph](image)

**Fig. 2.** Effects of the concentration of pentazocine on the cytotoxicity of CR lymphocytes against Ehrlich carcinoma cells. The mixture of Ehrlich cells and cortisone-resistant (CR) lymphocytes from thymus (○) or spleen (●) was incubated with 0.1 to 10 μM pentazocine for 120 min and inoculated s.c. into mice thereafter. The number of tumor cells in the cell mixture was smaller in the mixture of Ehrlich cells and thymic CR lymphocytes treated with 10 μM pentazocine for 90 min, as compared with that in the cell mixture treated with or without other drugs (tumor cells/200 lymphocytes: 11 ± 1 and 20 ± 1, n = 6). In the case of the splenic CR lymphocytes, however, there was no significant difference in the number of tumor cells between the cell mixture treated with and without pentazocine for 90 min (tumor cells/200 lymphocytes: 20 ± 1 and 20 ± 1, n = 6).

**Table 2.** Tumor takes in mice given the mixture of tumor cells and pentazocine-pretreated CR lymphocytes

<table>
<thead>
<tr>
<th>Origin of CR lymphocytes</th>
<th>Tumor takes</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>0 min</td>
</tr>
<tr>
<td>Thymus</td>
<td>25/30 (83%)</td>
</tr>
<tr>
<td>Spleen</td>
<td>31/33 (94%)</td>
</tr>
</tbody>
</table>

See Table 1 for explanations of the results. CR lymphocytes were pretreated with 10 μM pentazocine for 60 or 120 min before the cytotoxicity test (pentazocine-pretreated lymphocytes). The groups of mice inoculated with the cell mixture of tumor cells and pentazocine-untreated CR lymphocytes are expressed as 0 min.

**Table 3.** Tumor takes in mice given the mixture of CR lymphocytes and pentazocine-pretreated tumor cells

<table>
<thead>
<tr>
<th>Origin of CR lymphocytes</th>
<th>Tumor takes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 min</td>
</tr>
<tr>
<td>Thymus</td>
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</tr>
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<td>Spleen</td>
<td>31/33 (94%)</td>
</tr>
</tbody>
</table>

See Table 1 for explanations of the results. Tumor cells were pretreated with 10 μM pentazocine for 60 or 120 min before the cytotoxicity test (pentazocine-pretreated tumor cells). The groups of mice inoculated with the cell mixture of pentazocine-untreated tumor cells and CR lymphocytes are expressed as 0 min.

in 60 min, but the proportion was significantly lower than that in pentazocine-treated cells (Fig. 3 and Table 4).

The number of tumor cells in the cell mixture was smaller in the mixture of Ehrlich cells and thymic CR lymphocytes treated with 10 μM pentazocine for 90 min, as compared with that in the cell mixture treated with or without other drugs (tumor cells/200 lymphocytes: 11 ± 1 and 20 ± 1, n = 6). In the case of the splenic CR lymphocytes, however, there was no significant difference in the number of tumor cells between the cell mixture treated with and without pentazocine for 90 min (tumor cells/200 lymphocytes: 20 ± 1 and 20 ± 1, n = 6).

**DISCUSSION**

Pentazocine and its related compounds had no direct effect on the viability of Ehrlich carcinoma cells at a concentration of 10 μM. The result is consistent with our previous in vitro study in which there was only a slight difference in the proportion of viable cells among the groups of tumor cells treated with 10 μM pentazocine, its related drugs and without a drug (11).

The treatment of the mixture of Ehrlich cells and
Fig. 3. Proportion of the Ehrlich carcinoma cells conjugated with CR lymphocytes in the mixture of these cells. The tumor cells were mixed with cortisone-resistant (CR) lymphocytes from the thymus (A) or spleen (B) (tumor cells/lymphocytes: 1/10), and the cell mixture was incubated at 37°C for 15 to 90 min with 10 μM pentazocine (●) or 10 μM naloxone (▲). The cell mixture incubated without a drug was used as a control (○). After incubation, the tumor cells conjugated with CR lymphocytes were examined microscopically. Each value represents the mean ± S.E. of 8 experiments, except the values for naloxone (6 experiments). Asterisks indicate statistically significant differences from the control value (Student’s t-test, P < 0.01).

Table 4. Proportion of the tumor cells conjugated with CR lymphocytes

<table>
<thead>
<tr>
<th>Origin of CR lymphocytes</th>
<th>Pentazocine</th>
<th>Naloxone</th>
<th>Levallorphan</th>
<th>Eptazocine</th>
<th>Morphine</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymus</td>
<td>58.8 ± 1.5*</td>
<td>31.6 ± 1.1</td>
<td>31.7 ± 0.8</td>
<td>31.3 ± 1.3</td>
<td>31.2 ± 0.9</td>
<td>31.0 ± 0.7</td>
</tr>
<tr>
<td>Spleen</td>
<td>50.0 ± 1.2*</td>
<td>34.1 ± 1.6</td>
<td>34.2 ± 1.0</td>
<td>34.5 ± 1.2</td>
<td>32.0 ± 0.7</td>
<td>33.7 ± 0.7</td>
</tr>
</tbody>
</table>

The proportion of the tumor cells conjugated with CR lymphocytes was calculated as described in the legend for Fig. 3 and expressed as a percentage. Each value represents the mean ± S.E. of 6 experiments except the values for pentazocine and the control (8 experiments). *Significantly different from the control values (P < 0.01 by Student’s t-test).

Thymic CR lymphocytes with pentazocine enhanced the cytotoxicity of the CR lymphocytes to Ehrlich cells with an increase in the proportion of lymphocyte-conjugated tumor cells. With reference to the proportion of the conjugated tumor cells, the number of tumor cells in the cell mixture treated with pentazocine was about a half of that in the cell mixture treated with other drugs or without any drug, suggesting that the cytolysis of the tumor cells occurred. It has been suggested that the conjugation of target cells and cytotoxic lymphocytes (13–15), natural killer cells (16) or lymphokine activated killer cells (17) is very important for the cytolysis of target cells: that is, the proportion of the target cells conjugated with the cytotoxic lymphocytes is closely connected with the proportion of the lysed target cells. The present results suggest that pentazocine increases the proportion of CR lymphocyte-conjugated tumor cells when the cell mixture was incubated with the drug, resulting in the increase of the number of tumor cells to be lysed.

The pretreatment of CR lymphocytes or Ehrlich cells with pentazocine did not enhance the cytotoxicity of CR lymphocytes. In addition, the proportion of the conjugated tumor cells differed slightly between the cell mixture containing the pentazocine-pretreated cells and that containing untreated cells (data not shown). Thus it is likely that pentazocine does not modify the nature of CR lymphocytes nor Ehrlich carcinoma cells to be
more conjugative, but helps the conjugation of the lymphocytes and the tumor cells when the cell mixture is simultaneously incubated with pentazocine. The result also supports the conclusion that the conjugation of tumor cells with CR lymphocytes is important for the cytolyis of tumor cells in this effector-target cell system.

Although pentazocine significantly increased the proportion of the CR lymphocyte-conjugated tumor cells when incubated with splenic CR lymphocytes, it did not enhance the cytotoxicity of the splenic CR lymphocytes. These results indicate the possible heterogeneity of the CR lymphocytes. Heterogeneity in the mesenteric and splenic CR lymphocytes has been demonstrated in our previous report (5).

The mechanisms responsible for the increase in the cytotoxicity of the CR lymphocytes by the treatment with pentazocine remain unclear. Phosphoinositide breakdown or protein kinase C activation may be related to the mechanisms, since activation of protein kinase C has been suggested to be involved in the cytotoxic action of T lymphocytes (18). This point should be elucidated by further experiments.

Among the drugs tested, only pentazocine showed the enhancing effect on the cytotoxicity. The result may be explained by the difference in the chemical structure, since pentazocine differs markedly from morphine, naloxone, levallorphan or eptazocine in its chemical structure (19). There is little difference in the pharmacological properties between pentazocine and eptazocine (20). Therefore, the effect of pentazocine on the cytotoxicity of thymic CR lymphocytes does not seem to be related to the pharmacological properties reported.

Acknowledgments

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