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Life-Span Investigations for Carcinogenicity of some Immune-Stimulating, Immunodepressive and Neurotropic Substances in Sprague-Dawley-Rats

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Summary. In a long-term trial in a total of 858 Sprague-Dawley-rats of both sexes the possible carcinogenicity of 12 substances was investigated.
Complete Freunds' adjuvant which is among the immune-stimulating substances was found to decrease significantly the lifetime of the animals; whereas an increase in tumor incidences was not observed. Bacille Calmette-Guérin (BCG), human albumin, and vitamin-A-acid were neither carcinogenic, nor had they an influence on the survival times.
Rabbit-antirat lymphocytic serum which is a well-known immunodepressive agent caused a significant decrease in survival times, it was not found to be carcinogenic. The known carcinogenic properties of cyclophosphamide were verified. The applied dose of this compound significantly reduced the mean survival times of the animals. Hydrocortisone and amethopterin were found to have no carcinogenic effects.
Atropine, pilocarpine, nicotine, and phenyl-ethyl-barbituric acid (phenobarbital) which are known to have neurotropic effects were tested. Nicotine and phenobarbital were found to diminish the mean survival times. None of these neurotropic substances was found to be carcinogenic.

Chronische Carcinogenitätsuntersuchungen einiger immunstimulierender, immunodepressiver und neurotroper Substanzen bei Sprague-Dawley-Ratten

Zusammenfassung. In chronischen Langzeitversuchen an 858 Sprague-Dawley-Ratten beiderlei Geschlechts wurde die mögliche carcinogene Wirkung von 12 Substanzen untersucht.
Komplettes Freundsches Adjuvans, das zu den immunstimulierenden Substanzen gehört, zeigte eine signifikante Verminderung der Überlebenszeiten, jedoch keine Zunahme der Tumorraten. BCG, Humanalbumin und Vitamin A-Säure wirkten weder carcinogen, noch beeinflußten sie die Überlebenszeiten der Versuchstiere.
Kaninchen-Antiratten-Lymphozytenserum als immunodepressiv wirkende Substanz führte zu einer signifikanten Verkürzung der Überlebenszeiten,

wurde jedoch nicht als carcinogen befunden. Die bekannten carcinogenen Wirkungen von Cyclophosphamid wurden bestätigt. Die verabreichte Dosis dieser Verbindung führte zu einer deutlichen Verkürzung der mittleren Überlebenszeit der Tiere. Hydrocortison und Amethopterin zeigten keine chronisch toxischen Effekte bei Verabreichung über die gesamte Lebenszeit.
Atropin, Pilocarpin, Nikotin und Phenobarbital, die als neurotrop wirkende Substanzen bekannt sind, wurden untersucht. Nicotin und Phenobarbital verkürzten die Überlebenszeiten der Tiere. Keine der genannten neurotrop wirkenden Substanzen wurde als carcinogen befunden.

Introduction

In a previous publication (Schmähl et al., 1974) we reported on the influence of treatment with immune-stimulating, immunodepressive or neurotropic substances on the chemical carcinogenesis induced by ethylnitroso urea (ENU). The present study was aimed to show up chronically toxic effects of the above substances, which were applied during the induction period of ENU induced carcinomas.

Methods

Substances

The substances applied, suppliers, dosages and routes of application are presented in Table 1.

Table 1. Substances tested, suppliers, dosages and modes of application

Substance	Supplier	Dosage	Mode of application
Complete Freunds' adjuvant (c.F.A)	DIFCO-Laboratories, Detroit	0,2 ml/animal/week	s.c.
BCG-vaccine (BCG)	Behring-Werke, Marburg[a]	0,67 mg/kg/week	s.c.
Human albumine	Behring-Werke, Marburg[a]	85 mg/kg/week	i.p.
Vitamin A acid (vit. A.a.)	Hoffmann-La Roche, Grenzach[a]	5 mg/kg/week	i.p.
Rabbit-anti-rat lymphocytic serum (ALS)	own laboratory	1 ml titre 10/week	i.p.
Hydrocortisone	Farbwerke Hoechst, Frankfurt[a]	37,5 mg/kg/week	p.o.
Amethopterin (Methotrexat®)	Lederle, München[a]	0,625 mg/kg/week	i.p.
Cyclophosphamide (Endoxan®)	Asta-Werke, Brackwede[a]	4 mg/kg/week	i.p.
Atropine	Merck, Darmstadt	6 mg/kg/week	i.p.
Pilocarpine	Merck, Darmstadt	30 mg/kg/week	i.p.
Nicotine	Merck, Darmstadt	2 mg/kg/week	i.p.
Phenylethyl barbituric acid (Phenobarbital, Luminal®)	Bayer, Leverkusen[a]	2 mg/kg/week	i.p.

[a] We thank the above firms for providing us with the drugs.

Animals and Treatments

12 days old Sprague-Dawley-rats were at random divided into 13 groups of 36 males and 36 females each. They were maintained under conventional conditions and housed in macrolon cages in groups of 4 animals each. They were fed Altromin-pellets and tapwater ad libitum. Only those animals that lived longer than 200 days were considered in the study since at that time the first tumor arose in one of the groups (compare Table 2). 5% of the acute LD_{50} per week was used in all groups; this was found to be the maximal dose for juvenile animals (Schmähl et al., 1974). The rats were kept under observation up to their natural death. All animals were dissected and organs which appeared unusual were investigated histologically [1]. Statistical significance of the results was evaluated by the t-test and χ^2-test according to Cavalli-Sforza (1974) and Weber (1972).

Results

General Findings

The various treatments were well tolerated. Compared to the controls no differences in weight were seen during the first months. Later on a weight decrease was observed in those groups having reduced mean survival times. Animals that did not die from tumors died from intercurrent infections; enterocolitis, otitis media and pneumonia were most often seen. Treated animals as well as females of the control group developed adenofibromas mammae in an incidence of $11 \pm 2\%$ (total 52/460 animals). This applied virtually to all groups. This finding is in accord with the literature (Noble and Cutts, 1959).

Table 2 demonstrates the mean survival times and the number of animals that developed tumors. In Table 3 the induction periods and localizations of malignant tumors are given.

Controls

3 females out of a total of 33 developed adenocarcinomas of the mammary gland after a mean induction time of 717 ± 99 days. Among 36 males 1 animal developed a haemangioendothelioma of the liver after 637 days.

Immune-Stimulating Substances

11 of the animals treated with complete Freunds' adjuvant developed malignant tumors. In females we found 7 adenocarcinomas of the mamma and 2 subcutaneous fibrosarcomas. Adenocarcinomas of the mamma were predominantly observed, not only in this group but also in all other groups including the control. In males we detected 1 subcutaneous fibrosarcoma and 1 sarcoma of the prostate. The shortening of the life-spans was significant. 2 out of 66 BCG-treated animals developed tumors. One of them had a phaeochromocytoma, the other a had a haemangioendothelioma in the abdominal cavity. The life-spans were identical with those of the untreated controls. Human albumin or vitamin A-acid did not significantly alter tumor incidence or life-span.

Immunodepressive Substances

While in the group treated with rabbit-antirat lymphocytic serum no increase in tumor incidences was observed, there was a significant reduction of life-spans. Hydrocortisone and amethopterin had no effect on tumor incidences or mean

[1] We thank Prof. Dr. Kl. Goerttler (Institut für experimentelle Pathologie am Deutschen Krebsforschungszentrum) for histological examinations.

Table 2. Tumor incidence

Substance	Number of animals		Mean survival time (weeks) p		Animals bearing tumors			χ^2
			t_{50}		No	%	Sp[a]	
Control	69	♂ 36	96±17	—	1	3	±3	—
		♀ 33	94±15	—	3	11	±5	—
Immune-stimulating substances								
complete Freunds' adjuvant (c.F.a.)	69	♂ 36	87±17	<0,03	2	6	±4	2,69; p>0,05
		♀ 33	87±20	<0,05	9	27	±8	
BCG-vaccine (BCG)	66	♂ 34	87±13	>0.05	0	0	—	—
		♀ 32	86±15	>0.1	2	6	±4	—
human albumine	63	♂ 33	90±17	>0.1	1	3	±3	—
		♀ 30	92±12	>0.5	2	7	±5	—
Vitamin A acid (vit. A. a.)	68	♂ 33	90±15	>0.1	1	3	±3	—
		♀ 35	91±22	>0.4	2	6	±4	—
Immunodepressive substances								
Rabbit-antirat lymphocytic serum	67	♂ 35	81±16	<0.005	0	0	—	—
		♀ 32	82±22	<0.02	1	3	±3	—
Hydrocortisone	69	♂ 29	94±15	>0.5	0	0	—	—
		♀ 40	96±16	>0.6	2	5	±3	—
Amethopterin (Methotrexat®)	61	♂ 30	90±14	>0.5	0	0	—	—
		♀ 31	101±15	>0.1	1	3	3	—
Cyclophosphamide (Endoxan®)	68	♂ 32	87±18	<0.05	5	16	±6	8.31; p<0.05
		♀ 36	85±16	<0.02	12	33	±8	
							χ^2	♂ = 2.06; p>0.05
							χ^2	♀ = 4.61; p<0.05
Neurotropic ubstances								
Atropine	61	♂ 30	88±19	>0.05	0	0	—	—
		♀ 31	89±14	>0.3	3	10	±5	—
Pilocarpine	68	♂ 34	88±26	>0.05	1	3	±3	—
		♀ 34	82±25	>0.05	4	12	±6	—
Nicotine	67	♂ 35	85±16	<0.01	2	6	±4	—
		32	86±17	<0.05	2	6	±4	—
phenylethylbarbituric acid (Luminal)	62	♂ 32	80±23	<0.02	0	0	—	—
		♀ 30	85±17	<0.05	2	7	±5	—

[a] Standard deviation of percentage of frequency.

survival times. In the cyclophosphamide-treated animals a significantly increased tumor rate was observed. Though the number of tumors in females (12/36 = 33%) was increased compared to males (5/32 = 16%), this sex-dependent difference was not significant in comparison to that observed in the control. The life-span was reduced in both males and females.

Neutropic Substances

The application of atropine and pilocarpine had no influence on tumor frequencies and mean life-spans. Nicotine and phenobarbital did not influence tumor incidences, but reduced life-spans.

Discussion

The present investigations seemed to be important because the substances tested are wide-spread in our environment or are commonly used as drugs.

None of the immune-stimulating substances had carcinogenic activity. The fact that complete Freunds' adjuvant did not significantly increase the cancer incidence is supported by the work of Rubin (1971) who failed to show increased tumor induction in 30 DBA/2 mice with this compound. In the present rat study mainly tumors of the mammary gland were seen (7/11 = 64% of total tumors). This finding seems to be remarkable since combination treatment with ENU (transplacental application) and complete Freunds' adjuvant (postnatal application) revealed a significant increase in mammary tumors compared to the application of ENU alone (Schmähl et al., 1974). The question of a possible increase in the rate of spontaneous neoplasms by complete Freunds' adjuvant needs further investigation in a larger animal sample. The shortening of the life expectancies was caused by an increased rate of infections.

In the past many attempts have been made to demonstrate a possible tumor-suppressing effect of BCG. This compound is increasingly used in clinical immunotherapy (Ott et al., 1974). As far as we know BCG was examined in the present investigation for the first time in a large animal sample during lifetime. No significant differences were seen between BCG-treated rats and controls. Human albumin had no influence on tumor formation, life expectancies and body weight curves. In the tables (2 and 3) vitamin A-acid listed among the immune-stimulating agencts because of the current discussion on its possible influence upon the immune system (Dresser, 1968; Bollag, 1972; Tannock et al., 1972). No vitamin A intoxication was observed when a dosage of 5 mg/kg/week was applied. The lack of a distinctly systemic response is in good accord with the results of Rogers et al. (1972) and Cone and Nettesheim (1973).

In the group with immunodepressive treatment rabbit-antirat lymphocytic serum was found to most markedly affect the life-span. The percentage of infections was increased, though the rats were handled with particular care. This substance showed no influence on tumor formation. Gleichmann and Gleichmann (1973) reported comparable results in mice. The present findings are in good agreement with a work of Rubin (1971) with antithymocytic serum, who reported no differences in DBA/2 mice compared to the controls. The higher predisposition of animals treated with rabbit-antirat lymphocytic serum to infectious diseases diminished the mean survival times of the animals. No relevant effects in hydrocortisone-treated animals were noted. There was no correlation observed between immunosuppressive effects and carcinogenicity of hydrocortisone. No differences were seen between amethopterin-treated animals and controls. The life-span was not shortened. This finding is in contrast with the results of Schmähl and Osswald (1970). It is probable that this effect is dose-related since in the present study the dosage was reduced by $\sim 30\%$.

The lack of carcinogenic potential of amethopterin confirms the results of earlier investigations in rats by Schmähl and Osswald (1970) and in mice and hamsters by Shubik and Rustia (1973). The present findings are inconsistent with those described by Roschlau and Justus (1971), who reported a clear carcinogenic

Table 3. Induction period and localization of malignomas

	Substance	No.	Mamma		Ind. P. days	Leucosis	Ind. P. days	Suprarenal gland	Ind. P. days	Others
Control	—	4	♂							1[a]
			♀	3	717 ± 99					
Immune-stimulating substances	complete Freunds' adjuvant (c.F.a.)	11	♂							2[b,c]
			♀	7	567 ± 222					2[d,e]
	BCG-vaccine (BCG)	2	♂					1	711	
			♀							1[f]
	human albumine	3	♂					1	789	
			♀	1	713					1[g]
	vitamin A acid (vit.A.a.)	3	♂			1	345			
			♂	2	564 ± 274					
Immuno-depressive substances	rabbit-antirat lymphocytic serum (ALS)	1	♂							
			♀	1	433					
	hydrocortisone	2	♂							
			♀	1	603					1[h]
	amethopterin (Methotrexat®)	1	♂							
			♀	1	320					
	cyclo-phosphamide Endoxan®	17	♂					3	642 ± 152	2[i,k]
			♀	10	558 ± 103			1	722	1[l]
Neurotropic substances	atropine	3	♂							
			♀	2	644 ± 133	1	402			
	pilocarpine	5	♂			1	261			
			♀	3	497 ± 283					1[m]
	nicotine	4	♂		583	1	426			
			♀	2	545 ± 117					
	phenylethyl-barbituric acid (Luminal®)	2	♂					1	682	
			♀							1[n]

[a] Haemangioendothelioma of the liver (637 days).
[b] Subcutaneous fibrosarcoma (568 days).
[c] Sarcoma of the prostate (678 days).
[d] Subcutaneous fibrosarcoma (568 days).
[e] Subcutaneous fibrosarcoma (774 days).
[f] Haemangioendothelioma of the abdominal cavity (731 days).
[g] Uterine sarcoma (793 days).
[h] Sarcoma of the lung (772 days).
[i] Subcutaneous fibrosarcoma (475 days).
[k] Haemangioendothelioma of the salivary gland (743 days).
[l] Sarcoma in the region of the urinary bladder (596 days).
[m] Carcinoma of the uterus (683 days).
[n] Carcinoma of the nasal cavity (220 days).

activity of amethopterin in mice. Cyclophosphamide was established to be carcinogenic and to diminish the life-span. Comparable results were reported by Schmähl (1967), Schmähl and Osswald (1970) and Roschlau and Justus (1971). Schmähl and Osswald applied 13 mg/kg/week (p.o.) to rats and induced carcinomas in 17% of the animals. In the present experiments in 25% malignant tumors were found, though we injected only 4 mg/kg/week (i.p.). It is possible that the mean survival time in the animals given the greater dosage of cyclophosphamide was reduced to 72 weeks compared to 86 weeks in the lower dosage group. It is probable that the reduction of the life expectancies reduces the tumor rate (Schmähl and Osswald, 1970). The differences in strains and mode of application may also play a role (Schmähl, 1970). The high rate of mammary adenocarcinomas in females seen in these studies with complete Freunds' adjuvant was also observed in a combination treatment of ENU (transplacental application) and cyclophosphamide (postnatal application) (Schmähl et al., 1974). Since the immunogenic effects of these two compounds are quite different the possible increasing effect on the formation of mammary tumors cannot be explained by immunological reactions.

Atropine and pilocarpine neither showed any influence on the life expectancy nor had they carcinogenic effects. Nicotine proved to be noncarcinogenic in these studies. The carcinogenic activity of tobacco smoke condensate was often discussed and in part led back to nicotine. Schmähl and Osswald (1968) failed to verify the carcinogenicity of cotinine—a metabolic product of nicotine—found by Truhaut et al. (1964). Further investigations are in progress to elucidate whether the life-shortening effect of nicotine depends on changes in the cardiovascular system (Larson et al., 1961; Chevalier and Kersten, 1970; Wynder and Hoffmann, 1972; Dontenwill et al., 1973). Phenobarbital had no influence upon carcinogenicity. This result is in agreement with findings in rats reported by Peraino et al. (1971) and in mice (Kunz et al., 1969). The life-span in phenobarbital-treated animals was reduced compared to that of the untreated controls. We have so far no explanation for this finding. Reduction of the deposit of fat as described by Kunz et al. (1969) was not found, nor did we note a reduced uptake in food.

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