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Effects of phenytoin on cell-mediated immunity

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Summary. The effects of phenytoin on cellular immunity were examined in murine models. Fresh splenocytes were obtained from mice which had received 1 mg/day of phenytoin i.p. for 28 days. The serum concentration of phenytoin in these animals was 10–20 μg/ml. The proliferative response of splenocytes to mitogens was assessed by ^{3}H-thymidine incorporation. The cytotoxic activities of cells such as natural killer (NK) cells, cytotoxic T lymphocytes (CTL), and lymphokine-activated killer (LAK) cells were estimated by a 4-h ^{51}Cr release assay. The ^{3}H-thymidine incorporation of splenocytes was reduced significantly ($P < 0.01$) in phenytoin-treated mice. The NK and CTL activities of splenocytes from phenytoin-treated mice were significantly suppressed. However, the LAK activity of phenytoin-treated mice was equal to that of control mice.

Introduction

Seizures are sometimes the initial symptom of a brain tumor and in some instances, they can persist for years before the development of other signs. Anticonvulsant agents are also routinely administered to patients with brain tumors as prophylactic anticonvulsant therapy. Among various anticonvulsant drugs, phenytoin is highly effective and most widely used. During its long history of therapeutic use, a broad spectrum of side effects have been reported. It is very important to investigate the side effects of phenytoin on cellular immunity of patients who receive it as anticonvulsant therapy for a long time. We evaluated the effects of phenytoin on the cell-mediated immunity of mice which were given phenytoin i.p. for 28 consecutive days.

Materials and methods

Mice. Female C57BL/6(H-2^b haplotype) and C3H/HeN(H-2^k) mice were purchased from Charles River Laboratory, Kanagawa, Japan and female CBA/J(H-2^k) mice were purchased from The Jackson Laboratory, Bar Harbor, Me. All mice were used for these studies at 8 to 12 weeks of age.

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Target cells. The following 3 tumor cell lines were employed: a Molony leukemia virus-induced T cell lymphoma of A/Sn mouse origin (YAK-1), a Schmitt-Ruppin Rous sarcoma virus-induced malignant glioma of C3H/He mouse origin (RSV-M glioma), and a 20-methylcholanthrene-induced ependymoblastoma of C57BL/6 mouse origin (203 glioma).

Interleukin-2. Human recombinant interleukin-2(rIL-2) was kindly supplied by TAKEDA Chemical Industries, Ltd. for all the experiments.

Phenytoin treatment in mice. Phenytoin (1 mg, 130–140 mg/m^2 of body surface), dissolved in saline at a concentration of 10 mg/ml, was given to mice i.p. for 28 days. The serum concentration of phenytoin [5] was measured on day 14 and on day 28 in the experimental models, and ranged from 10 to 20 μg/ml. The daily phenytoin dose of 130–140 mg/m^2 body surface is the clinically achievable dosage. The same volume of saline was given i.p. to mice in the control group. There was no difference between total cell counts of splenocytes of phenytoin-treated mice and normal mice.

Proliferative response of lymphocytes to concanavalin A and rIL-2. The proliferative responses of lymphocytes to concanavalin A (ConA) and rIL-2 were evaluated on the basis of ^{3}H-thymidine incorporation. Fresh splenocytes ($0.5–2 \times 10^6$/ml) from phenytoin-treated and nontreated mice were suspended in complete medium containing RPMI 1640 with 0.1 m*M* nonessential amino acids, 1 μg/ml sodium pyruvate, 5×10^{-5} *M* 2-mercaptoethanol, 5.0 μg/ml gentamicin sulfate, and 5% heat-inactivated fetal calf serum (FCS) in addition to a final concentration of either 0.75 μg/ml of ConA or 1 unit of rIL-2. Then 200 μl was added to each well of plastic flat-bottomed microtiter plates, which were incubated for 3 days at 37° C in a humidified atmosphere containing 5% CO_2. Then 0.4 μCi of ^{3}H-thymidine was added to each well 18 h before cell harvest. The incorporated radioactivity was measured using a liquid scintillation counter. These experiments were performed in triplicate.

In vitro cytotoxic assay. The antitumor efficacy for natural killer (NK), cytotoxic T lymphocyte (CTL), and lymphokine-activated killer (LAK) activities were assessed by a standard 4-h ^{51}Cr release assay. Target cells (10^7) were sus-

pended in 1 ml of RPMI 1640 with 5% heat-inactivated FCS and labeled with 50 μCi of sodium ^{51}chromate at 37° C for 90 min. After washing with Hanks' balanced salt solution three times, these target cells were resuspended at a concentration of 10^5 cells/ml in RPMI 1640 with 5% FCS. The effector cells were also suspended at a concentration of $1-4 \times 10^6$/ml in RPMI 1640 with 5% FCS. Then 100 μl each of these cell suspensions were mixed in the microwells of round-bottomed 96-well microtiter plates. After 4 h of incubation, the supernatant from each well was collected, and the radioactivity measured using a gamma counter.

The cytotoxic activity was expressed as follows

$$\% \text{ Lysis} = \frac{\text{experimental CPM} - \text{spontaneous CPM}}{\text{maximal CPM} - \text{spontaneous CPM}} \times 100,$$

where experimental CPM was the ^{51}Cr released from injured targets, spontaneous CPM was the spontaneous release from the targets, and maximal CPM the total CPM in the target cells lysed with 0.1% Triton X.

NK assay. The NK activity was evaluated by the cytotoxicity of fresh splenocytes from CBA/J mice against NK-sensitive YAC-1 cells. The cytotoxicity was measured by a 4-h ^{51}Cr release assay.

CTL assay. Splenocytes from C57BL/6(H-2^b) mice were stimulated in vitro by splenocytes from C3H/HeN(H-2^k) mice which had previously been treated with mitomycin C [1]. The cytotoxicity of these CTL, which had cytocidal activities against cells possessing the H-2^k phenotype antigens on the cell membrane, was evaluated by a 4-h ^{51}Cr release assay in which RSV-M glioma cells (H-2^k) were the target cells.

LAK assay [14]. LAK cells were generated by placing 5×10^7 fresh C57BL/6 splenocytes in 10-cm diameter plastic dishes with 10 ml of complete medium and 5 units/ml of rIL-2 at 37° C in 5% CO_2 for 3 days. The LAK cells were then harvested and resuspended in RPMI 1640 with 5% heat-inactivated FCS for in vitro assay. The antitumor efficacy of these LAK cells against syngeneic RSV-M glioma and allogeneic 203 glioma cells was estimated in triplicate using a 4-h ^{51}Cr release assay.

Results

Proliferative response of lymphocytes to mitogens

The proliferative response of fresh splenocytes from phenytoin-treated and control mice to mitogens (ConA and rIL-2) was evaluated by ^{3}H-thymidine incorporation. As some previous reports have indicated, ^{3}H-thymidine incorporation by splenocytes from phenytoin-treated mice was significantly suppressed compared with those of control mice ($P < 0.01$) (Fig. 1). These findings confirmed previous reports [4, 8, 12] that the in vivo administration of phenytoin suppresses the proliferative response of lymphocytes to mitogens.

NK activity

The NK activity was assessed by the cytotoxicity of fresh splenocytes from CAB/J mice against NK-sensitive

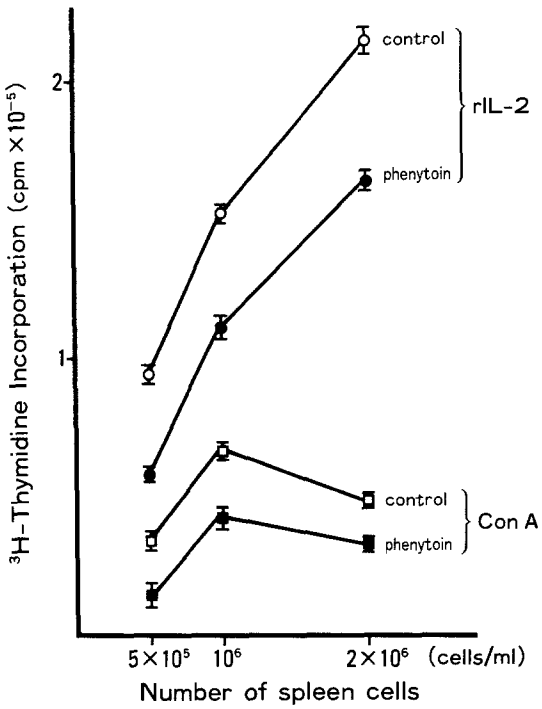

Fig. 1. ^{3}H-Thymidine incorporation by splenocytes in co-culture with 0.75 μg/ml of concanavalin A or 1.0 units/ml of recombinant interleukin-2. The *vertical bars* indicate the standard error of the mean (SE). O——O group of normal mice; ●——● group of phenytoin-treated mice ■——■

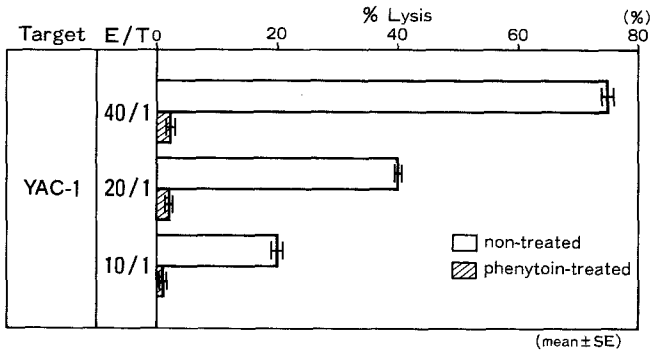

Fig. 2. The in vitro cytotoxicity of fresh CBA/J splenocytes from phenytoin-treated and normal mice against natural killer-sensitive YAC-1 cells in a 4-h ^{51}Cr release assay. (E: effector T: target)

YAC-1 cells. The cytotoxicities of splenocytes from control mice were 75%, 40%, and 20% at effector:target (E:T) ratios of 40:1, 20:1, and 10:1 respectively, whereas those from phenytoin-treated were less than 5% at any E:T ratio (Fig. 2). The NK activity was apparently suppressed in the phenytoin-treated mice.

CTL activity

The CTL activity in these experimental models was evaluated by the cytotoxicity of splenocytes from C57BL/6 mice against RSV-M glioma cells. It had previously been checked by flow cytometric analysis on a FACS IV (Becton Dickinson) that all RSV-M glioma cells expressed K^k antigens on their cell membrane. The cytotoxicities of splenocytes from control and phenytoin-treated mice

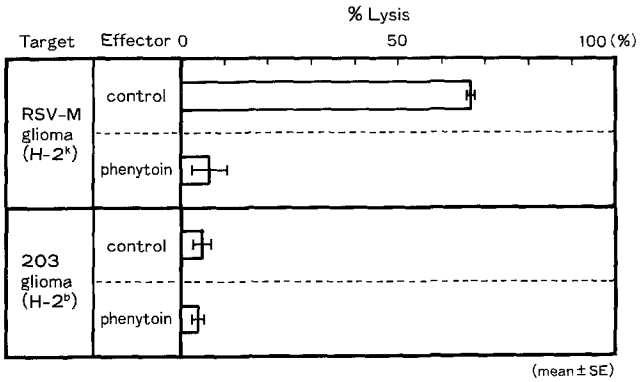

Fig. 3. The in vitro cytotoxicity of cytotoxic T lymphocytes from phenytoin-treated and normal mice against allogeneic RSV-M glioma and syngeneic 203 glioma in a 4-h ^{51}Cr release assay. E/T ratio = 40/1

against allogeneic RSV-M cells were 65% and a very small percentage, respectively (Fig. 3). Neither splenocytes from control nor those from phenytoin-treated mice killed syngeneic 203 glioma cells. Furthermore, we studied the CTL activity from splenocytes of C3H/HeN mice, which were stimulated with those of C57BL/6 mice, against 203 glioma cells. These results coincided with the previous results (data not shown). Thus the alloreactive CTL activity was severely suppressed in the phenytoin-treated mice.

LAK activity

The effector cells responsible for LAK activity have not been clearly identified, but at least most of these unique cells are not identical with NK cells or CTL cells. It is very interesting to investigate whether or not LAK activity is suppressed by phenytoin. LAK activity was estimated by the cytotoxicity of LAK cells induced from splenocytes of C3H/HeN mice cultured with rIL-2, against syngeneic RSV-M glioma and allogeneic 203 glioma. The cytotoxicities of LAK cells induced from control and phenytoin-treated mice against syngeneic RSV-M glioma were 68% and 66%, respectively. There was no statistical difference between these two values. The cytotoxicities of LAK cells from control and phenytoin-treated mice against allogeneic 203 glioma were 80% and 65%, respectively ($P < 0.05$) (Fig. 4). The cytotoxicity of LAK cells induced from phenytoin-treated mice was not very reduced compared with normal mice.

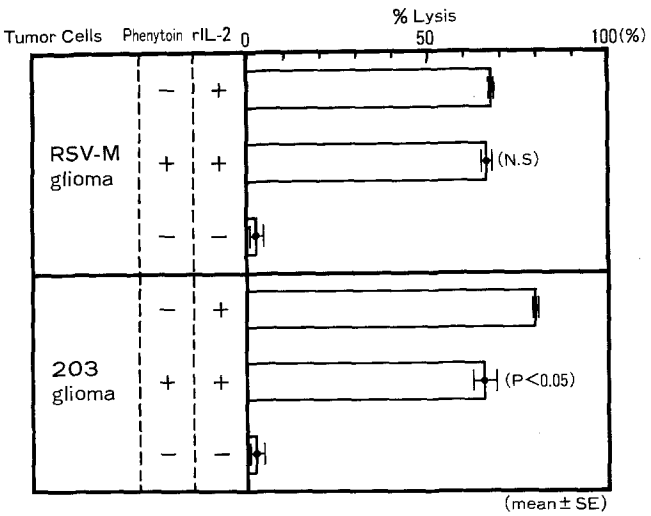

Fig. 4. The in vitro cytotoxicity of lymphokine-activated killer cells from phenytoin-treated and normal mice against syngeneic RSV-M glioma and allogeneic 203 glioma cells in a 4-h ^{51}Cr release assay. E/T ratio = 40/1 N. S.: not significant

Discussion

Anticonvulsants have been widely used and are indispensable as precautions against convulsions in patients with brain tumors. Phenytoin is said to be one of the most useful and effective of the anticonvulsant agents. Despite its efficacy, phenytoin causes numerous side effects such as visual disturbances, gingival hyperplasia, and several immunological abnormalities [2, 7, 9, 20, 22]. Patients with brain tumors have been reported to have impaired cell-mediated immunity [13, 17]. Some reports indicate that phenytoin may play some role in this immunosuppression in brain tumor patients [10].

It has been generally considered that neoplasms of the brain are more difficult to attack with chemotherapy or immunotherapy than neoplasms in other organs because of the presence of the blood-brain barrier and the absence of lymphatic tissue [3, 16]. A few nitrosourea agents such as 1-(4-amino-2-methyl-5-pyrimidinyl)methyl-3-(2-chloroethyl)-3-nitrosourea hydrochloride (ACNU) and methotrexate have been available for patients with brain tumors in addition to surgery and radiation. ACNU can be used only every 4–6 weeks because of its bone marrow suppression.

With progress in basic immunology, it has been found that HLA-DR antigens [6, 21], which are considered to be markers of antigen-presenting cells, are present in the brain parenchyma. This suggests that immunotherapy may be useful for brain tumors. Much attention has also been focused on biological response modifiers (BRM), which enhance the immunopotential of patients with malignant tumors. Clinical trials using some kinds of BRM such as interferon, rIL-2 have been undertaken in brain tumor patients. But many previous studies dealing with BRM failed to consider the immunological backgrounds of patients with malignant tumors.

In this study, we investigated the effects of phenytoin on cellular immunity in murine models. Our findings have confirmed some previous findings that phenytoin suppresses the proliferative response of lymphocytes to mitogens [11]. Furthermore, we obtained new findings that both NK activity and CTL activity were remarkably suppressed by phenytoin. Repeated i.p. injections may cause severe stress and malnutrition which result in depressed cellular immunity. But there was no difference in body weight between phenytoin-treated mice and normal mice. These depressed immune functions suggest a direct drug effect and not resulting from stress or malnutrition. On the other hand, the antitumor efficacy of LAK cells was induced in phenytoin-treated and normal mice. It has been reported that LAK cells are able to be induced by rIL-2 in peripheral blood lymphocytes from patients with malignant glioma and healthy subjects [18]. Brain tumor patients are currently being given adoptive immunotherapy with LAK cells [15, 19], as some of these patients are insensitive to several

anticancer agents and have severe immunosuppressive conditions induced by repeated radiation or chemotherapy.

The data in this paper suggest that BRM, which enhance NK and/or CTL activities, are not so effective in the patients who have been given phenytoin for long periods, and that adoptive immunotherapy with LAK cells is more useful in such patients.

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