**Immune mechanism of the retarded growth of tumor nodules in mice exposed to single low-level irradiations with X-rays**

**ANETA CHEDA, JOLANTA WREMBEL-WARGOCKA, EWA M. NOWOSIELSKA, MAREK K. JANIAK**

Department of Radiobiology and Radiation Protection, Military Institute of Hygiene and Epidemiology in Warsaw, Poland

**Abstract**

A number of epidemiological and experimental data indicate that exposures to low doses of low-LET ionizing radiation may trigger the activity of natural anti-tumour immune mechanisms and inhibit tumour growth. Natural killer (NK) cells and activated macrophages play an important role in the anti-tumour defence of the host. In our experiments, BALB/c mice were irradiated with single doses of 0.1, 0.2, or 1.0 Gy X-rays and then intravenously (i.v.) injected with L1 sarcoma cells. Cytotoxic activities of NK cells and macrophages were estimated in vitro using the classical 

**Key words:** X rays, low doses, tumour colonies, macrophages, NK cells, cytotoxic activity

(Centr Eur J Immunol 2006; 31 (1-2): 44-50)

Correspondence: Aneta Cheda, Department of Radiobiology and Radiation Protection, Military Institute of Hygiene and Epidemiology in Warsaw, Poland, e-mail: acheda@wp.pl
Material and methods

Animals and irradiation

Male BALB/c mice aged 6-8 weeks were used throughout. The animals, obtained from the Medical Research Centre, Polish Academy of Sciences, Warsaw, Poland, were whole-body irradiated (WBI) from the HS320 Pantak X-ray generator (230 kV, 20 mA) supplied with the 1-mm Al and Cu filters, at 2.2 Gy/h dose rate to obtain the absorbed doses of 0.1, 0.2 or 1.0 Gy per mouse (the absorbed doses were verified using thermoluminescent dosimeters implanted subcutaneously (s.c.) in the middle abdominal region). Control mice were sham-exposed (generator at the off-mode) in identical conditions. All the studies were carried out by permission of the Local Ethical Committee for Experimentation on Animals at the National Institute of Public Health in Warsaw.

Tumour cells

L1 sarcoma cells were obtained from the Maria Skłodowska-Curie Memorial Cancer Center and Institute of Oncology, Warsaw, Poland. These cells developed spontaneously in the lungs of a BALB/c mouse and have since been propagated in the in vitro culture [28]. YAC-1, a murine lymphoma cell line, was obtained from the Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wroclaw, Poland. The cells were grown in a culture medium (CM) composed of the RPMI-1640 medium (Sigma, Poznan, Poland), 10% FBS (GIBCO BRL, Karlsruhe, Germany), 100 U/ml penicillin, 100 μg/ml streptomycin (Polfa, Warsaw, Poland) and 2 mM L-glutamine (Sigma), and stabilized with Na2CO3 (Sigma).

Immunogenicity assay

Mice were intraperitoneally (i.p.) injected with 106 L1 sarcoma cells, 106 splenocytes obtained from allogeneic C57Bl/6 mice, or pure CM. Four and seven days later the animals were sacrificed and cells from the mesenteric lymph nodes were collected and pulled. After washing, the cells were resuspended in wells of the microtiter plates (Corning, Warsaw, Poland). The NK-enriched cell population was then added at the 100:1 effector-to-target (E:T) cell ratio; five samples were performed for each experimental group, resuspended in CM, and incubated on glass Petri dishes for 2 h at 37°C in a humidified atmosphere of 95% air and 5% CO2; in each case the cells were collected and pulled from at least three mice. The non-adherent cells were then collected, washed, and incubated for 30 s at room temperature in the ammonium chloride solution to lyse the erythrocytes. After washing and resuspending in CM the cells were passed through a nylon wool column and the wool-nonadherent cells were used for the NK cell-mediated cytolysis assay.

Preparation of the NK cell suspension

NK cells were purified as previously described [30]. Briefly, single cell-suspensions in CM were prepared from the spleens of both irradiated and sham-irradiated mice and incubated on glass Petri dishes for 40 min. at 37°C in a humidified atmosphere of 95% air and 5% CO2; in each case the cells were collected and pulled from at least three mice. The non-adherent cells were then collected, washed, and incubated for 30 s at room temperature in the ammonium chloride solution to lyse the erythrocytes. After washing and resuspending in CM the cells were passed through a nylon wool column and the wool-nonadherent cells were used for the NK cell-mediated cytolysis assay.

Preparation of the macrophage-enriched cell suspension

Two days before the collection of cells, mice were i.p. injected with 10% Sephadex G-25 (Pharmacia, Uppsala, Sweden). Peritoneal macrophages were collected on the third day post-irradiation, pulled from at least four mice per each experimental group, resuspended in CM, and incubated on glass Petri dishes for 2 h at 37°C in a humidified atmosphere of 95% air and 5% CO2. The glass-adherent macrophages were then harvested and resuspended in CM.

NK cell-mediated cytolysis assay

Cytotoxic activity of NK cells was measured on the second day post-irradiation using the standard in vitro 51Cr-release assay [31]. In brief, 105 YAC-1 target cells suspended in 0.1 ml CM were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO2 for one hour with 5.55 MBq of Na251CrO4 (Polatom). After the incubation, the cells were washed with PBS and resuspended in CM to the final concentration of 106 cells/ml. The lung tumour colony assay was performed as described previously [29]. Briefly, two hours after the irradiation mice were i.v. injected with 0.2 ml of the L1-cell suspension per mouse. Fourteen days later, the animals were sacrificed, their lungs were rinsed with India ink and total numbers of superficial macroscopic colonies per lung were counted using a magnifying glass. Each experimental group consisted of 12 mice.

Lung tumour colony assay

The assay utilizing L1 sarcoma cells was used as a mouse model of experimental tumour metastases. To obtain the cells for the assay, 14 days after s.c. transplantation of 105 L1 cells, the developed tumours were removed, minced, and incubated for 30 min. at room temperature with 0.25% trypsin-EDTA (GIBCO BRL) and standard DNase I enzyme solution (Sigma). After that, the cells were washed and resuspended in CM to the final concentration of 105 cells/ml. The lung tumour colony assay was performed as described previously [29]. Briefly, two hours after the irradiation mice were i.v. injected with 0.2 ml of the L1-cell suspension per mouse. Fourteen days later, the animals were sacrificed, their lungs were rinsed with India ink and total numbers of superficial macroscopic colonies per lung were counted using a magnifying glass. Each experimental group consisted of 12 mice.

Preparation of the NK cell suspension

NK cells were purified as previously described [30]. Briefly, single cell-suspensions in CM were prepared from the spleens of both irradiated and sham-irradiated mice and incubated on glass Petri dishes for 40 min. at 37°C in a humidified atmosphere of 95% air and 5% CO2; in each case the cells were collected and pulled from at least three mice. The non-adherent cells were then collected, washed, and incubated for 30 s at room temperature in the ammonium chloride solution to lyse the erythrocytes. After washing and resuspending in CM the cells were passed through a nylon wool column and the wool-nonadherent cells were used for the NK cell-mediated cytolysis assay.

Preparation of the macrophage-enriched cell suspension

Two days before the collection of cells, mice were i.p. injected with 10% Sephadex G-25 (Pharmacia, Uppsala, Sweden). Peritoneal macrophages were collected on the third day post-irradiation, pulled from at least four mice per each experimental group, resuspended in CM, and incubated on glass Petri dishes for 2 h at 37°C in a humidified atmosphere of 95% air and 5% CO2. The glass-adherent macrophages were then harvested and resuspended in CM.

NK cell-mediated cytolysis assay

Cytotoxic activity of NK cells was measured on the second day post-irradiation using the standard in vitro 51Cr-release assay [31]. In brief, 105 YAC-1 target cells suspended in 0.1 ml CM were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO2 for one hour with 5.55 MBq of Na251CrO4 (Polatom). After the incubation, the cells were washed with PBS and resuspended in CM to the final concentration of 106 cells/ml. The lung tumour colony assay was performed as described previously [29]. Briefly, two hours after the irradiation mice were i.v. injected with 0.2 ml of the L1-cell suspension per mouse. Fourteen days later, the animals were sacrificed, their lungs were rinsed with India ink and total numbers of superficial macroscopic colonies per lung were counted using a magnifying glass. Each experimental group consisted of 12 mice.
the rabbit anti-asialo GM1 antibody (GM1Ab; Wako Chemicals, Neuss, Germany) was used as a classical blocker of the activity of murine NK cells [33, 34]. For this purpose, one day before the irradiation and injection of the L1 cells mice were treated i.p. with GM1Ab (20 μl Ab in 0.5 ml PBS) or 0.5 ml PBS and two or 14 days later assayed for the activity of NK splenocytes and the number of the developed pulmonary tumour colonies, respectively. For each experimental group four (the NK cell-mediated cytotoxicity assay) and 12 (the tumour lung colonies assay) mice were used.

**Suppression of the macrophage-mediated activity**

To suppress macrophage functions in vivo carrageenan (CGN; Sigma) was used as a classical blocker of the activity of these cells [35]. Briefly, one day before the irradiation and four days before the collection of macrophages mice were i.p. injected with CGN (4 mg in 0.4 ml PBS per mice) or 0.4 ml PBS. The animals were then assayed for the number of pulmonary tumour colonies and the collected peritoneal cells were assessed for their cytotoxic activity.

**Statistical analysis**

Mann-Whitney U test for non-parametric trials was used for statistical analysis of the differences between the results obtained for each of the irradiated vs. sham-exposed groups and p values lower than 0.05 were regarded as significant.

**Results**

Immunogenic characteristics of the syngeneic L1 sarcoma cells and allogeneic C57Bl/6 cells are summarized in Table 1. Based on the study by Ryżewska et al [36], the examined cells can be regarded as immunogenic if the index of stimulation, i.e. the ratio of the activity of [³H]-thymidine incorporated into the mesenteric lymph nodes obtained from mice injected with the cells to the activity of the nodes dissected from mice given only culture medium, exceeds 3.0. Thus, the results shown in Table 1 clearly indicate that L1 sarcoma cells are not immunogenic for the BALB/c mice.

Figure 1 shows rates of the pulmonary tumour colonies (expressed as percentages of the control values obtained in the sham-exposed animals) that grown in mice after the single WBI with various doses of X-rays. As indicated in all the four separate experiments irradiation with 0.1 or 0.2 Gy led to the significant inhibition of the development of the colonies. In contrast, in most of the trials, no statistically significant reduction in the number of pulmonary tumour nodules could be detected when mice were pre-exposed to 1.0 Gy X-rays.

Figure 2 shows the results of the assessments of the in vitro cytolytic activity of NK lymphocytes obtained from the spleens of mice two days after exposure to 0.1, 0.2, or 1.0 Gy X-rays compared to the activity of NK splenocytes obtained from the control, sham-irradiated mice. As indicated, irradiation with each of the applied doses of X-rays resulted in the significant boosting of the cytotoxic function of the

### Table 1. Immunogenicity of the allogeneic (AC) and syngeneic (L1) cells

<table>
<thead>
<tr>
<th>Time after injection of cells</th>
<th>DPM/culture</th>
<th>Index of stimulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>CM</td>
<td>AC</td>
<td>L1</td>
</tr>
<tr>
<td>day 4</td>
<td>772±54</td>
<td>6021±165</td>
</tr>
<tr>
<td>day 7</td>
<td>562±49</td>
<td>5058±139</td>
</tr>
</tbody>
</table>

1 Disintegrations per minute (DPM) determined in a beta-counter in the cultures of the mesenteric lymph nodes obtained from BALB/c mice i.p. injected with pure culture medium (CM), splenocytes from C57Bl/6 mice (allogeneic cells, AC), or L1 sarcoma cells (L1) and incubated in the presence of [³H]-thymidine (as described in Materials and Methods); presented are means ± SD obtained from four replications of each suspension in the experiment; each experimental group consisted of three mice.

2 The ratio of DPM determined in the cultures of mesenteric lymphocytes obtained from mice injected with either AC or L1 cells to DPM of the mesenteric lymphocytes obtained from the CM-injected mice.
NK-type splenocytes. When mice were injected with GM1Ab, the activity of these cells tested two days later was totally abrogated and this inhibition could not be reversed by WBI with 0.1, 0.2 or 1.0 Gy X-rays.

As shown in figure 3, a single WBI of mice with either 0.1 or 0.2 Gy X-rays led to the significant elevation of the cytotoxic activity of the IFN-γ- and LPS-stimulated peritoneal macrophages against the L1 tumour targets on the third day post-exposure to X-rays compared to the activity of these cells obtained from both the sham-irradiated and 1.0 Gy exposed mice. Macrophages collected from mice pre-injected with CGN were significantly less cytotoxic against the L1 cells in vitro than macrophages obtained from the CGN-untreated animals in all the examined groups.

---

**Fig. 1.** Relative numbers (percentages of the control values indicated as solid line at 100%) of pulmonary L1 sarcoma cell colonies in mice exposed to 0.1, 0.2 or 1.0 Gy X-rays and two hours later i.v. injected with L1 sarcoma cells; presented are mean values ± SD. Results of four independent experiments (each experimental group consisted of 12 mice) are shown. * indicates statistically significant (p<0.05) difference from the control (100%) value.

**Fig. 2.** Cytotoxic activity of splenic NK cells (at 100:1 E:T ratio) on the second day after irradiation of mice with 0.1, 0.2 or 1.0 Gy X-rays. C – sham-exposed (control) mice; 0.1 Gy – mice exposed to a single WBI with 0.1 Gy X-rays; 0.2 Gy – mice exposed to a single WBI with 0.2 Gy X-rays; 1.0 Gy – mice exposed to a single WBI with 1.0 Gy X-rays; PBS – mice i.p. injected with phosphate buffered saline; Ab – mice injected with anti-asialo GM1 antibody. Presented are means ± SD from three independent experiments; each experimental group consisted of at least three mice. * indicates statistically significant (p<0.05) difference from the control value.

**Fig. 3.** Cytotoxic activity of peritoneal macrophages (at 20:1 E:T ratio) on the third day after irradiation of mice with 0.1, 0.2 or 1.0 Gy X-rays. C – sham-exposed (control) mice; 0.1 Gy – mice exposed to a single WBI with 0.1 Gy X-rays; 0.2 Gy – mice exposed to a single WBI with 0.2 Gy X-rays; 1.0 Gy – mice exposed to a single WBI with 1.0 Gy X-rays; PBS – mice i.p. injected with phosphate buffered saline; CGN – mice injected with CGN. Presented are means ± SD from three independent experiments; each experimental group consisted of at least three mice. * indicates statistically significant (p<0.05) difference from the control value.

**Fig. 4.** Relative numbers of pulmonary L1 sarcoma cell colonies in mice exposed to 0.1, 0.2 or 1.0 Gy X-rays and two hours later i.v. injected with L1 sarcoma cells. C – sham-exposed (control) mice; 0.1 Gy – mice exposed to a single WBI with 0.1 Gy X-rays; 0.2 Gy – mice exposed to a single WBI with 0.2 Gy X-rays; 1.0 Gy – mice exposed to a single WBI with 1.0 Gy X-rays; PBS – mice i.p. injected with phosphate buffered saline; Ab – mice injected with anti-asialo GM1 antibody. Presented are mean values ± SD. Results of three independent experiments (each experimental group consisted of 12 mice) are shown. * indicates statistically significant (p<0.05) difference from the control (C+PBS) value.
Figure 4 shows the relative numbers (expressed as percent of the control values measured in the sham-exposed animals) of the pulmonary tumour colonies developed in mice pre-treated with GM1Ab or CGN. Injection of the NK cell- or macrophage-blocker almost totally eliminated the differences in the numbers of tumour colonies between the irradiated and control groups. This effect was markedly more pronounced in the CGN- than in the GM1Ab-treated mice.

Discussion

The results of the present study indicate that development of the pulmonary tumour colonies is significantly retarded in mice pre-injected with L1 sarcoma cells and whole body-irradiated with 0.1 or 0.2 Gy of X-rays as compared to the sham-exposed as well as 1.0 Gy-irradiated mice. This observation corroborates the findings of Hosoi and Sakamoto [11] who detected a marked inhibition of both artificial and spontaneous pulmonary metastases in mice inoculated with tumour cells a few hours before or after the exposure to 0.15, 0.2 and 0.5 Gy X-rays. Likewise, significant reduction in the number of pulmonary tumour nodules was reported by Ju et al [12] who irradiated mice with single doses of X-rays ranging from 0.05 to 0.15 Gy 24 hours before the i.v. injection of B16 melanoma or Lewis lung cancer cells. Decreased incidence of lung and lymph node metastases was also reported by Hashimoto et al [14] who exposed rats to 0.2 Gy of γ-rays 14 days after s.c. implantation of hepatoma cells; the same dose, however, did not reduce the number of metastases after local irradiation of the primary tumour nor did it affect the number of pulmonary tumour colonies developed in mice exposed to 0.1, 0.2 Gy and 1.0 Gy. These results suggest that stimulation of the cytotoxicity of the IFN-γ- and LPS-treated peritoneal macrophages derived from mice exposed to 0.04 Gy of γ-rays was reported by Ikuki & Goto [18, 19] who used the P815 tumour cells as targets and assayed the effector macrophages already on the day of the exposure.

In the present study i.p. injection of both the anti-asialo GM1 antibody and CGN suppressed the cytolytic function of NK lymphocytes and macrophages, and abrogated the differences between the numbers of the lung tumour colonies developed in mice exposed to 0.1, 0.2 Gy and 1.0 Gy. These results suggest that stimulation of the NK cell- and macrophage-mediated activities was responsible for the retardation of the development of tumour metastases by the low doses of X-rays. Notably, injection of CGN appeared to be a more potent suppressor of the anti-neoplastic effect of the low-level exposures to X-rays than the GM1Ab. This observation may be explained by the possible suppression by CGN of the cytotoxic functions of both macrophages and NK cells. Indeed, Minarovits et al [41] demonstrated that concurrent application of the two inhibitors promoted tumour growth in mice transplanted with the SP94 adenocarcinoma and BaF1 fibrosarcoma cells to the same extent as did the sole injection of CGN. Moreover, several cytokines produced by macrophages (e.g., IL-12 and IL-18) are potent modulators of the activity of NK lymphocytes [42] and suppression of the activity of the former may compromise the function of the latter cells.

In conclusion, our present results indicate that suppression of artificial metastases by single low-level irradiations with X-rays may be causatively related to stimulation by such exposures of the cytotoxic functions of NK cells and macrophages. It remains to be explored in future studies whether other immune cells and/or reactions are also involved in the tumour-suppressive effect of the low-dose irradiations with low-LET radiation.
References


13. Cai L (1999): Research of the adaptive response induced by low-dose radiation: where have we been and where should we go? Hum Exp Toxicol 18: 419-425.


Footnote
1According to the UNSCEAR 1986 Report [2], acute doses above 2 Gy, between 2 and 0.2 Gy, and below 0.2 Gy are regarded as high, intermediate, and low, respectively