The Influence of Retinyl Acetate on the Postinitiation Phase of Preneoplastic Lung Nodules in Rats

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SUMMARY

The effects of retinyl acetate (RA) on the development of carcinogen-induced metaplastic lung nodules were investigated. Four mg of 3-methylcholanthrene were administered intratracheally to F344 rats maintained on a vitamin A-free diet and receiving 31.4 nmoles of RA per week by intragastric intubation. At 3, 5, and 10 weeks after intratracheal instillation of 3-methylcholanthrene, one group of 24 rats each was started on a weekly dose of 31,400 nmoles of RA; a control group was continued on the low-RA dose. Fifty-two weeks after 3-methylcholanthrene injection, the incidence of metaplastic lung nodules was found to be 3% in the combined high-RA-dose groups as compared with 42% in the low-RA-dose group. It is therefore concluded that RA has a significant effect on the postinitiation phase of preneoplastic lung nodules in rats.

INTRODUCTION

In recent years possible anticarcinogenic effects of vitamin A and vitamin A analogs have attracted increased attention (for review, see Ref. 2). It has been reported that carcinogen metabolism and carcinogen binding to DNA are altered by vitamin A compounds (10—12, 17), that the early cellular response to chemical carcinogens is inhibited in in vitro systems, and that induction and progression of preneoplastic and neoplastic lesions are inhibited or retarded in vivo (3, 5—8, 13, 14, 18, 19, 23).

We have recently reported that the development of 3-MCA\(^3\)-induced squamous cell nodules in the lung, from which squamous cell carcinomas have been shown to develop (21), is significantly inhibited in rats maintained on a high RA intake as compared with rats on low RA intake (6). These studies did not allow us to differentiate between effects on induction and effects on growth and progression of such lesions. The experiments reported here are designed to determine whether the development and growth of nodular squamous metaplasias in rat lungs could be influenced by high levels of RA achieved and maintained at different times after carcinogen administration. The advantages of the experimental system used are that a tumorigenic dose of carcinogen can be administered in 1 or 2 injections and that the carcinogen-induced metaplastic nodules can be readily observed, counted, and measured at different times after carcinogen administration (6, 21).

MATERIALS AND METHODS

Carcinogen. 3-MCA (Aldrich Chemical Co., Milwaukee, Wis.) was ground with a mortar and pestle and suspended in 0.9% NaCl solution containing 0.2% gelatin. The suspension was adjusted to a final concentration of 10 mg/ml of medium. The average crystal size of 3-MCA was 4.3 \(\mu\)m as determined by light microscopy. Carcinogens of smaller particle size had lower carcinogenic potency.

Carcinogen Extraction and Determination. Rats were killed at different times after i.tr. injection of 3-MCA. Their lungs were dried at 65°, minced in a filter-papier Soxhlet thimble, and extracted for 6 hr with 100 ml of spectrograde benzene (Burdick and Jackson Laboratories, Muskegon, Mich.). The spectrum of the resulting extract was obtained in an Acta II spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.), and the \(A_{295}\) nm was compared with that of a 3-MCA standard. The sample spectra were run after setting zero suppression with a lung blank at 295 nm.

Preparation and Storage of RA. Crystalline RA obtained as a gift from Hoffmann-La Roche (Nutley, N. J.) was stored under nitrogen gas at —60° in the dark. Solutions were prepared once a week, and RA was dissolved in chloroform and brought to volume with cottonseed oil (General Biological Chemicals, Inc., Chagrin Falls, Ohio); the final amount of chloroform did not exceed 1.2% (v/v). RA solutions were stored under nitrogen gas at 4° in the dark.

Determination of Liver and Serum Vitamin A. A modification of the procedure of Bligh and Dyer (1) was used to extract vitamin A from the livers of rats. Samples of liver (65 to 100 mg, wet weight) were homogenized with four 3.75-ml aliquots of chloroform:methanol (1:1). Each aliquot was transferred to a glass scintillation vial, and the contents were thoroughly shaken after the addition of 7 ml of water. After the vials were centrifuged for 10 min at 1700 rpm, we removed suitable aliquots of the chloroform layer for vitamin A assay by the trifluoroacetic acid procedure (9). When the vitamin A content of livers was expected to be low, liver extracts were assayed undiluted; 5.0 ml of the chloroform layer from these undiluted liver extracts were evaporated down and rediluted in 1.5 ml of chloroform. This procedure allowed detection of vitamin A above 0.006 nmole/mg, wet weight, of liver. Recovery was 94 to 98%
when 31 to 115 nmoles of retinyl palmitate were added to 40 mg of liver.

For determination of serum vitamin A, the trifluoroacetic acid procedure (4) was used with modifications described elsewhere. All procedures were carried out under General Electric gold fluorescence light.

Tissue Preparation and Scoring of Metaplastic Nodules. At 52 weeks after i.tr. injection of carcinogen or vehicle, all rats were killed and their lungs were fixed in buffered formaldehyde and examined under a dissecting microscope equipped with an ocular micrometer. Metaplastic lesions were easily recognized because of their slightly yellowish color and firm consistency. For size determination the longest diameter was measured, since most lesions were oval or near-spherical. The scored nodules were dissected out of the lungs and processed for histology.

Experimental Design. The design of the main study is summarized in Chart 1. Specific-pathogen-free female F344 rats were raised and maintained in a barrier facility on pasteurized 5010C Purina laboratory chow until weaning (i.e., 3 weeks of age). From weaning on, all rats received pasteurized vitamin A-free diet (TD 69389, General Biochemicals). Beginning at 5 weeks of age, all rats received 15.7 nmoles (5 µg) of RA twice a week by i.g. administration. At 7 weeks of age, 111 anesthetized (20) rats received 2 i.tr. injections, 24 hr apart, of 2 mg of 3-MCA. Twelve others received suspending medium without carcinogen.

At 3, 5, and 10 weeks after administration of carcinogen, 5 rats each were killed to determine the number of lung surface nodules detectable with the dissecting scope as well as the histological type of lung lesions present (6 sections/lung lobe, 200 µm apart). At the same time the weekly RA supplement was raised to 2 doses of 15,700 nmoles (5 µg) of RA twice a week by i.g. administration. At 7 weeks of age, 111 anesthetized (20) rats received 2 i.tr. injections, 24 hr apart, of 2 mg of 3-MCA. Twelve others received suspending medium without carcinogen.

Results

Prior to this study, several experiments in our laboratory had established: (a) that, in rats maintained on 31.4 nmoles of RA per week, 4 mg of 3-MCA induced scorable metaplastic lung nodules in 48% of i.tr. injection-treated rats while 2 mg of 3-MCA induced such lesions in only 18% of rats as determined at 6 months after carcinogen administration; (b) that a dose of 5 mg of 3-MCA given i.tr. resulted in death from lung cancer in 50 to 60% of the animals at 100 to 120 weeks and that the induction of metaplastic nodules as well as of squamous cell carcinomas in the lungs of rats with 3-MCA was a carcinogen-dose-dependent phenomenon; (c) that 31.4 nmoles of RA per week were sufficient to maintain female F344 rats without clinical or histopathological signs of vitamin A deficiency; (d) that 31,400 nmoles of RA per week did not cause signs of hypervitaminosis within 52 weeks of study; and (e) that no disease of infectious or other nature (not related to the carcinogen injection) occurred under the experimental conditions.

With this information at hand we set out to determine whether high RA levels would suppress the development and growth of lung squamous nodules induced by a dose of 4 mg of 3-MCA. At 3, 5, and 10 weeks after i.tr. administration of 3-MCA, 5 rats were killed and their lungs were examined macroscopically as well as microscopically to determine what carcinogen-induced lesions had developed at the time of change in RA levels. At 3 weeks, no metaplasias were detected histologically; only small granulomas associated with mildly hyperplastic terminal bronchioles [as previously described (21)] were seen in 1 of 5 rats, but they were too small to be seen with the dissecting scope. At 5 weeks, small metaplastic lesions were seen histologically in 3 of 5 rats. At 10 weeks, squamous metaplasias were detected in 5 of 5 rats histologically, only 1 of which was large enough to be detectable under the dissecting scope. The most advanced lesions detected are pictured in Fig. 1. The livers of these rats were analyzed for retinyl ester storage. Only 3 of the 15 rats had detectable retinyl ester levels (0.011, 0.011, and 0.303 nmoles/mg liver).

Table 1 shows that there were no noticeable differences in body weights and growth rates between the different groups maintained for 1 year on different RA levels. The growth rate is typical for female F344 rats maintained in our colony on regular diet, confirming the histological observations that the RA levels used cause neither toxicity nor deficiency. At the termination of the experiment, i.e., 52 weeks after carcinogen injection, liver and serum retinol and retinyl ester levels were determined in 6 to 9 rats each of
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Group 3 (31,400 nmoles of RA per week) and Group 5 (31.4 nmoles of RA per week) 3 days after the last RA administration. The average amount of retinol and retinyl esters in the liver and serum was 43.0 nmoles/mg of liver and 300 nmoles/100 ml of serum, respectively, in the rats receiving 31,400 nmoles of RA per week. In the rats receiving 31.4 nmoles of RA per week, the liver levels were below detectable levels and the serum contained 43.3 nmoles/100 ml. In comparison, rats maintained for 1 year on the regular stock diet (pasteurized Purina 5010C) have an average liver storage of 3.8 nmoles/mg and a serum concentration of 105 to 175 nmoles/100 ml of retinol and retinyl esters.

The lung nodule counts of the rats given i.tr. injections of 3-MCA are summarized in Table 2. It can be seen that only an occasional metaplastic nodule was detected under the dissecting scope in rats shifted to high levels of RA 3 to 10 weeks after carcinogen injection, the overall incidence being 3%. In contrast, rats maintained on a low RA dose showed an incidence of 42% (3 animals having 2 lung nodules each). Thus the incidence of carcinogen-induced lesions is 14-fold higher in the low-RA group than in the excess-RA group. Microscopic examination of the excised lung nodules showed that all scored lesions were nodular squamous metaplasias (Fig. 2) with keratinized centers. In most cases the epithelium was well differentiated with no, or only mild, cellular atypia (Fig. 3). In a few instances, evidence of resorption of the keratin by multinucleated cells was evident. While the lesions are composed of mostly keratinizing squamous cells, nests of less-differentiated, densely packed cells with high nuclear-cytoplasmic ratio (Fig. 4) were occasionally seen when multiple sections of individual nodules were made. The morphological features of the metaplastic nodules were not noticeably influenced by RA. The rats of Group 5 (no carcinogen, low RA) were also killed at 52 weeks after the other groups had received carcinogen. Multiple histological sections were prepared from salivary glands, nasal cavity, larynx, trachea, lungs, kidneys (renal pelvis), bladder, and liver to determine whether any lesions indicative of vitamin A deficiency, in particular squamous metaplasias, could be found. No macroscopic or microscopic pathological lesions were detected. The dose of 31.4 nmoles of RA per week must be considered "subnormal," but adequate to prevent overt signs of vitamin A deficiency in spite of the absence of detectable retinyl ester storage in the liver.

To determine what fraction of the initially administered 3-MCA dose was persisting in the lungs at the time the high RA intake levels were initiated, a carcinogen clearance study was performed. We attempted to duplicate the conditions of the previous study as closely as possible. Eighteen rats were maintained on 31.4 nmoles of RA per week. At 7 weeks of age they were given i.tr. injections of 2 mg of 3-MCA on 2 subsequent days. The amount of 3-MCA remaining immediately after and at 3, 7, 21, 35, and 70 days after the 2nd carcinogen instillation was determined. The data are summarized in Chart 2 and are expressed as percentage of 3-MCA remaining, based on the amount detected immediately after (i.e., within 15 min) the 2nd dose of carcinogen.
The experiments presented indicate that RA profoundly influences the response of respiratory tract epithelium to carcinogen. The development of 3-MCA-induced metaplastic lung nodules in rats maintained on subnormal RA intake (31.4 nmoles/week), but not low enough to cause deficiency symptoms, was markedly inhibited by administration of high (31,400 nmoles/week) but nontoxic RA doses. This inhibitory effect was observed when the high RA dose levels were initiated either at 3, 5, or as late as 10 weeks after the carcinogen administration, i.e., during the "postinitiation" phase. Since at this time 5 to 10% of the initial carcinogen dose was still recoverable from the lungs, an effect on carcinogen penetration into the target cells or on carcinogen metabolism (7, 11) cannot be completely ruled out. However, because only a small proportion of the initial carcinogen dose was still remaining, particularly at 10 weeks after 3-MCA, it appears reasonable to conclude that in this study the major effect of RA was an inhibition of progression and growth of transformed cells. Apparently, the high RA dose not only prevented the development of metaplastic foci (Group 1, RA intake shifted at 3 weeks; see Fig. 1a) but also inhibited the expansion and growth of already existing metaplasias or caused their regression (Groups 2 and 3, RA intake shifted at 5 and 10 weeks; see Fig. 1b and c). The metaplastic lesions histologically detectable at 5 and 10 weeks after 3-MCA were prevented to develop into grossly visible lung nodules in the groups receiving high RA levels. This finding supports and extends our previous observations (6) that rats maintained on high RA intake before, during, and after carcinogen exposure develop fewer metaplastic lung nodules than rats on low RA levels. With the data at hand, it is not possible to decide whether the observed effect is indicative of increased susceptibility to carcinogen due to inadequate vitamin A consumption or whether it suggests a protective effect of excess vitamin A intake. We chose the 2 extreme vitamin A dose levels, one at the fringes of vitamin A deficiency and the other at the fringes of vitamin A toxicity (but neither one detectably compromising the health of "normal" rats, i.e., rats not exposed to carcinogen), to secure the previously observed RA effect and, if possible, to magnify it. Studies are now in progress that include intermediate RA levels.

The question must be raised as to what significance these findings have in terms of carcinogenesis, since the lung lesions scored are not invasive cancers. In fact, we have reason to believe that some of these metaplastic nodules will not become invasive and that a few might actually regress, leaving only the keratin and a foreign-body reaction behind. We consider the squamous nodule in the rat lung to be a preneoplastic lesion, i.e., the precursor from which the squamous cell carcinoma, typically seen in rats following i.tr. 3-MCA injections, develops (21), if it does develop. Whether or not invasive neoplasia develops depends on "external" factors such as the carcinogen dose by which the aberrant cell population, which forms the squamous cell nodule, is induced and on host factors that may either favor or disfavor expression of the transformed state. The term preneoplasia is used here to mean a population of cells that differ from "normal" cells by their increased potential and increased probability to become neoplastic (or fully transformed), and from neoplastic cells—generally considered to be characterized by "autonomous" growth, by incomplete expression and realization of their neoplastic potential. There are several lines of evidence that justify considering these metaplastic lung nodules as (potential) precursors of squamous cell carcinomas. We showed in an earlier study, in which 25 mg 3-MCA were injected i.tr. into rats, that transplanted lung nodules become invasive cancers after a latency period of several months (21). This is proof that the squamous cell carcinomas induced by 3-MCA in the lungs of rats develop from the earlier-appearing metaplastic nodules; it does not mean, however, that every metaplastic nodule eventually develops into a carcinoma. Other evidence is that the incidence of death from lung cancer following i.tr. injection of a carcinogen dose similar to the one used in the present study, namely, 5 mg of 3-MCA, is 50 to 60% as compared to a 42% incidence of metaplastic lung nodules at 12 months. Since no lesions other than the squamous metaplastic nodules were detected at 6 and 12 months after 3-MCA injection, this provides further evidence for the morphogenetic relationship between the metaplasias and the carcinomas. The lung cancer incidence...
studies will be the subject of a later report. It seems justified to conclude from our studies that the development and growth of the precursor lesions of lung squamous cell carcinomas in rats are markedly influenced by the level of RA intake.

It remains to be explained why several other studies (15, 16, 24, 25) have failed to show inhibitory effects of vitamin A. There are a number of variables that appear to be essential in determining the outcome of such experiments. One is the severity of the carcinogenic insult. We believe that the main reason for the stronger RA effect in the present study as compared with the previous one (6) is the lower carcinogen dose used in the experiments reported here (namely, 4 instead of 10 mg of 3-MCA). Another variable is the "base level" of vitamin A used. In our experiments presented thus far, we have chosen probably the lowest possible base levels of vitamin A intake that prevent development of overt vitamin A deficiency.

Another variable that appears to be crucial in studies related to the modification of the tumor response by vitamin A and vitamin A analogs is the stage of tumor development at which the level of vitamin A intake is being changed. In a study recently reported by Smith et al. (25), RA had no inhibitory effect on respiratory tract tumor development in hamsters. However, a number of animals died from respiratory tract cancers several weeks before the series of carcinogen injections was completed and before the RA "treatment" was initiated. A cytological study previously conducted in our laboratory (22) suggests that, in the benzo(a)pyrene-Fe₃O₄ lung tumor induction system in hamsters, neoplasias develop in most of the animals anywhere from 10 to 20 weeks prior to death from cancer. It therefore seems likely that, in the study reported by Smith et al. (25), frank neoplasias were already present in many animals weeks before the administration of high RA doses was started. Thus, unless RA significantly alters the growth and invasive characteristics of cancer cells, an inhibition of tumor development by RA is not to be expected. This points to the desirability of an experimental model in which the carcinogen exposure is short and the tumor latency long. The rat lung tumor system used in these studies appears to be an improvement in this respect.

ACKNOWLEDGMENTS

We thank Hoffmann-La Roche (Nutley, N. J.) for the generous supply of RA.

REFERENCES

Fig. 1. Lung lesions detected in rats at different times after i.tr. injection of 4 mg of 3-MCA. Only the most advanced lesions are shown: (a) 3 weeks after carcinogen, small alveolar duct granulomas (encircled) with mildly hyperplastic terminal bronchiole (arrow); (b) 5 weeks after carcinogen, small alveolar squamous metaplasia; (c) 10 weeks after carcinogen, metaplastic lesions involving a group of alveoli, approximately 0.5 mm in diameter.
Fig. 2. Typical nodular squamous metaplasia with well-differentiated epidermoid cells on the periphery and keratin masses in the center of the lesions (52 weeks after i.tr. 3-MCA injection).

Fig. 3. Higher magnification of the highly cellular peripheral portion of a squamous nodule showing keratinizing epidermoid cells with few signs of atypia (52 weeks after i.tr. injection of 3-MCA).

Fig. 4. Squamous cell nodule, showing partial resorption of keratin and granuloma formation (left) and nonkeratinizing, less-differentiated cells with a high nuclear cytoplasmic ratio (right). This is a peripheral segment of a lesion (6 mm in diameter) which is otherwise composed of highly keratinizing squamous epithelium (52 weeks after i.tr. injection of 3-MCA).
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