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HYDANTOIN IMMUNOSUPPRESSION AND CARCINOGENESIS

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SUMMARY

The immunological response of mice submitted to hydantoin treatment was determined. Hydantoin reduced the absolute number of spleen cells in treated animals and did not modify spleen cells reactivity to concanavalin A, although the response to SRBC challenge, as measured by the Jerne plaque-forming cell technique, was significantly decreased.

Following these findings, the influence of hydantoin on carcinogenesis was evaluated by using the model of urethane-induced lung adenomas in SWR mice. Treatment with hydantoin significantly reduced the incidence of the induced adenomas.

We confirmed that hydantoin modifies the immune response of the host mainly by depressing its humoral function and we have shown that this effect was associated with an inhibitory effect on tumour induction.

INTRODUCTION

Hydantoin (sodium diphenylhydantoin), a widely used drug for the treatment of epilepsy and cardiac arrhythmias, has been associated with a lymphoproliferative disorder which ranges from benign lymphoid hyperplasia to malignant lymphoma (Gams, Neal & Conrad, 1968; Hyman & Sommers, 1966; Grob & Herold, 1972). In the light of this 'side effect', investigations were performed in order to find out whether this phenomenon was associated with or preceded by immunological derangement (Grob & Herold, 1972; Sorrell, Forbes, Burness & Rischbieth, 1971). It was suggested that hydantoin induced immunological disturbances consisting of definite impairment of the humoral immune response and probably damage of the cellular immune response (Grob & Herold, 1972; Sorrell *et al.*, 1971; Easton, 1972). It was even proposed that the lymphoproliferative disorder might be attributed to this immunological impairment (Easton, 1972).

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The present experiments were performed to test the influence of hydantoin on some parameters of the immune response and to evaluate its capacity to modify the response of mice to the carcinogenic effect of urethane.

MATERIALS AND METHODS

Animals

Four- to 6-week-old female inbred C57Bl/6 and SWR mice were used throughout the experiments. The animals were supplied by the Animal Breeding Center, Weizmann Institute of Science. Homozygosity was routinely tested by skin grafting.

Hydantoin or solvent treatment

0·02 ml of diphenylhydantoin (Dilantin) (0·5 mg/mouse) or its solvent (Parke–Davis) were injected either intraperitoneally (i.p.) or subcutaneously (s.c.) daily. This dosage of hydantoin corresponds per weight to the dosage commonly used for patients.

Induction of lung adenomas in SWR mice

SWR mice were injected once, i.p., with 5% aqueous solution of urethane (British Drug Houses, Ltd, Poole), on the basis of 0·5 mg/g body weight. Twelve weeks after urethane administration, the mice were killed, their lungs were examined with a low-power dissecting microscope and the number of lung adenomas recorded as previously described (Trainin *et al.*, 1967).

Preparation of spleen cells

Spleens were removed surgically, the tissues placed in cold Dulbecco's modified Eagle's medium (EM), and pressed through a fine stainless steel mesh. The cell suspensions obtained were washed and centrifuged twice at 600 *g* for 5 minutes, counted in a haemocytometer and resuspended in EM.

Lymphocyte transformation induced by concanavalin A (con A)

Five million nucleated spleen cells in 1 ml of EM + 10% foetal calf serum (FCS, Grand Island Biological Company, Berkley, California), were incubated for 72 hr in the presence of 2 μg of concanavalin A (con A) (Miles-Yeda, Rehovot, Israel). Two hours before the end of the reaction, 2 μCi of [^{3}H]thymidine (Amersham, Surrey) were added to the cultures, which were then gently shaken in a 37°C water bath. The cells were then poured onto fibreglass filters (Watman, England) and rinsed successively with saline, a cold solution of 5% TCA and absolute ethanol. The filters were dried, immersed into PPO–POPOP toluene, and counted in a Packard scintillation counter.

Plaque-forming cell (PFC) technique to SRBC

The response to sheep red blood cells (SRBC) was measured. SRBC stored in Alsever's medium were washed several times and mice were injected i.p. with 0·2 ml of a 10% cell suspension. The number of direct plaque-forming cells (PFC)/spleen to SRBC was determined 5 days later using the Jerne technique (Jerne, Nordin & Henry, 1963).

Statistical analysis

Student's *t*-test was used to analyse the statistical significance of the results.

RESULTS

Effect of hydantoin treatment on spleen cells reactivity to con A

The influence of hydantoin treatment on cellular (T-cell) function was evaluated by measuring the reactivity of spleen cells from treated mice to con A.

Hydantoin treatment consisted of either three i.p. or seven s.c. injections before the removal of the spleens for the *in vitro* assay. Control animals either received solvent injections according to the same schedule, or were left intact. As seen from Table 1 all groups of animals achieved the same degree of transformation with the mitogen; thus T-cell function, as measured by the reactivity to con A, was not affected by hydantoin treatment.

TABLE 1. Reactivity to con A of spleen cells from mice treated with hydantoin

Experiment number	Treatment*	Culture(−) Con A (ct/min ± s.e.)	Culture(+) Con A (ct/min ± s.e.)	Ratio of transformation
1	Hydantoin	1946 ± 44	12,717 ± 2348	6·5
	Solvent	2079 ± 33	10,606 ± 2853	5·1
	None	2104 ± 50	11,091 ± 1033	5·3
2	Hydantoin	2363 ± 212	13,148 ± 581	5·6
	Solvent	2761 ± 89	16,405 ± 308	5·9
	None	3307 ± 134	20,174 ± 1329	6·1
3	Hydantoin	1193 ± 118	22,625 ± 137	19·0
	Solvent	1274 ± 64	22,501 ± 592	17·7
	None	1002 ± 232	17,987 ± 517	18·0
4	Hydantoin	5060 ± 250	93,650 ± 4282	18·5
	Solvent	6025 ± 112	118,546 ± 6161	19·7

* Hydantoin or solvent treatment consisted in experiments numbers 1 and 3 of seven daily s.c. injections, in experiment number 2 of three daily i.p. injections, and in experiment number 4 of fourteen daily s.c. injections.

TABLE 2. Effect of hydantoin treatment on the response of mice to SRBC

Treatment*	Experiment 1		Experiment 2	
	PFC/spleen ± s.e.	*P*†	PFC/spleen ± s.e.	*P*†
None	151,500 ± 35,100	n.s.	139,200 ± 36,300	< 0·05
Solvent	168,600 + 27,000	< 0·005		
Hydantoin	66,600 ± 12,000		43,200 ± 9900	

n.s. = Not significant.

* Hydantoin or solvent treatment consisted of eleven daily s.c. injections, six before and five after challenge with SRBC.

† Probability was computed by Student's *t*-test.

Effect of hydantoin treatment on the immunological response of mice to SRBC

In the next experiment we tested the response of animals treated with hydantoin to SRBC, which is known to be mediated through the collaboration of B and T cells. Treatment consisted of eleven s.c. daily injections of hydantoin. On day 6 of this treatment mice were challenged with SRBC and 5 days later their spleens were removed and the number of PFC/spleen to SRBC was determined. As shown in Table 2 a marked decrease in the number of PFC to SRBC was observed in the experimental animals as compared with the response of the untreated controls or controls treated with solvent only.

TABLE 3. Effect of hydantoin treatment on the spleens of treated mice

	Experiment 1	Experiment 2	Experiment 3
Treatment*	10^6 spleen cells (number ± s.e.)	10^6 spleen cells (number ± s.e.)	10^6 spleen cells (number ± s.e.)
Hydantoin	57·75 ± 7·43	80 ± 7·99	108 ± 7·99
None	99·5 ± 6·65†		
Solvent		119 ± 11·99‡	143 ± 5·99‡

* Hydantoin or solvent treatment consisted in experiments numbers 1 and 2 of seven daily s.c. injections and in experiment number 3 of eleven daily s.c. injections.

Probability was computed by Student's *t*-test and indicated as follows: † $P < 0{\cdot}005$; ‡ $P < 0{\cdot}05$.

Effect of hydantoin treatment on the spleens of mice

The following experiment was performed in order to evaluate the direct effect of hydantoin on the spleen cell population of treated mice. Mice were daily injected s.c. with either hydantoin or solvent for 7–10 days. At the end of this treatment their spleens were removed, cell suspensions were prepared, and the number of live nucleated cells was determined by

TABLE 4. Influence of hydantoin treatment on the incidence of urethane-induced* lung adenomas in SWR mice

Treatment†	Number of animals	Number of adenomas per animal (mean ± s.e.)	*P* value ‡
Solvent	15	4·66 ± 0·583	—
None	15	4·53 ± 0·576	n.s.
Hydantoin	14	2·93 ± 0·559	< 0·025

n.s. = Not significant.

* Urethane, 0·5 mg/g of body weight, one single i.p. injection.

† Hydantoin or solvent treatment consisted of seven daily s.c. injections before and after urethane administration.

‡ Computed according to Student's *t*-test.

using the Trypan Blue exclusion technique. As shown in Table 3 hydantoin treatment significantly decreased the number of spleen cells of the treated mice.

Influence of hydantoin treatment on the incidence of urethane-induced lung adenomas in SWR mice

In the following experiment we used the model of urethane-induced lung adenomas in SWR mice in order to evaluate the influence of hydantoin treatment on tumour induction. Mice received seven daily s.c. injections of either hydantoin or solvent before and after a single dose of urethane. Twelve weeks after urethane administration animals were killed and their lungs examined for the presence of adenomas. As seen in Table 4 the incidence of lung adenomas was significantly lower in the group of mice submitted to treatment with hydantoin (2·9) as compared to recipients of solvent (4·7) or intact controls (4·5).

DISCUSSION

Sorrell *et al.* (1971) studied the immunological function in sixty-three patients who were on long-term phenytoin therapy. Their data indicated that diphenylhydantoin therapy can suppress both cellular and humoral immune responses. Later Grob & Herold (1972) reported on immunological abnormalities in twenty patients who were receiving hydantoins. Their findings were similar to those reported by Sorrell's group and suggested hydantoin-induced immunological disturbances of various degrees caused by impairment of the humoral immune response and, equivocally, impairment of the cellular immune response.

However, the immunological evaluation of the patients in both groups was based on indirect and inaccurate methods which did not permit quantitative measurement of the immune response. In the present experiments we tried to evaluate the immunological effects of hydantoin by using two established *in vitro* techniques in an animal experimental model. We used the concanavalin assay to measure the cellular (T-cell) function of the host, since this mitogen is known to be an exclusive T-cell mitogen in mice (Greaves & Janossy, 1972). Spleen cells taken from mice treated with hydantoin achieved the same degree of transformation with con A as those taken from control animals which were treated with solvent only. Thus T-cell function was apparently intact in these animals.

In the next experiment we used the SRBC plaque-forming cell technique (Jerne, Nordin & Henry, 1963) to measure the response to SRBC. Since this response is mediated by the collaboration of both B and T cells (Katz & Benacerraf, 1972) we measured both functions by this technique, but since the T-cell function was found to be preserved, the assay mainly reflected the B-cell function of the host.

We found that the response to SRBC in the treated animals was markedly reduced, which suggests that hydantoin mainly affected the B-cell function of these animals. We also found that hydantoin treatment significantly decreased the number of spleen cells of the treated mice. Since this effect was associated with a decrease in the humoral function, hydantoin probably selectively reduced the B-cell population of these spleens.

Since 1959 when the immunological surveillance theory was first proposed by Thomas and later by Burnet (1964), evidence that the immunological system has a regulatory function on tumour growth has accumulated. According to the prevalent concept the cellular (T-cell) function is usually responsible for tumour rejection (Hellström & Hellström, 1969;

Rollinghoff & Wagner, 1973), whereas the humoral (B-cell) function, by antagonizing the T-cell function, enhances tumour growth (Hellström & Hellström, 1970; Amos, Cohen & Klein, 1970). Because of the effects of hydantoin treatment on the immunological response of the host we wanted to check whether such treatment has any effect on carcinogenesis. For this purpose we used the model of urethane-induced lung ademonas in SWR mice. It has previously been shown that this model conforms to the general concept stated above, since depression of T cells in these animals leads to an increased incidence of lung adenomas (Trainin *et al.*, 1967; Trainin & Linker-Israeli, 1970). In the present experiments it was found that hydantoin treatment had the opposite effect, since it reduced the incidence of lung adenomas. It is possible that this tumour-protective effect of hydantoin is mediated by its modifying effect on the immunological system of the host.

The following possibilities are suggested: (a) hydantoin inhibits tumour growth by diminishing B-cell function with the result that fewer enhancing antibodies are produced and consequently there is less antagonism to T-cell function; (b) hydantoin enhances T-cell function either directly or secondarily to the decreased B-cell function; (c) hydantoin selectively eliminates a subpopulation of T cells which are able to enhance tumour growth. The existence of such populations has recently been established (Prehn, 1972; Ilfeld *et al.*, 1973). The possibility of a direct inhibitory effect of hydantoin on tumour cells cannot be excluded, although evidence for such a mechanism has not been reported.

Further research should be done in order to elucidate these problems. We are currently investigating the effect of hydantoin on transplanted tumours. In view of the tumour-protective effect of hydantoin, the increased incidence of benign and even malignant lymphomas observed in patients treated with hydantoin could not be explained on the basis of an immunological impairment.

Possibly they are part of a lymphoproliferative disorder which is secondary to the direct effect of hydantoin on the lymphatic cells. Hydantoin, by the selective elimination of several lymphoid subpopulations, may stimulate other subpopulations and thus either directly, or through the activation of latent viruses, may lead to the lymphoproliferative disorder.

We confirmed by our experimental model that hydantoin modifies the immune response of the host mainly by depressing its humoral function and we have shown that this effect is associated with an inhibitory effect on tumour growth.

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