EFFECTS OF PASSIVE IMMUNIZATION WITH RABBIT ANTI-PHENOBARBITAL IgG ON CYCLOBARBITAL-INDUCED SLEEPING TIME AND HEPATIC ENZYME ACTIVITIES OF C3H MICE

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Accepted August 12, 1975

Abstract—Effects of normal rabbit IgG (N-IgG) and phenobarbital specific IgG (Ab-IgG) on cyclobarbital-induced sleeping time were studied. Ab-IgG was specifically purified by Immunoadsorbent from rabbit antisera obtained by immunization with p-azophenobarbital bovine gamma globulin. N-IgG was purified from normal rabbit sera by DEAE-cellulose chromatography. The aggregate-free IgG were passively immunized to C3H mice 1 hr before intraperitoneal injection of cyclobarbital which has a high binding affinity to the antibodies. The pretreatment with N-IgG prolonged significantly cyclobarbital-induced sleeping time as compared with that of saline treated group; however, in the Ab-IgG treated group the duration of the sleeping was much the same as that seen in the saline group. To determine whether Ab-IgG has any influence on barbiturate tolerance, effects of N-IgG and Ab-IgG on the activities of hepatic enzymes in 9,000 g supernatant (alkaline RNase and aminopyrine demethylase) of the mice were examined at 40 hours after cyclobarbital administration. The activities of both enzymes which could be induced by cyclobarbital were decreased significantly in Ab-IgG treated group as compared with those of N-IgG group. These results are discussed with relation to barbiturate tolerance.

The development of tolerance by repeated administration of barbiturates has been principally explained in terms of induction of liver microsomal drug metabolizing enzyme systems and partly in adaptation of the cells of the central nervous system.

On the other hand, reports on barbiturate-induced hypersensitivity suggest that repeated administration of barbiturates may produce humoral antibodies (1–3). If the antibody can specifically bind the drugs, a modification of the pharmacologic action may occur and there may be such an immune mechanism as one component of barbiturate tolerance.

Berkowitz and Spector reported that the effect of morphine was diminished in mice immunized with morphine immunogen (4). However, there are at least two problems which occur when effects of specific antibodies on the pharmacologic activity of barbiturates are studied using animals which are producing specific antibodies by active immunization.

The first is stimulation of the reticulo-endothelial system by immunization with adjuvant, since the functional state of this system reportedly affects the duration of the action of barbiturates (5, 6).

The second problem is the effect mediated through a regulating factor of protein synthesis on the drug metabolizing enzyme systems, as significant elevation of serum protein
level occurs by nonspecific globulin production accompanied with specific antibody formation (in preparation). Therefore, in this study the effects of barbiturate specific antibody on the biological actions of cyclobarbital were examined using mice passively immunized with specifically purified rabbit anti-phenobarbital IgG. Aggregate-free rabbit IgG obtained by ultracentrifugation was passively immunized to C3H/He mice which were reported to be immunologically tolerant to soluble rabbit IgG (7). As described in a previous paper, the average binding affinity of phenobarbital against the antibodies used was $1.4 \times 10^8$ l/mole and relative affinity of cyclobarbital was 0.4 when the binding affinity of phenobarbital was standardized as 1.0 (8). In the studies of cyclobarbital-induced biological action, activities of two enzymes present in 9,000 x g supernatant of mouse liver were also determined 40 hr after induction of sleep. Aminopyrine demethylase is known to be induced by barbiturates and alkaline ribonuclease (RNase) might be one of the regulating factors of drug metabolizing enzyme induction (9, 10).

MATERIALS AND METHODS

Specific purification of anti-phenobarbital antibodies

The preparation of rabbit anti-phenobarbital antisera (Ab-IgG) and the characterization of the binding sites were performed as previously reported (8). For specific purification of anti-phenobarbital antibodies, immunoabsorbent was prepared by coupling of p-azophenobarbital rabbit serum albumin with CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals AB, Uppsala, Sweden). The procedure is summarized in Fig. 1. The specific isolation of the antibodies was carried out as follows; the globulin fraction obtained by salt fractionation at 33% saturation of ammonium sulfate from the antisera was dialyzed against 0.1 M Tris-HCl buffer, pH 7.6, containing 0.5 M NaCl and applied on the immunoabsorbent column (i.d.; 2 cm, height; 10 cm), after the elution of non-specific globulins the

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**Fig. 1**

Preparation of Immunoabsorbent

CNBr-activated Sepharose 4B: 14 g

| washing with 3 l of 0.001 M HCl |
| CNBr-activated Sepharose 4B in borate buffer: 300 ml |
  | (0.1 M, pH 8.5 + 0.5 M NaCl) |
  | p-Azophenobarbital rabbit serum albumin: 500 mg |
  | shaking for 4 hr at room temp. |
  | washing with borate buffer |
  | Inactivation of the Sepharose |

| by 1 M ethanolamine |
| Alternate washing with borate buffer (pH 8.5) and |
  | acetate buffer (pH 4) |
  | Suspension of Sepharose 4B coupled with the protein |
  | in Tris-HCl buffer (0.1 M, pH 7.6 + 0.5 M NaCl) |
  | Column preparation |
column was washed well with the 0.5 M NaCl containing Tris-HCl buffer, and when O.D. at 280 nm was less than 0.002 the specific antibodies were eluted with saturated phenobarbital in the same solvent and further with pH 2.4 glycine-HCl buffer to obtain a good yield, and the peak was collected and used as specific antibodies after condensation and exhaustive dialysis.

**Preparation of normal rabbit IgG (N-IgG) and globulin fraction**

N-IgG was prepared from a single batch of pooled normal rabbit sera by means of precipitation with 33% saturated ammonium sulfate, followed by DEAE-cellulose column chromatography. The protein fraction eluted from the column in 0.005 M sodium phosphate buffer at pH 7.5 was used as normal rabbit IgG. Mouse globulin fraction (MGG) was prepared from pooled C3H mouse sera by salt fractionation twice with 33% saturation of ammonium sulfate. Gel filtration (Sephadex G-50) and dialysis was performed to eliminate any contamination of ammonium sulfate. Concentration of the protein was determined spectrophotometrically. The extinction coefficient $E_{280\text{nm}}^{1\%} = 13.5$ was used (11).

**Immunoelectrophoresis**

To verify the presence of phenobarbital specific antibodies, immunoelectrophoresis was employed using goat anti-rabbit serum (MILES, Kankakee, Illinois, U.S.A.). As shown in Fig. 2, phenobarbital specific globulin was identified as IgG.

**Passive immunization of C3H/HeSLC mice**

C3H/HeSLC mice (C3H mice) were obtained from the Shizuoka Laboratory Center (Shizuoka, Japan). Mice were 6 weeks of age at the time of the first injection. Normal and specific IgG in saline were centrifuged at 105,000×g for 30 min and the upper half of the tube was used for injection after filtration through Millipore membrane (pore size; 0.45 μ). Both the aggregate-free IgG solutions were passively immunized in a dose of 1.2 mg/10 g body weight by i.v. injection. MGG in saline was filtered through Millipore membrane after centrifugation of 12,000×g for 20 min and i.v. administered (1 mg/10 g) 30 min before cyclobarbital 100 mg/kg i.p. injection. Rabbit serum albumin (RSA) Fr. V; MILES) in saline was i.v. injected (12 mg/10 g) 2 hr before cyclobarbital 70 mg/kg i.p. injection.

**Sleeping time**

The hypnotic response of C3H mice induced by i.p. administration of cyclobarbital (100 mg/kg) at 1 hr after passive immunization was estimated. Cyclobarbital was chosen as a barbiturate which induces adequate sleeping time in mice and has a high binding affinity to anti-phenobarbital IgG. The time which elapsed from loss of the righting reflex to its recovery was considered duration of the response.
Preparation of hepatic enzyme source

Mice were decapitated, the livers were removed, rinsed in 0.25 M sucrose, blotted and homogenized in 0.25 M sucrose solution (1:5). The homogenate was centrifuged at 9,000 g for 20 min and this supernatant was used for determination of aminopyrine demethylase and RNase activities. Protein was determined by modified Biuret method of Yonetani (12).

Aminopyrine demethylase assay

Demethylation of aminopyrine was estimated by assaying for formed formaldehyde as described by Nash (13).

The incubation mixture (2.5 ml) contained: 0.5 /mole aminopyrine, 10 /mole semicarbazide, 10 /mole nicotinamide, 1.25 /mole glucose-6-phosphate, 1 /mole NADP, 12.5 /mole MgCl2, 0.5 ml 9,000 g supernatant, and 1.25 ml 0.3 M phosphate buffer, pH 7.4. Incubation was performed in air at 37 C in a shaking incubator for 1 hr and the reaction was terminated by addition of 0.5 ml of 20% ZnSO4 and 0.75 ml of saturated Ba(OH)2. Two ml of the supernatant by centrifugation was reacted with 1 ml of acetylacetone reagent.

RNase assay

The assay of RNase activity was carried out as described by Louis-Ferdinand and Fuller (10) except p-chloromercuribenzoate (PCMB) addition. The incubation medium (1 ml) contained 1.2 mg highly polymerized yeast RNA, 0.1 ml 9,000 g supernatant, 0.1 ml PCMB solution (10⁻² M) and 0.7 ml Tris-HCl buffer, pH 7.6. PCMB was added to inhibit endogenous RNase inhibitor normally present in liver soluble fraction, since in this study soluble fraction was not separated (14).

RESULTS

Sleeping time

Mean sleeping times of three experimental groups are shown in Table 1. Pretreatment with N-IgG prolonged significantly cyclobarbital induced sleeping time as compared with that of the saline group. In Ab-IgG group, however, the significant prolongation was not observed (p 0.1). The significant prolongation of cyclobarbital-induced sleeping time was also observed in C3H mice administered isologous gamma globulin fraction i.v. suggesting

| Pretreatment | No. of mice | Time (min) sleeping induction from administration | Duration of sleeping time (min) |
|--------------|-------------|-----------------------------------------------|--------------------------------|
| Saline       | 4           | 7.6 ± 0.6                                    | 42.5 ± 5.7                      |
| N-IgG        | 4           | 5.5 ± 0.3                                    | 79.3 ± 3.8                      |
| Ab-IgG       | 4           | 6.5 ± 0.3                                    | 51.8 ± 2.9                      |

* Values are expressed as the mean SE
a Significantly different from control (p = 0.01)
b Not significantly different from control (0.1 p 0.2)
TABLE 2. Effect of isologous gamma globulin fraction on cyclobarbital-induced sleeping time

| Pretreatment | No. of mice | Duration of sleeping time (min) |
|--------------|-------------|--------------------------------|
| Saline       | 4           | 33.7 ± 1.3                     |
| MGG          | 4           | 61.5 ± 10.3                    |

* Mice were 17 weeks of age.

TABLE 3. Effect of rabbit serum albumin (RSA) on cyclobarbital-induced sleeping time

| Pretreatment | No. of mice | Duration of sleeping time (min) |
|--------------|-------------|--------------------------------|
| Saline       | 5           | 0                              |
| RSA          | 5           | 43.6 ± 3.6                     |

* Mice were 13 weeks of age.

that the prolongation was not a specific phenomenon by heterologous protein (Table 2). Effect of a high dose of rabbit serum albumin on the sleeping time was also investigated. As shown in Table 3, significant induction of the pharmacological action of cyclobarbital was found with the serum protein administration, though sleep was not induced in the saline pre-treated group given a dose of 70 mg/kg. Thus, phenobarbital specific IgG did not modify apparently cyclobarbital-induced sleeping time, whereas N-IgG and serum albumin which is considered a main drug binding protein in the blood enhanced the pharmacological action.

**Hepatic RNase and aminopyrine demethylase**

Mice of three experimental groups as shown in Table I were kept for 40 hr after cyclobarbital administration and the livers were utilized to determine hepatic enzyme activities. Two groups treated with saline or N-IgG alone were also kept as control groups. The RNase activities of the five groups are shown in Fig. 3. N-IgG treated controls showed a significant depression against the saline control group (p<0.05). There was a tendency for the RNase activity to increase in the saline and cyclobarbital combination group (saline + cycl. group) but there was statistically no significant difference in the activities between N-IgG + cycl. group and saline + cycl. group (0.05<p<0.1). On the other hand, Ab-IgG and cyclobarbital combination group (Ab-IgG + cycl. group) showed a significant depression in the activity as compared with the saline + cycl. group (p<0.01).

The results of aminopyrine demethylase estimation are shown in Fig. 4. In the N-IgG group, the demethylase activity showed some depression as compared with that of saline control group (0.05<p<0.1). In the saline + cycl. group, significantly higher activity was observed (p<0.05) as compared to the saline group, and there was no significant difference in the activities between N-IgG + cycl. group and saline + cycl. group (p>0.2). The Ab-IgG + cycl. group, however, showed significantly lower activity than that of the saline + cycl. group (p<0.05).
FIG. 3. Effects of normal and phenobarbital specific rabbit IgG on 9000 × g supernatant RNase activity affected by cyclobarbital administration (100 mg/kg). Values represent mean ± SE of at least 4 animals per group.

FIG. 4. Effects of normal and phenobarbital specific rabbit IgG on aminopyrine demethylase activity affected by cyclobarbital administration.

In order to investigate the effects of N-IgG and Ab-IgG administration on the two enzymes, the time course of the activities of RNase and aminopyrine demethylase after cyclobarbital administration was examined. As shown in Fig. 5, biphasic stimulation of aminopyrine demethylase activities was observed within a week after cyclobarbital administration (100 mg/kg). On the other hand, modification of RNase activities was inversely related to variations in the demethylase activities, preceding the change of demethylase activities. Thus, it was confirmed that both the enzyme activities were in the induction phase at 40 hr after cyclobarbital administration.

DISCUSSION

Neutralization of the pharmacological effect by drug specific antibodies has been reported in a steroid hormone (15), and chloramphenicol (16). In our study, specific IgG
did not reduce cyclobarbital-induced sleeping time compared with saline, but it significantly reduced the pharmacological action compared with normal IgG.

The clinically useful barbiturates are all bound to varying degrees to plasma. Goldbaum and Smith who measured binding of a series of barbiturates to bovine serum albumin in vitro, found them to range from barbital (5% bound) through phenobarbital (20% bound) and pentobarbital (37% bound) to thiopental (65% bound), the same rank-order previously observed with lipid solubility as the criterion (17). Binding of barbiturates to plasma protein is an unstable type of bond, readily reversible with change in concentration and it may be considered a means of barbiturate transport in the blood stream, somewhat analogous to oxygen transport by hemoglobin (except that oxyhemoglobin is a chemical compound, while albumin and the barbiturate form a loose structural complex) (18). Tissue proteins also bind barbiturates, to about the same extent as the binding to plasma proteins (17). Thus it seems that the two key factors in barbiturate pharmacokinetics are the lipid solubility of the drug and the concentration of unbound, undissociated molecules at whatever site is being examined. In order to obtain information on the mechanisms of the prolongation of sleeping time by N-IgG immunized, the tissue distribution (Fig. 6) and serum concentration change (Fig. 7) of 14C-phenobarbital pretreated with the IgG were examined. As shown in Fig. 6, at 1 hr after 80 mg/kg administration, 14C concentration in brains as well as livers and kidneys was higher in the IgG treated groups than that in the saline group. The prolongation of cyclobarbital-induced sleeping time in N-IgG group might be related to the high distribution of the drug in the brain where the effective form of the drug may be released according to the lipid solubility and/or the dissociation constant against the tissue or serum protein.

The significantly swift onset of sleeping time following i.p. injection of cyclobarbital in the N-IgG treated group com-
pared with that in the saline group as shown in Table 1 might also be elucidated in terms of rapid transport of the drug to the brain by the IgG immunized.

On the other hand, the fact that Ab-IgG did not enhance the sleeping time might be explained by the high binding affinity of the drug to the specific antibodies. The blood concentration of the binding sites of the specific IgG was calculated to be about 0.04 μmole/ml. The concentration seems too small compared with the mole concentration of the drug in the whole body to elicit an effective modification of the pharmacologic activity. This fact suggests that a considerable amount of the drug is bound to serum or tissue proteins in a stable type of bonding as described for thiopental by Taylor et al. (19), although in our experiment, rabbit serum albumin did not block the pharmacologic activity.

REFERENCES
1) Muranaka, M., Okumura, H., Takeda, K., Suzuki, S. and Koizumi, K.: Japan. J. Allergology 18, 562 (1969) (in Japanese)
2) Tanioku, K.: J. Clinical Science 7, 662 (1971) (in Japanese)
3) Hattori, K.: Clinic All-Round 22, 1922 (1973) (in Japanese)
4) Berkowitz, B. and Spector, S.: Science 178, 1290 (1972)
5) Woofes, W.R. and Borzelleca, J.F.: J. Reticuloendothel. Soc. 3, 41 (1966)
6) Dipasquale, G., Whal, P. and Rasselt, C.L.: Res. Commun. Chem. Pathol. Pharm. 9, 253 (1974)
7) Fujiiwa, M.: Japan. J. exp. Med. 41, 59 (1971)
8) Satoh, H. and Kuroiwa, Y.: J. Biochem. Tokyo 76, 1293 (1974)
9) Lechner, M.C. and Pousada, C.R.: Biochem. Pharmacol. 20, 3021 (1971)
10) Lechiner, R.T. and Fuller, G.C.: Toxic. appl. Pharmacol. 23, 492 (1972)
11) Sober, H.A. and Harte, R.A.: Handbook of Biochemistry, p. C-80, The Chemical Rubber Co., Cleveland, Ohio (1970)
12) Yonetani, T.: J. biol. Chem. 236, 1680 (1961)
13) Nash, T.: Biochem. J. 55, 416 (1953)
14) Shiozawa, T.: Biochim. Biophys. Acta 55, 88 (1962)
15) Neri, R.O., Tolksdorf, S., Beiser, S.M., Frlanger, B.F., Agafe, F.J. Jr. and Liebelman, S.: Endocrinology 74, 593 (1964)
16) Hamburger, R.N. and Douglas, J.H.: Immunology 17, 599 (1969)
17) Goldsbaum, L.R. and Smith, P.K.: J. Pharmacol. exp. Ther. 111, 197 (1954)
18) Mark, L.C., Parel, J.M., Brand, L. and Dayton, P.G.: Anesthesiology 31, 384 (1969)
19) Taylor, J.D., Richards, R.K., Davin, J.C. and Asher, J.: J. Pharmacol. exp. Ther. 112, 40 (1954)