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DEPRESSION OF IMMUNE COMPETENCE BY PHENYTOIN AND CARBAMAZEPINE

STUDIES *IN VIVO* AND *IN VITRO*

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SUMMARY

Depression of one or more parameters of cellular and/or humoral immune responses was found in 60% of general hospital patients treated with phenytoin and 47% of patients treated with carbamazepine. Phenytoin-treated patients failed to manifest delayed hypersensitivity (DHS) reactions to common antigens, and to make antibody to *Salmonella typhi* and tetanus toxoid. Serum levels of IgA and IgM, DNA synthesis in circulating leucocytes, and phytohaemagglutinin (PHA) induced deoxyribonucleic acid synthesis were also low. Depression of IgA, DHS reactivity and antibody responsiveness to *S. typhi* were shown to develop after the commencement of phenytoin therapy in a study of eleven patients. The presence of immunological defects was independent of the dosage of drug, its serum concentration, the duration of therapy and the sex of the subject. Studies *in vitro* provided evidence that immunosuppression was the result of a direct effect of phenytoin on the metabolism of lymphoid cells. Carbamazepine was shown to have a similar but less potent direct effect. Pharmacological concentrations of phenytoin caused a significant depression of DNA synthesis in PHA-stimulated and non-stimulated blood cell cultures *in vitro*. High concentrations in addition caused depression of cell counts, lymphocyte blastogenesis, ribonucleic acid and protein synthesis. Phenytoin was not cytocidal at concentrations of up to 125 μg/ml. Depression of DNA synthesis by phenytoin was maximal when phenytoin was added within 4–8 hr of the addition of PHA. PHA-induced DNA synthesis was not significantly affected by pre-incubation with phenytoin. *In vivo*, the presence of immunological defects was not related to phenytoin-induced folic acid deficiency. High concentrations of carbamazepine, but not phenobarbitone or diazepam caused a significant depression of PHA-stimulated DNA synthesis in blood cell cultures.

The data show that immunosuppression is a common side-effect of phenytoin therapy, and that lymphoma is rare. They suggest that in the presence of phenytoin-

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induced immunosuppression another factor, or factors are required to induce the formation of lymphoma.

INTRODUCTION

The occurrence of Hodgkin's disease and other lymphomas has been documented in a significant number of patients treated with the anticonvulsant drug, phenytoin sodium (diphenylhydantoin), and other hydantoins (Hyman & Sommers, 1966; Anthony, 1970; Brown, 1971). It is known that many patients with lymphoma are immunosuppressed (Brown *et al.*, 1967; Sutherland, Inch & McCredie, 1971). Immunological deficiencies, but not lymph node abnormalities, were found in a preliminary survey of institutionalized epileptic patients treated with phenytoin but not in epileptic patients treated with barbiturates (Sorrell *et al.*, 1971).

This paper reports studies of the immunological status of a group of general hospital patients treated with phenytoin and of patients treated with a newer anticonvulsant, carbamazepine, and studies *in vitro* of the direct effect of phenytoin on lymphocytes.

MATERIALS AND METHODS

Patients

(a) Sixty-three patients (thirty-five males and twenty-eight females, mean age 35·0 years, range 14–70 years) had been ingesting phenytoin regularly for at least 1 month, most commonly in a dose of 300 mg/24 hr. At the time of study, phenytoin was the only anticonvulsant taken by thirty-seven of these patients.

(b) Control data were obtained from 137 patients.

Data for DNA synthesis were obtained from ninety healthy persons (fifty-one males, thirty-nine females, mean age 30·6 years, range 14–65 years). Controls for other data were drawn from fifty of these healthy persons, twenty patients with vascular disease, fifteen with untreated epilepsy, and fifteen with various diseases not known to be associated with immunological abnormality (fifty-five males, forty-five females, mean age 36·0 years, range 14–78 years. The epileptic subgroup was composed of eight males and seven females, mean age 29·7, range 15–56 years).

(c) A number of immunological parameters was measured in eleven patients (nine males and two females) before the commencement of, and during, therapy with phenytoin.

(d) Fifteen epileptic patients (seven males and eight females) were taking carbamazepine as their only anticonvulsant. Their mean age was 26·3 years, with a range of 14–58 years.

Clinical assessment

Patients were examined for lymph node enlargement and hepatosplenomegaly, and chest X-rays were reviewed. Patients with evidence of infection were excluded from the study.

Immunological assessment

Serum concentrations of immunoglobulins IgG, IgA and IgM, and the C3 component of complement (β^{IC}/IA) were determined using Behringwerke immunodiffusion plates. Patients were immunized with 0·1 ml of monovalent *Salmonella typhi* suspension (Commonwealth

Serum Laboratories, Australia) and with 0·5 ml of alum-precipitated tetanus toxoid (Commonwealth Serum Laboratories). Sera collected 2 weeks later were tested for the presence of antibodies to *S. typhi* H antigen and tetanus toxoid, and for the presence of antinuclear antibody, using an immunofluorescent technique (Forbes, 1971). *Candida albicans* (1%, Commonwealth Serum Laboratories) in 0·1 ml, 10 units of streptokinase plus 2·5 units streptodornase (Varidase, Lederle) in 0·1 ml of physiological saline, and 0·1 ml of killed mumps antigen (Eli Lilly, Indianapolis), were injected intradermally to test delayed hypersensitivity reactions. These were read at 48 hr.

Circulating lymphocyte counts were derived from leucocyte counts (Coulter counter, Model S) and estimates of the percentage of lymphocytes in stained blood smears. PHA-induced lymphocyte DNA synthesis was measured in triplicate cultures, by a modification of the method of Junge *et al.* (1970). Cultures contained 0·2 ml of heparinized blood 0·4 ml of serum (autologous serum or foetal calf serum (FCS) (Commonwealth Serum Laboratories), 0·02 ml of reconstituted PHA (Wellcome, batch 3716) and 3·38 ml of medium 199. Tritiated thymidine [^{3}H-methyl]thymidine, 2·5 μCi (specific activity 500 mCi/mmol) (Radiochemical Centre, Amersham), was added to cultures after incubation for 92 hr at 37°C. The amount of [^{3}H]thymidine incorporated into trichloracetic acid-precipitable DNA after 96 hr of culture was measured in a Packard liquid scintillation spectrometer. Results were corrected for quenching using an automatic external standard, and expressed as the number of disintegrations per minute per culture (d/min/culture). DNA synthesis in circulating leucocytes was measured in triplicate cultures containing 0·2 ml of freshly drawn heparinized blood, 3·8 ml of medium 199 and 2·5 μCi of [^{3}H]thymidine. Cultures were processed for liquid scintillation counting after 4 hr of incubation at 37°C.

In vitro studies

Preparation of drug solutions. Phenytoin for intravenous use (Parke Davis, Michigan), 250 mg, was dissolved in a sterile solution of propylene glycol (40%), ethyl alcohol (10%) and distilled water (50%), adjusted to pH 12 with sodium hydroxide. Phenobarbitone (Farmer Hill, Australia) and diazepam pure substance (Roche, Switzerland) were dissolved in the same solvent except that the pH was not adjusted. Carbamazepine pure substance (Geigy, Australia) was dissolved in propylene glycol (50%) and ethyl alcohol (50%), and sterilized by passage through a millipore filter.

Measurement of inhibition of DNA synthesis by drugs

Phenytoin, carbamazepine, phenobarbitone, diazepam, or solvent (0·01 ml), were added to triplicate culture tubes and mixed with reconstituted PHA (Wellcome, 0·02 ml) in HEPES-buffered medium 199 (2·98 ml, pH 7·5 at 37°C). Blood suspension (0·2 ml in 0·4 ml of FCS, and 0·4 ml of medium 199) was added separately. Cultures were resuspended daily during incubation at 37°C. Phenytoin dose–response curves were also constructed for non-PHA-stimulated cultures. [^{3}H]Thymidine or [^{3}H]deoxyuridine (2·5 μCi, specific activity 500 mCi/mmol), 0·1 ml, was added at 92 hr. At 96 hr, cultures were processed as described above.

Measurement of inhibition of ribonucleic acid (RNA) synthesis by phenytoin

RNA synthesis was measured in lymphocyte cultures of greater than 98% purity, as recommended by Cline (1966). Lymphocytes were separated from heparinized blood by

sedimentation over methyl-cellulose–Hypaque (Hulliger & Blazkovek, 1967), followed by passage through a cotton wool column (Lamvik, 1966). Triplicate cultures containing $1{\cdot}5 \times 10^6$ lymphocytes were incubated for 24 hr with PHA (0·02 ml) FCS (0·2 ml) and medium 199 such that the culture volume was 2·0 ml. RNA synthesis was determined by the incorporation of [^{3}H]uridine (3·2 μCi, specific activity 6·4 Ci/mmol, 0·1 ml/culture, added at 20 hr of incubation) into RNA extracted from the cells by the Schmidt–Thannhauser technique as modified by Fleck & Munro (1962), using potassium hydroxide (0·3 M at 37°C for 1 hr) to digest the RNA. An aliquot of the RNA extract (0·2 ml) was shaken with toluene–ethanol-based scintillation fluid, and the radioactivity determined as for DNA synthesis.

Measurement of inhibition of protein synthesis by phenytoin

Protein synthesis was measured in lymphocyte cultures of greater than 95% purity to prevent contamination with erythrocyte protein during processing. Lymphocytes were obtained from heparinized blood by centrifugation through a column of Ficoll–Hypaque (Froland & Natvig, 1970). Triplicate PHA-stimulated cultures (2·0 ml) contained $2{\cdot}0 \times 10^6$ lymphocytes. [^{14}C]Leucine (1·0 μCi, specific activity 344 mCi/mMol, in medium 199) was added at the beginning of culture. At 24 hr, cultures were frozen and thawed twice, precipitated three times in trichloroacetic acid (final concentration, 5%) dissolved in Soluene (1·0 ml), mixed with toluene-based scintillation fluid and counted in the Packard spectrometer.

Determination of cell counts in culture

PHA-stimulated and non-stimulated blood cell cultures were incubated with phenytoin, carbamazepine or phenobarbitone for 96 hr. The cytoplasm was stripped from the cultured cells by vigorous shaking with filtered counting fluid (18·0 ml) to which cetrimide solution (2·0 ml) had been freshly added (Stewart & Ingram, 1967). Cell nuclei were counted in a Coulter counter Model A, orifice diameter 100 μm, current setting 7, threshold 15.

Lymphocyte viability

This was determined by the trypan blue dye exclusion technique (Ling, 1968).

Drug assays

The serum concentration of phenytoin was measured by a gas chromatographic technique (Dill *et al.*, 1971).

Carbamazepine concentrations were determined from its ultraviolet absorption at 257 nm after extraction by the method of Curry (1969). Serum concentrations were measured 2–3 hr after the morning dose.

Ultraviolet absorption curves were identical for the phenytoin extracted from blood cell cultures at 1 and 96 hr of incubation (concentrations ranged from 5–125 μg/ml), confirming that phenytoin remained stable throughout the period of culture. Similarly, identical curves were obtained for supernatants of phenytoin-medium 199 incubation mixtures and aliquots of the resuspended mixture, indicating that phenytoin remained in solution. Similar results were obtained for carbamazepine (concentrations 5–62·5 μg/ml).

Determination of serum folate concentration

Serum folate concentrations were measured by a modification of the method of Baker *et al.* (1959).

Statistical methods

The distributions of results for serum immunoglobulin concentrations, circulating leucocyte DNA synthesis, and PHA-induced lymphocyte transformation (DNA synthesis) were tested for goodness of fit to a Gaussian curve by using Fisher's coefficients of skewness and kurtosis (Snedecor, 1959) in the control sample. The means of the normally distributed groups were compared with test means using Student's *t*-test, after F-testing had established that there was no significant difference between the variances of the respective groups. The value of immunoglobulins IgG, IgA and IgM, and lymphocyte counts, measured before and during therapy with phenytoin, were compared using the Wilcoxon test for pair differences (Diem, 1962).

Differences in antibody responsiveness and DHS reactivity were tested for by Fisher's exact test (Bailey, 1968).

RESULTS

Clinical examination

There was no evidence of enlargement of lymph nodes, liver or spleen in any patient. Retrospective study of case records did not suggest that any patient had a predisposition to infection.

Immunoglobulin and complement concentrations (Table 1)

IgA and IgM concentrations were depressed in phenytoin-treated patients in comparison with controls ($P<0{\cdot}001$ in each case). IgA was depressed to a greater extent in females than in males ($P<0{\cdot}01$). IgM was depressed only in males. The IgA level was significantly depressed in carbamazepine-treated patients. Serum complement concentrations were within the normal range in both groups.

TABLE 1. Immunoglobulin concentrations (mg/100 ml)

Group	Number	IgG	IgA	IgM*
Control	87	1190 ± 311	197 ± 77	162 ± 70
Phenytoin	63	1235 ± 370	121 ± 78†	136 ± 63† (61)
Carbamazepine	15	1235 ± 370	138 ± 58†	138 ± 67
Control (male)	48	1210 ± 297	212 ± 76	163 ± 74 (51)
Phenytoin (male)	36	1287 ± 412	150 ± 95† (37)	126 ± 48†
Control (female)	37	1161 ± 326	180 ± 75 (39)	161 ± 63 (38)
Phenytoin (female)	27	1150 ± 288	90 ± 61† (26)	151 ± 76 (26)

The values shown are the arithmetic means and their standard deviations. The number of determinations is shown in parentheses when they differ from the number shown in the second column.

* IgM was log-normally distributed.

† Significantly different from control (see text).

Antibody responses

Six of forty-six phenytoin-treated patients (four males and two females), and one of thirteen carbamazepine-treated patients failed to develop measurable antibody to *S. typhi*. The number of non-responders in the phenytoin-treated group was significantly greater than that in the control group, all of whom made this antibody ($P<0{\cdot}001$). Ten of forty-two phenytoin-treated patients (four males and six females) did not make antibody to tetanus toxoid ($P<0{\cdot}0001$); two of thirteen carbamazepine-treated patients also failed to make this antibody ($P<0{\cdot}02$).

Antinuclear antibody

This was found in the serum of one of the sixty-three phenytoin-treated patients.

Delayed hypersensitivity reactions

Three of forty-six phenytoin-treated patients, one of fifteen carbamazepine-treated patients and one of ninety-four controls did not react to any test antigen. These differences were not significant. Non-reactive patients developed an inflammatory reaction to the non-specific irritant, croton oil (Johnson, Maibach & Salmon, 1971). However, the total number of positive reactions to the three test antigens was significantly reduced in the phenytoin-treated and carbamazepine-treated groups (Table 2). Reactions were depressed in males and females in the phenytoin-treated group.

TABLE 2. DHS reactions in hospital patients

Group	Total tests	Reactions (number)	Reactions (percentage positive)	Significance
Control	293	220	75	
Epileptic control	44	30	68	
Phenytoin	139	85	61	$P<0{\cdot}005$*
Carbamazepine	45	26	58	$P<0{\cdot}01$
Control (male)	165	122	74	
Phenytoin (male)	87	46	53	$P<0{\cdot}001$
Control (female)	128	98	77	
Phenytoin (female)	62	39	63	$P<0{\cdot}025$

* Compared with control patients.

Lymphocyte counts and DNA synthesis in circulating leucocytes

Mean lymphocyte counts (cells per microlitre of blood), measured on three occasions, were normal in the phenytoin-treated group, and significantly low in the carbamazepine-treated patients ($P<0{\cdot}005$).

There was a significant depression of circulating leucocyte DNA synthesis in phenytoin-treated patients ($P<0{\cdot}025$). This was due to low results in the female but not the male subgroup.

PHA-induced DNA synthesis

DNA synthesis was depressed in phenytoin-treated hospital patients ($P<0{\cdot}05$), the depression being confined to the female patients.

PHA-induced DNA synthesis by lymphocytes of these patients in the presence of foetal calf serum was lower than that in the controls, but the difference was not significant, and the results were independent of the sex of the patient.

Extent of immunological abnormality in test patients

One or more immunodeficiencies was found in thirty of fifty phenytoin-treated patients, seven of fifteen carbamazepine-treated patients and seven of eighty-seven control subjects. Abnormalities were present in a significant number of male and female patients taking phenytoin, and in patients taking phenytoin alone, or in combination with other anticonvulsants. A particular pattern of immunodeficiency was not evident in the test population, although deficiencies of IgA concentrations and tetanus antibody responses were especially common.

Mean serum phenytoin concentrations were similar in thirty-four males ($0{\cdot}85 \pm 0{\cdot}72$ mg/100 ml) and twenty females ($0{\cdot}84 \pm 0{\cdot}77$ mg/100 ml). There was no relationship between serum phenytoin concentrations and the presence of one or more immunological deficiencies. Similarly, the duration of phenytoin therapy was not related to the presence of immunoogical deficiencies.

Serum folate concentration

The mean serum folate concentration was normal in the hospital patients on phenytoin. There was no correlation between immunological deficiency and serum folate concentration.

Measurement of immunological function before and after commencement of therapy with phenytoin

The results obtained from eleven patients tested before and after the commencement of phenytoin therapy are summarized in Table 3. There was a significant depression of IgA concentration associated with phenytoin therapy ($P<0{\cdot}01$, Wilcoxon's test for paired samples), but not of IgG or IgM, or circulating lymphocyte counts. One patient, who developed a primary antibody response to *S. typhi* immediately before the commencement of therapy did not make antibody after a second challenge 2 months later (i.e. at rechallenge no antibody was detected in the pre- and post-immunization sera). This depression of responsiveness was not statistically significant. The total number of positive DHS reactions obtained in ten untreated patients was significantly higher than the number of reactions to the same antigens, measured after the commencement of therapy ($P<0{\cdot}01$). One patient, who was reactive to *Candida* and mumps antigens immediately before the commencement of phenytoin, was non-reactive 12 months later.

The effect of phenytoin, carbamazepine, phenobarbitone, and diazepam on DNA synthesis

In PHA-stimulated cultures there was a significant concentration-dependent decrease in the incorporation of [^{3}H]thymidine into DNA in the presence of phenytoin and carbamazepine (Fig. 1a). No inhibition was caused by diazepam concentrations of up to 3·0 μg/ml, the maximum concentration attained in therapy (Clarke, 1969). The incorporation

TABLE 3. Immunological function before and during therapy with phenytoin

Patient number	Sex	Age	Time lag (months)	IgG		IgA		IgM		*S. typhi*		DHS reactions		Lymphocyte count	
				Pre-Rx	Post-Rx	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx
1	M	49	15	675	690	198	35	46	35	n.d.	n.d.	n.d.	n.d.	1932	1740
2	F	45	6	930	840	100	80	90	45	n.d.	n.d.	n.d.	n.d.	1672	1728
3	M	41	3	1250	940	300	215	110	110	⩾640	320	3	3	3152	2520
4	M	16	5	930	980	145	135	175	125	⩾640	320	2	2	2449	2142
5	M	20	3	900	900	110	110	80	95	80	0	3	2	2296	2706
6	M	46	12	1641	1210	73	85	170	175	⩾640	320	2	0	1840	2828
7	M	33	9	792	695	190	140	78	65	⩾640	160	1	1	1218	1682
8	F	26	2	1080	1260	195	70	160	165	⩾640	⩾640	1	0	896	946
9	M	56	3	960	1200	180	150	115	110	⩾640	⩾640	1	1	1092	1120
10	M	30	3	1115	860	130	85	215	150	n.d.	n.d.	1	0	1650	1560
11	M	33	2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	⩾640	40	2	1	n.d.	n.d.

Pre-Rx = before phenytoin; post-Rx = after phenytoin; n.d. = not determined.

of [^{3}H]deoxyuridine into cell DNA was depressed to 50% of control values by pharmacological concentrations of phenytoin (10–20 μg/ml) (Buchthal & Svensmark, 1971).

The depression of DNA synthesis by phenytoin and carbamazepine, but not phenobarbitone, was significantly greater than the depression of cell counts (Fig 1b). Counts were depressed in phenytoin-treated cultures at drug concentrations greater than 40 μg/ml. DNA synthesis was also depressed by phenytoin in non-stimulated cultures.

Serum obtained from a patient at intervals after he had taken an overdose of phenytoin was incubated with PHA-stimulated cultures of his peripheral blood, obtained when the serum phenytoin had returned to 3 μg/ml. Control cultures from the patient were incubated with PHA and the same concentration range of commercially available phenytoin solution

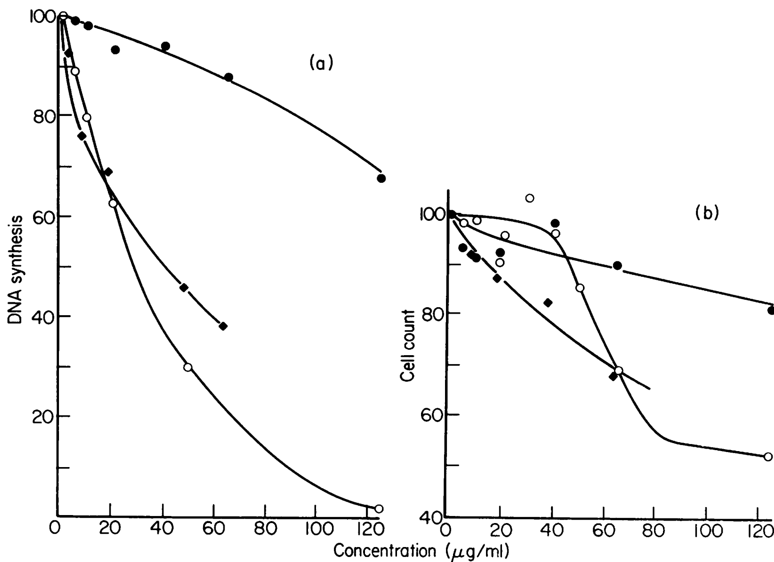

FIG. 1. (a) Depression of PHA-induced DNA synthesis by anticonvulsant drugs *in vitro*, expressed as a percentage of that of the control cultures. (b) Cell counts in PHA-stimulated cultures, expressed as a percentage of control. (●) Phenobarbitone. (♦) Carbamazepine. (○) Phenytoin.

in the presence of FCS (Fig. 2). There was a similar, concentration-dependent decrease in DNA synthesis in cultures containing phenytoin from the two sources (cultures containing 30% serum, Fig. 2 (■) and (▲)). (●) Shows the results obtained in cultures containing 10% autologous serum. There is a relatively greater depression of DNA synthesis by a given concentration of phenytoin in cultures containing less serum (10% compared with 30%).

Kinetics of depression of PHA-induced DNA synthesis by phenytoin

[^{3}H]Thymidine was added to all cultures at 92 hr of incubation and DNA synthesis was determined at 96 hr. Triplicate blood cell cultures were incubated with phenytoin (50 μg/ml) or phenytoin solvent for various times before the addition of PHA. The degree of depression of DNA synthesis was not significantly affected by pre-incubation with phenytoin. Phenytoin (50 μg/ml) or solvent was then added at specified times after the initiation of PHA-stimulated

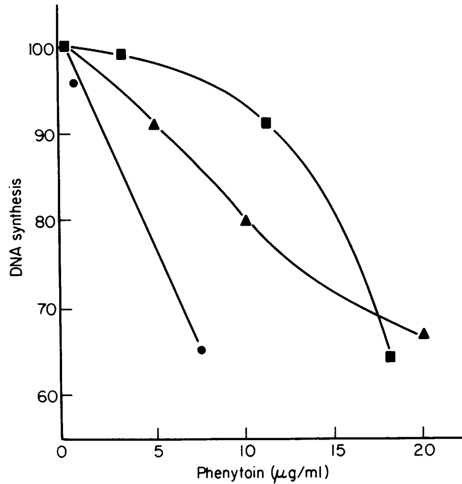

FIG. 2. Depression of PHA-induced DNA synthesis by phenytoin present in autologous serum (30% (■); 10% (●)) or added *in vitro* to cultures containing foetal calf serum (30%, ▲).

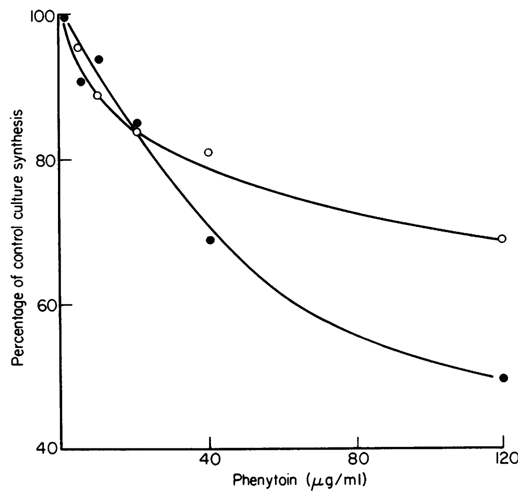

FIG. 3. The effect of phenytoin on PHA-stimulated RNA synthesis (●) and protein synthesis (○) expressed as a percentage of that in control cultures.

cultures. Depression of DNA synthesis was maximal when phenytoin was added within 4–8 hr of the initiation of culture.

The effect of phenytoin on lymphocyte blastogenesis

Lymphocyte blastogenesis (expressed as the percentage of transformed cells) was decreased in the presence of phenytoin concentrations greater than 30 μg/ml.

The effect of phenytoin on RNA and protein synthesis (Fig. 3)

The incorporation of [^{3}H]uridine into RNA was depressed to 50% of control values at

phenytoin concentrations of 100 μg/ml; at this concentration the incorporation of [^{14}C]-leucine into protein was depressed to 70% of control values.

DISCUSSION

The data indicate that depression of cellular and/or humoral immunity is common in patients treated with phenytoin sodium, and that these defects develop after the commencement of therapy.

At least one abnormality was found in 60% of patients in this series of general hospital-treated epileptics. In a preliminary survey, at least one defect was found in 40% of a group institutionalized, intellectually retarded patients (Sorrell *et al.*, 1971). Immunodeficiencies in phenytoin-treated patients have been reported independently by Grob & Herold (1972) and MacKinney & Booker (1972).

Therapy with carbamazepine, an anticonvulsant used in the treatment of temporal lobe epilepsy was associated with immunological defects in some patients, but in lower frequency than in the phenytoin-treated group.

Phenytoin appeared to accentuate sex differences in several immunological parameters. IgA levels were depressed to a greater extent in females than in males; IgM was low only in males. These are accentuations of reported differences (Buckley & Dorsey, 1971). Depression of PHA-induced DNA synthesis, and the rate of DNA synthesis in circulating leucocytes was observed only in females, accentuating trends found in the control series used in this study (Sorrell, 1974). The presence of immunological abnormalities was independent of the serum concentration of phenytoin, the duration of therapy, the sex of the subject, and the use of additional anticonvulsants. Similarly, there was no relationship between serum folate concentrations (which may be low in patients treated with phenytoin) (Klipstein, 1964), and immunological deficiencies.

PHA-induced DNA synthesis was depressed in cultures containing autologous serum but not foetal calf serum. DNA synthesis in circulating leucocytes was also depressed. These results are consistent with the presence of an inhibitory factor in the serum of phenytoin-treated patients, probably phenytoin itself. This hypothesis is supported by the fact that serum from a patient who had taken an overdose of phenytoin caused depression of DNA synthesis in PHA-stimulated blood cell cultures to an extent similar to that obtained in cells cultured with the same concentrations of phenytoin added *in vitro*.

In vitro studies also showed that depression of DNA synthesis by PHA-stimulated lymphocytes was significant at pharmacological concentrations of phenytoin (10–20 μg/ml) (Buchthal & Svensmark, 1971), and that concentrations of up to 125 μg/ml were not cytocidal. The depression of DNA synthesis *in vitro* varied inversely with the serum concentration (Sorrell, 1974), suggesting that only unbound phenytoin was effective.

Pharmacological concentrations of phenytoin did not cause a significant depression of cell counts, lymphocyte blastogenesis, RNA synthesis, or protein synthesis. MacKinney & Vyas (1972) measured DNA, RNA and protein synthesis in 72-hr leucocyte cultures and found that DNA synthesis was selectively depressed by pharmacological concentrations of phenytoin.

The early steps in the synthesis of thymidylic acid and uridylic acid include a common pathway. In the present study, pharmacological concentrations of phenytoin were shown to inhibit the incorporation of [^{3}H]deoxyuridine and [^{3}H]thymidine into DNA, but not

[^{3}H]uridine into RNA. It is probable that DNA synthesis is inhibited by phenytoin at a point distal to the formation of thymidine monophosphate, perhaps by depression of the activity of DNA polymerase. Kinetic studies suggested that phenytoin maximally affects the early stages of induction or onset of DNA synthesis, causing greatest depression of PHA-stimulated lymphocyte DNA synthesis when added in the first 8 hr of culture.

The data obtained in this study are consistent with the hypothesis that phenytoin-induced depression of DNA synthesis is significant *in vivo*. This may be a major mechanism by which the drug causes immunosuppression. Depression of DNA synthesis by carbamazepine *in vitro* is significantly less than that by phenytoin, in parallel with the lesser immunosuppressive effect observed *in vivo*. Barbiturates, which are not immunosuppressive *in vivo* (Sorrell *et al.*, 1971) cause insignificant depression of DNA synthesis *in vitro*.

Immunosuppression by phenytoin may be important in the pathogenesis of lymphoma occurring in patients taking this drug. Sensitivity to phenytoin as manifested by induction of DNA synthesis in cultured lymphocytes was demonstrated in a patient who developed Hodgkin's disease after treatment with phenytoin for 3 years (Sorrell & Forbes, 1975). It has been suggested that both immunosuppression and hypersenitivity are involved in the induction of phenytoin-associated lymphoma (Kruger & Harris, 1972).

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REFERENCES

Anthony, J.J. (1970) Malignant lymphoma associated with hydantoin drugs. *Arch. Neurol.* **22,** 450.

Bailey, N.J.T. (1968) *Statistical Methods in Biology*, 4th edn. English University Press, London.

Baker, H., Herbert, V., Frank, O., Pasher, I., Hutner, S.H., Wasserman, L.R. & Sobotka, H. (1959) A microbiologic method for detecting folic acid deficiency in man. *Clin. Chem.* **5,** 275.

Brown, J.M. (1971) Drug-associated lymphadenopathies with special reference to the Reed–Sternberg cell. *Med. J. Aust.* **i,** 375.

Brown, R.S., Haynes, H.A., Foley, H.T., Godwin, H.A., Berard, C.W. & Carbone, P.P. (1967) Hodgkin's disease—immunological, clinical and histological features of 50 untreated patients. *Ann. intern. Med.* **67,** 291.

Buchthal, F. & Svensmark, O. (1971) Serum concentrations of diphenylhydantoin (Phenytoin) and phenobarbital and their relation to therapeutic and toxic effects. *Psychiat. Neurol. Neurochir.* **74,** 117.

Buckley, C.E. & Dorsey, F.C. (1971) Serum immunoglobulin levels thoughout the life-span of healthy man. *Ann. intern. Med.* **75,** 673.

Clarke, E.G.C. (1969) *Isolation and Identification of Drugs in Pharmaceuticals, Body Fluids and Postmortem Material*, p. 496. The Pharmaceutical Press, London.

Cleaver, J.E. (1967) *Thymidine Metabolism and Cell Kinetics* (ed. by A. Neuberger and E. L. Tatum), p. 44. North-Holland, Amsterdam.

Cline, M.J. (1966) Isolation and characterization of RNA from human leukocytes. *J. Lab. clin. Med.* **68,** 33.

Curry, A.S. (1969) *Poisons Detection in Human Organs*, p. 93. C.C. Thomas, Springfield, Illinois.

Diem, K. (1962) *Documenta Geigy Scientific Tables*, 6th edn. Geigy Pharmaceuticals, St Leonards, Australia.

DILL, W.A., CHUCOT, L., CHANG, T. & GLAZKO, A.J. (1971) Simplified benzophenone procedure for determination of diphenylhydantoin in plasma. *Clin. Chem.* **17,** 1200.

FLECK, A. & MUNRO, H.N. (1962) The precision of ultraviolet absorption measurements in the Schmidt–Thannhauser procedure for nucleic acid estimation. *Biochim. biophys. Acta* (*Amst.*), **55,** 571.

FORBES, I.J. (1971) Measurement of immunological function in clinical medicine. *Aust. N.Z. Med.* **1,** 160.

FROLAND, S.S. & NATVIG, J.B. (1970) Effect of polyspecific rabbit anti-immunoglobulin antisera on human lymphocytes *in vitro*. *Int. Arch. Allergy*, **39,** 121.

GROB, P.J. & HEROLD, G.E. (1972) Immunological abnormalities and hydantoins. *Brit. med. J.* **ii,** 561.

HULLIGER, L. & BLAZKOVEK, A.A. (1967) A simple and efficient method of separating peripheral blood leukocytes for *in vitro* studies. *Lancet*, **i,** 1304.

HYMAN, G.A. & SOMMERS, S.C. (1966) The development of Hodgkin's disease and lymphoma during anticonvulsant therapy. *Blood*, **28,** 416.

JOHNSON, M.W., MAIBACH, H.I. & SALMON, S.E. (1971) Skin reactivity of patients with cancer. Impaired delayed hypersensitivity or faulty inflammatory response? *New Engl. J. Med.* **284,** 1255.

JUNGE, U., HOEKSTRA, J., WOLFE, L. & DEINHARDT, F. (1970) Microtechnique for quantitative evaluation of *in vitro* lymphocyte transformation. *Clin. exp. Immunol.* **7,** 431.

KLIPSTEIN, F.A. (1964) Subnormal serum folate and macrocytosis due to anticonvulsant drug therapy. *Blood*, **23,** 68.

KRUGER, G.R.F. & HARRIS, D. (1972) Is phenytoin carcinogenic? *Lancet*, **i,** 323.

LAMVIK, J.O. (1966) Separation of lymphocytes from human blood. *Acta Haemat.* (*Basel*), **35,** 294.

LING, N.R. (1968) *Lymphocyte Stimulation*, p. 45. North-Holland, Amsterdam.

MACKINNEY, A.A., JR & BOOKER, H.E. (1972) Diphenylhydantoin effects on human lymphocytes *in vitro* and *in vivo*. *Arch. intern. Med.* **129,** 988.

MACKINNEY, A.A. & VYAS, R. (1972) Diphenylhydantoin-induced inhibition of nucleic acid synthesis in cultured human lymphocytes. *Proc. Soc. exp. Biol.* (*N.Y.*), **141,** 89.

SNEDECOR, G.W. (1959) *Statistical Methods*, 5th edn, p. 201. Iowa State College Press, Ames, Iowa.

SORRELL, T.C. (1974) *Anticonvulsants in immunosuppression and carcinogenesis*. M.D. Thesis, University of Adelaide.

SORRELL, T.C. & FORBES, I.J. (1975) Phenytoin sensitivity in a case of phenytoin-associated Hodgkin's disease. *Aust. N.Z. J. Med.* (In press.)

SORRELL, T.C., FORBES, I.J., BURNESS, F.R. & RISCHBIETH, R.H.C. (1971) Depression of immunological function in patients treated with phenytoin sodium (diphenylhydantoin). *Lancet*, **ii,** 1233.

STEWART, C.C. & INGRAM, M. (1967) A method for counting phytohemagglutinin-stimulated lymphocytes. *Blood*, **29,** 628.

SUTHERLAND, R.M., INCH, W.R. & MCCREDIE, J.A. (1971) Phytohemagglutinin (PHA)-induced transformation of lymphocytes from patients with cancer. *Cancer*, **27,** 574.