7-Allyl-8-oxoguanosine (loxoribine) inhibits the metastasis of B16 melanoma cells and has adjuvant activity in mice immunized with a B16 tumor vaccine

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Abstract. We have shown previously that loxoribine exhibits adjuvant activity for B cells, activates natural killer (NK) cells, and enhances the activation of lymphokine-activated killer cells by interleukin-2 (IL-2). In this study, we examined loxoribine for protective effects in a B16 melanoma lung tumor metastasis model. Significant inhibition of B16 metastasis was seen in mice given a single injection of 2 mg loxoribine as late as day 3 of tumor growth but the greatest inhibition (96%) was seen in mice given four injections of loxoribine on alternate days starting the day before tumor injection. In experiments in which both IL-2 and loxoribine were administered, both agents were active when tested alone, but the combination of IL-2 and loxoribine gave significantly greater inhibition of metastasis. Loxoribine partially inhibited the development of tumors in mice that had been depleted of NK cells by the administration of anti-asialo-GM1 or anti-NK1.1 antibodies and in NK-deficient beige mice. In all cases, protection was seen only when smaller tumor inocula were injected. Taken together, these data suggest that both NK and non-NK cell populations or effector mechanisms with antitumor activity were activated by loxoribine. Since substituted guanosine analogs have been shown to have adjuvant activity in B cell systems, we evaluated whether loxoribine was active as an adjuvant in a tumor protection model. Mice immunized with both irradiated tumor cells and loxoribine developed a significantly lower number of lung tumors when challenged by live B16 tumor cells, whereas mice injected with either vaccine or loxoribine alone were not protected. There was a clear dose response seen with both loxoribine and the vaccine preparations. These data suggest that loxoribine may be useful in tumor therapy as an immunomodulator or as an adjuvant for use with tumor vaccines.

Key words: Tumor metastasis – Immunomodulator – Tumor vaccine adjuvant – NK cells

Introduction

The role of the immune response in surveillance against newly arising and metastasizing neoplasms has been the subject of much debate and many experimental studies, particularly over the past two decades. In general, there are two classes of immune responses that are thought to be of importance in antitumor immunity: non-specific or natural immunity and tumor-antigen-specific immunity. Numerous experimental and clinical studies have suggested that natural immunity plays an important role in both immunosurveillance and the blockade of metastases from primary tumors [1, 2]. The relevant effector mechanisms have been identified as natural killer (NK) cells [3-8], lymphokine-activated killer (LAK) cells [9, 10], and macrophages [11-13]. The role of the antigen-specific immune response in tumor resistance is less well documented, but tumor-infiltrating lymphocytes (TIL) [14-16] and antibodies [17, 18] appear to mediate resistance in specific tumor models. There is also evidence that non-specific and specific immune responses may interact beneficially to provide resistance against tumors. An example of this has been the enhancement of LAK cell activity by antibody-dependent cellular cytotoxic (ADCC) responses induced by tumor-specific antibodies [17, 19].

A variety of strategies have been used in tumor immunotherapy. Agents that activate NK or LAK cells have been administered in an attempt to activate endogenous effector cells and, in doing so, to inhibit tumor metastasis [20-24]. A second strategy has been to adoptively transfer exogenously activated LAK or TIL cells and to maintain in vivo activation by co-administration of cytokines [25-29]. A third strategy has been to immunize actively with tumor vaccines to stimulate the production of antigen-specific immune effector mechanisms [30, 31]. In the latter case, co-administration of adjuvants or agents that block tumor-activated immunosuppressive mechanisms has been incorporated into trials to enhance the protective effects over those seen with vaccine alone [32, 33]. More recently, tumor cells with transfected cytokine genes have been used...
in an attempt to provide immunostimulation in conjunction with tumor antigen sensitization [34–37].

A great number of immunomodulatory agents have been used, in both experimental models and human trials, as agents that activate non-specific immune mechanisms. The most commonly used have included (a) bacterial preparations such as bacillus Calmette-Guérin, streptococcal extracts, or Corynebacterium parvum [20, 22, 38]; (b) viral products [39, 40]; (c) cytokines such as interferon α or γ [18, 29, 41], interleukin-1 (IL-1) [42, 43], IL-2 [21, 24, 26, 28], and tumor necrosis factor (TNF) [23, 44]; and (d) interferon-inducers such as bropiramine [45, 46] or 7-thia-8-oxoguanosine [47]. Some of these agents have also been used as adjuvants in combination with tumor vaccines in order to enhance antigen-specific immune responses [48].

We have shown previously that substituted guanosine compounds, including 7-allyl-8-oxoguanosine (loxoribine), have adjuvant activity for B cells [49], activate murine NK cells [50], and enhance LAK activation in response to IL-2 [51]. Here we show that loxoribine inhibits the metastasis of murine B16 melanoma cells through NK-dependent and NK-independent mechanisms and, in addition, has adjuvant activity for a murine melanoma vaccine.

Materials and methods

**Mice.** Male C57BL/6J and C57BL/6J-bg/bg mice were obtained from Jackson Laboratories (Bar Harbor, Me.). All mice were used at 8–12 weeks of age. They were fed Purina rodent laboratory chow and tap water ad libitum.

**Cell lines.** The B16-F10 (B16) melanoma cell line was obtained from the American Type Culture Collection (ATCC, Rockville, Md.). It was maintained in culture in Dulbecco’s modified Eagle’s medium (Flow Laboratories, McLean, Va.) supplemented with 10% fetal bovine serum (FBS, Gibco, Grand Island, N.Y., or HyClone Laboratories, Logan, Utah), 100 IU/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine (all from Flow Laboratories). The PK 136 and S-S.1 hybridoma cell lines were obtained from the ATCC and were maintained in culture in RPMI-1640 medium (Flow Laboratories) supplemented as above.

**Reagents.** Loxoribine was dissolved in 1 M NaOH, diluted to the appropriate concentration with saline, adjusted to pH 7.0 with 1 M HCl, and filter-sterilized. Mice were injected i.v. or i.p. with 0.5-ml volumes. The anti-asialo-GM1 antibody was a polyclonal antibody produced in rabbits (Wako Chemicals, Tex.), which was diluted 1:50 in saline and injected i.p. in a volume of 0.5 ml. Normal rabbit serum, diluted as above, was used as a control. The anti-NK1.1 antibody was an IgG2a monoclonal mouse anti-NK1.1 [52] produced by the PK 136 hybridoma. Culture supernatants collected from confluent cultures were filter-sterilized and administered i.p. in a volume of 1 ml. As a control for the anti-NK1.1, culture supernatants from the S-S.1 hybridoma were used. This cell line secretes an IgG2a anti-(sheep red blood cell) antibody. Both the anti-asialo-GM1 and NK1.1 antibody preparations completely inhibited NK activity when administered to mice that had been stimulated 24 h previously with polyinosinic-polycytidylic acid to enhance the NK activity [51].

**B16 lung tumors.** B16 cells were removed from culture vessels by trypsinization, washed once with culture medium and once with saline, and resuspended in saline at 8 × 10⁶ cells/ml. Mice were injected i.v. on day 0 of the experiment with 0.5 ml tumor cells. Fourteen days later, the lungs were removed from mice and examined under a dissecting microscope, and the total number of tumors in the lungs were counted.

**B16 vaccine.** B16 cells were removed from culture vessels by trypsinization and washed once with culture medium and once with saline. Cells were irradiated in a cesium source with a total dose of 600 GY and adjusted to a concentration of 2 × 10⁶ cells/ml. Mice were injected i.p. with 0.5 ml irradiated cells 21 and 14 days before i.v. challenge with live B16 cells.

**Data presentation.** Data for all experiments are presented as the mean number of tumors per mouse ± the standard error (SE) for groups of 8–11 mice. To determine whether the differences between experimental groups were significant, data were analyzed using parametric or non-parametric Dunnett’s tests or the Wilcoxon test. In one figure with data pooled from four experiments, a randomized block design was used. In all cases, the normality of the data for each experiment was assessed using the Wilk-Shapiro test for normality. A parametric test was used if the data were normally distributed.

Results

**Loxoribine inhibits the metastasis of B16 melanoma cells to the lungs of mice**

Our first objective in this study was to determine whether loxoribine treatment of mice would block the establishment of lung tumors following i.v. administration of B16 tumor cells. To do so, loxoribine was administered at a dose of 2 mg/mouse (approximately 80 mg/kg) as single injections given either 1 day before (−1) or 1, 3, or 6 days after the i.v. injection of the B16 cells. One group of mice was given injections on all 4 days of the study. As can be seen from Fig. 1, when evaluated 14 days after tumor inoculation, mice injected with vehicle developed an average of 197 tumors in their lungs. Mice developed significantly lower numbers of tumors if given a single dose of loxoribine