Induction of Natural Killer (NK) Activity in Mice by Injection of Chromomycin A3

MARIKO KAMBE, RYUNOSUKE KANAMARU, YASUSHI MITACHI and AKIRA WAKUI

Department of Clinical Cancer Chemotherapy, the Research Institute for Tuberculosis and Cancer, Tohoku University, Sendai 980

KAMBE, M., KANAMARU, R., MITACHI, Y. and WAKUI, A. Induction of Natural Killer (NK) Activity in Mice by Injection of Chromomycin A3. Tohoku J. Exp. Med., 1991, 163 (4), 279-288 —— Intravenously or intraperitoneally administered Chromomycin A3 (CHRM), an anticancer drug, augmented natural killer (NK) activity of both spleen cells and peritoneal exudate cells in BALB/c mice. When CHRM was administered intravenously, NK activity increased to about five fold that in nontreated mice on the 3rd to the 5th day, then rapidly decreased by the 7th day. On the other hand, when CHRM was administered by the intraperitoneal route, a peak of increased NK activity was observed on 5th to 7th day followed by a more gentle decrease. Augmentation of NK activity by CHRM was enhanced by additional administration of Interferon-γ (IFN-γ). Experimental evidence that NK activity could be augmented by CHRM in various strains of mice, independent of H-2 haplotype, suggested that involvement of genetic control within class I region of major histocompatibility complex could be excluded. When BALB/c mice inoculated subcutaneously with Meth A cells were treated with i.p. injection of CHRM, or CHRM in combination with IFN-γ, the growth of the tumor cells was inhibited, indicating in vivo significance for the increased NK activity. Since this inhibitory effect was decreased by the injection of anti Asialo GM1 antibody (a-ASGM1), the effector cells presumably exerting killing activity against Meth A cells were concluded to be Asialo GM1 antigen positive. —— chromomycin A3; NK activity

The immunomodulating activity of anticancer drugs was first reported by Mihich (1969). They suggested that anticancer drugs might exert their effects by altering the regulatory mechanisms involved in development and maintenance of immune response. Subsequently, several investigators confirmed that certain anticancer drugs have true immunomodulating activity, this feature now being well recognized (Gale et al. 1971; Mizushima et al. 1981; Hancock and Kilburn 1982; Shindo et al. 1985). Schwartz and Grindey (1973) reported that when P388 leukemia cells transplanted to DBA/2 mice were treated with Adriamycin (ADR) or Daunorubisin (DNR), the former agent showed greater therapeutic effects than the latter although these two drugs have similar chemical structures

Received November 27, 1990; revision accepted for publication March 22, 1991.
and biological actions. They concluded that this difference could be attributed to differences in splenic toxicity, because DNR is more toxic to the spleen and therefore exerts a stronger immunosuppressive effect than ADR.

When these two drugs were applied individually to Moloney Sarcoma Virus induced tumor cells, which are known to be highly antigenic, ADR also showed a greater tumoricidal effect than DNR. In contrast, no significant differences were observed regarding MS-2 tumor cells whose antigenic properties are relatively limited (Giuliani et al. 1974).

These studies thus provided strong evidence that the therapeutic effects of ADR are related to cooperative interactions with host defence mechanisms against tumors (Mihich and Ehrke 1984). Further, natural killer (NK) cell function of PECs was found to be augmented by a single i.p. injection of ADR, while NK activity in spleen was reduced dose-dependently after i.p. or i.v. administration (Santoni et al. 1980). Spleen cells from ADR treated mice, when cultured for 5 days, also showed lower NK activity than spleen cells from untreated mice, this reduction requiring the presence of adherent cells and their product, Prostaglandin E₂ (Cohen et al. 1982b; Ehrke et al. 1982).

Thus investigation of the immunological properties of anticancer drugs has become of major interest to workers in the field of the cancer chemotherapy. We previously found that Chromomycin A₃ (CHRM), an anthracyclin anticancer antibiotic which inhibits DNA dependent RNA polymerase by intercalation into double stranded DNA (Kaziro and Kamiyama 1965), also have some immunomodulating effect on tumor bearing mice. Thus BALB/c mice, pretreated with a low dose of CHRM, obtained the ability to reject a second Meth A cell challenge (Kambe et al. 1989). This finding suggested that the tumoricidal effect of CHRM against Meth A cells was not via a direct killing action but rather indirectly by activating some part(s) of the host defence system. The present experiments were designed to cast light on how CHRM influences immunological mechanisms.

**MATERIALS AND METHODS**

Mice and tumor cells. Inbred male BALB/c, DBA/2, BDF₁, C₅7BL/6, C₅7BL/10, CBA, AKR and C₃H/He mice, 4-6 weeks age, were obtained from the Funabashi Animal Center (Chiba).

A Meth A transplantable fibrosarcoma induced by 3-Methylcholanthrene in a BALB/c mouse was maintained in ascites form.

Reagents. Chromomycin A₃ (CHRM; Takeda Chemical Industries Ltd., Osaka) was a commercial product for clinical use, and was administered intraperitoneally (i.p.) or intravenously (i.v.) at the concentrations described in the text. Murine IFN-γ was supplied by Chugai Pharmaceutical Co., Ltd.(Tokyo) and was administered i.p. at 1-2 x 10⁴ U in RPMI 1640 medium (Nissui Pharmaceutical Co., Ltd., Tokyo). Rabbit anti Asialo GM₁ antibody (α-ASGM₁) was supplied by Wako Pure Chemical Industries Co., Ltd.(Osaka), and was administered i.p. at 500 μg/mouse.

Preparation of cell suspensions. Spleens were aseptically removed and cell suspensions
were prepared by mechanical disruption between glass slides in RPMI 1640 medium containing 100 units/ml penicillin and 100 µg/ml streptomycin supplemented with 10% heat-inactivated fetal bovine serum (PBS; Gibco, Grand Island, NY, USA). The spleen cells were treated with Gey's solution to eliminate red blood cells, and washed three times with RPMI 1640 medium.

Peritoneal exudate cells (PECs). PECs were harvested from peritoneal cavity by washing with phosphate buffer solution (PBS), and three times with RPMI 1640 medium, successively.

Cytolytic assay. YAC-1 lymphoma target cells were maintained in suspension culture in RPMI 1640 medium, and labeled with 50 µCi of Na251CrO4 (Amersham, Arlington Heights, IL, USA) for 60 min at 37°C. One million effector cells were mixed with 1×10^4 251Cr-labeled YAC-1 cells in 0.2 ml of RPMI 1640 medium in round bottom microtiter plate wells (Nunc, Raskilde, Denmark). Each test was performed in triplicate. After 4 hr's incubation at 37°C, the radioactivities of 0.1 ml supernatants were counted using a gamma counter (Auto-Gammma 500C; Packard Instrument Company Subsidiary of Ambac Industries Inc., IL, USA). Percentage cell mediated lysis values were calculated according to the following equation:

\[
\text{cytotoxicity} = \frac{\text{CPM}_{\text{exp}} - \text{CPM}_{\text{spont}}} {\text{CPM}_{\text{max}} - \text{CPM}_{\text{spont}}} \times 100
\]

where CPM_{spont}, CPM_{max} and CPM_{exp} are the radioactivity counts for back ground, whole YAC-1 cells broken in 5% Triton X-100, and cells killed in the experiment, respectively.

In vivo tumor growing assay. BALB/c mice were intraperitoneally administered 20 µg of CHRM/kg. Five or 7 days after injection of CHRM, they were treated with IFN-γ, or a combination of IFN-γ and α-ASGM1. Then, 1×10^5 of Meth A cells were inoculated subcutaneously (s.c.) into the mice, and the subsequent tumor development was serially measured. Controls were inoculated with 1×10^5 Meth A cells without pretreatment with CHRM or other agents. Mean tumor size was calculated using the following equation (Mohammed and David 1966);

\[
\text{Mean tumor size (mm^2)} = 0.4 \times a \times b^2
\]

where “a” and “b” are major and minor diameters (mm), respectively.

RESULTS

Effect of CHRM on NK activity of spleen cells and PECs

The effect of CHRM injected i.p. into BALB/c mice on NK activity of spleen cells was determined by assaying cytolysis of YAC-1 cells. Spleen cells were extracted 7 days after administration of CHRM. As shown in Fig. 1, NK activity expressed as lytic potential was maximal with a dose of 20 µg/kg. Subsequent experiments were therefore carried out by administrating 20 µg/kg of CHRM.

After the intravenous injection of CHRM, trace of NK activity of spleen cells of BALB/c mice with time showed a steep convex curve with maximum activity being observed at the 3rd to 5th days (Fig. 2).

Similar experiments were carried out using the spleen cells or PECs from BALB/c mice as effector cells after i.p. injection of CHRM. NK activities in both spleen cells and PECs also increased with time, until reaching a maximum on the 7th day followed by gentle decrease (Fig. 3).
Single or double repeated administrations of $2 \times 10^4$ U of murine IFN-γ by i.p. injection was given to two groups of mice, one comprising untreated control and the other being pretreated with intraperitoneally administrated CHRM 7 days previously. The next day, NK activity of prepared PECs was measured.

As shown in Fig. 4, IFN-γ itself demonstrated potential for increasing NK
Induction of NK Activity by Chromomycin A₃

Fig. 3. Kinetics of NK activity for spleen cells and PECs from BALB/c mice injected i.p. with CHRM (20 μg/kg). •, spleen cells of CHRM treated mice; ○, PECs of CHRM treated mice; ■, spleen cells of control mice; □, PECs of control mice. The values are means ± s.e. of data from 5 mice.

Fig. 4. Effects of murine IFN-γ injection in vivo alone or combination with CHRM on the level of NK activity. PECs were prepared from BALB/c mice, either injected i.p. with CHRM (20 μg/kg) 7 days previously (▲), or non treated (△), one day following administration of IFN-γ (1 ~ 2×10⁴ U/mouse) once or twice. Data points are means ± s.e. of data from 5 mice. *p < 0.05 (paired t-test).
Table 1. NK activity of PECs from various strains of mice

<table>
<thead>
<tr>
<th>Strain</th>
<th>H-2 haplotype</th>
<th>1 Control*</th>
<th>CHRM*</th>
<th>2 Control*</th>
<th>CHRM*</th>
</tr>
</thead>
<tbody>
<tr>
<td>DBA/2</td>
<td>d</td>
<td>11.7±0.3</td>
<td>24.7±0.7**</td>
<td>29.7±0.2</td>
<td>45.2±1.0**</td>
</tr>
<tr>
<td>BALB/c</td>
<td>d</td>
<td>8.8±0.3</td>
<td>12.1±0.7**</td>
<td>12.3±0.7</td>
<td>32.5±1.3**</td>
</tr>
<tr>
<td>BDF1</td>
<td>bd</td>
<td>9.9±0.3</td>
<td>17.4±0.8**</td>
<td>15.1±0.9</td>
<td>18.9±0.9**</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>b</td>
<td>2.3±0.3</td>
<td>29.2±1.4**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57BL/10</td>
<td>b</td>
<td>8.5±0.9</td>
<td>17.9±1.2**</td>
<td>9.2±0.4</td>
<td>17.1±0.9**</td>
</tr>
<tr>
<td>C3H/He</td>
<td>k</td>
<td>2.4±0.8</td>
<td>2.4±0.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CBA</td>
<td>k</td>
<td>1.7±0.4</td>
<td>1.9±0.2</td>
<td>4.8±0.5</td>
<td>3.8±0.7</td>
</tr>
<tr>
<td>AKR</td>
<td>k</td>
<td>8.6±0.6</td>
<td>14.3±1.5**</td>
<td>9.7±0.6</td>
<td>27.5±3.2**</td>
</tr>
</tbody>
</table>

*PECs were harvested from control mice. **PECs were harvested 7 days after i.p. injection of CHRM (20 μg/kg).

Values are means±s.e. **p<0.01 vs. control (paired t-test).

Fig. 5. Effects of CHRM or murine IFN-γ on the growth of Meth A tumor cells inoculated s.c. (■) in controls; (○) in CHRM (20 μg/kg) injected mice treated 7 days before Meth A inoculation; (▲) in murine IFN-γ (1~2×10⁴ U/mouse) injected mice treated on the day previous to Meth A inoculation; (▲) in mice injected with both CHRM and IFN-γ before Meth A inoculation. Each group consisted of 5~6 mice, and data points are means±s.e. at 5 weeks after injection of Meth A. *p<0.05; **p<0.01 (paired t-test).
activity of PECs, the augmentation being remarkable CHRM-pretreated animal.

**Genetic control of NK activity induced by CHRM**

In order to investigate whether the induction of NK activity by CHRM was restricted within major histocompatibility complex (MHC), PEC cytolytic activities for YAC-1 cells were studied using various strains of mice. As shown in Table 1, in C3H/He mice, as well as in CBA mice, for which the H-2 haplotype is \( \kappa \), enhancement of PEC NK activity was not observed even 7 days after i.p. injection of CHRM. However, in AKR mice which possess the same \( \kappa \) H-2 haplotype as that of C3H/He mice and CBA mice, NK activity of PECs was increased by CHRM treatment. Therefore, augmentation of NK activity by CHRM was not restricted genetically by class I gene products of H-2 haplotype in MHC.

**In vivo inhibition of tumor growth by CHRM**

The effects of PECs on the growth of Meth A cells (1 \( \times \) 10^5) inoculated s.c. into BALB/c mice were studied. Mice were separated into two groups, one pretreated with CHRM 5-7 days before inoculation of Meth A cells (1 \( \times \) 10^5) and another without pretreatment. As shown in Fig. 5, the growth of Meth A cells was inhibited in the pretreated mice. This inhibition was more pronounced when murine IFN-\( \gamma \) was administered in combination with CHRM one or 3 days prior

![Fig. 6. Effect of \( \alpha \)-ASGM, on inhibition of tumor growth by CHRM. The growth of Meth A cells in: (■) control mice; (○) mice treated with CHRM before Meth A inoculation as described for Fig. 5; (▲) mice injected with CHRM followed by i.p. administration of \( \alpha \)-ASGM, (1 mg/mouse) on the day before Meth A inoculation. **\( p < 0.01 \) (paired \( t \)-test).]
to inoculation of Meth A cells. In mice injected with CHRM followed by \( \alpha \)-ASGM, the inhibitory activity of CHRM was decreased (Fig. 6). The evidence suggests that the effector cells which kill Meth A cells are \( \alpha \)-ASGM, antigen positive.

**DISCUSSION**

The present results showed that the treatment of mice with CHRM enhanced NK activity in both PECs and spleen cells. The degree of the enhancement as expressed by the lytic activity of those cells on tumor cells was dependent upon the dose and timing of CHRM pretreatment, being maximal after i.p. injection in spleen cells at a dose of 20 \( \mu \)g/kg, i.e. 1/100 dose of the LD\(_{50}\) for mice. This dose is roughly equal to the human therapeutic dose /kg and one tenth of the therapeutic dose in mice. The ability to enhance NK activity of cells by CHRM was not restricted to those characterized by class I gene products of MHC in mice (Table 1). A similar phenomenon might be expected in human cancer bearers, whose MHC are heterozygous.

Measurement of the NK activity of peripheral blood lymphocytes (PBLs) from cancer patients pretreated with CHRM using K-562 human myelogeneous leukemia cells as target cells, revealed significant increase from 5 to 7 days after the treatment, this activity persisting upon additional administration of CHRM (data were not shown).

This is in line with recent reports by several investigators that potent NK activities appear in the peripheral blood after administration of some kinds of anticancer drugs such as Adriamycin or Mitomycin (Ehrke et al. 1982; Cohen et al. 1982a; Ogura et al. 1982; Shindo et al. 1985). It was further demonstrated that NK cells could inhibit metastatic spread of cancer cells from primary sites (Gorelik et al. 1979; Hanna and Burton 1981; Santoli et al. 1981; Kiyohara et al. 1988).

The results obtained from the present experiments show that the NK activity enhanced by a low dose of CHRM was extremely high and its activity lasted pretty long. The evidence would suggest that enhanced NK activity by CHRM pretreatment might play certain role on destruction of tumor cells in cancer bearers. So we examined whether or not CHRM suppresses the growth of Meth A cells, and observed that the growth of Meth A cells could be inhibited by treatment with CHRM, alone or in combination with IFN-\( \gamma \). Since this inhibition was diminished by injection of \( \alpha \)-ASGM, into host animals, the cells which were responsible for the influence on the growth of Meth A cells are presumably ASGM, antigen positive.

But NK cells separated from PECs by Percoll Hypaque gradient, derived from BALB/c mice administered with CHRM, could not efficiently kill Meth A cells as evaluated by \(^{3}\)H-Udr release assay in vitro compared with the case of untreated BALB/c mice. Therefore, the effector cells, which suppressed the
growth of Meth A cells in vivo, would be either or in all of monocytes, some T lymphocytes, some LAK cells and some NK cells. However, the precise mechanisms by which injection with CHRM suppressed the growth of Meth A cells or brings about the augmentation of NK activity is not known. Experiments are now under way to clarify these points.

In conclusion, the present data indicate that in some cases, choice of dose and timing of anticancer drug administration should be taken into consideration from the immunological standpoints in design of chemotherapy schedules.

References


