Enhanced Cytotoxic Activity of Macrophages and Suppressed Tumor Metastases in Mice Irradiated with Low Doses of X-rays

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Low-dose X-rays/Macrophages/Cytotoxicity/Tumor colonies/Nitric oxide.

We showed in our previous report that a single exposure of mice to 0.1 or 0.2 Gy X-rays led to the significant inhibition of the development of artificial tumor metastases in the lungs and that the effect was related to the enhanced activity of natural killer cells. In the present study, a possible involvement of cytotoxic macrophages in the anti-metastatic effect of the low-level X-ray exposures was investigated. We now demonstrate that irradiation of mice with either of the two low doses of X-rays significantly stimulates the macrophage-mediated cytolysis of the susceptible tumor targets and that the effect coincides with the enhanced production of nitric oxide in the collected effector cells. We also show that suppression of the in vivo function of macrophages by carrageenan eliminates the inhibitory effect of the two low doses of X-rays on the development of pulmonary tumor colonies as well as significantly suppresses the macrophage-mediated cytotoxicity and nitric oxide production. Finally, aminoguanidine added to the culture medium of the assayed macrophages not only shuts down the nitric oxide synthesis in these cells but also significantly suppresses their cytolytic activity. Overall, the obtained results indicate that inhibition of the tumor metastases by a single exposure of mice to 0.1 or 0.2 Gy X-rays results, to a large extent, from the radiation-induced stimulation of the cytoidal activity of macrophages which secrete enhanced amounts of nitric oxide.

INTRODUCTION

As suggested by the accumulating epidemiological and experimental evidence both acute and protracted exposures to low doses\(^1\) of low-LET ionizing radiation inhibit rather than precipitate the development of some malignant neoplasms in humans and laboratory animals.\(^2-6\) Anti-tumor effects of the low-level exposures are generally detectable when whole bodies of the subjects are irradiated and, in case of the animal studies, the irradiations are performed before inoculation of the malignant cells.\(^3\) In contrast, local irradiations of the tumor neither inhibit the inception of metastases nor stimulate migration of lymphocytes to the neoplastic tissue.\(^3,7\) These findings emphasize a role of the anti-neoplastic surveillance system in the inhibitory effect of the exposures to radiation.\(^3\)

In fact, it has been reported that absorption by mice or rats of low doses of X- or \(\gamma\)-rays markedly stimulated proliferation and/or activity of NK cells and macrophages obtained from the irradiated animals.\(^7-12\) Recently, we demonstrated\(^13,14\) that a single exposure to either 0.1 or 0.2 Gy X-rays inhibited the development of artificial pulmonary tumor nodules in mice and that the effect was related to the enhanced cytotoxic activity of NK lymphocytes. However, in the lungs it is the cytoidal function of macrophages which constitutes a primary non-specific barrier against the invasion and/or growth of tumor cells.\(^15\) This activity of macrophages relies, to a large extent, on the production of nitric oxide (NO), a pleiotropic modulator of various physiological and pathological processes including cancer development and growth.\(^16,17,18\)

In view of the above, we aimed in the present investigation to determine if the tumor-inhibitory effect of low-level exposures to radiation is associated with the cytotoxic function of macrophages. The results indicate that single irradiations of mice with 0.1 and 0.2 Gy of X-rays significantly

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\(^1\) According to the UNSCEAR 1986 Report\(^1\), acute doses above 2 Gy, between 2 and 0.2 Gy, and below 0.2 Gy are regarded as high, intermediate, and low, respectively.

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stimulate cytolysis of tumor cells by macrophages which produce elevated amounts of NO and that the enhanced cytotoxicity of these cells plays a major role in the anti-neoplastic effect of the irradiations.

**MATERIALS AND METHODS**

**Animals and Irradiation**

Male BALB/c mice aged 6–8 weeks were used throughout. The animals, obtained from the Institute of Biochemistry and Biophysics, Warsaw, Poland, were whole-body irradiated (WBI) from the HS320 Pantak X-ray generator (230 kV, 20 mA) supplied with the Al and Cu filters, at 2.2 Gy/h dose rate to obtain the absorbed doses of 0.1 or 0.2 Gy per mouse (the absorbed doses were verified using thermoluminescent dosimeters implanted s.c. in the middle abdominal region). Control mice were sham-exposed (generator at the off-mode) in identical conditions. All mice were maintained under specific pathogen-free 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.

**Tumor Cells and Cell Lines**

L1 sarcoma (the cell line derived from a tumor spontaneously developed in a BALB/c mouse) and P815 mastocytoma 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).

**Lung Tumor Colony Assay**

The assay employing syngeneic L1 sarcoma cells was used as described previously. Briefly, two hours after the irradiation mice were i.v. injected with the L1 cell suspension and fourteen days later the animals were sacrificed, their lungs injected with India ink and superficial macroscopic tumor colonies were counted.

**Preparation of the Macrophage-Enriched Cell Suspensions**

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

**Macrophage-Mediated Cytotoxicity Assay**

Cytotoxic activity of the macrophages was measured as described previously, using the L1 sarcoma or P815 mastocytoma cells as targets. Briefly, 4 × 10⁴ L1 or P815 cells were suspended in 2.5 ml CM supplemented with 0.8 µCi [³H]thymidine and incubated in standard conditions for 20 h. The macrophages were then added at 20:1 effector to target cell ratios (E:T) with or without the addition of 50 U/ml IFN-γ (Sigma) and 100 ng/ml LPS (Sigma). After the 48-hour incubation in wells of a microtiter plate, viable adherent cells were lysed, harvested, and their radioactivity was determined as described above.

**Production of Nitric Oxide by Peritoneal Macrophages**

Nitric oxide (NO) synthesized in macrophages was quantitated based on measurements of the level of the NO⁻ anion. Briefly, peritoneal macrophages were incubated in wells of a microtiter plate for 48 h in standard conditions with or without the addition of 50 U/ml IFN-γ (Sigma) and 100 ng/ml LPS (Sigma). After that, the supernatant was mixed with the same volume of the Griess reagent and kept in the dark for 10 min. at room temperature. Absorbance at 540 nm was then measured using the SpectraCount™ microplate reader.

**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. Briefly, one day before the irradiation mice were i.p. injected with CGN (4 mg in 0.4 ml PBS per mice). Then, the animals were assayed for the number of pulmonary tumor colonies 14 days post-exposure and i.v. injection of L1 sarcoma cells, whereas peritoneal macrophages were collected three days post-irradiation and assessed for their cytotoxic activity and production of NO in vitro as described above.

To suppress the synthesis of NO, aminoguanidine (AG; Sigma) was used as a classical inhibitor of the activity of inducible nitric oxide synthase (iNOS). Briefly, AG was added to the cultures of macrophages collected on the third day post-irradiation and incubated in wells of a microtiter plate for 48 h in standard conditions with or without the addition of 50 U/ml IFN-γ (Sigma) and 100 ng/ml LPS (Sigma), the final concentration of AG equaled to 2 mM. After that the cytotoxic activity and production of NO were estimated as described above.

**Statistical Analysis**

The Mann-Whitney U test for non-parametric trials was...
used for statistical analysis of the results and p values less than 0.05 were regarded as significant.

RESULTS

As shown in Fig. 1, 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-boosted peritoneal macrophages against both the L1 (Fig. 1A) and P815 (Fig. 1B) tumor cells. The effect was most pronounced between the third and fifth days post-exposure to X-rays and then gradually declined. Somewhat lower, but kinetically similar, stimulation of the cytotoxicity was noted in the cells incubated in the absence of IFN-γ and LPS (Fig. 2A and 2B).

Since the cytolytic activity of macrophages against the L1 sarcoma cells was well pronounced and kinetically similar to that against the P815 mastocytoma cells, and since the former cells were used in the present experimental system for the induction of pulmonary colonies, the L1 cells were chosen as targets in all the forthcoming cytotoxicity assays.

Exposure of mice to either of the two low doses of X-rays also resulted in the significant elevation of the production of NO in the IFN-γ- and LPS-treated peritoneal macrophages (Fig. 3A). As in the case of the cytotoxic function, this effect was most pronounced between the third and fifth days post exposure and declined to the baseline level six days later. Notably, the enhanced synthesis of NO in the cultured macrophages, whether obtained from the sham-exposed or X-ray-irradiated mice, could not be detected without the addition of IFN-γ and LPS to CM (Fig. 3B).
The two tumor cell lines used in the present experiments appeared to be comparably potent stimulators of the production of NO in macrophages. In fact, when either the L1 or P815 cells were incubated with the collected peritoneal macrophages the latter cells produced significantly elevated levels of NO (Fig. 4). The effect was much more pronounced in macrophages assayed in the presence of IFN-γ and LPS but the untreated macrophages incubated with the tumor cells also produced significant amounts of NO. Importantly, the levels of NO produced by the tumor cells themselves were low and comparable to the level detected in macrophages incubated alone in the absence of IFN-γ and LPS (Fig. 4).

As indicated in Fig. 5, development of the tumor colonies was significantly inhibited in the lungs of mice irradiated with either 0.1 or 0.2 Gy X-rays as compared to the non-irradiated control animals. Importantly, pretreatment of mice with CGN totally abrogated the inhibitory effects of the irradiations. In fact, no statistical differences were noted in the numbers of pulmonary tumor colonies between the CGN-treated X-ray-irradiated and the CGN treated sham-exposed mice (Fig. 5).

As shown in Fig. 6 macrophages collected from mice pre-injected with CGN were significantly less cytotoxic against the L1 cells than macrophages obtained from the CGN-untreated animals. When the effector cells were collected from mice exposed to either of the two low doses of X-rays...
the reduction of the cytotoxicity was more pronounced than in cells obtained from the sham-exposed animals. The inhibitory effect of CGN was detectable in both the untreated (Fig. 6A) and the IFN-γ and LPS-treated (Fig. 6B) macrophages but it was less significant in the latter case. Even more pronounced reduction of the cytotoxic function of macrophages was detected in the cells obtained from the CGN-untreated mice and incubated in vitro in the presence of AG (Fig. 6). In fact, inhibition of the production of NO resulted in the highly significant suppression of the macrophage-mediated cytotoxicity regardless of whether the effector cells were incubated or not in the presence of IFN-γ and LPS. As in the case of the CGN-mediated inhibition, the AG-induced reduction of the cytotoxicity was more pronounced in macrophages obtained from the X-ray-irradiated mice than in the cells collected from the sham-exposed mice. Although the tendency indicated in Fig. 6 may suggest otherwise, there was no significant difference between the cytotoxic activity of macrophages obtained from mice pre-treated with CGN and irradiated with X-rays and that of macrophages collected from the CGN-treated and sham-exposed animals.

As indicated in figure 7, production of NO in macrophages obtained from both the sham- and X-ray-exposed mice pre-treated with CGN was almost totally suppressed, in spite of the fact that the cells were incubated in the presence of IFN-γ and LPS (Fig. 7). Importantly, macrophages obtained from the CGN-treated control and irradiated mice produced comparable low amounts of nitric oxide even though the exposure to either of the two X-ray doses significantly stimulated the NO synthesis in these cells. As expected, addition of AG to the incubation medium of the collected macroph-

![Fig. 6. Cytotoxic activity against the L1 sarcoma cells of the untreated (A) or IFN-γ- and LPS-treated (B) peritoneal macrophages obtained from mice pre-injected with CGN or incubated in vitro with AG tested on the third day after WBI. Mean values ± SD obtained from three independent experiments are presented. C – sham-exposed 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; M – macrophages obtained from the CGN-untreated mice; M + CGN – macrophages obtained from mice pre-treated with CGN; M + AG – macrophages obtained from the CGN-untreated mice and incubated in vitro in the presence of AG. *indicates statistically significant (P < 0.05) difference from the results obtained in group CM.

![Fig. 7. CGN and AG-induced inhibition of the production of NO in the IFN-γ- and LPS-treated peritoneal macrophages tested on the third day after WBI. Mean values ± SD obtained from three independent experiments are presented. C – sham-exposed 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; M – macrophages obtained from the CGN-untreated mice; M + CGN – macrophages obtained from mice pretreated with CGN; M + AG – macrophages obtained from the CGN-untreated mice and incubated in vitro in the presence of AG. *indicates statistically significant (P < 0.05) difference from the results obtained in group CM.](https://academic.oup.com/jrr/article-abstract/47/3-4/229/957487)
ages led to the significant inhibition of the production of NO whose level was very low regardless of whether the effector cells were obtained from the sham- or X-ray-exposed animals (Fig. 7).

**DISCUSSION**

The results of the present investigation demonstrate that macrophages obtained from mice whole body-irradiated with either 0.1 or 0.2 Gy X-rays exhibit the significantly enhanced cytolytic activity *in vitro* against both the P815 mastocytoma (a classical target for murine macrophages in the standard cytotoxicity assay) and L1 sarcoma cells (used by us for the induction of pulmonary tumor colonies). The effect was well detectable regardless of whether the effector cells were boosted or not with IFN-γ and LPS, although the cytotoxic activity was higher in the former than in the latter case. In both instances, the stimulation was pronounced between the third and fifth days post exposure to X-rays and remained elevated for the next two to three days (Fig. 1 and 2). This effect coincides with the previously reported by us, and confirmed in the present study (Fig. 5), significant radiation-induced reduction of the number of artificial tumor nodules in the lungs. In fact, in this experimental system the nodules develop within 10 to 14 days after the intravenous injection of syngeneic invasive tumor cells and activated macrophages play an important role in elimination of the invaders within the pulmonary parenchyma.

To our knowledge, this is the first demonstration of the association of the suppressed development of experimental tumor colonies with the stimulated cytotoxic function of macrophages, the two effects resulting from a single low-level exposure to X-rays.

Activated macrophages may kill susceptible target cells by secreting a number of cytotoxic factors of which nitric oxide has been shown to play a major role in the tumor cell by secreting a number of cytotoxic factors of which nitric oxide has been shown to play a major role in the tumor cell death and inhibition of tumor progression.

Interestingly, in the present investigation CGN appeared to be a more potent suppressor of the anti-neoplastic effect of the low-level exposure to X-rays than the previously employed by us anti-asialo GM1 antibody, a specific blocker of the NK cell-mediated cytotoxic activity; the suppressive effect of the former treatment was several fold greater than that of the latter. This observation may be explained by a possible shut-down or reduction by CGN of the cytotoxic functions of macrophages and indirectly NK cells. In fact, it has been shown that several cytokines produced by macrophages, such as IL-12 and IL-18, are potent modulators of the activity of NK lymphocytes, and inhibition of the activity of the former cells may affect the function of the latter cells *in vivo*. The possible CGN-mediated...
suppression of both macrophages and NK lymphocytes is also implied by the observation that although, in the present study, the former cells obtained from the CGN-pre-treated mice retained some of their cytotoxic function in vitro (Fig. 6), the CGN-induced denial of the tumor-inhibitory effects of 0.1 and 0.2 Gy of X-rays (Fig. 5) was much more pronounced than that previously described by us for the anti-asialo GM1 antibody.\textsuperscript{13}

In addition to the promotion of tumor growth and inhibition of the macrophage-mediated cytotoxicity, injection of mice with CGN resulted in the almost total suppression of the synthesis of nitric oxide in the collected macrophages, regardless of whether the cells were obtained from the irradiated or sham-exposed mice (Fig. 7). This finding supports the above discussed possible involvement of NO in the mechanism of the macrophage-mediated anti-tumor effect of the low-level exposures to X-rays. However, macrophages collected from the CGN-treated animals still exhibited cytotoxic activity in vitro, even in the absence of IFN-\(\gamma\) and LPS in the incubation medium (Fig. 6A). This can be explained by the above described stimulation of the synthesis of NO in the effector macrophages by the target cells (Fig. 4). Indeed, suppression of the activity of iNOS by AG markedly reduced the cytolytic function of the effector cells obtained from both the sham-exposed and irradiated mice (Fig. 6). Since, however, the AG-induced reduction of the cytotoxicity was not complete, it is possible that other cytolytic factor(s) were involved in the macrophage-mediated elimination of the L1 cells in the present experimental system. In fact, as shown by the results of Iwamoto and McBride,\textsuperscript{27} and as indicated by our preliminary findings (data not shown), irradiation of the isolated cells with \(\gamma\)-rays or the whole mice with X-rays, respectively, stimulated macrophages to secrete elevated amounts of tumor necrosis factor-\(\alpha\), an important mediator of the anti-neoplastic function of these cells in vitro and in vivo.\textsuperscript{28} The role of this cytokine in the mechanisms of anti-tumor effects of the low-level exposures to the low-LET radiation will be explored in our future studies.

In conclusion, the results of the present investigation indicate that single exposures of mice to low doses of X-rays significantly stimulate tumoricidal functions of macrophages and that the enhanced activity of these cells may, to a large extent, account for the tumor-inhibitory effect of such exposures. Moreover, the obtained results clearly suggest that the X-ray-stimulated cytotoxicity of macrophages is related to the synthesis of nitric oxide by these cells. The present findings supplement and extend the previously reported by us involvement of natural killer lymphocytes in the low-dose X-ray-induced suppression of pulmonary tumor nodules\textsuperscript{13,14} by emphasizing the role of yet another non-specifically cytotoxic cell population in this phenomenon. Since cooperation of macrophages and NK cells may be necessary for the efficient control of the development of both primary and secondary tumors in vivo\textsuperscript{29} it will be interesting to explore in future studies the possible interactions of the two effector cell types in the mechanism(s) of tumor surveillance in subjects exposed to single and multiple irradiations with low total doses of X- or gamma-rays.

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