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Evaluation of Mizoribine as an Immunosuppressant in Subrenal Capsule Assay Using Immunocompetent Mice

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We studied the application of mizoribine (MZR) to normal immunocompetent mice in subrenal capsule assay (SRCA) by means of tumor growth curve determination, histological analysis and autoradiography. At 400 mg/kg, MZR prolonged the actual tumor growth and moderately reduced the host reaction. Doses below 200 mg/kg did not effectively suppress the host reaction. The maximal weight loss of mice in the 400 mg/kg group reached 29%, but did not exceed 10% within 8 days. Hence, we applied 400 mg/kg of MZR to SRCA for up to eight days for cancer chemotherapy testing. This dose of MZR did not affect the labeling index of tumor cells compared with the control.

Key words: Mizoribine — Subrenal capsule assay — Immunosuppression

Subrenal capsule assay (SRCA)[2] was first reported by Bogden and his coworkers[1] in 1978. This method permits precise *in situ* measurement of transplanted tumors by using a stereomicroscope with an ocular micrometer. SRCA is also advantageous for delivery of nutrients, drugs and other substances to tumors. As compared with subcutaneous xenografts in athymic nude (AN) mice, SRCA allows highly precise measurements of tumor size and requires less time to obtain clinically useful data for individual cancer chemotherapy.

Since normal immunocompetent (NIC) mice were used by Bogden *et al.* for SRCA in a six-day protocol,[2] large numbers of clinical materials have been tested similarly. Edelstein *et al.*,[3] however, carried out a histological examination, and reported that massive host reactive cells appeared within six days and thus macroscopic evaluation had been affected by cell infiltration. Accordingly, four-day SRCA with a complex multiparameter histological score was proposed by Levi *et al.*[4,5] On the other hand, some immunosuppressive maneuvers such as treatment with cyclophosphamide,[3,6] cortisone,[3] or cyclosporin A,[6–8] or total body irradiation[3,6] have been implemented. The application of the immunosuppressant mizoribine (4-carbamoyl-1-β-D-ribofuranosylimidazolium-5-olate) has not been investigated in detail. Here we report the effects of mizoribine in SRCA.

MATERIALS AND METHODS

Animals Male BDF_1 (C57BL/6×DBA/2 F_1 hybrid) mice, 6–8 weeks old, were purchased from Funabashi Farm Co., Ltd. Mice were kept in a laminar air flow system at constant temperature (24–26°C) and humidity (60–70%), and received food and water *ad libitum*. They were kept under specific pathogen-free conditions. Each group consisted of five to six mice.

Tumor cell line Human gastric carcinoma H-111 (highly differentiated adenocarcinoma) was used in this study. This tumor has been maintained by serial transplantation in male BALB/c *nu/nu* mice in our department.

Drugs Mizoribine (MZR) was donated by Toyo Jozo Co., Ltd. as a powder and was dissolved in 0.9% sterile saline just prior to injection. MZR was given subcutaneously 6 h before the tumor implantation and daily until the termination of the experiment.

Method of SRCA The methodology of SRCA used in this study followed that of Bogden *et al.*[1,2] The tumor was removed from the subcutaneous tissue of an AN mouse and put into RPMI1640 cell culture medium including 10% fetal bovine serum, streptomycin and penicillin in a sterile petri dish. After trimming, the tumor was minced with scalpels into fragments of about 1 mm^3. Mice were anesthetized by intraperitoneal injection of pentobarbital sodium and inhalation of diethylether. The body weight of every mouse was recorded. An incision about 0.7–0.8 cm long was made on the left flank of the mouse and the left kidney was exteriorized, then a small slit was made in the renal capsule. A single fragment of tumor was inserted through the slit, and the longest diameter (L) and another diameter in the middle perpendicular to the longest diameter (W) were measured under a stereomicroscope with an ocular micrometer. Finally the kidney was reinserted into the body cavity and the incision was sutured. Processing 40–60 mice usually required approximately 3 h.

At the termination of the experiment, all mice were weighed and killed with diethylether. The kidneys con-

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[2] Abbreviations: SRCA, subrenal capsule assay; MZR, mizoribine; NIC, normal immunocompetent; AN, athymic nude.

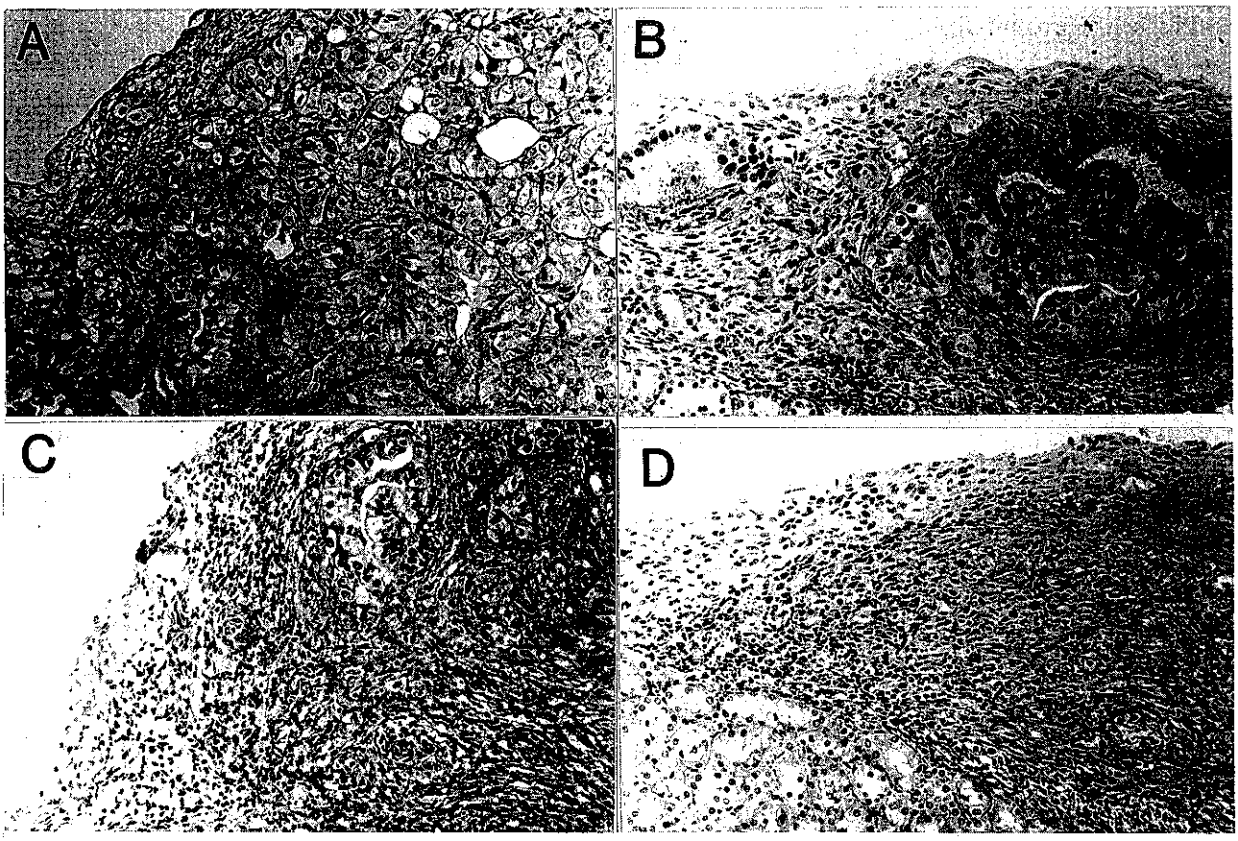

Fig. 1. Examples of histological classification in this study (see "Materials and Methods"). Figures 1A, 1B, 1C and 1D are examples of classes 0, 1, 2 and 3, respectively.

taining the tumor were removed and final tumor diameters were measured. An estimated tumor weight (ETW) was calculated by using the formula for a spheroid[9]: $ETW = L \times W^2/2$. Relative tumor weight (RTW) on day n was calculated by using the following formula: RTW = ETW on day n/ETW on day 0. Body weight change on day n was expressed as body weight on day n − body weight on day 0/body weight on day 0×100 (%).

Histological evaluation Three slides were prepared from one specimen at intervals of 100–300 μm and stained with hematoxylin-eosin (H-E). On the basis of the actual tumor growth and the host reaction in the microscopic findings, each specimen was assigned to one of four classes. 0: No lymphocytic invasion with well preserved tumor cells and stroma (Fig. 1A). 1: Some lymphocytic invasion, but neither tumor cells nor stroma were affected by the host reaction (Fig. 1B). 2: More extensive lymphocytic invasion and the volume of tumor cells and stroma was apparently decreased (Fig. 1C). 3: Massive lymphocytic invasion and tumor cells and stroma could hardly be recognized (Fig. 1D).

Autoradiography Each mouse was injected intravenously with 1480 kBq (40 μCi) of [6-^{3}H]TdR. Half an hour after the injection, mice were killed and kidneys containing tumors were fixed in 10% formalin, then slides were prepared. These slides were dipped into Sakura NR-M2 autoradiographic emulsion and exposed for sixty days. All slides were developed and fixed, followed by H-E staining. We counted over 1000 tumor cells to determine the labeling index (LI).

RESULTS

Growth characteristics Growth curves at four different doses of MZR (20, 100, 200 and 400 mg/kg) are shown in Fig. 2. The values of maximal relative tumor weight (RTW) were different e.g., 13.5 in the 100 mg/kg-treated group and 2.90 in the 400 mg/kg-treated group. Different doses of MZR also resulted in different patterns of growth: in the 20 mg/kg-treated group the tumor grew until day 6, whereas in the other groups, the tumor grew until day 8 to 10. All control groups showed a decrease in

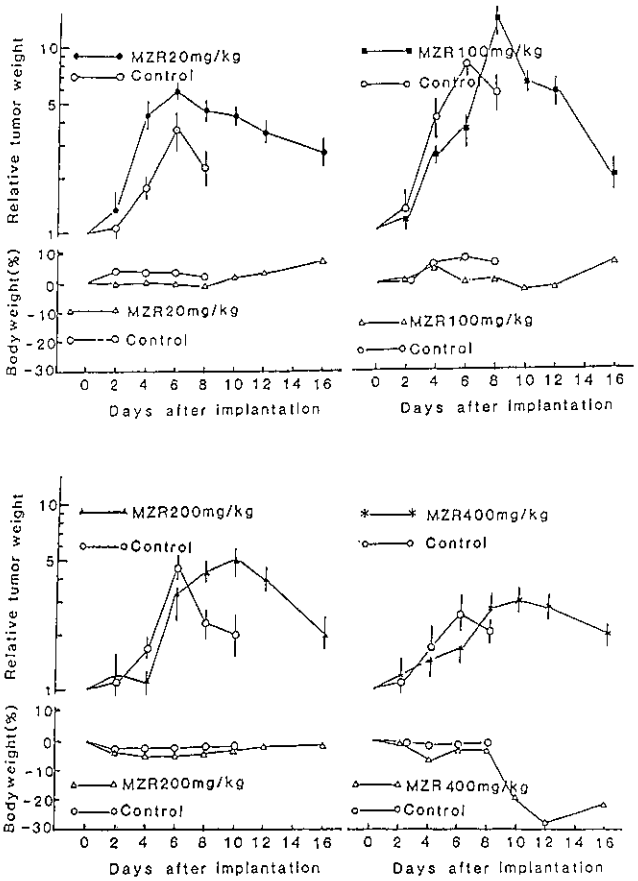

Fig. 2. Growth curves of H-111 tumor implanted under the renal capsule of BDF_1 mice, and body weight changes (control and treated groups, mean ± SE).

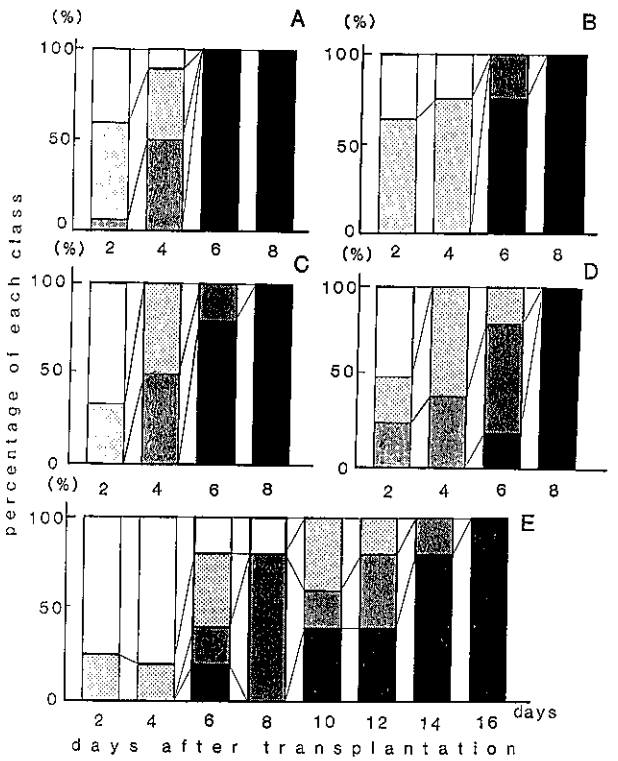

Fig. 3. Histological analysis of 498 samples. The percentage in each class is shown at various times after transplantation. Class 0, □; class 1, ▨; class 2, ▩; class 3, ■. A, control; B, MZR 20 mg/kg; C, MZR 100 mg/kg; D, MZR 200 mg/kg; E, MZR 400 mg/kg.

diameter after day 6. We found that only the 200 mg/kg-treated group exhibited statistically significant macroscopic tumor growth compared to the control on day 10 ($P<0.01$, Student's t test).

Alteration of mouse body weight The alteration of body weight is also represented in Fig. 2. In the 20,100 and 200 mg/kg-treated groups, weight loss did not exceed 10%. In the 400 mg/kg-treated group, however, maximal weight loss was over 29% on day 12, although weight loss did not exceed 10% until day 8 in this group.

Histological evaluation Histological evaluation was performed on 225 specimens. Each kidney containing a tumor was processed to obtain slides at three different sites (see "Materials and Methods"). Finally, 498 slides among 675 could be evaluated (73%). The results for controls and the 20, 100 or 200 mg/kg-treated groups are represented only until day 8 in Fig. 3, because all slides were assigned to class 3 in our classification after day 8. Fig. 3 demonstrates that only 400 mg/kg of MZR prevented a massive host reaction up to day 8.

Autoradiography Autoradiography was performed on only the 200 and 400 mg/kg-treated and control groups. The LI was the highest on day 4 in each group. There was no statistically significant difference of LI among these three groups (Fig. 4).

DISCUSSION

Mizoribine was found to act directly on both B and T lymphocytes, suppressing their proliferation, and to promote long-term survival of grafted kidney, heart, thyroid gland and skin in animal models.[10-13] In the present study we found that mizoribine prolonged macroscopic tumor growth in the 200 and 400 mg/kg-treated groups until day 10.

Microscopic findings, however, revealed that there were many host reactive cells in the graft of the 200 mg/kg-treated group on day 8. It is supposed that infiltration of mouse cells contributed to the mass effect of the graft

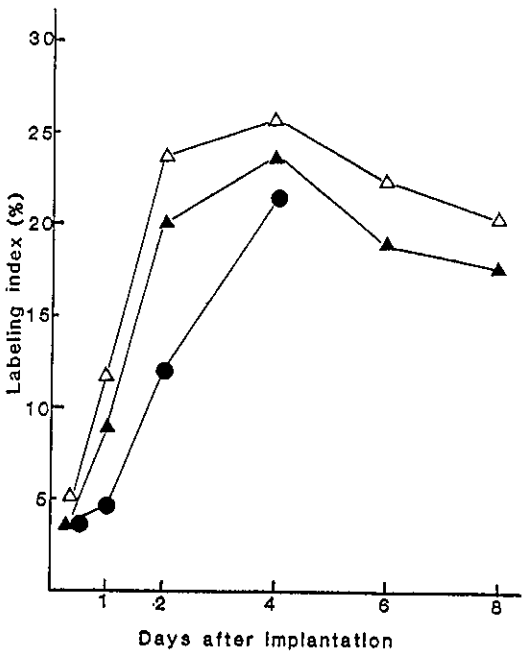

Fig. 4. Labeling index of H-111 tumor cells implanted under the renal capsule of BDF_1 mice. Control group (●), and 200 mg/kg (▲), and 400 mg/kg (△) mizoribine-treated groups.

and thus constituted a source of error in the macroscopic evaluation, i.e., host reactive cells instead of actual tumor cells made the graft size larger after day 6 in the 100 and 200 mg/kg-treated groups. It appeared that only 400 mg/kg of MZR could act effectively on the basis of the macroscopic and microscopic examinations. The autoradiograms showed that 400 mg/kg of MZR daily did not affect the labeling index until day 8. We therefore presume that this dose of MZR did not suppress the actual tumor growth. MZR is assumed to inhibit the change of inosylate to guanylate.[11] But SRCA using 400 mg/kg of MZR must be terminated by day 8, because of toxicity (severe weight loss), as we demonstrated in this study.

Many immunosuppressive maneuvers such as treatment with cyclophosphamide (CPM),[3,6] or cyclosporin A (CSA),[6,8] or total body irradiation (TBI)[3,6] have been proposed. Our previous study demonstrated that the immunosuppressive effects were in the order of CSA > CPM ≒ TBI.[6] The dose of 400 mg/kg of MZR daily appeared to be as effective as CPM. But even in the 400 mg/kg-treated group, severe host reaction was observed ten days after the beginning of treatment. Drug metabolism may have been activated after daily administration of MZR. In a previous paper, we reported that daily use of 80 mg/kg of CSA could not prevent the host reaction after day 14 in SRCA using NIC mice.[6] We indicated that CSA was more effective than CPM on immunosuppression, but this might have been a consequence of antitumor activity.[14] Using AN mice in SRCA would be more appropriate[15] but is expensive. The present results suggest that mizoribine is a suitable immunosuppressive tool in SRCA.

Although SRCA has been used in many institutions for various purposes, its use should be limited to the identification of active drugs against established tumors, not freshly obtained tumors. Futhermore, the results of SRCA should be compared with those on drug sensitivity in tumors transplanted into AN mice. SRCA is of limited value as a drug screening test for finding new compounds and of no value for the identification of active drugs against tumors of individual patients.

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