Effect of Vitamin A on Pulmonary Macrophage Mobilization in Hamsters Exposed to Cigarette Smoke

Takanori Harada¹, Paul D. Meade², and Parvathi K. Basrur²

¹Toxicology Division, Institute of Environmental Toxicology and ²Department of Biomedical Sciences, Ontario Veterinary College, University of Guelph

Abstract: Male Syrian golden hamsters fed either vitamin A-free diet or normal stock diet with or without vitamin A supplementation were subjected to cigarette smoke inhalation for 6 weeks to study the relationship between the pulmonary cell response and vitamin A intake. All surviving animals after the last smoke exposure were killed for analysis of plasma and liver vitamin A contents and histopathology of the lung. Plasma and liver vitamin A levels were apparently lower in hamsters fed vitamin A-free diet than those maintained on normal stock diet. Liver vitamin A content was significantly higher in vitamin A-supplemented hamsters than those on stock diet alone, although there was no significant difference in plasma vitamin A level. Histopathologically, the number of free macrophages was counted in lung sections stained with hematoxylin and eosin and the count was used as the index to evaluate smoke-related insult at different levels of vitamin A. The number of macrophages was significantly greater in the smoke-exposed hamsters on vitamin A-free diet than those on normal stock diet. However, there was no significant difference in the macrophage count between vitamin A-supplemented and unsupplemented hamsters on stock diet. These results indicate that vitamin A deficiency and smoke exposure appear to enhance pulmonary macrophage mobilization. It is possible that the respiratory epithelium of vitamin A-deficient hamsters loses its integrity and becomes susceptible to injury by irritants such as those present in cigarette smoke. The elevated pulmonary free macrophage count may be a response to the damage caused by vitamin A deficiency and smoke exposure. (J Toxicol Pathol 1997; 10: 193~198)

Key words: Vitamin A, Macrophage, Cigarette smoke, Lung, Hamster

Introduction

Pulmonary macrophage plays an important role in the lung clearance by its principal function as a phagocytic cell¹⁻⁵. Human smokers^{6,7} and cigarette smoke-exposed animals⁸⁻¹¹ are known to have increased numbers of free macrophages in the lung. Vitamin A is generally believed to have a protective effect on various epithelia against toxic substances including carcinogens¹²⁻¹⁵. Various effects of vitamin A on the respiratory tract have been reported in hamsters by several investigators¹⁶⁻²⁰. In addition, it has been shown in rats that vitamin A may activate the function of alveolar macrophages^{21,22}. However, information concerning the influence of vitamin A on pulmonary macrophage mobilization is still limited. This report concerns a quantitative evaluation of pulmonary free macrophages in hamsters maintained on different levels of vitamin A intake and exposed to cigarette smoke.

Materials and Methods

For this experiment, 90 male Syrian golden hamsters (*Mesocricetus auratus*) were obtained from Engles Laboratory Animals Inc. (Farmersburg, Indiana, USA). As shown in Table 1, they were divided into three equal groups at 7-8 weeks of age. One group was fed vitamin A-free diet (ICN Lab Sciences, Cleveland, Ohio, USA) for 100 days prior to and during smoke inhalation period and the other two

groups were fed a standard stock diet, Purina Lab Chow (Ralston Purina, St. Louis, MO, USA) with one of the groups receiving a single supplement of 25,000 I.U. (13,750 µg) of retinyl palmitate (Sigma Chemical CO., St. Louis, MO, USA) in 0.5 ml of corn oil intragastrically 24 hours prior to initiation of smoke inhalation. These groups were further divided into three subgroups: cage-held control, sham-smoked control, and smoke-exposed groups (Table 1). All

Table 1. Study Protocol

Pretreatment period	Smoke inhalation period ^a
100 days	49 days
Vitamin A-free diet group (30) ^b	Cage-held control (10)
	Sham-smoke control (10)
	Smoke-exposed (10)
Normal stock diet + Vitamin A supplement group* (30)	Cage-held control (10)
	Sham-smoke control (10)
	Smoke-exposed (10)
Normal stock diet group (30)	Cage-held control (10)
	Sham-smoke control (10)
	Smoke-exposed (10)

^a Including one-week orientation period.

^b Number in parenthesis indicates number of animals used.

* Receiving a single oral administration of vitamin A 24 hours prior to initiation of smoke inhalation.

原田孝則

Accepted for publication: September 15, 1997

Mailing address: Takanori Harada, Institute of Environmental Toxicology, 4321 Uchimoriya-cho, Mitsukaido-shi, Ibaraki 303, Japan.

animals were housed individually in suspended cages with wire-mesh bottoms and kept in an animal room controlled at a temperature of 21°C and a relative humidity of 60% with a 12-hour light/dark cycle. Food and water were provided *ad libitum*. These animals were handled during the course of the experiment according to the guidelines of the Canadian Laboratory Animals Guide (Care of Experimental Animals: A guide for Canada, Canadian Council on Animal Care, 151 Slater Street, Ottawa, Canada, 1973).

The smoke inhalation procedure consisted of a one-week orientation period starting with one cigarette per day and gradually increasing it to eight cigarettes per day, followed by a six-week period of smoke inhalation (5 days exposure per week). Smoke inhalation was performed using an ORNL-Maddox intermittent smoking machine²³ which delivered a two-second 35 ml puff of smoke diluted to 350 ml to produce a 10% aerosol that remained in the chamber for 30 seconds, the chamber was then purged with fresh air for 30 seconds before another puff was delivered and the cycle was repeated until the cigarette reached a butt length of 19 mm (about 10 puffs). The test cigarette was a Canadian research cigarette (Monitor C) and its tar and nicotine contents were 18.91 mg and 1.51 mg, respectively. Some animals died during the course of the study and they were excluded from evaluation of this experiment.

At the termination of this experiment, all surviving animals were anesthetized with sodium pentobarbital (0.4 ml/100 g body weight) given intraperitoneally and blood was collected via cardiac puncture with a 6.0 ml heparinized syringe for vitamin A analysis. Plasma samples of the blood and liver tissues from these animals were subjected to vitamin A analysis by the method of Neeld and Peason²⁴ using trifluoroacetic acid (J.T. Baker Chem. Co., Phillipsburg, NJ, USA) as the colorimetric reagent.

After necropsy, the lungs were fixed with McDowell's fixative²⁵ containing 4% formaldehyde and 1% glutaraldehyde in phosphate buffer (pH 7.4). The fixed lung tissues were processed routinely for light and electron microscopy. Five pieces of the lung (one section from each lobe) from each animal were embedded in paraffin and the paraffin sections (5 μ m) were stained with hematoxylin and eosin (HE) for light microscopic examination. Additional samples from each

animal were embedded in Epon 812 for electron microscopy. Semithin sections (1 μ m) were stained with 1% toluidine blue and examined under a light microscope to select objective fields. Ultrathin sections of selected specimens were stained with uranyl acetate and Reynold's lead citrate and subjected to ultrastructural observation by a transmission electron microscope, JOEL 100S. According to the previously published method^{10,26}, macrophage count was performed using a light microscope at a magnification of 200 $\times$ on the lung sections stained with HE. Only free phagocytic cells which were observed in bronchiolar and alveolar spaces were recorded for a total of 35 randomly selected fields per animal. The area of each field was 0.65 mm². The fields occupied by bronchi and large blood vessels were eliminated from sampling areas.

The results of vitamin A analysis and macrophage count were subjected to statistical analysis by Student's *t* test or Aspin-Welch test.

Results

Plasma and liver vitamin A analysis

Plasma and liver vitamin A contents in hamsters fed vitamin A-free diet were approximately 30% and 80% lower than those in animals on normal stock diet alone, respectively (Table 2). There was no significant difference in the plasma vitamin A level between the vitamin A-supplemented and unsupplemented hamsters on stock diet, but the liver vitamin A content was significantly higher in the group receiving vitamin A supplement (Table 2). The plasma and liver vitamin A contents in the smoke-exposed hamsters on any diet were significantly lower than those in the respective cage-held or sham-smoked controls (Table 2).

Histopathology of the lung and macrophage count

Macrophages observed in the lung sections stained with HE were typically engorged with golden-brown pigments and had a tendency to gather around bronchiolar-alveolar spaces in the smoke-exposed hamsters (Figs. 1 and 2). Such phagocytic cells were also noted at a low frequency in cage-held and sham-smoked controls. In the smoke-exposed

Table 2. Plasma and Liver Vitamin A Contents in Hamsters Receiving Different Treatments

Treatment group	Vitamin A content (Mean $\pm$ S.E.)		
	Vitamin A-free diet	Stock diet and vitamin A supplement	Stock diet alone
Plasma (μ g retinol/100 ml)			
Cage-held control	18.7 $\pm$ 1.4 (9) ^a	27.6 $\pm$ 1.7 (10)	26.3 $\pm$ 1.8 (10)
Sham-smoked control	17.9 $\pm$ 0.8 (8) ^a	27.6 $\pm$ 1.4 (8)	26.1 $\pm$ 1.6 (10)
Smoke-exposed	14.2 $\pm$ 0.7 (9) ^{a,*}	24.4 $\pm$ 1.0 (9) [*]	23.4 $\pm$ 1.5 (10) [*]
Liver (μ g retinol/liver)			
Cage-held control	245 $\pm$ 9.8 (9) ^a	2,626 $\pm$ 104 (10) ^a	1,383 $\pm$ 37 (10)
Sham-smoked control	228 $\pm$ 15.8 (8) ^a	2,438 $\pm$ 74 (8) ^a	1,255 $\pm$ 41 (10)
Smoke-exposed	188 $\pm$ 16.7 (9) ^{a,*}	1,803 $\pm$ 112 (9) ^{a,*}	924 $\pm$ 41 (10) [*]

Number in parenthesis indicates number of animals examined.

^a Significantly different from the group on stock diet alone ($p < 0.05$).

^{*} Significantly different from the respective cage-held or sham-smoked control ($p < 0.05$).

Fig. 1 Alveolar free macrophages laden with golden-brown pigments in the lung of a smoke-exposed hamster on stock diet alone. A multinucleated giant macrophage is noted. HE, $\times 340$.

Fig. 2 Semithin section showing the macrophages filled with numerous inclusions in the bronchio-alveolar space of a smoke-exposed hamster on stock diet alone. Toluidine blue, $\times 340$.

Fig. 3 Electron micrograph of the macrophage from a smoke-exposed hamster on stock diet alone showing the cytoplasmic inclusions contained fiber-like structures (arrows). Bar = $1\ \mu\text{m}$.

Table 3. Number of Free Macrophages Counted in Lung Sections from Hamsters Receiving Different Treatments

Treatment group	Mean $\pm$ S.E./Field (0.65 mm ²)		
	Vitamin A-free diet	Stock diet and vitamin A supplement	Stock diet alone
Cage-held control	3.4 $\pm$ 0.7 (9) ^a	1.9 $\pm$ 0.4 (10) ^c	2.9 $\pm$ 0.4 (10) ^a
Sham-smoked control	2.9 $\pm$ 0.5 (8) ^a	1.7 $\pm$ 0.3 (8) ^c	1.8 $\pm$ 0.3 (10) ^c
Smoke-exposed	9.6 $\pm$ 0.9 (9) ^b	5.6 $\pm$ 0.6 (9) ^d	7.2 $\pm$ 0.6 (10) ^d

Number in parenthesis indicates number of animals examined.

Values with different superscripts are significantly different from each other ($p < 0.05$).

hamsters, the macrophages were scattered throughout the lung tissues, and occasionally clumps of the macrophages and/or multinucleated giant cells were observed (Fig. 1). These changes were most evident in the smoke-exposed hamsters fed vitamin A-free diet. Electron microscopic examination revealed that the macrophages in the smoke-exposed hamsters were filled with numerous cytoplasmic inclusions which contained fiber-like structures (Fig. 3). Such fiber-like structures were not observed in the cage-held or sham-smoked controls.

Quantitative analysis of macrophages in the lung sections stained with HE revealed the number of free macrophages in the smoke-exposed hamsters was apparently higher than that in the cage-held or sham-smoked controls (Table 3). In the groups of smoke-exposed or sham-smoked hamsters, the mean macrophage number in animals fed vitamin A-free diet was significantly higher than that in animals on normal stock diet with or without vitamin A supplementation (Table 3).

Discussion

The present study confirms that the number of pulmonary macrophages is increased by exposure to cigarette smoke, which is consistent with previous reports⁶⁻¹¹. The increase in macrophage number may be a response to inhaled smoke particles in the lung. It has been suggested that the increased deposition of inert or infectious particles in the pulmonary phagocytic cells may facilitate the recruitment of additional macrophages¹. In this experiment, most macrophages observed in the lungs from smoke-exposed hamsters were engorged with many golden-brown pigments. Since such macrophages were observed even in the lungs from cage-held or sham-smoked controls, some of the phagocytic cells may contain endogenous materials such as hemosiderin and lipids. Dontenwill *et al.*⁸ had considered that the pulmonary phagocytic cells, the so-called "smoke cells" were not smoke-specific, because they also appeared in animals not exposed to smoke. However, it was reported that the ultrastructure of alveolar macrophages from smokers was different from that of non-smokers in that the smoker's macrophages contained distinctive fiber-like inclusions in the cytoplasm⁶. It was also reported that the needle-like structures were noted only in smoker's macrophages²⁷. Brody and Craighead²⁸ have suggested that such needle or fiber-like structures may have a disc or plate-like configuration, since cross sections of the structures have never been observed in electron micrographs. The authors suggested that the structures might be derived from inhaled tobacco smoke and

could well represent aluminum kaolinate. Matulionis and Traurig²⁶ reported that cigarette smoke-exposed mice showed increases in number, variety, and size of residual bodies in pulmonary macrophages with prolongation of exposure time which resulted in an increase in the size of macrophages and appearance of multinucleated macrophages. In addition, electron diffraction analysis demonstrated that crystal-like inclusions noted in the macrophages from smoke-exposed mice was kaolinite. In the present study, we have also observed similar needle-like structures in alveolar macrophages and multinucleated giant cells in the smoke-exposed hamsters. Although the exact origin of the pulmonary free macrophages could not be determined in the present study, it has been suggested that the elevated macrophage population in smoke-exposed animals appears to be due to immigration of cells from bone marrow rather than *in situ* division of resident macrophages²⁹.

Statistical evaluation of macrophage counts revealed that the number of free macrophages in the lung sections from hamsters maintained on vitamin A-free diet was significantly higher than that in animals on normal stock diet with or without vitamin A supplementation in the smoke-exposed and sham-smoked groups. It is possible that the respiratory epithelium of vitamin A-deficient hamsters loses its integrity and becomes susceptible to injury caused by the irritants present in cigarette smoke. The importance of vitamin A for the local epithelial defences has been recognized and the lack of vitamin A has been shown to reduce local resistance to toxic or infectious injuries through the epithelial changes in various organs¹²⁻¹⁵. Such epithelial alterations in the respiratory tract are characterized principally by squamous cell metaplasia and reduced mucous production^{12,17,20}. DeLuca *et al.*^{30,31} reported that vitamin A deficiency caused a decrease in the number of goblet cells in the small intestine of the rat. The authors have concluded that protein synthesis of intestinal mucosa is depressed under the conditions of vitamin A deficiency and a measurable shift in the type of glycoprotein synthesized is associated with loss of mucous cells. In contrast, it has been shown that extra amount of vitamin A may activate the function of alveolar macrophages, which are probably stimulated by fat soluble vitamins coming into the lung via lymphatic route from the intestine, and will be especially important in preventing pulmonary disease^{21,22}. In the present study, however, vitamin A supplementation resulted in no significant effect on macrophage mobilization.

Yeung³² reported that cigarette smoking increased the levels of plasma vitamin A in human. However, our vitamin A analyses showed that plasma vitamin A levels in the smoke-exposed hamsters were significantly lower than that of

cage-held or sham-smoked controls. It was reported that serum vitamin A levels dropped sharply in patients undergoing severe stress^{33,34}. It is possible that exposure to cigarette smoke provokes a kind of stress reaction in these hamsters. Since epidemiological studies³⁵⁻³⁷ suggest that low carotene intake and low levels of serum vitamin A may be associated with an increased risk of lung cancer, further experiments should be done to clarify the relationship between smoking and vitamin A.

The present study suggests that the increased number of pulmonary free macrophages in the smoke-exposed hamsters on vitamin A-free diet may be a response to the damage caused by vitamin A deficiency which in turn is accentuated by smoke exposure.

Acknowledgements: We are indebted to Professor E.V. Evans, Department of Nutrition, University of Guelph, for his co-operation and to Mr. E. Reyes, Mr. C. Ackerley, Mrs. Helen Randall, and Miss Janice G. Hicks for their technical assistance in this work. This investigation was supported by a Grant-in-Aid of Research from the Non-Medical-Use of Drugs Directorate, Health Protection Branch, National Health and Welfare, Canada.

References

- Brain JD. Free cells in the lungs. *Arch Intern Med* 1970; **126**: 477-87.
- Sorokin SP. Properties of alveolar cells and tissues that strengthen alveolar defenses. *Arch Intern Med* 1970; **126**: 450-63.
- Fogelmark B, Rylander R, Sjöstrand M, and Reininghaus W. Free lung cell phagocytosis and the effect of cigarette smoke exposure. *Exp Lung Res* 1980; **1**: 131-38.
- Holt PF. Dust elimination from pulmonary alveoli. *Environ Res* 1980; **23**: 224-27.
- Adamson IYR and Bowden DH. Dose response of the pulmonary macrophagic system to various particulates and its relationship to transepithelial passage of free particles. *Exp Lung Res* 1981; **2**: 165-75.
- Harris JO, Swenson EW, and Johnson III JE. Human alveolar macrophages: Comparison of phagocytic ability, glucose utilization and ultrastructure in smokers and non-smokers. *J Clin Invest* 1970; **49**: 2086-96.
- Hoidal JR and Niewoehner DE. Lung phagocyte recruitment and metabolic alterations induced by cigarette smoke in humans and in hamsters. *Am Rev Respir Dis* 1982; **126**: 548-52.
- Dontenwill W, Chevalier HJ, Harke HP, Lafrenz U, Reckzeh G, and Schneider B. Investigations on the effects of chronic cigarette smoke inhalation in Syrian golden hamsters. *J Natl Cancer Inst* 1973; **51**: 1781-832.
- Bernfeld P, Homburger F, Soto E, and Pai KJ. Cigarette smoke inhalation studies in inbred Syrian golden hamsters. *J Natl Cancer Inst* 1979; **63**: 675-89.
- Harada T, Maita K, Nakashima N, Odanaka Y, and Shirasu Y. Quantitative studies of biological responses in hamsters exposed to tobacco smoke and effects of vitamin C supplement on smoke inhalation toxicity. *Jpn J Vet Sci* 1983; **45**: 613-26.
- Henry CJ and Kouri RE. Chronic inhalation studies in mice. II. Effects of long-term exposure to 2R1 cigarette smoke on (C57BL/Cum x C3H/AnfCum) F₁ mice. *J Natl Cancer Inst* 1986; **77**: 203-12.
- McLaren DS. Present knowledge of the role of vitamin A in health and disease. *Trans Roy Soc Trop Med Hyg* 1966; **60**: 436-62.
- Nettesheim P, Topping DC, and Jamasbi R. Host and environmental factors enhancing carcinogenesis in the respiratory tract. *Ann Rev Pharmacol Toxicol* 1981; **21**: 133-63.
- Sporn MB and Newton DL. Retinoids and chemoprevention of cancer. In: *Inhibition of Tumor Induction and Development*, MS Zedeck, and M Lipkin (eds), New York: Plenum Press, pp. 71-100, 1981.
- Ong DE and Chytil F. Vitamin A and cancer. *Vitamins and Hormones* 1983; **40**: 105-44.
- Sally JJ and Bryson WF. Vitamin A deficiency in the hamsters. *J Dent Res* 1957; **36**: 935-44.
- Saffiotti U, Montesano R, Sellakumar AR, and Borg SA. Experimental cancer of the lung: Inhibition by vitamin A of the induction of tracheobronchial squamous metaplasia and squamous cell tumors. *Cancer* 1967; **20**: 857-64.
- Kaufman DG, Baker MS, Smith JM, Henderson WR, Harris CC, Sporn MB, and Saffiotti U. RNA metabolism in tracheal epithelium: Alteration in hamsters deficient in vitamin A. *Science* 1972; **177**: 1105-08.
- Dontenwill W, Chevalier HJ, Marke HP, Klimisch HJ, Kuhnigk C, Reckzeh G, and Schneider B. Studies on the effect of chronic cigarette smoke inhalation in Syrian golden hamsters and the importance of vitamin A on morphological alterations after smoke exposure. *Z Krebsforsch* 1977; **89**: 153-80.
- Mead PD, Yamashiro S, Harada T, and Basrur PK. Influence of vitamin A on the laryngeal response of hamsters exposed to cigarette smoke. *Prog Exp Tumor Res* 1979; **24**: 320-29.
- Kishino Y, Takama S, and Kitajima S. Ultrastructure of tumor cell interaction with alveolar macrophages stimulated by vitamin A. *Virchows Arch [Cell Pathol]* 1985; **50**: 81-89.
- Kishino Y and Moriguchi S. Nutritional factors and cellular immune responses. *Nutrition and Health* 1992; **8**: 133-41.
- Guerin M, Maddox WL, and Stockley JR. Tobacco smoke inhalation exposure: concepts and devices. *Proceedings of the Tobacco Smoke Inhalation Workshop*, DHEW Publication No. (NIH) 75-906, pp. 32-44, 1975.
- Neeld JB and Pearson WN. Macro- and micromethods for the determination of serum vitamin A using trifluoroacetic acid. *J Nutr* 1963; **79**: 454-62.
- McDowell EM and Trump BF. Histologic fixatives suitable for diagnostic light and electron microscopy. *Arch Pathol Lab Med* 1976; **100**: 405-14.
- Matulionis DH and Trauring HH. *In situ* response of lung macrophages and hydrolase activities to cigarette smoke. *Lab Invest* 1977; **37**: 314-26.
- Pratt SA, Smith MH, Ladman AJ, and Finley TN. The ultrastructure of alveolar macrophages from human cigarette smokers and nonsmokers. *Lab Invest* 1971; **24**: 331-38.
- Brody AR and Craighead JE. Cytoplasmic inclusions in pulmonary macrophages of cigarette smokers. *Lab Invest* 1975; **32**: 125-32.
- Matulionis DH. Reaction of macrophages to cigarette smoke. II. Immigration of macrophages to the lungs. *Arch Environ Health* 1979; **34**: 298-301.
- Deluca L, Little EP, and Wolf G. Vitamin A and protein synthesis by rat intestinal mucosa. *J Biol Chem* 1969; **244**: 701-08.
- Deluca L, Schumacher M, and Wolf G. Biosynthesis of a fucose-containing glycopeptide from rat small intestine in normal and vitamin A-deficient conditions. *J Biol Chem* 1970; **245**: 4551-58.
- Yeung DL. Relationships between cigarette smoking, oral contraceptives, and plasma vitamin A, E, C, and plasma triglycerides and cholesterol. *Am J Clin Nutr* 1976; **29**: 1216-21.
- Chernov MS, Hale Jr HW, and Wood M. Prevention of stress ulcers. *Am J Surg* 1971; **122**: 674-77.

34. Kasper H, Brodersen M, and Schedel R. Concentration of vitamin A, retinol-binding protein and prealbumin in serum in response to stress. *Acta Hepato-Gastroenterol* 1975; **22**: 403-08.
35. Mettlin C, Graham S, and Swanson M. Vitamin A and lung cancer. *J Natl Cancer Inst* 1979; **62**: 1435-38.
36. Kark JD, Smith AH, Switzer BR, and Hames CG. Serum vitamin A (retial) and cancer incidence in Evans County, Georgia. *J Natl Cancer Inst* 1981; **66**: 7-16.
37. Willett WC, Polk BF, Underwood BA, Stampfer MJ, Pressel S, Rosner B, Taylor JO, Schneider K, and Hames CG. Relation of serum vitamin A and E and carotenoids to the risk of cancer. *N Engl J Med* 1984; **310**: 430-34.