AROMATIC RETINOID RO 40-8757 REDUCES IMMUNOTOXICITIES OF CYCLOPHOSPHAMIDE AS REVEALED BY IMMUNOHISTOCHEMICAL STAINING OF LYMPHOID TISSUES AND GENERAL PATHOLOGIC EXAMINATIONS

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ABSTRACT — The aromatic retinoid (arotinoid) Ro 40-8757 (4-[2-[*p*-(*E*)-2-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthyl)propenyl]phenoxy]ethyl]-morpholine), a compound with antitumor activities, has been studied in a combination therapy with the cytostatic antitumor drug cyclophosphamide (CPA), and was found to protect bone marrow from the toxic effects of CPA. To evaluate its protective effects against CPA toxicities on lymphoid systems, we treated BDF1 mice with Ro 40-8757 orally for 1 to 5 weeks in combination with CPA intraperitoneally. After the combination treatment, mice were subjected to immunohistochemical analysis with antibodies against cell surface markers (Thy1.2, Lyt-2, L3T4, Kappa chain, and Ia) and, in addition, general pathologic examinations were done. The protective effects of Ro 40-8757 on CPA toxicities were observed. The lymphocyte reductions (both in T cells and B cells) in lymphoid organs by CPA were apparently less severe. In particular, recovery of immature T cells in the thymic cortex was greater in combination treatment with Ro 40-8757 and CPA than in treatment with CPA alone. From these results, it can be concluded that Ro 40-8757 protects the lymphoid organs (thymus, spleen, and lymph node) from the immunotoxicity of CPA, and the protective effect is evident, especially in the thymic cortical lymphocytes.

KEY WORDS: Antitumor drugs, Retinoids, Cell surface markers, Cyclophosphamide, Protection of toxicities, Lymphoid tissues

INTRODUCTION

The arotinoid Ro 40-8757 is a compound which has antitumor activities (Eliason *et al.*, 1990; Teelmann *et al.*, 1993), and combination therapies with cytostatic antitumor drugs are expected. Cyclophosphamide (CPA), a cytostatic antitumor drug in the nitrogen mustard group, is known to suppress the immune system and selectively deplete B cells in lymphoid tissues and thymic cortical lymphocytes (Turk and Poulter, 1972; Loc *et al.*, 1981). CPA is also known to deplete T suppressor/cytotoxic cells (CD8 positive cells), particularly in the lymph node and thymus in rats (El-Sady *et al.*, 1986) and mice (Miyauchi *et al.*, 1990). We found protective effects of Ro 40-8757 against the lethal effect

and myelotoxicity of CPA by combination with Ro 40-8757 in a rat tumor model (Eliason *et al.*, 1993). In this experiment, the protective effects of the arotinoid Ro 40-8757 on CPA immunotoxicities were assessed in normal BDF1 mice, particularly on lymphoid organs by immunohistochemical staining of cell surface markers of lymphoid cells and, in addition, general pathologic examinations.

MATERIALS AND METHODS

Chemicals

The arotinoid Ro 40-8757 was obtained from F. Hoffmann-La Roche AG (Basel, Switzerland). The cyclophosphamide (CPA), Endoxan[®]; mixture of CPA

+NaCl = 100:45) from Shionogi & Co., Ltd. (Osaka, Japan) was used.

Animals

Male BDF1 (C57BL/6×DBA/2 F1) mice (6 weeks old) were obtained from Japan SLC (Shizuoka, Japan). The animals were housed in the animal facility for one week prior to use and weighed 23-28 grams at the start of the treatment. The animals were maintained on a 12-hr light/dark cycle under conditions of constant temperature ($22 \pm 2^\circ\text{C}$) and humidity ($55 \pm 10\%$) and were provided with food (CRF-1) and water *ad libitum*. The animal experiment was approved by the Institutional Committee for Animal Experiment.

Treatment of mice and observations

Endoxan was dissolved in distilled water at a concentration of 20 mg CPA/ml, diluted with physiological saline, and administered intraperitoneally at daily doses of 0 (vehicle), 25, or 50 mg/kg (1 ml/100 g body weight) for 35 days (from day 0 to day 34). Ro 40-8757, suspended in rape seed oil, was administered in combination with CPA orally by intubation at daily doses of 0 (vehicle) or 100 mg/kg (0.5 ml/100 g body weight) for 35 days (from day 0 to day 34). The Ro 40-8757 suspension and the CPA solution were prepared twice a week. The treatment groups are listed in Table 1. Clinical signs were observed daily, and body weight was measured twice a week.

General pathology

On days 7, 14, 21, 28, and 35, six animals/group (total of 36 animals/stage) were sacrificed and subjected to necropsy. Internal organs of the necropsied animals were observed for gross changes, and were weighed. The peripheral blood was collected and hematologically analyzed with the Technicon H*1 autoanalyzer (Tarrytown, NY, U.S.A.). The reticulocytes were counted after new methylene blue-staining of the peripheral blood smear samples.

Table 1. Treatment groups.

Treatment group	A	B	C	D	E	F
The arotinoid Ro 40-8757 (mg/kg/day)	0	0	0	100	100	100
Cyclophosphamide ; CPA (mg/kg/day)	0	25	50	0	25	50

Immunohistochemistry

On days 7, 14, 21, 28, and 35, six animals/group (total of 36 animals/stage) were subjected to immunohistochemistry. The spleen, thymus, and mesenteric lymph node were embedded in O.C.T. media (Miles Inc., IN, U.S.A.), frozen in 2-methylbutane-dry ice, and sectioned at $6\mu\text{m}$. The frozen sections were stained by the avidin-biotin-peroxidase method. The frozen sections were fixed for 3 min in ethanol and successively treated with 1.5% normal rabbit serum, primary antibody, biotinylated rabbit-derived anti-rat IgG antibody (Vector Laboratories, CA, U.S.A.), 1 mg/ml periodic acid, 0.2 mg/ml sodium borohydride, avidin-biotinylated horseradish peroxidase complex (Vector Laboratories, CA, U.S.A.), 1 mg/ml 3,3'-diaminobenzidine tetrahydrochloride with 0.01% hydrogen peroxide, and counter-stained with hematoxylin. Primary antibodies used in the staining were anti-Thy1.2 (Pan-T cells, Ledbetter *et al.*, 1980) (Becton Dickinson, CA), Lyt-2 (CD8, T suppressor/cytotoxic cells, Ledbetter *et al.*, 1980) (Becton Dickinson, CA), L3T4 (CD4, T helper/inducer cells, Dialynas *et al.*, 1983) (Becton Dickinson, CA), Kappa chain (B cells, Sebra *et al.*, 1984) (Meiji-nyuugyou, Japan), and Ia (MHC class II, Bhattacharya *et al.*, 1981) (Boehringer Mannheim, IN, U.S.A.).

Statistical analysis

Pitman test (Lehman, 1974) or t-test with F-test (JIS, 1965) was employed for analysis of treatment control (with Ro 40-8757 and without Ro 40-8757) differences. Results were expressed as mean values $\pm$ S.D.

RESULTS

Body and lymphoid organ weights

No significant difference in body weight alterations was observed between the combination of Ro 40-8757 plus CPA and CPA alone (Fig. 1). Body weight gain was dose-dependently lower with CPA both in combination with Ro 40-8757 and in CPA alone. The reduction of the body weight gain was most severe during days 7 to 17 of the administration, which tended to recover.

The recovery of the thymus weight was slightly greater for the combination of Ro 40-8757 plus CPA than for CPA alone (Fig. 2a). The thymus weight was severely reduced with CPA both in combination with Ro 40-8757 plus CPA and in CPA alone. This reduction progressed for 2 or 3 weeks, but tended to recover with the combination of Ro 40-8757 plus CPA. The

recovery of the spleen weight was greater and approximately 1 week earlier for the combination of Ro 40-8757 plus CPA than for CPA alone (Fig. 2b). The spleen weight was reduced during weeks 1 and 2 of CPA alone. These then tended to recover, approaching the control level during week 3 for 25 mg/kg of CPA alone and week 4 for 50 mg/kg of CPA alone, but week 2 for the combination with Ro 40-8757 plus 25 mg/kg of CPA and week 3 for the combination with Ro 40-8757 plus 50 mg/kg of CPA.

Hematology

The recovery of the peripheral white blood cell count (WBC) was earlier for the combination with Ro 40-8757 plus CPA than for CPA alone (Fig. 2c). The WBC was severely reduced with CPA and tended to recover during week 3 of administration, especially for the combination with Ro 40-8757 plus CPA. The reduction of the peripheral red blood cell count (RBC) was clearly less severe for the combination with Ro 40-8757 plus CPA than for CPA alone, especially for 50 mg/kg of CPA (Fig. 2d). The RBC was reduced with CPA; this reduction progressed for 3 weeks. The peripheral blood reticulocyte count was reduced with CPA during week 1 of the administration; this recovered during week 2 for the 25 mg/kg CPA and during week 4 for the 50 mg/kg CPA (Fig. 2e). The recovery

of the reduced reticulocyte number was 1 week earlier for the combination treatment of Ro 40-8757 plus 50 mg/kg CPA than for 50 mg/kg CPA alone. After the reticulocyte number was recovered, red blood cells were macrocytic (mean corpuscular volume and mean corpuscular hemoglobin were increased. The results were not indicated.). These hematological examinations revealed that Ro 40-8757 assists in the early recovery of the hematological alterations, especially assisting those resulting from the 50 mg/kg administration of CPA.

Immunohistochemistry

For the low-dose (25 mg/kg) CPA, in 5-week co-administration with Ro 40-8757, an effect of Ro 40-8757 on thymocytes was apparent (Photo 1). For the administration of CPA alone, thymocytes, particularly immature cortical $CD8^+$ ($Lyt-2^+$) and $CD4^+$ ($L3T4^+$) (by staining with anti-CD4, results similar with CD8 were obtained) thymocytes were almost depleted and the meshwork structure of Ia positive epithelial cells had disappeared. On the other hand, with the combination of Ro 40-8757 plus CPA, the immature thymocytes were only slightly reduced. From these results, the protective effect of Ro 40-8757 on the thymus was evident, as it rescued the immature cortical thymocytes from the toxic effects of CPA.

Fig. 1. Alterations of body weight gains for each treatment group. The control and cyclophosphamide (CPA) alone groups are indicated by open symbols; Ro 40-8757 (Ro) alone group and Ro 40-8757 plus cyclophosphamide (Ro+CPA) groups are indicated by closed symbols. No statistically significant difference ($p < 0.05$) was observed between mice treated with Ro 40-8757 (100 mg/kg/day) and mice treated without Ro 40-8757 in each week and each dose of CPA (0, 25, 50 mg/kg/day).

The protection of lymphocytes by the arotinoid in secondary lymphoid organs was also observed. Both T and B cells in secondary lymphoid organs were decreased with CPA, and the decrease was progressive during administration. In the mesenteric lymph nodes (Photo 2), T cells (Thy1.2⁺) were reduced and B cells (Kappa chain⁺) were almost completely depleted in 3-week administration with the 50 mg/kg CPA alone. With the co-administration of Ro 40-8757 and CPA, many T cells remained and some B cells were still present. In the spleen (Photo 3), T cells (Thy1.2⁺) and B cells (Kappa chain⁺) were severely reduced in 5-week administration with the 50 mg/kg CPA alone. On the other hand, the combination of Ro 40-8757 plus CPA showed that some T cells and B cells remained. From

these results, the degree in the reduction of lymphocytes caused by the CPA administration was more severe for B cells than for T cells both in the lymph node (Photo 2) and the spleen (Photo 3). These reductions of the lymphocytes were apparently less severe for the combination treatment of Ro 40-8757 plus CPA than for CPA alone.

DISCUSSION

The aromatic retinoid Ro 40-8757 (the arotinoid class of retinoids) has an antitumor activity in rats with chemically induced breast tumor (Eliason *et al.*, 1990; Teilmann *et al.*, 1993). The combination therapy of Ro 40-8757 with CPA showed additive antitumor activity

Fig. 2. Alterations of thymus weight (a), spleen weight (b), white blood cell count (c), red blood cell count (d), and peripheral blood reticulocyte count (e) for each week and each treatment group. Statistical significance between mice treated with Ro 40-8757 (Ro, 100 mg/kg/day) and without Ro 40-8757 in each week and each dose of cyclophosphamide (CPA, 0, 25, 50 mg/kg/day); *: p<0.05, **: p<0.01

Protection of cyclophosphamide immunotoxicities.

Photo 1. Immunohistochemically stained thymus sections from control mice (a,b), from mice treated with 25 mg/kg/day of cyclophosphamide (CPA) for 5 weeks (c,d), and from mice treated with 100 mg/kg/day of Ro 40-8757 plus 25 mg/kg/day of CPA for 5 weeks (e,f), stained with anti-Lyt-2 (a,c,e) or stained with anti-Ia (b,d,f). Original magnification $\times 200$. Bar = 100 μm .

Photo 2. Immunohistochemically stained lymph node sections from control mice (a,b), from mice treated with 50 mg/kg/day of cyclophosphamide (CPA) for 3 weeks (c,d), and from mice treated with 100 mg/kg/day of Ro 40-8757 plus 50 mg/kg/day of CPA for 3 weeks (e,f), stained with anti-Thy1.2. (a,c,e) or stained with anti-Kappa light chain (b,d,f). Original magnification $\times 200$. Bar = 100 μm .

Protection of cyclophosphamide immunotoxicities.

Photo 3. Immunohistochemically stained spleen sections from control mice (a,b), from mice treated with 50 mg/kg/day of cyclophosphamide (CPA) for 5 weeks (c,d), and from mice treated with 100 mg/kg/day of Ro 40-8757 plus 50 mg/kg/day of CPA for 5 weeks (e,f), stained with anti-Thy1.2. (a,c,e) or stained with anti-Kappa light chain (b,d,f). Original magnification $\times 200$. Bar = 100 μm .

in a chemically induced rat tumor model, and also showed protective effects on the lethal effect in the rat tumor model and protective effects on hematotoxicity of CPA in normal mice (Eliason *et al.*, 1993). Our experiment was conducted to clarify the protective effects of Ro 40-8757 on the lympho-hematopoietic organs in the normal mice without effects of tumors. For our experiment, the protective effects of Ro 40-8757 on CPA toxicities in lymphoid organs were revealed by immunohistochemical staining of lymphocyte subsets in normal mice.

In this experiment, CPA toxicities were characterized by the immunohistochemical staining of the lymphoid organs. All of the T helper cells, T suppressor/cytotoxic cells, cortical thymocytes, and B cells were depleted by repeated daily administration of CPA (25 or 50 mg/kg/day of CPA for 1 to 5 weeks), but B cells were more severely reduced than T cells. These characteristics of CPA toxicities in this investigation were consistent with the alteration of lymphocyte subsets in animals by a single administration of a high dose of CPA. The single administration of a high dose of CPA (300 mg/kg) selectively depleted B cells in mice and guinea pigs (Turk and Poulter, 1972), and the reduction of B cells was long-lasting in mice administered 400 mg/kg of CPA (Stockman *et al.*, 1973). The cortical thymocyte depletions were also observed in the rat thymus by 200 mg/kg of CPA (Loc *et al.*, 1981; Miyauchi *et al.*, 1990) and by 100 mg/kg of CPA (El-Sady *et al.*, 1986).

In the thymus, the reduction of the weight by CPA was less severe in the combination treatment with Ro 40-8757 plus CPA, particularly in the lower dose (25 mg/kg/day of CPA). The immunohistochemical staining of the thymus revealed protective effects of Ro 40-8757 which were mainly on immature cortical thymocytes. CD8⁺(Lyt-2⁺) and CD4⁺(L3T4⁺) thymocytes remained in the combination groups even though CD8⁺ and CD4⁺ thymocytes were almost depleted in the group receiving CPA alone. A single high dose of CPA was shown to cause thymocyte apoptosis in mice (Hiramine *et al.*, 1996). The cell death of cortical thymocytes was thought to be less severe in the combination treatment with Ro 40-8757 plus CPA than in CPA alone.

In the spleen and the lymph node, the decrease in the number of mature T and B lymphocytes by CPA alone was less severe in the combination treatment group of Ro 40-8757 and CPA. In all groups, the reductions of lymphocytes in the spleen and the lymph node were less severe than that of cortical thymocytes. This

finding indicates that immature thymocytes in the thymic cortex were more susceptible to CPA than mature lymphocytes, but the protective effects of Ro 40-8757 on CPA toxicities were observed both in immature thymocytes and mature lymphocytes, including B lymphocytes.

Retinoids have been shown to have direct effects on lymphocytes. Retinoids inhibit growth and prevent apoptosis of normal human B cells mediated via retinoic acid receptors (RARs) (Lomo *et al.*, 1998). Retinoids enhance interleukin-2 receptor alpha and beta expression of a mature human T cell line (Sidell *et al.*, 1997). Further, retinoids increase mature T cells in the thymus and inhibit thymocyte depletion induced by staphylococcal enterotoxin B in murine fetal thymic organ cultures, possibly via RAR alpha (Yagi *et al.*, 1997). The inhibition of T cell receptor-mediated apoptosis of murine T cell hybridoma by retinoids requires both RAR and retinoid X receptor (RXR) activation (Bissonnette *et al.*, 1995). From these effects of retinoids on lymphocytes, Ro 40-8757 is thought to have protective effects on lymphocyte depletion by CPA via direct action on lymphocytes by prevention of lymphocyte apoptosis. However, in contrast to other retinoids, Ro 40-8757 (the arotinoid class of retinoids) does not bind to known nuclear retinoic acid receptors RAR alpha, RAR beta, or RAR gamma (Teelmann *et al.*, 1993). Recently, 14-hydroxy-4,14-retro-retinol was reported to inhibit cell death in lymphocytes by a mechanism not involving nuclear retinoid receptors (O'Connel *et al.*, 1996), and a synthetic retinoid CD437 induced growth arrest and apoptosis in a variety of human T cell lymphoma cells in which the RAR and RXR selective retinoids are unable to induce apoptosis (Adachi *et al.*, 1998). These observations suggest a possibility that Ro 40-8757 showed protective effects on CPA immunotoxicity by mechanisms not mediated by RAR or RXR.

The protective effects of Ro 40-8757 (100 mg/kg/day) on bone marrow toxicities in normal mice treated with CPA given 50 mg/kg/day for 3 to 5 weeks were reported (Eliason *et al.*, 1993). In the mice receiving CPA alone, the total progenitor-cell numbers (CFU-GM, BFU-E and CFU-Mix) in the spleen were reduced at week 3. In the bone marrow of these animals, progenitor-cell numbers were increased at all times. The combination with Ro 40-8757 greatly enhanced the increase in marrow progenitors at weeks 3 and 4. In the spleen of the combination, a great increase was observed at week 5. These previous results indicated that the protection of CPA bone marrow toxicities by

Ro 40-8757 occurred in progenitor-cell levels. The progenitors of lymphocytes in bone marrow might also be protected by Ro 40-8757, and the recruitment of the progenitor cells from the bone marrow to the lymphoid organs might increase. Both the direct action of the lymphocytes in lymphoid organs and the action on the progenitor cells in bone marrow would be involved in the mechanism of protection of the lymphoid organs by Ro 40-8757.

In conclusion, the immunohistochemical staining of lymphocyte subsets revealed that Ro 40-8757 protects the lympho-hematopoietic organs (thymus, spleen, and lymph node) from the immunotoxicity of CPA, and the protective effect is evident, especially in the thymic cortical lymphocytes.

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