Barbiturate inhibition of lymphocyte function

Differing effects of various barbiturates used to induce coma

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The present studies evaluated the effect of phenobarbital, pentobarbital, and thiopental at concentrations comparable to those attained during therapeutic barbiturate-induced coma, on in vitro mitogen-induced lymphocyte activation. Lymphocytes from normal volunteers were incubated for 72 hours in culture medium containing mitogen (phytohemagglutinin) and a range of concentrations of the barbiturates (5 to 833 µg/ml). Three parameters of lymphocyte activation (mitogen-induced blast transformation, $^3$H-thymidine incorporation, and cell proliferation) were all suppressed by the barbiturates. The suppression was dose-dependent. The greatest suppression was caused by the short-acting barbiturate, thiopental. Lymphocyte responses were much less affected by the long-acting barbiturate, phenobarbital. The intermediate-acting barbiturate, pentobarbital, was also intermediate in its ability to inhibit lymphocyte activation. The twofold to threefold difference between the effects of thiopental and pentobarbital on lymphocyte function may have direct clinical relevance, since it is primarily these two agents that are employed to induce therapeutic "barbiturate coma." Since lymphocyte suppression appears to be much more marked in the presence of thiopental, these observations support a role for the other barbiturates in programs of induced coma.

KEY WORDS • barbiturate • lymphocyte activity • thiopental • phenobarbital • pentobarbital • coma induction

RECENT observations have focused attention on the clinical application of barbiturates in the management of brain injury. To date, their use has been limited to two categories of neurological disease: cerebral vascular disease and intracranial hypertension. Experimental models of stroke have shown improved neurological function and decreased evidence of neuropathological damage when large doses of barbiturates were given either immediately before or following transient (up to 6 hours) focal ischemic insult. The beneficial effect of barbiturates appears to be dose-dependent and therapeutically significant only when massive amounts of drug are administered. The clinical evidence that high-dose barbiturate therapy is useful in cerebrovascular disease is tenuous, presumably because of the time delay between the cerebral vascular insult and drug delivery. An exception has been the successful intraoperative use of barbiturates immediately before temporary occlusion of one of the major cerebral arteries.

The use of barbiturate coma to treat intracranial hypertension is based on the observation that high doses of barbiturates rapidly decrease elevated intracranial pressure. Standard therapy and barbiturate coma therapy were compared by retrospective analysis in patients with severe head injuries. With standard therapeutic measures, 25% of patients in one series still exhibited uncontrollable intracranial hypertension, which would normally be fatal. When barbiturate coma was added to the management program, 10 of 19 patients who responded returned to a productive life, two remained moderately disabled, two are severely disabled, one is vegetative, and four are dead.

Currently, interest focuses upon the type of barbiturate employed and the potential side effects of high-dose barbiturate therapy. For instance, Levin has...
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noted that patients managed with barbiturate coma frequently developed myelosuppression and infections (unpublished data). The present studies began as an investigation into the effect of barbiturates on white cell function. This communication summarizes the effect of three barbiturates (phenobarbital, pentobarbital, and thiopental) on in vitro lymphocyte activation in response to the T-cell mitogen, phytohemagglutinin. The effect of therapeutic concentrations of these three barbiturates on the suppression of mitogen responsiveness was examined by measuring their effect on three distinct aspects of lymphocyte response: blast cell transformation as measured by an increase in cell volume, lymphocyte 3H-thymidine incorporation, and actual lymphocyte proliferation.

Materials and Methods

Isolation and Purification of Human Peripheral Blood Lymphocytes

Lymphocytes for study were obtained from 50 ml of blood drawn into a heparinized syringe and diluted with 2 volumes of sterile saline (0.9%). Ficoll-Hypaque gradients were prepared using 40 ml of the above blood-saline mixture and 10 ml of Ficoll-Hypaque solution in 50-ml polypropylene conical tubes.* After centrifugation at 200 G for 45 minutes at room temperature, the lymphocyte-rich layer at the plasma-Ficoll-Hypaque interface was removed by aspiration and washed three times in saline (0.9%). The cells were resuspended in RPMI medium,† which was supplemented with L-glutamine (2 mM/ml), penicillin (200 U/ml), and gentamicin (10 μg/ml), and is hereafter referred to as “complete culture medium.”

The cells were then counted on a ZBI Coulter Counter,‡ and the cellular morphology of the purified lymphocyte suspension was evaluated by examining Wright-stained smears. Viability was determined with trypan blue dye exclusion. The final cell yield from 50 ml of blood ranged from 1 x 10⁷ to 9 x 10⁷, and was composed of 95% mononuclear cells of which 90% were viable.

Assays of Lymphocyte Mitogen Responsiveness

Three techniques were used to evaluate lymphocyte responsiveness. One evaluated the incorporation of radiolabeled pyrimidine nucleoside (tritiated thymidine) as a parameter of the cells’ entry into the DNA synthetic phase. The second evaluated changes in cell volume as a marker of blast transformation. Finally, lymphocyte proliferation was measured by counting the number of cells in culture after incubation with mitogen.

Short-Term Lymphocyte Culture on Microtiter Plates

Mitogen-induced lymphocyte tritiated thymidine (3H-Tdr) incorporation was measured in a microtiter plate system. Cells (5 x 10⁴ per well) were cultured in round-bottom microplates,§ using complete culture medium (0.2 ml), with 20 μl of heat-inactivated human serum (diluted 1:1 with normal saline). To each well, 20 μl of phytohemagglutinin (PHA: 5.0 μg/ml) was added; the final volume in each well was 0.24 ml.

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 (specific activity 6.7 Ci/m mole) was added to each well. At 90 hours, the cells were harvested on glass fiber filters, and unincorporated radiolabeled precursor was removed by washing with an automatic multiple-sample harvester.† Radioactivity was measured in a liquid scintillation counter.‡ Tritiated thymidine incorporation was quantitated as mean count of (mH) 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.

To evaluate the effects of thiopental, pentobarbital, and phenobarbital on mitogen-induced lymphocyte activation, concentrations ranging between 5 and 1000 μg/ml (final concentration) were added to the individual cultures. Normal saline was used as the diluent.

Short-Term Lymphocyte Culture in Tubes

Two other measures of lymphocyte activation were the measurement of cellular proliferation and determination of cell volume in response to the mitogenic stimulus. Since these measurements require a large culture volume, studies were performed in a tube culture system. To each sterile tube (17 x 100 mm polypropylene culture tubes), 4 x 10⁶ cells were added in 1.6 ml of complete RPMI 1640 medium containing 0.2 ml of heat-inactivated pooled normal human serum (diluted 1:1 with normal saline). Phytohemagglutinin (0.2 ml, 5 μg/ml) and various concentrations of the barbiturates were then added. The pH of each culture before and after incubation was determined to

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* Ficoll-Hypaque solution obtained from Pharmacia Fine Chemicals, Piscataway, New Jersey.
† RPMI (Roswell Park Memorial Institute) medium obtained from M. A. Bioproducts, Walkersville, Maryland.
‡ ZBI Coulter Counter manufactured by Coulter Electronics, Hialeah, Florida.
§ Microplates manufactured by Dynatech Laboratories, Alexandria, Virginia.
¶ Phytohemagglutinin (PHA) obtained from Wellcome Reagents Ltd., Beckenham, England.
* 3H-thymidine obtained from New England Nuclear, Boston, Massachusetts.
† Glass fiber filters and automatic multiple-sample harvester manufactured by Microbiological Associates, Walkersville, Maryland.
‡ Beckman Model LS-150 liquid scintillation counter manufactured by Beckman Instruments, Palo Alto, California.
be 7.2 to 7.6. Blastogenesis and cellular proliferation were evaluated with a ZBI Coulter Counter and Coulter Channelyzer following 90 hours of incubation at 37°C in a humidified atmosphere of 5% CO₂. The Coulter Counter settings were as follows: amplification 1/4; 1/aperture current 1/2; lower threshold 20; window width 100; count range 1K; and count control-stop at full scale. With these settings, Channel 1 corresponds to a cell diameter of 9.05 μ, Channel 20 to a diameter of 11.2 μ, and Channel 99 to a diameter of 16.1 μ.

Aliquots from each culture tube were evaluated for cell size and cellular proliferation. The number of cells under the Channelyzer curve between Channels 1 and 19 were integrated, as were those between Channels 20 and 99. Since the vast majority of normal lymphocytes, before or after culture, were observed to be assigned to Channels 1 to 19, Channel 20 was arbitrarily selected as the upper normal limit of volume for unstimulated normal lymphocytes. The percentage of cells above and below Channel 20 was then calculated for each experimental sample, and this was compared with the percentage obtained from control samples. Cellular proliferation was calculated by averaging multiple counts for each sample and calculating a proliferation ratio by dividing the number of cells in unstimulated (control) cultures into the cell number from experimental (mitogen-stimulated) cultures.

### Results

#### Effect of Barbiturates on Mitogen-Induced Lymphocyte Activation

The incorporation of ³H-thymidine into normal human lymphocytes in response to PHA was affected by the presence of each of the barbiturates studied (Fig. 1). Mitogen-induced lymphocyte ³H-thymidine incorporation was reduced by each, but was most affected by the presence of the lipid-soluble barbiturate, thiopental. Lymphocyte activation was inhibited by 50% when the thiopental concentration was 40 to 50 μg/ml. Phenobarbital required a concentration of 250 μg/ml to suppress lymphocyte blastogenesis to this same degree. The intermediate-acting barbiturate, pentobarbital, was also intermediate in its ability to inhibit blastogenesis. That is, 50% inhibition of lymphocyte activation required a drug level in the culture medium of 150 μg/ml.

#### Effect of Barbiturates on Mitogen-Stimulated Cell Proliferation

Another result of lymphocyte activation is cellular replication. This end-point can be expressed as a proliferation ratio defined by the cell numbers in each experiment divided by the number of cells in the control chamber containing no PHA. As shown in Table 1, PHA increased the proliferation ratio to 2.12 ± 0.07 (mean ± SEM). Suppression of proliferation was seen with each of the barbiturates (Table 1), and the pattern of suppression was clearly dose-related. Again, thiopental produced the greatest degree of suppression. It is of interest that this parameter of lymphocyte activation did not recognize differences between the effects of phenobarbital and pentobarbital, as was evident when ³H-thymidine incorporation was the measure of stimulation.

#### Effect of Barbiturates on Cell Volume

The Coulter Channelyzer provides a distribution of cells according to their volume. Over 80% of normal lymphocytes, either at the time of isolation or following a 72-hour period of incubation in the absence of mitogen, are found between Channels 1 and 20, which correspond to cell diameters of 9.1 μ to 11.2 μ. Only

* The proliferation ratio (mean value ± standard error of the mean) was calculated by dividing the cell number in each experimental culture by the number of cells in the control culture which contained no phytohemagglutinin (PHA).

† These values are statistically different (p ≤ 0.05) from the control values (PHA/no drug) using the t statistic for two means.
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20% of normal unstimulated human lymphocytes were found in Channels 20 to 100 (Fig. 2). In contrast, 68% of the lymphocytes cultured for 72 hours in the presence of PHA were found between Channels 20 and 100, corresponding to cell diameters of 11.2 μ to 16.1 μ. Barbiturates suppressed this mitogen-induced change in cell volume (Table 2 and Fig. 2). Again, the suppression was most extensive at any given dose when thiopental (Table 2 and Fig. 2 upper left) was the barbiturate utilized. Thiopental actively suppressed lymphocyte responsiveness at a final concentration of 50 μg/ml (Fig. 2 upper left). All three barbiturates markedly suppressed the expected mitogen-induced increase in cell volume when tested at 500 μg/ml (Fig. 2).

Effect of Protein Binding on the Barbiturate

Routine culture medium contains 5% serum, providing a low protein concentration in vitro relative to that in vivo. To evaluate the possible protective effect of protein binding upon the inhibitory effect of the barbiturates on mitogen-induced lymphocyte activation, studies were carried out with cells cultured in the

![Graphs showing effects of barbiturates on lymphocyte function](image)

**TABLE 2**

Effects of barbiturates on mitogen-induced increases in normal human lymphocyte cell size

<table>
<thead>
<tr>
<th>Experiment</th>
<th>% Activated Lymphocytes*</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>20.9 ± 2.2</td>
</tr>
<tr>
<td>no PHA</td>
<td>67.8 ± 2.4</td>
</tr>
<tr>
<td>PHA/no drug</td>
<td>39.3 ± 4.5†</td>
</tr>
<tr>
<td>thiopental sodium</td>
<td>23.0 ± 2.5†</td>
</tr>
<tr>
<td>50 μg</td>
<td>24.2 ± 5.5†</td>
</tr>
<tr>
<td>100 μg</td>
<td>60.9 ± 3.0</td>
</tr>
<tr>
<td>500 μg</td>
<td>59 ± 3.9</td>
</tr>
<tr>
<td>pentobarbital sodium</td>
<td>25.4 ± 4.4†</td>
</tr>
<tr>
<td>50 μg</td>
<td>64.2 ± 3.4</td>
</tr>
<tr>
<td>100 μg</td>
<td>59.8 ± 4.9</td>
</tr>
<tr>
<td>500 μg</td>
<td>27.9 ± 6.2†</td>
</tr>
</tbody>
</table>

* Activated lymphocytes, as defined in the Methods section, are those cells assigned to Channels 20 to 100 on the Coulter Channelizer (that is, they have a cell diameter of 11.2 to 16.1 μ). The numbers represent mean values ± standard error of the mean.
† These values are statistically different (p < 0.05) from the control values (phytohemagglutinin (PHA)/no drug) using the t statistic for two means.
presence of increasing concentrations of serum. As shown in Fig. 3, increased serum concentrations did have some protective effects against the barbiturate suppression. Protein binding appeared to be more of a factor with thiopental than with the other two barbiturates (Fig. 3). However, with all three barbiturates, little difference was seen when the protein concentration was increased beyond 10%, suggesting that protein binding has only a small effect on barbiturate suppression of mitogen-induced lymphocyte activation.

**Discussion**

The present studies provide evidence that barbiturates depress responsiveness of human lymphocytes to mitogenic stimulation. The significance of this suppression is supported by the evidence derived from three separate measures of lymphocyte activation, that of incorporation of a pyrimidine nucleoside precursor of DNA synthesis, change in cell size preliminary to replication, and the actual increase in lymphocyte number. In addition, the suppressive effects of the barbiturates were dose-related. To date, only limited data exist concerning depression of lymphocyte responsiveness induced by such agents. Formeister, et al., did observe that, at anesthetic levels, pentobarbital suppressed *in vitro* mitogen-induced lymphocyte activation.

The two- to threefold difference between the thiopental and pentobarbital effects on lymphocyte function described in the current studies may have additional clinical significance, since it is primarily these two agents that are employed to induce therapeutic barbiturate coma. The potential importance of this difference is accentuated by the fact that, when barbiturate coma is induced with pentobarbital, therapeutic blood levels generally range from 25 to 35 μg/ml, whereas, with thiopental, levels range from 60 to 580 μg/ml. Thus, barbiturates inhibit lymphocyte function, and this inhibition can be shown *in vitro* at barbiturate concentrations which are clinically observed in the serum of patients placed in barbiturate coma. Furthermore, these studies suggest that pentobarbital may be a superior drug for the therapeutic induction of coma, since lower serum levels of pentobarbital are required to attain an adequate degree of barbiturate-induced coma and since, at such levels, pentobarbital does not inhibit lymphocyte function, in contrast to thiopental. This choice may lessen the likelihood of immunosuppression and the risk of infection for patients placed in barbiturate coma. The lymphocyte suppressive effect of thiopental may also contribute to the immunosuppression associated with general anesthesia.

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**References**

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3. Levin AB: Personal communication, 1981

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