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The effect of vitamin A deficiency on the initiation and postinitiation phases of benzo(a)pyrene-induced lung tumourigenesis in rats

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Summary The present investigation shows the effect of vitamin A deficiency on the initiation and postinitiation phases of benzo(a)pyrene-induced lung carcinogenesis in male Wistar rats. Lung tumours were induced by giving three intratracheal instillations, one week apart, of 10 mg benzo(a)pyrene per instillation. Maximum tumour incidence (100%) and tumour burden per rat was found in rats which were kept on vitamin A deficient diet for 4 weeks prior to the first administration and 8 weeks after the last administration of benzo(a)pyrene. Rats in which vitamin A deficiency was terminated after the last administration of the carcinogen had 83% tumour incidence, whereas corresponding control pairfed animals had 39% incidence of tumours. These data represent the values obtained 32 weeks after the last administered dose of the carcinogen and indicate the role of vitamin A, both in the initiation as well as in the postinitiation phases of lung carcinogenesis.

Today vitamin A is well known for its importance in general growth and differentiation of epithelial tissues and its deficiency has been shown to lead to metaplastic changes in the epithelia of the respiratory, urogenital and gastrointestinal tracts (Harris *et al.*, 1972; Wolback, 1954). These metaplastic changes appeared by light microscopy to be morphologically similar to the changes found in certain precancerous lesions caused by carcinogen administration (Harris *et al.*, 1972; Hayes, 1971). This similarity between histological features of epithelial tissues of vitamin A deficient animals and certain precancerous lesions was the starting point for further investigations in this area. Several investigators have confirmed and extended the early observations and have shown a prophylactic effect of vitamin A on the development of carcinogen-induced epithelial tumours (Bollag & Matter, 1981; Lotan, 1980; Nettesheim & Williams, 1976). However, supplementation with retinoids as a prophylactic agent has resulted in variable effects and these are being related to the problem of distribution and toxicity of vitamin A (Sporn, 1976).

Nettesheim *et al.* (1976) investigated the influence of retinyl acetate supplementation at high dose on the initiation and postinitiation phase of 3-methylcholanthrene induced preneoplastic lung nodules in rats. In this study the incidence of metaplastic lung nodules was found to be 3% in the combined high retinyl acetate dose groups as compared with 42% in the low retinyl acetate dose group, and it was concluded that retinyl acetate treatment had a significant inhibitory effect on the postinitiation phase of preneoplastic lung nodules in rats. However, the effect of vitamin A deficiency on the initiation and postinitiation phases of carcinogenesis has not been evaluated. Therefore, the primary objective of the present investigation was to study and compare the effect of benzo(a)pyrene, a potent environmental lung carcinogen, on tumour incidence in vitamin A deficient rats, both at the initiation and postinitiation phases of pulmonary carcinogenesis.

Materials and methods

Male weanling rats (45–60 g) of our Institute's colony (Wistar derived) were used. They were maintained for 4 weeks on vitamin A deficient casein based diet composed of vitamin-free casein (20%), corn starch (39.8%), dextrose (30%), refined peanut oil (5%), salt mix (4%), choline chloride (0.2%), and vitamin A-free vitamin mix (1.0%). An identical diet supplemented with retinyl acetate (20,000 IU kg^{-1}) was given to control animals. Animals had free access to water and food during the first 4 weeks, thereafter during the next 2 weeks the amount of diet supplied to the control rats was reduced in proportion to that consumed by the deficient group.

Equal amounts of benzo(a)pyrene (BP) and Fe_2O_3 were mixed in a mortar and ground together

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for 2~4h, yielding a fine, homogeneously distributed mixture of BP and Fe_2O_3 (1:1) by weight. This mixture was prepared freshly every week before instillation and was then placed in a 25ml flat-bottomed conical flask to which 0.15M NaCl (sterile) was added to make the final concentration of the mixture 50mg ml^{-1}. After maintaining rats on their respective diets for 4 weeks, a total of three intratracheal instillations were given to each rat with a gap of one week between each administration. Each instillation consisted of 20mg mixture of BP-Fe_2O_3/0.4ml/rat. Animals were instilled under light ether anaesthesia. One day after the third instillation, rats from the vitamin A deficient group were randomly divided into two groups i.e. groups 2 and 3, to receive diet either containing 20,000 IU kg^{-1} or 1000 IU kg^{-1} of retinyl acetate respectively. Rats from group 3 were maintained on vitamin A deficient diet (1000 IU kg^{-1}) for a further 8 weeks after the last intratracheal instillation of the carcinogen and then were shifted to normal diet containing 20,000 IU retinyl acetate kg^{-1} diet. In addition to BP-Fe_2O_3 treated rats, weight matched rats from normal and vitamin A deficient groups were given 30mg of Fe_2O_3 intratracheally (10mg per instillation, instillations one week apart) to serve as controls. Rats were weighed and food consumption was determined twice a week.

Animals were checked twice daily. Some rats from each group died during study duration and were found cannibalized or exhibited tissue autolysis. These were not evaluated in the study. Twenty or more rats from each group were sacrificed at different time intervals (24, 28 and 32 weeks) after the last instillation of carcinogen. In each case, lungs were removed along with the trachea. Squamous nodules in lungs, >0.5cm or <0.5cm were scored as end points in the study. Lungs were fixed in 10% neutral buffered formalin, processed for routine histological staining with hematoxylin and eosin and examined microscopically.

Liver vitamin A was estimated by the method of Dugan *et al.* (1964). Statistical analysis of differences was done by Student's *t* test.

Results

Table I presents the body weight and hepatic vitamin A content of control and vitamin A deficient groups at various time intervals in the study. Initially, all groups were weight matched at 49±6g per rat. Weight gain in group 3 was slightly less than that in the other groups, but with the overlap of standard deviation between group 3 and other groups, this difference was not significant. Hepatic vitamin A content in rats maintained on vitamin A deficient diet for 4 weeks was reduced to less than 2 μg g^{-1} and was found to be 5 μg g^{-1} in the group of rats (Group 3) which was maintained on a low vitamin A containing diet for a further 8 weeks after the final intratracheal instillation of BP. However, hepatic vitamin A content in all the groups sacrificed on 24, 28, and 32 weeks after the last instillation of the carcinogen, did not show any significant difference.

Table II shows tumour incidences in the different groups, sacrificed at weeks 24, 28 and 32 after the

Table I Mean body weight and hepatic vitamin A content of rats maintained on diets with or without vitamin A

	Average bodyeight (g) at						*Hepatic vitamin A content (μg g^{-1})[a] at*				
*Group**	*4 wk*	*6 wk*	*14 wk*	*30 wk*	*34 wk*	*38 wk*	*4 wk*	*14 wk*	*30 wk*	*34 wk*	*38 wk*
1 (90)	148	170	251	300	315	320	90±12 (4)	74±13 (4)	71±14	66±11	69±13
2 (85)	142	162	242	285	303	302	1.8±0.5 (4)	66±11 (4)	70±12	65±9	64±7
3 (85)	—	—	233	292	305	302	—	4.2±0.6 (4)	62±9	63±8	62±9
4 (70)	151	175	264	315	330	341	87±10 (4)	76±12 (4)	75±14	74±9	71±12
5 (70)	144	169	249	298	312	318	1.6±0.6 (4)	6.4±1.2 (4)	68±10	70±13	68±11

[a]Mean±s.d. Number of rats used for estimating vitamin A content in liver at 4 and 14 weeks is given in parentheses. Groups 1 through 3 received three intratracheal instillations, one week apart, of 10mg BP at 4 weeks after rats were maintained on diets either containing 20,000 IU kg^{-1} of retinyl acetate (group 1) or on vitamin A deficient diet (groups 2 and 3). At 6 weeks group 2 was shifted to diet containing 20,000 IU of retinyl acetate kg^{-1}, whereas group 3 was fed diet containing 1000 IU of retinyl acetate kg^{-1} till 14 weeks and then this group was also shifted to the diet given to groups 1 and 2. Groups 4 and 5 were maintained on diets given to groups 1 and 3 respectively and received Fe_2O_3 instead of BP+Fe_2O_3 so as to serve as controls. Weeks 30, 34 and 38 mentioned here corresponds to weeks 24, 28 and 32 after the termination of BP+Fe_2O_3 or Fe_2O_3 treatment.

*Values in parentheses are the number of animals in each group at first intratracheal instillation of BP+Fe_2O_3 or Fe_2O_3.

Table II Lung tumour incidence after intratracheal instillation of 30 mg BP in normal and vitamin A deficient rats

		24 wk				28 wk				32 wk			
		Rats bearing tumour with mean diameter (%)				Rats bearing tumour with mean diameter (%)				Rats bearing tumour with mean diameter (%)			
Group	Mortality*	Incidence (%)	<5 mm	>5 mm	T/TBA (ratio)	Incidence (%)	<5 mm	>5 mm	T/TBA (ratio)	Incidence (%)	<5 mm	>5 mm	T/TBA (ratio)
1	6	30 (20)	20	10	1.3	40 (25)	24	16	1.2	39 (31)	23	16	1.6
2	9	60 (20)	20	40	2.3	75 (20)	25	50	2.1	83 (23)	35	48	2.6
3	11	80 (25)	24	56	2.5	100 (20)	10	90	2.6	100 (25)	12	88	3.0
4	2	0 (20)	—	—	—	0 (20)	—	—	—	0 (20)	—	—	—
5	2	0 (20)	—	—	—	0 (20)	—	—	—	0 (20)	—	—	—

Groups 1 through 3, after being maintained on diet containing 20,000 IU retinyl acetate kg^{-1} (group 1) or on vitamin A deficient diet (groups 2 and 3), at 4 weeks received three intratracheal instillations, one week apart, of 10 mg BP and were killed 24, 28 and 32 weeks later. Rats in groups 4 and 5 maintained on diets as groups 1 and 3 respectively received 30 mg of Fe_2O_3 in place of BP + Fe_2O_3 and served as controls. There were 20 or more rats per group.

*Number of animals which died during 34 week period after the first intratracheal instillation of BP + Fe_2O_3 or Fe_2O_3. These animals were not included in tumour incidence studies.

last BP-Fe_2O_3 intratracheal instillation. Tumours were verified histopathologically to be mainly squamous cell carcinomas (Figure 1). Detailed typing of tumours was not carried out. The majority of the lung tumours were roughly spherical and symmetrical (Figure 2). The mean tumour diameter and number of tumours per tumour-bearing animal (TBA) indicate the relative degrees of tumour burden in the various groups. Fe_2O_3 treated groups (Groups 4 and 5) in which rats were maintained on the same diets as the rats in groups 1 and 3 respectively, did not reveal any tumours even after 32 weeks. At 24 weeks, rats from group 1 had the lowest tumour incidence and T/TBA ratio, whereas these parameters were higher in groups 2 and 3, showing maximum incidence in group 3 which was kept on low vitamin A diet after the last BP-Fe_2O_3 dose. At 28 weeks, tumour incidence increased in all three groups but it still remained far below that in group 1 as compared to groups 2 and 3. Rats in group 3 showed 100% tumour incidence, however the T/TBA ratio in all the groups remained almost the same as it was at 24 weeks. At 32 weeks, group 1 did not show any further increase in tumour incidence, whereas it increased from 75% to 83% in group 2, and was 100% in group 3. At this point the T/TBA ratio was increased in all groups, thereby revealing an increased tumour burden per rat. The mortality due to intratracheal instillations of BP-Fe_2O_3 was found

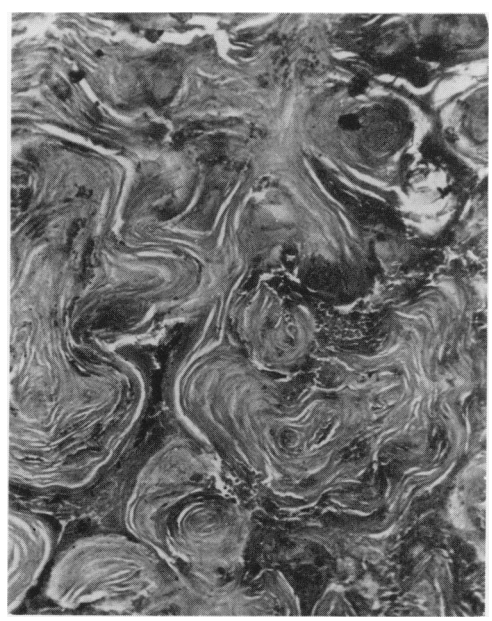

Figure 1 Well differentiated squamous cell carcinoma with horny pearls. H & E (×33).

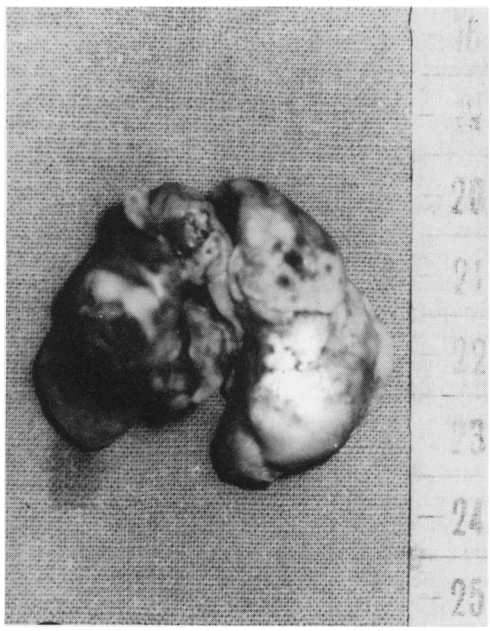

Figure 2 Gross appearance of a large squamous cell tumour in the middle portion of the right lung and with multiple tumours in the left lung. Rats received three intratracheal instillations, one week apart, of 10 mg BP. Experimental period 28 weeks.

to be maximal in animals kept on vitamin A deficient diet.

Discussion

The role of vitamin A deficiency in tumourigenesis has been studied by several investigators (Nettesheim & Williams, 1976; Rogers *et al.*, 1973; Sporn & Newton, 1981). However, the exact mechanism by which vitamin A influences this process has not been well delineated. In our present study, on periodically killing of rats treated with BP, we have observed that the tumour incidence in vitamin A deficient animals was more than double that in normal pairfed rats. This enhanced susceptibility of the lung to BP-induced carcinogenesis in vitamin A deficiency could possibly be related to a number of factors. Firstly, as changes inflicted by vitamin A deficiency have been shown to be similar to precancerous lesions, vitamin A deficiency may therefore, elevate the initiation and progression of tumourigenic process in animals exposed to chemical carcinogens, as has been observed in our study. Secondly, predisposition of the lung to chemical carcinogenesis may be due to an altered pattern of enzymes involved in the metabolism of these carcinogens, both in lung and liver. BP, like many other polycyclic aromatic hydrocarbons, requires metabolic activation by the action of mixed-function oxidases and vitamin A status has been shown to alter the activities of these enzymes (Becking, 1973; Miranda *et al.*, 1979; Siddik *et al.*, 1980). Our earlier studies in this area (Dogra *et al.*, 1982; Dogra *et al.*, 1983) suggested that imbalance between activation and conjugation processes in lung and liver in vitamin A deficiency might result in an increased yield of carcinogenic metabolites and their slow elimination from the body. This might, in turn, produce a favourable environment for the enhanced binding of reactive carcinogenic metabolites with critical cellular macromolecules like DNA. Earlier, we observed enhanced binding of [^{3}H]-BP to lung DNA in vitamin A deficient rats in *in vivo* and *in vitro* studies (Dogra *et al.*, 1984). These studies give support to the argument that mixed-function oxidase enzymes play a very crucial and important role in determining organ susceptibility to tumourigenesis.

In the present study vitamin A deficiency has been found to enhance the process of tumourigenesis, both at the initiation and post-initiation phases. However, the effect of vitamin A deficiency was more prominent at the initiation phase as rats from group 2, killed at 32 weeks after the termination of BP instillation had 83% tumour incidence compared to 39% in normal pairfed rats (group 1). Moreover, the tumour burden per rat was also greater in vitamin A deficient rats than in normal pairfed rats. Furthermore, the enhancing effect of vitamin A deficiency on carcinogenesis during the postinitiation phase was evident from the data on group 3 as rats which were kept on vitamin A deficient diet during and after the challenge with BP, not only had a higher percentage tumour incidence but also the tumour burden per rat was even greater. Also, this higher incidence of tumours was achieved earlier than in the other two groups (groups 1 and 2). This could be due to lack of vitamin A as an essential factor for the normal differentiation of cells during the post initiation phase of tumourigenesis. The other responsible factor could be the slow elimination of carcinogen (given as BP-Fe_2O_3) from the lung. Therefore, an effect of vitamin A deficiency on the metabolism of the carcinogen and the penetration of the carcinogens into the target cells could not be ruled out.

In conclusion this study has revealed that vitamin A deficiency enhances the process of lung carcinogenesis induced by benzo(a)pyrene both at the initiation and postinitiation phases. However, the more pronounced effect was seen at the initiation stage.

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