Increased Cytotoxic Sensitivity of YPC-1 Tumor Cells from Mice Treated with Nitrosoureas

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Abstract

The relative cytotoxic sensitivity of the YPC-1 tumor target cells from untreated mice and from animals treated with nitrosoureas was determined. The amount of 51Cr released from target cells increased significantly when the cells were obtained from treated mice. On the basis of the results of cold-target cytotoxicity inhibition assay, this enhancement was shown to be haplotype specific.

The amount of 51Cr released from target cells of mice treated with N,N'-bis(chloroethyl)-N-nitrosourea decreased significantly when the tumor cells were first incubated with fibrinogen and transglutaminase. Based on these results and other published data, a model system is suggested. The model is based on the observation that tumors, and thus tumor antigens, at the cell surface are partly or completely covered by fibrinogen or fibrin. The enzyme transglutaminase is involved in the binding of the fibrinogen or fibrin to the cell surface. Accordingly, it is hypothesized that the nitrosoureas have a dual mode of immunotherapeutic activity. The carbamoylation properties inhibit the fibrin-binding activity of transglutaminase, thus preventing fibrin from covering up or coating the tumor cells and preventing the ability of sensitized effector cells to recognize the tumor-specific antigens in association with self H-2 antigens. The alkylation property of the nitrosoureas mainly concerns reactivity with the DNA of the tumor cells.

Introduction

It is generally accepted that one of the effective modalities for cancer immunotherapy is the ability to activate the host immune system, specifically those cells which mediate cytotoxicity towards the tumor. Thus, passive transfer of syngeneic lymphoid cells preimmunized against tumor tissues or tumor-specific antigens into tumor-bearing hosts has led to prolonged survival of the host and concurrent decrease in the growth of the tumor (19). Another approach has been to select chemotherapeutic agents which are preferentially cytotoxic for neoplastic tissues. Nitrosoureas such as BCNU and CCNU have been shown to be cancerostatic against certain neoplastic tissues (23) and are undergoing experimental and clinical trials, although the precise mechanism by which this effect is mediated is not clearly understood. It is reasoned that a combination of effective immunotherapy and chemotherapy may be one approach for effective arrest of tumor growth. Perhaps the ideal antitumor therapeutic regimen would consist of a chemotherapeutic agent which would specifically activate the host tumor-specific cytotoxic cells and also lead to the arrest in growth of the tumor.

Experiments were conducted to examine whether the cancerostatic effect of nitrosoureas (BCNU and NPCNU) was due to their effect on the sensitivity of tumor cells to cell-mediated cytotoxicity as well as on the metabolism of the tumor cells. Data are presented which indicate that pretreatment of tumor-bearing animals with nitrosoureas leads to tumor cells which are highly sensitive to the cytotoxic effects of tumor-specific cytotoxic killer cells. The data also provide a basis for the possible mechanism of the immune-associated cancerostatic action of nitrosoureas.

Materials and Methods

Drugs and Chemicals. BCNU was obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md. NPCNU (a recently developed nitrosourea) was supplied by the National Foundation for Cancer Research at the American University Chemistry Department, Washington, D. C. Bovine fibrinogen was purified by the method of Laki (10), and transglutaminase was prepared from guinea pig liver according to the method described by Connellan et al. (3).

Animals. Female mice of the inbred strains CAF-1, BALB/c, and C57BL/6, 8 to 12 weeks old, were purchased from The Jackson Laboratory, Bar Harbor, Maine.

Tumor Cells. The YPC-1 tumor, which is a C-type virus-induced plasmacytoma of CAF-1 mice (24), was maintained in ascites form by serial i.p. passage of 1 x 10^6 tumor cells in CAF-1 mice, while the EL-4 tumor cells were maintained in ascites form in C57BL/6 mice.

Generation of Effector Lymphocytes. Various strains of mice were immunized i.p. with 1 x 10^7 mitomycin C-treated tumor cells (100 µg of mitomycin C were used to treat 1 x 10^7 tumor cells). Three weeks after initial immunization, spleens were removed, and lymphoid cells were obtained by gentle teasing in balanced salt solution, suspended, and washed. Subsequently, 1 x 10^7 lymphocytes were cultured in 16-mm-well, flat-bottomed Linbro plates, in a volume of 2.0 ml, along with 2 x 10^5 mitomycin C-treated appropriate tumor cells which were used for stimulation. Roswell Park Memorial Insti...
tute Medium 1640 supplemented with l-glutamine, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer solution (25 mm, pH 7.2), gentamicin (50 mg/ml), 2-mercaptoethanol (5 × 10⁻⁵ M), and 10% heat-inactivated fetal calf serum served as the culture medium for the experiments. After a total of 6 days of incubation at 37°, in a humidified atmosphere of 10% CO₂, the lymphoid cells were washed and used as effector cells. The viability of the effector lymphocytes was determined by the Trypan Blue dye exclusion technique prior to the assay.

**Treatment of Ascites Tumor-bearing Mice before Harvesting Target Cells.** A group of CAF-1 mice were given injections of 1 × 10⁵ YPC-1 ascites tumor cells. Fourteen days following tumor injection, groups of 6 mice each were treated as follows: (a) 0.5 mg in 0.15 M NaCl-BCNU s.c. 6, 12, or 18 hr before sacrifice; (b) 0.12 mg BCNU administered in 3 aliquots s.c., with injections being given 6, 12, and 18 hr prior to sacrifice; (c) 1.0 mg NPCNU s.c. 6, 12, or 18 hr before sacrifice; and (d) 0.15 M NaCl (control). These doses of BCNU and NPCNU were determined to be optimal for the expression of increased cytotoxicity as compared to those from 0.15 M NaCl-treated mice, respectively. In marked contrast, the mice presensitized in vivo and in vitro to mitomycin-treated YPC-1 tumor cells showed significant enhanced cytotoxicity when target tumor cells were from mice pretreated with 0.5 mg of BCNU as compared to those from similar animals treated with 0.15 M NaCl.

**Isolation and ⁵¹Cr Labeling of Tumor Cells.** The YPC-1 tumor was carried in CAF-1 mice in ascites form by an i.p. injection of 1 × 10⁵ cells and passed every 2 weeks. For preparation of target cells for the cytotoxicity assay, the ascites cells were obtained 14 days after passage, washed once, layered over a Ficoll-Hypaque gradient (specific gravity, 1.096), and centrifuged at 750 × g. Subsequently, the cells at the interface were collected and washed 3 times before labeling. These cells were always 95% viable (as assayed by Trypan Blue). Cells (1 × 10⁵) in a volume of 0.5 ml media were incubated with 100 µCi of ⁵¹Cr (sodium chromate; specific activity, 200 to 500 Ci/g; New England Nuclear, Boston, Mass.) at 37° for 45 min. The tumor cells were then washed 3 times, adjusted to contain 5 × 10⁴ viable tumor cells/ml, and used in the cytotoxicity assay. Target cells usually consisted of ⁵¹Cr-labeled ascites tumor cells from a pool of at least 3 untreated mice and from a similar pool of BCNU- or NPCNU-treated mice.

**In Vitro Treatment of Target Cells with Fibrinogen and Transglutaminase.** ⁵¹Cr-labeled tumor cells (5 × 10⁵) from BCNU-treated mice (0.5 mg BCNU, 6 hr before sacrifice) were incubated with 250 µg fibrinogen and 125 µg transglutaminase at 37° for 30 min in 5 ml of Tris-0.15 M NaCl as described previously by Fesus and Laki (5). Following incubation, the cells were washed 3 times and harvested in新鲜 media, and used as target cells in the cell-mediated cytotoxicity assay.

**Cytotoxicity Assay.** All assays were carried out in triplicate in Linbro 96 round-bottomed wells. Varying numbers of effector cells were incubated with 5 × 10⁴ ⁵¹Cr-labeled tumor cells in a total volume of 0.2 ml of Roswell Park Memorial Institute Medium 1640 containing 10% fetal calf serum. After an incubation period of 4 hr at 37°, the plates were centrifuged for 10 min at 500 × g. The cpm were determined for 0.1 ml of the supernatant fluid using a Nuclear Chicago gamma counter. The cold-target cytotoxicity inhibition assay was carried out in a manner similar to the cytotoxicity assay, except that varying numbers of unlabeled ("cold") target cells were added in appropriate wells. Net specific cytotoxicity (%) was calculated as follows:

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\text{% of net specific cytotoxicity} = \frac{\text{cpm experimental} - \text{cpm spontaneous}}{\text{cpm maximal} - \text{cpm spontaneous}} \times 100
\]

Mean maximal release was determined from triplicate cultures containing ⁵¹Cr-labeled target cells which were incubated with Triton-X in a similar total volume of media. The mean cpm for spontaneous ⁵¹Cr release was calculated from ⁵¹Cr-labeled target cells incubated with media alone.

**Statistics.** The significance of the difference was calculated by the Student's t test.

**RESULTS**

**Increased Lytic Susceptibility of Tumor Cells from Mice Pretreated with Nitrosoureas.** The YPC-1 ascites tumor cells from 0.15 M NaCl-pretreated CAF-1 mice and from CAF-1 mice pretreated with 0.5 mg of BCNU at 6, 12, and 18 hr prior to tumor cell harvest were compared for their susceptibility to lysis using cytotoxic effector cells from CAF-1 and BALB/c mice presensitized in vivo and in vitro to mitomycin-treated YPC-1 tumor cells. As seen in Table 1, the use of effector cells from CAF-1 and BALB/c mice resulted in a net cytotoxicity of 12.7 ± 1.2 (S.E.) and 14.7 ± 1.1% for YPC-1 target cells from 0.15 M NaCl-treated mice, respectively. In marked contrast, the same effector cells from CAF-1 and BALB/c mice were found to result in as high as 34.3 ± 5.6, 40.4 ± 5.6, and 40.4 ± 2.7% net specific ⁵¹Cr release, respectively, when YPC-1 tumor cells from mice pretreated with 0.5 mg of BCNU were used as targets. The pretreatment of mice with BCNU to give increased cytotoxicity could be administered from 6 to 18 hr prior to harvest of the ascites target cells (see Table 1). A similar effect was observed with NPCNU if it was given 6 hr prior to harvest of the ascites target cells (Table 1). The effector cells were found to be T-cells in these experiments (data not shown). Their sensitivity to treatment in vitro with anti-Thy-1.2 serum plus complement. The increased lysis was seen with all effector cell:target cell ratios (data not shown). These data clearly indicate that effector cells mediating lysis of both syngeneic (CAF-1 anti-YPC-1) and semiallogeneic (BALB/c anti-YPC-1) target cells show significant enhanced cytotoxicity when target tumor cells are obtained from animals treated previously with BCNU or NPCNU as compared to those from similar animals treated with 0.15 M NaCl.

**Specificity of the Effect of BCNU.** It was reasoned that the increased susceptibility of the target cells from BCNU-treated mice could be due to: (a) nonspecific increase in the fragility of the target cell; (b) effect of a carryover of BCNU on effector cells; (c) effect of BCNU on mononuclear immunocytes present in the peritoneal fluid, i.e., cells which might cooperate with effector killer lymphocytes; or (d) unmasking of target cell antigens against which the cytotoxic T-cells are directed. Experiments were therefore conducted to demonstrate target cell specificity of the effector cells. Thus, CAF-1 effector cells previously sensitized to mitomycin C-treated YPC-1 tumor cells were assessed for their ability to lyse ⁵¹Cr-labeled YPC-1 target cells on EL-4 (H-2b) target cells from mice which were either pretreated with 0.15 M NaCl or BCNU. These data revealed...
that CAF-1 effector cells were cytotoxic only toward the YPC-1 tumor target cell but not toward the EL-4 cells (data not shown).

A cold-target cell inhibition experiment was performed, whereby varying numbers of unlabeled YPC-1 or EL-4 tumor target cells were mixed with $^{51}$Cr-labeled YPC1 tumor target cells before the cell-mediated cytotoxicity experiment was carried out. As seen in Chart 1, the addition of increasing numbers of unlabeled YPC-1 tumor target cells, but not unlabeled EL-4 target cells, resulted in the specific inhibition of $^{51}$Cr release mediated by CAF-1 effector cells. Similar additions of unlabeled 129P H-2k ascites cells or unlabeled bacterial lipopolysaccharide blasts from mice of the H-2b, H-2d, and H-2k haplotype failed to inhibit the cytotoxic specificity of the effector cells for the YPC-1 tumor target cells. Further, there was no increased spontaneous release manifested by the target cells obtained from either the BCNU- or NPCNU-treated tumor-bearing mice and used in the assays described in Tables 1 and 2 and Chart 1. The spontaneous release for target cells from 0.15 M NaCl-treated CAF-1 mice in Table 1 was 238 cpm as compared to 212 for target cells from BCNU- and 187 for target cells from NPCNU-treated tumor-bearing mice. The values for target cells used in the experiments listed in Table 2 and Chart 1 fell essentially in the same range.

These data demonstrate several points: (a) the cytotoxic cells show target cell specificity; (b) the increase in cytotoxicity is also target cell specific; (c) there is a nonspecific effect of a carryover of BCNU on effector cells; otherwise, some residual cytotoxicity would have been evident against control target cells from BCNU-treated mice. Similarly, it makes it unlikely that BCNU pretreatment merely increases the fragility of the tumor cells, since (a) the increase in cytotoxicity was found to be specific, and (b) the use of mixtures of $^{51}$Cr-labeled specific YPC-1 target cells with cold or labeled target cells from BCNU-treated C57BL/6 mice carrying the EL-4 tumor did not show any reduction in cytotoxicity. These data demonstrate again that the cytotoxicity seen was specific and was not due to some special affinity of effector cells for BCNU-treated target cells.

Role of Transglutaminase and Fibrinogen in Tumor Cell-mediated Cytotoxicity. It has been demonstrated previously that the surface of YPC-1 tumor cells become coated with fibrinogen if the cells are incubated in vitro in the presence of transglutaminase and fibrinogen (5). Experiments were carried out to investigate whether this modulation of the cell surface will lead to change of tumor cell sensitivity against killer lymphocytes. Thus, experiments were carried out whereby YPC-1 tumor target cells were obtained from 0.15 M NaCl- or BCNU-treated CAF-1 mice. These cells were respectively labeled with $^{51}$Cr. The cells obtained from BCNU-treated mice were incubated in vitro with (a) Tris-0.15 M NaCl, (b) transglutaminase, (c) fibrinogen, (d) fibrinogen and transglutaminase. These target cells were then evaluated in the cell-mediated cytotoxicity assay using CAF-1 and BALB/c effector cells presensitized both in vivo and in vitro against the YPC-1 mitomycin C-treated tumor cells as described previously. As seen in Table 2, both CAF-1 and BALB/c effector cells, as expected, showed greater cytotoxicity against YPC-1 tumor cells from 0.15 M NaCl-treated mice, but there was a marked enhancement against YPC-1 tumor cells from BCNU-treated CAF-1 mice. In contrast, when the YPC-1 target cells from BCNU-treated CAF-1 mice were incubated with transglutaminase plus fibrinogen, there...
was a significant inhibition of cytotoxicity (see Table 2). This was not seen in target cells treated with transglutaminase or fibrinogen alone, denoting the necessity for treatment with both transglutaminase and fibrinogen for the inhibition of cytotoxicity.

**DISCUSSION**

It is generally accepted that tumor cells possess specific antigenic determinants which appear as neoantigens on the cell surface (17, 21). Several observations indicate that tumors elicit an immune response from the tumor-bearing host; however, in spite of the tumor-specific response, progressive tumors grow unchecked (7, 14). Several investigations have pointed out that tumor-specific immunity can be observed in tumor-bearing hosts subsequent to chemotherapy (1, 9, 18). Mihich observed in 1975 that the beneficial effect of chemotherapy was abrogated by prior immunosuppression of mice with whole-body irradiation (14).

In the present study, we found that effector lymphocytes were more effective in vitro against target cells from tumor-bearing animals treated with BCNU or NPCNU prior to harvest of the target cells. Similar effects of BCNU pretreatment were observed if repeated low doses of BCNU were given (e.g., 0.04 mg at 6, 12, and 18 hr prior to sacrifice, data not shown). The increase in the amount of 5^1Cr release from the targets from BCNU-treated mice was specifically inhibited by cold (unlabeled) tumor target cells, which indicates that the increased 5^1Cr release is the result of a specific immune reaction. These data suggest that nitrosoureas have effects other than a chemotherapeutic one on target cells. It seems from the data presented in this communication that the tumor cells are more susceptible to lysis by immune T-cells. Therefore, nitrosoureas can be considered as drugs with a special immunchemotherapeutic effect. The therapeutic effects observed using nitrosoureas, besides the cancerostatic effect, could also be the result of enhanced tumor-specific antigen recognition.

Numerous observations indicate that fibrin accumulates in high levels around a wide variety of mammalian tumors and persists in the tumor area throughout tumor development (2, 4, 8, 11, 13, 15). O'Meara (16) has shown that the fibrin network around tumor cells aids their nutrition by providing a constantly renewed source of available protein. It was suggested that the tumor-associated fibrin interferes with the free circulation of tissue fluids in the tumor area, thereby impeding the influx of immunocompetent lymphoid cells. In support of this hypothesis, it has been shown that fibrin or activated fibrinogen can induce neoplastic cells to clump together and that these cells are more resistant to attack by immunocompetent cells in vitro (6). Furthermore, Schlager and Dray (20) observed that rabbit antibody to FFE cured guinea pigs when 0.75 or 1.0 mg of an IgG preparation was given 6 and 16 days after the injection of a lethal dose of tumor cells. The regression of tumor cells was complete in all animals, while no significant antitumor effect was seen when normal-rabbit IgG or smaller doses of anti-FFE preparation were used. The long-term tumor-free survival of the animals and their resistance to subsequent tumor challenge indicated that the therapy led to systemic tumor immunity. A histological study of the tumor area, 7 hr after the anti-FFE injection, revealed a mononuclear cell infiltration into the tumor cell mass (20).

Transglutaminase is an important enzyme of proliferating tissues. Although this enzyme can cause aggregation of various proteins, its major substrates have been shown to be fibrin and fibrinogen. When transplanted tumor cells proliferate in experimental animals, the level of this enzyme drops significantly. Furthermore, it was observed that administration of transglutaminase to tumor-bearing mice increased the metastasis of Lewis lung carcinoma-bearing mice 3-fold (13).

Investigators on the effect of different nitrosoureas on transglutaminase have shown that, if it is incubated with BCNU, CCNU, or NPCNU, the enzyme becomes inactivated. The uptake of labeled CCNU can be prevented by cold cyclohexyl isocyanate, indicating that it is the isocyanate moiety which inactivates the enzyme (22). Although native BCNU lasts only for a short time in the body, its isocyanate moiety reacts with transglutaminase and keeps it inactivated long after BCNU has been shown to be detectable in vivo (12).

Fesus and Laki (5) observed that the surface of YPC-1 tumor cells become coated with fibrinogen when the cells are incubated with transglutaminase and fibrinogen. In the present study, we found that the sensitivity of tumor cells from BCNU-treated mice to cell-mediated cytotoxicity dropped significantly when the tumor cells were incubated with transglutaminase and fibrinogen. These observations are in agreement with the previously suggested hypothesis that, in the case of certain tumors, the tumor-associated fibrin prevents the tumor cells from being recognized and eliminated by the immune system (6). Transglutaminase may play an important role in the concealment of tumor cells by fibrin or fibrinogen. Thus, the inactivation of transglutaminase by nitrosoureas may result in the elimination of the tumor cells by the immune system. Accordingly, nitrosoureas can be considered as drugs with an immunochemotheorapeutic effect. The alkylating moieties...
mainly react with the DNA of cells, while the carbamoylating parts inhibit the effect of transglutaminase, leading to the unmasking of tumor-specific antigen(s).

These experiments do not rule out the possibility that treatment of ascites tumor-bearing mice with nitrosoureas increases the residual activity or the frequency of a subpopulation of cells in the ascites fluid that are able to induce enhanced activation of effector cells in the in vitro phase or elimination of suppressor cells present in the target cells population. Further experiments are in progress to ascertain the role of accessory cells in the enhanced expression of T-cell-mediated cytotoxicity against tumor cells from nitrosourea-treated mice.

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