Induction of Tumor Immunity and Natural Killer Cell Cytotoxicity in Mice by 5-Halo-6-phenyl Pyrimidinone

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ABSTRACT

We have investigated the effect of pyrimidinone molecule 2-amino-5-iodo-6-phenyl-4 pyrimidinone (AIPP) on natural killer (NK) cell lytic potential and on the growth of ascitic mammay adenocarcinoma, ACA-755, in B6D2F1 mice. Our studies demonstrated that AIPP was effective in both the prophylaxis and the therapy of this tumor and that the antitumor effect was mediated via induction of NK cell lytic activity. In vitro characterization studies showed that the AIPP-induced cytotoxic cells were not macrophages and exhibited characteristics of NK cells such as morphology of the large granular lymphocytes and sensitivity to asialo GM-1 antibody. Analysis of the mechanism of potentiation of NK cell cytotoxic function by AIPP indicated that the enhancement of cytotoxicity was accomplished by recruitment of NK cell tumor-binding potential (primarily those with large granular lymphocytic morphology) as well as by increased frequency of lytic NK cells. These studies implicate NK cells in the defense against malignant tumors and suggest that regional therapy with AIPP may represent a new therapeutic modality for treatment of cancer.

INTRODUCTION

NK1 cells have been implicated as one of the defense mechanisms against neoplasia and various types of infections (1–4); moreover, these cells have been shown to play a role in regulation of hemopoiesis and lymphopoiesis (3, 5, 6). Consequently, NK cell deficiency of the individual may result in a higher susceptibility to malignant growth and infectious diseases as well as in several types of hemo- and lymphopathies. For these reasons, potentiation of NK cell activity is of importance.

We have shown previously that 2-amino-5-iodo-6-phenyl-4 pyrimidinone and 2-amino-5-bromo-6-phenyl-4 pyrimidinone augmented or induced substantial levels of NK cell-mediated cytotoxicity in various body compartments of rodents (7, 8). Such potentiation of NK cell activity was relatively long lasting and importantly was induced not only to NK cell-sensitive, but also to NK cell-resistant tumors (7, 8). Tumors of syngeneic, allogeneic, and xenogeneic origin were equally sensitive to pyrimidinone-mediated NK cell cytotoxicity (7, 8). Pyrimidinones were furthermore shown to display antitumor and antiviral activity and IFN-inducing capacity (9, 10). Because of IFN and NK cell-stimulating properties, pyrimidinones may have a utility in therapy of cancer, infectious diseases, and perhaps also in disorders of hemopoietic and lymphoid systems.

In this study we investigated the prophylactic and therapeutic effect of one of the pyrimidinones, AIPP, against semisynthetic ascitic mammary adenocarcinoma tumor ACA-755; furthermore, we analyzed the mechanism of AIPP-mediated antitumor immunity and NK cell-potentiating activity.

MATERIALS AND METHODS

Mice. Twelve- to 14-week-old female C57BL/6 × DBA/2 F1 (hereafter called B6D2F1) hybrid mice were obtained from Cumberland View Farms, Clinton, TN. The mice were maintained in our animal facilities for at least 1 week prior to experimentation to allow for recovery from shipping trauma.

AIPP Treatment. AIPP was suspended in saline and was injected in a single dose or multiple doses of 250 mg/kg i.p. at various times prior to experimentation. Because of its poor solubility, this agent was ground and vigorously vortexed to achieve a uniform suspension. AIPP was kindly provided by the Upjohn Company, Kalamazoo, MI.

Preparation and Separation of Effector Cells. PE cell suspensions were prepared as described in detail in previous publications (7, 8, 11). Effector cells were separated on NW columns by placing 50 to 150 × 10^6 PE cells onto columns consisting of 0.8 g of NW (Fenwal Laboratories, Deerfield, IL) and incubating them for 45 min at 37°C. Nonadherent cells were collected by washing the columns with 20 ml of warm S-RPMI 1640 medium.

For depletion of macrophages, effector cells were treated with carbonyl iron. Fifty million PE cells were incubated for 30 min at 37°C with 20 mg of carbonyl iron filings. Iron-ingesting cells were then removed by multiple passages of the cell suspension over a magnet. Residual suspension was found to contain <2% macrophages, as determined morphologically or by nonspecific esterase staining (12).

Antibody Treatment. For depletion of NK cells, mice were given i.p. injections of one or two doses of 0.2 ml of NK 1.1 antisem (1:8 dilution) prepared by Dr. Sylvia Pollack (6, 13). Thy 1.2 monoclonal antibody (New England Nuclear, Boston, MA) was used to deplete T cells (6). This antibody was injected i.p. in one or two doses of 0.2 ml (1:100 dilution). Asialo GM-1 antibody, prepared by Dr. Nabil Hanna (14), was used for depletion of NK cells in vitro. Briefly, NW-filtered PE cells (5 × 10^6/ml) were incubated with asialo GM-1 antibody (in a final concentration of 1:250) and rabbit complement (1:10 dilution) for 30 min at 37°C. The cells were then washed and resuspended in S-RPMI 1640 medium.

Tumor Cells. The murine ascitic adenocarcinoma-755, ACA-755, was derived from a spontaneous tumor originating in C57 mice (15). This tumor grows as an ascites and has been maintained in our laboratory by continuous passage in vivo in B6D2F1; mice. The murine T-cell lymphoma YAC-1 and mastocytoma P815 were also used in some experiments; these tumors were maintained as continuous cell lines in S-RPMI 1640 medium. All tissue culture cell lines have been found to be Mycoplasma free.

51Cr Release Assay. The 51Cr release assay was performed as described previously (8, 16). Briefly, 5 × 10^4 target cells suspended in 0.5 ml of 150 μCi of 51Cr (Amersham Corp., Arlington, IL) for 30 min at 37°C, washed, and resuspended in S-RPMI 1640. The various numbers of effector cells and 2 × 10^4 of target cells were plated in quadruplicate, in the total volume of 150 μl of S-RPMI 1640 in the wells of microtiter plates and incubated for 4 h. The percentage of cytotoxicity was calculated by the following formula:

\[
\text{cpm experimental release} - \text{cpm spontaneous release} \times 100.
\]

Total cpm incorporated into the cells

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2 To whom requests for reprints should be addressed.

3 The abbreviations used are: NK, natural killer; AIPP, 2-amino-5-iodo-6-phenyl-4 pyrimidinone; IFN, interferon; PE, peritoneal exudate; NW, nylon wool; S-RPMI 1640, RPMI 1640 medium supplemented with 10% fetal calf serum, antibiotics, and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer; TBC, tumor-binding cells; CTBC, cytotoxic tumor-binding cells; LGL, large granular lymphocyte.
Spontaneous release ranged from 5 to 7% for all targets tested.

Single-Cell Assay. To determine the number of TBC and CTBC, a single-cell assay was performed (16). Equal numbers (5 × 10⁶) of NW-filtered PE and tumor cells were mixed in 1 ml of S-RPMI 1640 and incubated for 5 min at 37°C. The cells were then centrifuged for 5 min, resuspended in 0.5% agarose, plated in 0.5 ml volume into poly-L-lysine-coated 30-mm plastic Petri dishes and incubated for 4 h at 37°C in a 5% CO₂ humidified atmosphere. The plates were then stained with 0.1% trypan blue, washed, and fixed with 0.5% formaldehyde. The percentage of TBC was determined by counting 200 lymphocytes; the percentage of CTBC was ascertained by counting the number of dead tumor cells in 100 conjugates and correcting for spontaneous tumor cell death. The frequency of active NK cells, i.e., the effector cells that killed the tumor, was calculated by the formula, % TBC × % CTBC.

Analysis of Morphology of TBC. The conjugate assay as described previously (7, 11) was used to evaluate the percentage of TBC. In brief, after incubation of tumor cells and NW-filtered PE cells for 5 min at 37°C and centrifugation, 2 × 10⁶ cells in 0.2 ml volume were centrifuged onto slides using a CytoSpin 2 (Shandon Southern Instruments, Inc., Sewickley, PA). The slides were air-dried and stained with May-Grünwald and Giemsa. The morphology of at least 100 conjugates was evaluated.

Statistical Analysis. The difference between experimental and control groups was evaluated by a Student’s t test and P was calculated.

RESULTS

Modulation of Tumor Growth Kinetics and NK Cell Activity by AIPP. Initially we investigated the effect of AIPP on the growth kinetics of ACA-755 tumor. AIPP was given as a single injection of 250 mg/kg i.p. to B6D2F1 mice 3 days prior to regional injection of 10² or 10³ tumor cells. Mice given injections of tumor cells only served as controls. As illustrated in Fig. 1, after a lag period of 2 days, the tumor grew quite progressively in control mice, resulting in the death of all animals within 3 weeks. In contrast, B6D2F1 mice, given injections of AIPP neither developed tumors nor showed any signs of morbidity within the observation period of at least 100 days. These data strongly indicated that AIPP displayed antitumor effect via host defense mechanism.

To determine the effect of AIPP on NK cell cytotoxic potential to NK cell highly sensitive tumor, YAC-1, we measured cytotoxicity of control mice and tumor-bearing mice, either untreated or given injections of AIPP, in a ⁵¹Cr release assay. No NK cell activity was detected in PE of untreated mice; however, high levels of cytotoxicity (as high as 70%) were seen in the same tissue 3 days after AIPP stimulation (Table 1). Similarly, in the studies on antitumor immunity, control mice (tumor free, AIPP untreated) did not display any PE-NK cell cytotoxicity to YAC-1. Mice given injections of 10² or 10⁴ tumor cells exhibited elevated but transitory (only within 1 to 4 days) NK cell activity (Fig. 2). In contrast, mice treated with AIPP 3 days prior to tumor inoculation displayed substantial and relatively long-lasting NK cell cytotoxic levels (ranging from 20 to 65% after injection of 10² or 10⁴ ACA-755 tumor cells) (Fig. 2). These observations suggested that NK cells may be responsible for AIPP-mediated antitumor effect.

In the light of this postulation, it was of importance to determine whether induction of antitumor immunity to ACA-755 in vivo will be reflected by NK cell reactivity to the same tumor in vitro; indeed, whereas no lysis of ACA-755 was displayed by unstimulated PE lymphocytes, significant levels of killing were observed after AIPP treatment (Fig. 3).

Since at least some of the murine NK cells have been shown to exhibit morphology of LGLs (2, 3), we analyzed the percentage of LGLs in the peritoneal cavity of B6D2F1 mice, prior to and post-AIPP treatment. It can be clearly seen from Fig. 4 that treatment with AIPP resulted in an increase in the percentage of LGLs as well as in the total number of LGLs in the peritoneal cavity. These results suggest that AIPP stimulation may be responsible for induction of the in vivo role of NK cells in reactivity against ACA-755 tumor.

### Table 1

<table>
<thead>
<tr>
<th>Target-to-effector cell ratio</th>
<th>Untreated</th>
<th>AIPP-treated*</th>
</tr>
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<tbody>
<tr>
<td>1:2</td>
<td>1.8 ± 0.9</td>
<td>43.2 ± 3.2</td>
</tr>
<tr>
<td>1:12</td>
<td>1.3 ± 0.7</td>
<td>30.3 ± 2.9</td>
</tr>
<tr>
<td>1:25</td>
<td>2.3 ± 0.8</td>
<td>56.8 ± 2.9</td>
</tr>
<tr>
<td>1:50</td>
<td>0.9 ± 0.1</td>
<td>72.9 ± 2.1</td>
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* AIPP was administered in a dose of 250 mg/kg i.p. 72 h prior to assay.

#<br>

Fig. 2. Effect of AIPP on NK cell activity of tumor-bearing B6D2F1 mice. AIPP-treated (D) or untreated (D) mice were given injections of 10² or 10⁴ ACA-755 tumor cells and tested at various time intervals posttumor inoculation for NK cell activity (target:effector cell ratio, 1:25) in a 4-h ⁵¹Cr release assay. AIPP was administered as indicated in legend of Fig. 1. I : I, mean ± SE (bars) of 4 mice. C, mice not treated with either AIPP or tumor. Significant increase in NK cell activity was observed at days 1 to 14 in tumor-injected and AIPP-treated groups (P < 0.001).
clearly indicated that NK cells are involved in in vivo antitumor activity against ACA-755 ascites.

In the next series of experiments we characterized the AIPP-induced cytotoxic cells mediating lysis of tumor cells in vitro. We used asialo GM-1 antibody, which has been shown to deplete NK cells (17), and tested its effect on AIPP-induced NK cell cytotoxicity against 3 different tumor cell lines, YAC-1, ACA-755, and P815. As can be seen from Fig. 6, AIPP-augmented NK cell activity against all 3 tumors was completely abrogated with asialo GM-1 antibody. As we have shown previously, treatment with this antibody resulted also in decrease of LGL (18) indicating involvement of NK cells with LGL morphology in cytotoxic reactivity. Cytotoxicity of AIPP-stimulated PE effector cells against any of the above-described tumors was not diminished by carbonyl iron treatment; this suggests that macrophages (phagocytic cells) do not play a role in cytotoxic function, as measured in a short-term cytotoxicity assay (Fig. 7).

Analysis of the Mechanism of in Vitro NK Cell Potentiation by AIPP. NK cell potentiation by AIPP could have occurred

755 tumor, we used the advantage of antibodies depleting selectively NK or T-cells. Specifically, we treated B6D2F1 mice with AIPP (3 days prior to tumor inoculation) and then individual groups of mice were given injections in vivo with either NK 1.1 antiserum, which has been shown previously to deplete selectively NK cells (6, 13) and/or Thy 1.2 monoclonal antibody, directed against T-cells. Normal mouse serum was used as a control. Treatment with antibodies was done either 4 h prior to or 4 h prior to and 24 h postinoculation of B6D2F1 mice with 10^3 tumor cells. Since no difference was observed between these two antibody treatment schedules, the data were pooled. It is illustrated in Fig. 5 that all mice inoculated only with ACA-755 and the majority of mice (80%) inoculated with AIPP and NK 1.1 antiserum succumbed to the tumor. In contrast, groups of mice treated with AIPP and Thy 1.2 antibody or normal mouse serum survived without any signs of tumor development for 100 days of observation period. This

Fig. 3. AIPP-induced NK cell activity against ACA-755 tumor in peritoneal cavity of B6D2F1 mice. O, •, mean percentage of cytotoxicity ± SE (bars) of 9 mice, as determined in a 4-h ^51Cr release assay. AIPP was injected as indicated in legend of Fig. 1. Increase in NK cell cytotoxicity after AIPP treatment was observed at all target to effector cell ratios (P ranged from <0.01 to <0.02).

Fig. 4. Increase in LGL content in peritoneal cavity of B6D2F1 mice following AIPP treatment. Mice were either untreated (C) or given injections of AIPP (see legend of Fig. 1). The percentage of LGLs was determined by analysis of May-Grünwald- and Giemsa-stained cytocentrifuge slides (7); total number of LGLs was determined by the equation % LGL x total number of PE cells. □, ◆, mean ± SE (bars) of 10 mice.

Fig. 5. Abrogation of the antitumor effect of AIPP by NK 1.1 antibodies. Groups of 6 to 10 B6D2F1 mice were given injections of AIPP alone, AIPP and normal mouse serum (NMS), AIPP and Thy 1.2 antibodies, or AIPP and NK 1.1 antiserum (for specific treatments, see "Materials and Methods"). All mice received a challenge of 10^3 ACA-755 tumor cells i.p. on day 0. □, mean percentage of survivors. AIPP was injected 3 days prior to administration of tumor cells.

Fig. 6. Abrogation of AIPP-induced NK cell activity in peritoneal exudate of B6D2F1 mice by asialo GM-1 antibody treatment. NW-filtered PE cells of control (C) and AIPP-treated mice (for treatment, see legend of Fig. 2) were tested for cytotoxicity to various tumor cell lines in a 4-h ^51Cr release assay; target:effector cell ratio was 1:3 for YAC-1, and 1:50 for ACA-755 and P815. □, no treatment; ■, rabbit complement treatment; ◆, asialo GM-1 antibody and rabbit complement treatment (for treatment, see "Materials and Methods"); ◂, mean percentage of cytotoxicity ± SE (bars) of 2 experiments. Significant decrease in NK cell cytotoxicity against all target cells was observed following asialo GM-1 antibody treatment (P < 0.001).
The results corresponding with decreased NK cell killing observed in 
$^{51}$Cr release assay after asialo GM-1 treatment (Fig. 6). The analysis of NK cell-ACA-755 tumor conjugates on cytocentrifuge slides showed that decline in TBC capacity after asialo GM-1 treatment could be attributed to the depletion of tumor-binding cells with LGL morphology (Fig. 10).

Therapeutic Effect of AIPP against ACA-755. Next we 
investigated the therapeutic potential of AIPP against ACA-755 tumor. The mice were given injections i.p. with the tumors ranging from $10^2$ to $10^6$ cells and 1 to 7 days later were regionally treated with 2 to 3 consecutive injections of AIPP. The results of these studies are illustrated in Fig. 11. It can be

on at least 2 levels; this agent could potentiate an ability of NK cells to bind to the tumor cells or it could augment the NK cell lytic activity. Using a single-cell assay, we analyzed the changes in the percentage of TBC as well as in the percentage of TBC which kill the tumor, CTBC, after AIPP stimulation. We demonstrated that AIPP was effective in both, recruiting TBC and in augmenting CTBC against ACA-755 tumor. This resulted in an overall increase in the frequency of CTBC (Fig. 8). Morphological analysis of TBC using the conjugate assay on cytocentrifuge slides revealed that increased tumor-binding activity was primarily due to the recruitment of TBC with LGL morphology (Fig. 8).

We also tested the effect of asialo GM-1 antibody on TBC, CTBC, and frequency of CTBC in PE of AIPP-treated mice. It can be seen that after treatment of effector cells of AIPP-injected mice with asialo GM-1 antibody NK cell ability to bind to ACA-755 tumor declined and was only 32% of that of untreated AIPP-stimulated mice; however, the capability of residual TBC to kill the tumor was virtually unchanged (Fig. 9). The frequency of CTBC was dramatically reduced (63%),
The AlPP-induced NK cell activation was mediated by IFN-
was, however, dependent on the time of its administration and
and on the dose of tumor cells injected. Specifically, the majority
mice given injections of AlPP early after tumor inoculation
survived challenge with high doses of the tumor cells (in the
range of $10^6$ to $10^8$). The percentage of surviving mice chal-
enged with high doses of tumors declined with the extended
time interval between tumor inoculation and AlPP treatment.
It is, however, notable that a substantial number of mice dis-
played extended survival even when treated with AlPP as late
as 4 and 5, or 6 and 7 days posttumor inoculation. Considering
that at day 4 to 7 after inoculation of, for instance, 10⁴ tumor
cells, the number of these cells in the animals reached the
concentration of $10^8$ to $4 \times 10^8$ (as extrapolated from the dose
response curves; see Fig. 1), the antitumor activity of AlPP was
quite effective.

**DISCUSSION**

We have shown that AlPP displayed both prophylactic and
therapeutic effects against ascitic mammary adenocarcinoma-
A575 after regional administration into B6D2F₁ mice. The effect
of AlPP was quite impressive, since it cured 70 to 100% of mice
given injections of relatively high doses of tumor ($5 \times 10^5$
to $10^6$ cells) when administered 1 to 3 days posttumor inoc-
ulation; furthermore, prolongation of survival of mice was ac-
complished when this agent was injected as late as 3 to 7 days
posttumor inoculation, especially when the mice were chal-
enged with lower doses of tumor cells. These data clearly
indicate that regional administration of AlPP may have ther-
apeutic effects in man. Tumors such as ovarian or colon carci-
noma or those tumors with metastatic ascitic growth to the
peritoneal cavity may be proper candidates for regional ther-
apy with this agent. The effect of AlPP may be even more enhanced
when more than 2 injections are administered or when com-
bined with other therapeutic modalities such as surgery, radia-
tion therapy, or chemotherapy. In fact, it has been shown recen-
tly by Li et al. (9) that another pyrimidinone, which
exhibited minimal therapeutic effectiveness when administered
alone, was quite effective after combination with chemotherapy.
We are currently investigating the effect of multiple treatments
with AlPP as well as its effect in combination with other
therapeutic modalities.

The analysis of the mechanism of antitumor activity mediated
by AlPP demonstrated that such an effect was accomplished
via induction of NK cells. This was shown by concomitant
induction of NK cell lytic activity and antitumor immunity in the
peritoneal cavity and by abrogation of antitumor effect by the
NK cell-depleting reagent, NK 1.1 antiserum. Characteri-
ization studies showed that AlPP-induced cytotoxicity in PE
was abrogated by asialo GM-1 antibody (antibody directed
against NK cells), substantiating the role of NK cells in ACA-
575 tumor-directed lytic activity. The fact that asialo GM-1
injection resulted also in depletion of LGL indicates that at
least some of the murine NK cells which are involved in the
defense against this tumor are of LGL morphology. The studies
on the mechanism of NK cell potentiating by AlPP revealed
that AlPP induced recruitment of TBC, particularly those of
LGL morphology, and also increased the frequency of CTBC.
The AlPP-induced NK cell activation was mediated by an IFN-
dependent mechanism, since anti-IFN antibody abrogated NK

cell potentiating effect in previous studies (18). It is also notable
and therapeutically important that AlPP-activated NK cells
displayed reactivity against a broad spectrum of target cells (8).
Even though our studies suggest that NK cells are involved in
both in vitro and in vivo AlPP-induced antitumor activity, they
do not completely eliminate the stimulatory effect of AlPP on
other effector cell populations, e.g., macrophages.

Our studies resulted in 2 important observations: (a) AlPP is a
potent NK cell inducer and when used regionally may
represent an effective therapeutic agent in the treatment of
ascitic tumors; and (b) the observation that the antitumor effect
was mediated by NK cells indicates the importance of this cell
population in antitumor immunity. The latter observation is in
agreement with studies of other investigators implicating NK
cells in resistance to primary and metastatic tumors (19–21).

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