Importance of antitumor immunity for complete cure of highly drug-sensitive leukemia in mice *

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Summary. Seven transplantable leukemia lines were established from spontaneous leukemias and screened for \(3\text{-}(4\text{-amino}-2\text{-methyl}-5\text{-pyrimidinyl})\text{-methyl}-1\text{-}(\text{chloroethyl})\text{-1-nitrosourea hydrochloride (ACNU)}\) sensitivity in DDD mice. Three of them were classified as highly sensitive, two as sensitive and two as resistant to ACNU. A highly sensitive line, DL812, was extensively characterized from a therapeutic point of view. DL812 cells were so invasive as to produce enlargement of spleens and lymph nodes but not local tumors when injected s.c., markedly sensitive to in vitro ACNU exposure and moderately immunogenic. The invasion process of DL812 cells differed with the status of host immunity. Advanced DL812 leukemias were macroscopically completely cured with normalization of spleen and lymph node sizes 3-7 days after an i.p. injection of 0.5 mg ACNU, but more ACNU-resistant leukemias with splenomegaly and enlarged lymph nodes recurred thereafter. Recurring DL812 cells were approximately four times more resistant to in vitro ACNU exposure but maintained similar immunogenicity as compared to the original ones. Permanent cures of advanced leukemias were achieved by ACNU treatment plus subsequent adoptive transfer of immune splenocytes in 15% of diseased mice. The results suggest the importance of host antitumor immunity for permanent cures of highly drug-sensitive leukemias, overcoming drug resistance and relapse.

Key words: Mouse leukemias – ACNU sensitivity – Host immunity – Chemotherapy – Adoptive immunotherapy

Introduction

Over 30 chemotherapeutic agents have been approved for the treatment of cancer in many countries (Powis 1985). They have been used mostly in combination, with varying degrees of success in cancer patients. Chemotherapy is frequently effective in obtaining cures in leukemia. However, the success of the therapy has been hampered by the twin problems of relapse and the emergence of drug resistance (Moscow and Cowa 1988). It is therefore very important to develop more adequate methods for applying chemotherapeutic agents so that they can fully manifest their anticancer activities while overcoming these problems. The development of animal models is prerequisite for this purpose, and the current study was conducted to achieve it. A water-soluble nitrosourea, ACNU, was taken up as an anticancer agent because of its extensive application in treatment of leukemia in man (Takubo et al. 1978, 1981), and spontaneous lymphatic leukemias from DDD mice were screened for sensitivity to the drug. As a result, the unique transplantable leukemia line, DL812, which is highly sensitive to ACNU, was obtained. The biological, therapeutic and immunological characterization of this line is described in this report.

Materials and methods

Drug and reagents

ACNU was obtained from Sankyo Company (Tokyo, Japan). FITC-anti-Thy-1, FITC-anti-Lyt-1, FITC-anti-Lyt-2, FITC-anti-(mouse Ig), anti-L3T4 and FITC-anti-(rat \(\kappa\) chain) antibodies were purchased from Becton Dickinson (Mountain View, Calif). M199 was obtained from Nissui Company (Tokyo, Japan). All other chemicals were commercial preparations of analytical grade.

Animals

DDD mice used were bred and maintained in the mouse colony operated under the specific pathogen-free conditions by the Laboratory Animal Research Center of our institute (Tanaka et al. 1987). This
strain of mice has been characterized by the moderate incidence of leukemias and the low incidence of mammary tumors (Matsuzawa et al. 1970). Male mice, 8–10 weeks old, were used at the start of all experiments. They had free access to F-2 pellets (Funabashi Nojo Company, Funabashi-city, Japan) and tap water, and were housed 5–8/cage in a temperature-controlled room with a 12-h light/12-h dark cycle.

Leukemias

DDD mice older than 6 months sporadically develop spontaneous leukemias, characterized by splenomegaly combined with enlargement of the thymus and/or mesenteric and other lymph nodes. Leukemias can be easily transplanted to fresh syngenic mice by i.p. or s.c. injection of mechanically dissociated cells from enlarged spleens (Matsuzawa et al. 1970). Seven leukemia lines freshly established in this manner were examined for sensitivity to ACNU within ten passages.

Screening of leukemias for sensitivity to ACNU

The s.c. injected leukemic cells produced a local tumor mass and/or enlargement of the spleen and lymph nodes. The local tumor mass or enlarged spleen was mechanically dissociated with scissors and forceps in PBS, and filtered through nylon mesh to obtain a single-cell suspension. Viable cells were counted by the trypan blue dye exclusion test. Fresh mice were inoculated s.c. with 10^6 viable cells in 0.1 ml PBS at the right inguinal site and randomly divided into two groups. Immediately, one group of mice was injected i.p. with 0.1 ml physiological saline as control and the other with 0.5 mg ACNU in 0.1 ml physiological saline. When clearly visible tumorous masses at the injected site and/or significant splenomegaly were detected by palpation in control mice, animals of both groups were killed by chloroform overdose to determine the weights of the local tumor mass and spleen in each. The leukemia was considered highly sensitive to ACNU when significant local tumor formation and/or splenomegaly occurred in the control group and no leukemic lesions were visible at autopsy in the treated group. The leukemia was considered sensitive or resistant to ACNU when the local tumor and/or spleen weights were significantly larger in the control than in the treated group or were similar in both groups, respectively. The difference was statistically evaluated by the Student's t-test and considered significant at P<0.05. In the course of screening, the DL812 leukemia line was found to be unique in growth pattern and extremely high sensitivity to ACNU. Thus, further characterization and therapeutic studies were carried out in this line.

Growth pattern of DL812 leukemic cells

Single cells were prepared from enlarged spleens as mentioned above. Mice were given a s.c. inoculation of 10^5 viable cells at the inguinal site and killed on days 4, 7, 9, 11, and 14 after inoculation to determine the weights of the local tumor, spleen and mesenteric lymph nodes.

Surface markers of DL812 cells

The single DL812 cell suspension was treated with TRIS-buffered NH_4Cl solution (pH 7.2) to remove erythrocytes. After washing, 5 x 10^6 cells were treated with FITC-anti-Thy-1, FITC-anti-Lyt-1, FITC-anti-Lyt-2 or FITC-Ig antibodies in PBS containing 10% fetal calf serum and 10 mM sodium azide. Indirect staining was performed for the determination of L3T4 positivity. After incubation with anti-L3T4 monoclonal antibody, the cells were washed and incubated with anti-(rat IgG). The percentage of positive cells was determined using 10^5 FITC-labeled cells with a fluorescence-activated cell sorter (Becton Dickinson, Mountain View, Calif) with the scatter gate between 110 and 161.

Examination of sensitivity to ACNU of DL812 cells

Mice were inoculated with 10^6 DL812 cells, immediately divided into six groups at random and given an i.p. injection of 0.1 ml physiological saline or 0.1, 0.2, 0.3, 0.5 or 1.0 mg ACNU in 0.1 ml physiological saline. On day 16 of ACNU treatment, mice were killed to determine spleen weights.

To examine the direct effect of ACNU on DL812 leukemia, a DL812 single-cell suspension in M199 was prepared from enlarged spleens on day 14 after inoculation; the cells were washed twice with M199 and resuspended at a concentration of 10^7 viable cells/ml in M199 containing ACNU at 0, 0.5, 1.0, 2.0, 4.0, 8.0 or 15.0 μg/ml. Two ml of each cell suspension was incubated in 5% CO_2 in air at 37 °C for 1 h in a humidified chamber. Cells were then washed twice with M199 and resuspended in 2 ml PBS. Mice were inoculated s.c. with 0.1-ml aliquots of the cell suspension. Splenomegaly was checked by weight determination on day 10 of inoculation.

Recurrent DL812 cells were also prepared from the spleens that had enlarged again after a macroscopically complete cure, induced by a single i.p. injection of 1 mg ACNU (see Results), and processed in the same manner to investigate the acquisition of resistance to ACNU by these cells.

Immunication of mice with DL812 cells

A DL812 cell suspension was prepared from enlarged spleens and irradiated with gamma rays at 4000 rad by using a 137Cs γ irradiator (Gammacell 1000, Atomic Energy of Canada). Mice were immunized by four s.c. injections of 2 x 10^7 or 5 x 10^7 irradiated cells at weekly intervals or by three s.c. injections of 2 x 10^7 cells at weekly intervals followed by an injection of 5 x 10^7 of these cells 2 weeks later. The last immunization procedure was the best and therefore used in all experiments except for the early ones. Since some mice developed leukemia in the course of immunization, the immunized mice that had been confirmed by palpation to be free from the disease for 3 weeks after the last injection were used as immune mice.

Challenge of immune mice with DL812 cells

Immune mice were challenged with a s.c. inoculation of 10^6, 10^5 or 10^4 viable DL812 cells at the inguinal site. Unimmunized normal mice were challenged in the same way for comparison. Thereafter, these mice were observed for local tumor formation and splenomegaly by palpation and for death.

In addition, fresh immune mice and the immune mice which had survived the challenge with DL812 cells (see Results) were challenged with 10^5 recurrent DL812 cells and observed similarly, to clarify whether or not the same antigenicity was preserved after exposure to ACNU.

Winn assays

To examine the effector function of the spleen cells of immune mice, the in vitro neutralization assay of Winn (1961) was used. Spleens from normal and immune mice were mechanically disrupted in M199, filtered through nylon mesh, washed three times with M199 and resuspended at a concentration of 10^7 or 5 x 10^7 normal or immune splenocytes, respectively. A 0.1-ml sample of the mixture, consisting of 10^5 DL812 cells alone or 10^5 or 5 x 10^5 normal or immune splenocytes, was injected s.c. into each normal recipient at the inguinal site. Spleen weights were determined 11 days later.

Treatment of advanced DL812 leukemia with ACNU

Mice were inoculated s.c. with 10^6 viable DL812 cells and injected i.p. with 0.5 mg ACNU 14 days later, when leukemic cells were disseminated throughout the body with marked splenomegaly and enlarged lymph nodes. Some of them were given an additional i.p. in-