Immunosuppression by phenytoin: implication for altered immune competence in brain-tumor patients

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This investigation was conducted to examine the immunosuppressive potential of phenytoin in vivo and to document a correlation between phenytoin therapy and depressed lymphocyte responsiveness to mitogens. It was thought that phenytoin, the most widely used anticonvulsant agent, may play some role in the immunosuppression seen in brain-tumor patients. The effect of phenytoin on mitogen-stimulated lymphocyte function was evaluated by tritiated (3H)-thymidine incorporation and lymphocyte nuclear size distribution. Lymphocytes from either phenytoin-treated or normal rabbits were incubated for 90 hours in culture medium in the presence of three mitogens: phytohemagglutinin (PHA), concanavalin A (Con A), and pokeweed mitogen (PWM). Significant suppression of mitogen-induced activation of the lymphocytes from treated animals was demonstrated. The present studies suggest a possible connection between phenytoin therapy and altered immune competence in brain-tumor patients.

KEY WORDS • phenytoin • dilantin • anticonvulsant agent • immunology • brain tumor

Several studies have demonstrated depressed cell-mediated immunity in patients with brain tumors. To date, the pathogenesis of this depressed cell-mediated immunity is unknown. We have previously investigated 22 patients with benign and malignant intracranial neoplasms in order to delineate the factor(s) responsible for this anergy. A possible relationship has been suggested in these in vitro studies between impaired cellular immunity in brain-tumor patients and concomitant anticonvulsant (phenytoin and phenobarbital) therapy. Phenytoin and phenobarbital are widely used as anticonvulsant drugs for the management of brain-tumor patients. In vitro studies have shown that high therapeutic as well as toxic levels of phenytoin could depress mitogen-induced lymphocyte blastogenesis. It was also found in our previous studies that brain-tumor cyst fluid contains immunosuppressive factors, suggesting that brain-tumor cells may locally produce suppressive factor(s) capable of inhibiting lymphocyte activation. The purpose of the present investigation was to document the possible correlation between phenytoin therapy and depressed lymphocyte responsiveness to mitogens in vivo.

Materials and Methods

Phenytoin Treatment in Rabbits

Female New Zealand rabbits (each weighing 3.5 to 5.0 kg) were used for the present investigation. In the chronic animal studies, after blood was withdrawn by cardiac puncture for baseline lymphocyte evaluation, an intraperitoneal dose of 250 mg phenytoin* was administered (50 to 70 mg/kg). The same dose was administered daily as a maintenance dose for 2 weeks prior to the lymphocyte evaluation. The serum phenytoin level was assayed at that time to ensure a concentration of greater than 10 µg/ml. The rabbits were then tapered off phenytoin over a 3-week period, after which time lymphocyte function was again evaluated.

In the acute animal studies, phenytoin (85 mg) was infused intravenously into the rabbit lateral ear vein over 5 minutes. One hour after phenytoin administration, cardiac puncture was performed to obtain blood for the assessment of lymphocyte responsiveness to mitogens and to measure the serum phenytoin level.

Measurement of Serum Phenytoin Levels

The serum phenytoin level was measured by enzyme immunoassay† at the time that the in vitro lymphocyte responsiveness to mitogens was evaluated. The lower limits of sensitivity of this assay are 2.5 µg/ml. These evaluations were performed in the clinical laboratory.

* Phenytoin obtained from Elkins-Sinn, Inc., Cherry Hill, New Jersey.
† EMIT phenytoin assay manufactured by Syva Corp., Palo Alto, California.
at the Veterans Administration Hospital, Portland, Oregon.

**Preparation of Rabbit Lymphocytes**

Forty ml of blood was withdrawn by cardiac puncture with a heparinized syringe and diluted 2:5 with 0.9% normal saline. Aliquots (35 ml) of the blood-saline mixture were placed in 50-ml polypropylene conical tubes, and Ficoll-Hypaque (15 ml) was layered below the blood-saline mixture using a spinal needle. After centrifugation at 200 G for 45 minutes at room temperature, the cloudy lymphocyte-rich layer at the plasma-Ficoll-Hypaque interface was removed by aspiration with a Pasteur pipette and washed three times in sterile normal saline. The cells were resuspended in RPMI 1640 culture medium supplemented with L-glutamine (2 mM/ml), penicillin (200 units/ml), streptomycin (200 µg/ml), and gentamicin (10 µg/ml). This is hereafter referred to as “complete RPMI 1640 culture medium.” The cells were then counted on a ZBI Coulter counter, and the cell morphology of the purified lymphocyte suspension was evaluated by examining Wright-stained smears and viability was determined by the trypan blue dye exclusion technique. The final cell population was 80% to 85% mononuclear in character and approximately 95% of the cells were viable.

**Assessment of Mitogen-Induced Lymphocyte Activation**

Assays for in vitro lymphocyte responsiveness to mitogens were performed using two parameters as follows: 1) tritiated (3H)-thymidine incorporation into deoxyribonucleic acid (DNA), and 2) lymphocyte nuclear size distribution analysis. 3H-thymidine incorporation into DNA was evaluated using a microtiter culture system. Nuclear size distribution was evaluated on a ZBI Coulter counter and Coulter Channelyzer using a tube culture system.

**Analysis of 3H-thymidine Incorporation**

Lymphocytes were cultured in triplicate using round-bottomed microtiter plates. Media (200 µl) containing 1.0 x 10⁶ cells in suspension were added to each well of the plates with an optimal concentration of either phytohemagglutinin (PHA: 10 µg/ml), concanavalin A (Con A: 100 µg/ml), or pokeweed mitogen (PWM: 500 µg/ml), in a volume of 20 µl.* Twenty µl of rabbit serum (either autologous rabbit serum obtained simultaneously with lymphocytes or pooled normal rabbit serum), previously heat-inactivated at 56°C for 30 minutes, was also added to the wells; the final volume in each well was 240 µl. Cell cultures were incubated at 37°C in a humidified atmosphere containing 5% CO₂ and 95% air for 72 hours. Then 1.0 µCi of 3H-thymidine (6.7 Ci/m mole) was added to each well. Eighteen hours later the cells were harvested on glass fiber filters and the unincorporated radiolabeled precursor was removed by washing with an automatic multiple-sample harvester. Radioactivity was measured in a liquid scintillation counter. Responsiveness was quantitated as mean count of 3H per minute (cpm) per filter disc, where the mean was that of the triplicate assay. Where the average cpm from any well deviated by more than 50% of the median value of the three wells, that value was discarded.

**Evaluation of Nuclear Size Distribution**

Lymphocyte cultures were also performed using sterile culture tubes (17 x 100-mm polypropylene culture tubes) to evaluate changes in nuclear size, since these studies could not be done with the microtiter cultures described earlier. To each sterile tube, 1.0 x 10⁶ cells were added in 1.6 ml of complete RPMI 1640 culture medium with 0.2 ml of heat-inactivated rabbit serum (autologous rabbit serum, diluted 1:1 with 0.9% normal saline). Then PHA (0.2 ml: 10 µg/ml) was added to a final volume of 2.0 ml. Lymphocyte nuclear size distribution was evaluated on a ZBI Coulter counter and Coulter Channelyzer after 90 hours of incubation at 37°C in a humidified atmosphere of 5% CO₂ and 95% air.

Lymphocyte nuclei were characterized with respect to size after the addition of Zap-Oglobin II.§ After 5 minutes to allow cell lysis to be complete, the Coulter counter and Coulter Channelyzer were set as follows to evaluate each experimental culture for nuclear size distribution: amplification ½; 1/aperture current □ 89; lower threshold 2; upper threshold 27; base channel threshold 1; window width 20; count range 1K; and count control stop at full scale. With these settings, our preliminary studies indicated that the majority (approximately 80%) of unstimulated normal rabbit lymphocyte nuclei were observed between Channels 1 and 19. Channel 1 corresponds to a nuclear diameter of 3.36 µ, Channel 20 to a diameter of 5.40 µ, and Channel 100 corresponds to a diameter of 8.71 µ. Channel 20 was arbitrarily selected as the upper normal limit of volume for unstimulated normal rabbit lymphocyte nuclei. Thus, activated lymphocytes were defined as those whose nuclei were assigned to Channels 20 to 100 on the

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Coulter Channelyzer after 90 hours of incubation with PHA. The number of nuclei between Channels 1 and 19 and those between Channels 20 and 99 were separately integrated, and the percentage of nuclei above Channel 20 (defined as percent activated lymphocytes) was calculated for each experimental culture.

Results

Pharmacokinetics after Phenytoin Administration

Figure 1 shows phenytoin serum concentrations for the time course of 24 hours after either acute or chronic phenytoin administration. Immediately after the intravenous infusion (250 mg), the serum concentration of phenytoin increased up to 60 μg/ml and it declined quite rapidly within 24 hours. Phenytoin was not detected in the serum after 24 hours. Intravenous administration of a single dose of phenytoin (85 mg) resulted in a serum concentration of approximately 20 μg/ml. This value was achieved 1 hour after the phenytoin administration, and was assumed to be the upper therapeutic level for antiepileptic action.

On the other hand, an intraperitoneal dose of 250 mg of phenytoin in rabbits already treated with the same regimen for 2 weeks provided sustained serum phenytoin levels during 24 hours, reaching a peak concentration of approximately 20 μg/ml. It was noted on hematological evaluation that the lymphocyte count was reduced, but not to a significant extent (p > 0.05), during phenytoin treatment in comparison to control studies (data not shown). It should also be mentioned that the rabbits treated with intraperitoneal administration of phenytoin for 2 weeks became anorexic and had weight loss of approximately 0.5 kg (10% of body weight) during the course of therapy. This prompted us to perform acute animal studies in an attempt to eliminate the effect of malnutrition on lymphocyte function.

Effect of Phenytoin on Mitogen-Induced Lymphocyte Activation

Figure 2 shows the inhibitory effect of phenytoin treatment on \(^3\)H-thymidine incorporation into DNA of mitogen-stimulated rabbit lymphocyte cell cultures. Three common mitogens were used in order to evaluate the lymphocyte responsiveness before, during, and after the phenytoin treatment.

The overall suppression of \(^3\)H-thymidine incorporation was 73% with PHA, 86% with Con A, and 78% with PWM in the presence of autologous serum at a mean serum phenytoin level of 20.4 μg/ml, when compared to control lymphocyte cultures performed prior to phenytoin treatment. Simultaneously, pooled nor-
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### TABLE 1

<table>
<thead>
<tr>
<th>Cells Studied</th>
<th>³H-thymidine Incorporation (cpm)</th>
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<tbody>
<tr>
<td></td>
<td>PHA</td>
</tr>
<tr>
<td>lymphocytes from control rabbits</td>
<td>47,510 ± 6618</td>
</tr>
<tr>
<td>lymphocytes from treated rabbits</td>
<td>45,104 ± 29,210</td>
</tr>
</tbody>
</table>

* Values are means ± standard error of the means for three rabbits in each group. PHA = phytohemagglutinin; Con A = concanavalin A; and PWM = pokeweed mitogen.

† The rabbits were treated with a single phenytoin dose (85 mg) intravenously. The mean serum phenytoin concentration was 23.1 μg/ml in three rabbits.

### TABLE 2

<table>
<thead>
<tr>
<th>Phenytoin Level</th>
<th>³H-thymidine Incorporation (cpm)</th>
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<tbody>
<tr>
<td>none (pooled normal rabbit serum)</td>
<td>59,892 ± 17,958</td>
</tr>
<tr>
<td>therapeutic (10–20 μg/ml)†</td>
<td>32,494 ± 5971</td>
</tr>
<tr>
<td>toxic (30–50 μg/ml)†</td>
<td>27,364 ± 2094</td>
</tr>
</tbody>
</table>

* Values are means ± standard error of the means for four rabbits in each group. Statistical significance is shown using Student’s t-test comparison to control cultures containing pooled normal rabbit serum. PHA = phytohemagglutinin; Con A = concanavalin A; and PWM = pokeweed mitogen.

† Serum with therapeutic phenytoin levels was obtained as described in the Methods section. Serum with toxic levels was obtained by additional intraperitoneal injection of phenytoin prior to the experiments.

### TABLE 3

<table>
<thead>
<tr>
<th>Lymphocyte Response</th>
<th>% Activated Lymphocytes†</th>
</tr>
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<tbody>
<tr>
<td>before therapy</td>
<td></td>
</tr>
<tr>
<td>no PHA</td>
<td>17.9 ± 1.9</td>
</tr>
<tr>
<td>PHA + 0.2 ml autologous serum</td>
<td>56.6 ± 5.0</td>
</tr>
<tr>
<td>during therapy§</td>
<td></td>
</tr>
<tr>
<td>PHA + 0.2 ml autologous serum</td>
<td>38.5 ± 6.2‡</td>
</tr>
</tbody>
</table>

* Values are means ± standard error of the means for four rabbits in each group. PHA = phytohemagglutinin.

† Activated lymphocytes (as defined in the Methods section) are those cells with nuclei assigned to Channels 20 to 100 on the Coulter Channelizer. The percentage of cells above Channel 20 is referred to as % activated lymphocytes.

‡ This value is statistically different from the control value (p < 0.05) (PHA/autologous serum) using Student’s t-test.

§ Mean serum phenytoin level was 30.5 μg/ml in four rabbits, which was achieved by a daily intraperitoneal dose of 250 mg phenytoin for 2 weeks.

Discussion

The present studies have confirmed previous findings that phenytoin, the most widely used anticonvulsant agent, has immunosuppressive potential. Two parameters (lymphocyte nuclear size and DNA synthesis) demonstrated this suppression; a correlation is illustrated in comparing Figs. 2 and 3. One of the most extensive studies regarding the association between immunosuppression and anticonvulsant therapy was done by Sorrell and Forbes. They reported depression of normal rabbit serum was used instead of autologous serum to evaluate for the presence of an intrinsic abnormality in lymphocytes which could be caused by phenytoin treatment. Lymphocyte responsiveness to PHA and Con A, as evaluated by ³H-thymidine incorporation, was again markedly suppressed in cultures containing pooled normal rabbit serum during phenytoin treatment. In contrast, an apparent inhibition was not observed when PWM was used.

Of particular interest is the observation that suppression of lymphocyte function during phenytoin treatment, as evidenced by ³H-thymidine incorporation, seemed to disappear approximately 3 weeks after the treatment was discontinued. To control for the effect of malnutrition on lymphocyte function, acute studies were performed. However, a suppressive effect of acute phenytoin treatment on rabbit lymphocytes was seen only with Con A as the mitogen (Table 1).

Subsequently, to evaluate for the serum factors, normal rabbit lymphocytes were cultured with mitogens in the presence of either pooled normal rabbit serum or allogeneic serum from phenytoin-treated animals (Table 2). There seems to be a phenytoin concentration-dependent inhibition of mitogen-induced lymphocyte activation as evaluated by ³H-thymidine incorporation. It appears that therapeutic as well as toxic levels are associated with a suppression of lymphocyte activation in response to at least one of the three mitogens. The hyporesponsiveness was more significant with both Con A and PWM than with PHA.

Effect of Phenytoin on Mitogen-Induced Increase in Lymphocyte Nuclear Size

As another means of evaluating the effect of phenytoin therapy on lymphocyte activation, the nuclear size distribution analysis was performed on the Coulter Channelizer, with the settings described in the Methods section using cells cultured in tubes rather than microtiter plates. As shown in Table 3, approximately 60% of normal rabbit lymphocyte nuclei (following 90 hours of incubation in the presence of PHA) were found between Channels 20 and 100, corresponding to a nuclear diameter of between 5.40 and 8.71 μ. This compares to 40% activated lymphocytes during phenytoin therapy (Fig. 3). Thus, significant suppression of mitogen-induced lymphocyte activation was observed during phenytoin treatment.

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Fig. 3. Effect of phenytoin treatment on mitogen-induced increase in rabbit lymphocyte nuclear size. Channel number as indicated in the Methods section is a measure of lymphocyte nuclear size. PHA = phytohemagglutinin.

one or more parameters of cellular and/or humoral immune response in 38 of 63 patients receiving phenytoin. MacKinney, et al.,13-15 using cultured human lymphocytes in vitro, showed that phenytoin significantly inhibited DNA synthesis of PHA-stimulated lymphocytes in the therapeutic range (10 to 20 µg/ml). On the other hand, Gabourel, et al.,9 suggested that phenytoin, used clinically to prevent seizures, did not depress lymphocyte responses to mitogens, but rather that there was a significant tendency to increased lymphocyte response to mitogens while taking phenytoin. In our previous studies using three different parameters of lymphocyte activation,19 we have demonstrated that phenytoin suppressed PHA-induced lymphocyte blastogenesis in a dose-related manner after adding the anticonvulsant directly to the in vitro short-term normal human lymphocyte cultures. This was further extended in the current studies which were designed to document in vivo evidence of an immunosuppressive effect in experimental animals treated with phenytoin.

It was demonstrated in the present investigation that both acute and chronic phenytoin treatment can induce functional alterations in lymphocytes and therefore cause abrogation in cellular immunity in experimental animals. Also of interest was the observation that the suppression of lymphocyte function caused by phenytoin treatment seemed to disappear after phenytoin treatment was stopped. It may be argued, however, that the daily phenytoin dose of 50 mg/kg employed for rabbits in the current investigation was exceedingly high in comparison with the actual clinical situation in man. Preliminary pharmacokinetic studies were performed in an attempt to delineate the optimal dosage of phenytoin that would maintain the phenytoin serum concentrations at greater than 10 µg/ml. It was found that the daily intraperitoneal injection of phenytoin at 50 mg/kg, but not less, successfully induced the therapeutic serum concentration of phenytoin in rabbits. Masuda, et al.,17 reported marked species differences in effective doses of phenytoin eliciting therapeutic effects against seizures. Furthermore, they showed in their studies using five different species that in rabbits the therapeutic serum concentrations of phenytoin were obtainable only with doses greater than 300 mg/kg of phenytoin, and yet no evidence of neurotoxic effects was observed at this dosage. It is also argued that studies involving the addition of phenytoin to lymphocyte cultures may have little application to possible phenytoin effects on the lymphocytes of patients taking phenytoin to prevent seizures. This problem appears to be solved in the current studies in which a therapeutic level of phenytoin serum concentration was obtained in experimental animals. The possibility that the sensitivity of rabbit lymphocytes to a therapeutic level of phenytoin may be different from that of human lymphocytes remains to be determined.

Since the animals receiving chronic phenytoin lost weight, the effect of malnutrition must be considered. There are numerous reports suggesting a correlation between depressed cellular immunity and malnutrition.5,20 One explanation for impaired cellular immunity in malnutrition is the reduced number of T lymphocytes in the peripheral blood.7 Acute studies were also performed in the current investigation in an attempt to eliminate the effect of malnutrition on lymphocyte function. These studies demonstrated that acute administration of phenytoin could also cause hyporesponsiveness of lymphocytes to at least one mitogen. A direct effect was also suggested in chronically treated animals, since the hyporesponsiveness with autologous (phenytoin-containing) serum was greater than with normal pooled rabbit serum. This was best exemplified with the PWM response, but was also observed with PHA and Con A. These results suggest a drug effect and not malnutrition.

Phenytoin is a highly effective anticonvulsant agent that is widely used in the management of patients with epileptic disorders and brain tumors. However, in previous studies in which patients with brain tumors were found to have depressed cell-mediated immunity, little mention was made of anticonvulsant drugs, with the exception of the work by Roszman and Brooks.21 The primary significance of our present communication together with our previous report19 is the implication that one important basis of impaired lymphocyte function and anergy observed in brain-tumor patients may be, at least in part, iatrogenic, secondary to drug-induced lymphocyte suppression. Phenytoin-treated patients have been shown to have moderate lymphopenia,2 which was considered to be due to a suppressive effect of phenytoin upon lymphocyte proliferation. More recent studies1-3,8 have shown that T lymphocytes are qualitatively as well as quantitatively affected by phenytoin therapy. Dosch, et al.,8 observed the development of abnormal suppressor T cells and hypogammaglobulinemia in a patient receiving phenytoin,
suggesting a pathogenic role of drug-induced abnormal T cell activity on B cell immunity. Phenytoin treatment does seem to interfere with both humoral and cellular immunity. However, at present it is impossible to decide to what extent anergy in brain-tumor patients is drug-induced as opposed to being directly related to underlying tumor. Our previous studies\(^2\) showed that human brain-tumor cyst fluid contained an immunosuppressive factor(s) which is not phenytoin, suggesting that brain-tumor cells may locally produce suppressive factor(s) capable of inhibiting lymphocyte activation. The relative importance of phenytoin and/or immunosuppressive factors produced by tumor cells will necessitate further investigation before an answer can be found.

References


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