PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 161, 600–604 (1979)

Effect of Vitamin A Deficiency on Susceptibility of Rats to *Angiostrongylus cantonensis* (40605)[1]

MOHAMED D. DARIP,[2] STITAYA SIRISINHA,[3] AND ADRIAN J. LAMB[4]

Department of Microbiology, Faculty of Science, Mahidol University, Bangkok 4, Thailand

Although it has been recognized for years that vitamin A deficiency predisposes the host to virtually all types of infection, the exact role of vitamin A in host defense is still far from clear (1). The role of vitamin A in the maintenance of the functional integrity of the epithelium and the mucosa is of obvious importance in this regard, in that these structures act as a physical barrier to infection. The ability however of vitamin A-deficient hosts to clear an infection once the tissue has been invaded also appears to be reduced (1). Thus, several lines of evidence suggest that both humoral and cell-mediated immune responses may be defective in vitamin A deficiency (1–4). A marked depletion of lymphocytes from several lymphoid organs including the thymus has also been reported (5), this depletion being more severe in deficient animals with concomitant viral infection (6). Moreover, both circulating antibody and antibody-forming cells in the spleen of vitamin A-deficient animals were found to be reduced when compared with those of pair-fed controls (7). As with other physiological lesions in vitamin A deficiency, the molecular mechanism(s) involved in these changes are not understood, but altered membrane-associated functions or impaired cellular differentiation in the deficiency state have been proposed and have extensive experimental support (8, 9). Another problem that one encounters in evaluating defects reported to be associated with vitamin A deficiency is the extent to which changes are complicated by secondary inanition. To overcome this problem we have adopted a rearing system (10) which permits the rapid and synchronous induction of vitamin A deficiency. The short time course of such experiments enable the force-feeding of animals, and hence rigorous control of not only vitamin A status but also dietary protein and energy input.

The purpose of the present study was to use this novel system to investigate the effect of uncomplicated vitamin A deficiency on the course of infection by *Angiostrongylus cantonensis*. This tissue nematode, a rat lungworm that occasionally causes a human infection known as eosinophilic meningoencephalitis (11), is a useful model to evaluate the role of both tissue barrier and host immune responses in vitamin A deficiency. Moreover, the immune responses to infection by *A. cantonensis* have been well characterized in recent years, thus making it highly feasible to use this host–parasite system for immunological investigation (for review, see Ref. (12)). The results show that vitamin A deficiency has a profound influence on the course of infection by *A. cantonensis*.

Materials and methods. Induction of vitamin A deficiency. Albino rats of Fisher–Wistar cross weighing 270–320 g were made vitamin A deficient by the withdrawal of retinoic acid from the diet of retinoate cycled, stringently vitamin A-deficient animals as previously described (10, 13). In essence, male weanling rats were fed *ad libitum* a vitamin A-free diet for 3 weeks until early growth plateau. Thereafter they were fed a diet first supplemented with and then lacking in 5 μg retinoic acid per gram diet in repeating 18-day/10-day cycles. After a minimum of four such cycles, the level of retinoate supplementation was reduced to 2 μg per gram diet for the last 8–10 days prior to being used in any experiment. Animals selected as A^+ controls were given either 500 or 1000 μg retinyl palmitate in split doses 2 days (T_{-2}) and 1 day (T_{-1}) prior to retinoate withdrawal (T_0). Animals selected

[1] Supported in part by the National Research Council of Thailand and by Grant AM 11367 from the National Institutes of Health.

[2] Postdoctoral fellow on leave from the Departments of Microbiology, Faculty of Medicine, University of North Sumatra, Medan, Indonesia.

[3] To whom reprint requests should be addressed.

[4] Department of Biochemistry, Faculty of Science, Mahidol University.

0037-9727/79/090600-05$01.00/0

as A^- were given oil mixture only. Following the withdrawal of retinoic acid, animals were tube-fed twice daily with 5 g dry wt stock 18% casein vitamin A-free diet with sucrose in place of starch.

Infection of rats with A. cantonensis. The strain of *A. cantonensis* used in the present study was originally obtained from Dr. M. Kamiya (then at the Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand) and was propagated by cycling it through adult rats and *Biomphalaria glabrata* snails. The inoculation of rats with third-stage larvae (L_2) and snails with first-stage larvae (L_1) was as previously described (14). In rats, L_3 migrate via the blood to the central nervous system (CNS) where they molt to become fourth-stage larvae (L_4) within 8–10 days of infection. L_4 molt and develop further to become young adults which later migrate to the heart and then to the lungs. From around Day 28 onward, sexually mature adult worms can be recovered from the pulmonary arteries and lungs of infected animals. The LD_{50} values for adult rats as calculated from dose–response curves of animals infected orally and parenterally by a subcutaneous injection into skin over the back were found to be 180 and 85, respectively (12). Normal rats infected with a sublethal dose of L_3 appear healthy and remain alive for several months and continue to shed L_1 in their feces throughout this period (11).

Results. Effect of vitamin A deficiency on the susceptibility of rats to infection with 50 third-stage larvae. The effect of vitamin A deficiency on the susceptibility of animals to a normally sublethal dose of *A. cantonensis* third-stage larvae (L_3) was determined by inoculating retinoate cycled, stringently vitamin A-deficient and control animals subcutaneously or orally with 50 L_3 6 days after the fourth cyclical withdrawal of retinoic acid (T_6). Thereafter, the animals were fed retinoate-free diet for a further 5 days (from T_6 to T_{10}). Secondary nutritional imbalances that might have developed during this period were overcome by the tube feeding of deficient (A^-) and retinyl palmitate treated control (A^+) animals with vitamin A-free diet twice daily as described. Subsequently, all animals were transfered to standard supplementation–deprivation regimen while allowing the parasite to complete its development. In this preliminary experiment therefore the primary difference between these two groups of animals occurred at the time of initial inoculation and during the early phase of parasitic development. Throughout this time animals were monitored daily for clinical manifestations of infection until death or until Day 60 of infection, at which time all surviving animals were killed and scored for worm recovery.

Under the condition described above, vitamin A deficiency led to increased susceptibility to infection by *A. cantonensis* (Table I). This was particularly obvious after a subcutaneous challenge when all A^- animals died with an average survival time of only 39 days. In sharp contrast, all A^+ controls were found to be alive and healthy throughout the observation period. The number of worms recovered from the pulmonary arteries and lungs of A^- animals, determined immediately after death, was also significantly higher than that of the A^+ controls ($P < 0.05$, Table I). Nonetheless, in both groups of animals the worms were able to develop to mature adults, as evidenced from the presence of L_1 in the feces. No worms were recovered from the CNS of any animals, either at the time of death or at Day 60 when all remaining animals were sacrificed. A similar general pattern was observed when rats were infected orally (Table I). However, due to a lower infection rate, none of the animals in the A^- or A^+ groups died (Table I). Although the

TABLE I. EFFECT OF VITAMIN A DEFICIENCY ON SUSCEPTIBILITY TO *Angiostrongylus* INFECTION

Vitamin A status	No. of animals	Challenge				Worm recovery		
		Route	No. of larvae	Mortality (%)	Survival (days)	Total	%	*P* value
A^-	4	Subcutaneous	50	100	39.0 ± 4.5[a]	38.0 ± 1.0	76.0 ± 2.0	<0.05
A^+	4	Subcutaneous	50	0	>60	30.2 ± 3.1	60.5 ± 6.2	
A^-	4	Oral	50	0	>60	6.3 ± 2.0	12.6 ± 4.0	
A^+	4	Oral	50	0	>60	3.5 ± 0.5	7.0 ± 1.0	

[a] Values indicate means ± SE of the mean.

ratio of worm recovered in A^- animals compared with A^+ controls (6.3/3.5) was higher than when animals were infected subcutaneously (38.0/30.2), the low worm recovery in both orally infected A^+ and A^- animals precluded meaningful statistical analysis.

Worm development in vitamin-A deficient rats infected with lethal doses of infective larvae. The increased susceptibility of long-term retinoate cycled A^- rats to *A. cantonensis* (Table I) might conceivably have been due to a difference in the ability of these worms to penetrate the intestinal mucosa (oral infection) or to migrate through the tissues to the CNS (oral and subcutaneous infections), or to some unfavorable alteration in the defense mechanism(s) of such animals. To help distinguish between these possibilities, five A^- and three A^+ animals were infected subcutaneously with a larger challenging dose (150 L_3) on Day 3 (T_3) following the withdrawal of retinoic acid. Because it takes between 7 and 10 days for L_3 to develop into L_4 (unpublished observation), animals were therefore force-fed the retinoate-free diet twice daily until T_{12} (Day 9 of infection) when all were killed and the numbers of L_4 in the CNS were determined. Thus, in this experiment animals were kept vitamin A-deficient continuously throughout the early phase of larva migration and development.

Animals were killed by ether 9 days after infection (T_{12}). The brain and meninges were removed immediately and macerated lightly in the presence of 10 ml of warm phosphate-buffered saline (37°C). The L_4 migrated from the macerated tissues into the surrounding fluid within a few minutes. The larvae were then picked up one by one under a dissecting microscope and the total larvae recovered from each animal were scored. The number of L_4 recovered from A^+ and A^- rats infected by a subcutaneous route were similar (Table II). Seemingly, therefore there is no differences in deficient animals with regard to the early phase of worm migration and development, i.e., from L_3 to L_4 larval stages. On the other hand, when these animals were challenged by an oral route with 350 L_3, a significant difference in the number of L_4 recovered from A^+ and A^- rats was observed (Table II). In that there was no difference in the early migration and development of worms when L_3 were deposited subcutaneously into these two groups of rats, the difference following oral challenge is most probably due to altered penetrability of the gastrointestinal mucosa of these animals. Taken together, the data presented in Tables I and II suggest that vitamin A is an important factor controlling infection at the site of entry, but that once the parasites have invaded the tissue, they are able to migrate and develop normally, at least during the early phase of worm development, regardless of the vitamin A status of the host.

Effect of vitamin A deficiency on the induction of protective immunity. It has been demonstrated previously by several groups of investigators including ourselves that a low degree of infection by *A. cantonensis* larvae confers a solid immunity to reinfection by this parasite (14–16). To determine whether the difference in the final outcome of infection following the subcutaneous challenge of A^+ and A^- animals shown in Table I was in any way due to a reduced capacity of A^- rats to mount a specific immune response necessary to control the development of the parasite during the late stages of infection, the following experiment was performed. Force-fed retinyl palmitate-treated A^+ and A^- animals were given a primary challenge orally with 50 L_3 6 days after the withdrawal of retinoic acid from the diet (T_6) and both groups were force-fed a retinoic acid-free diet

TABLE II. EFFECT OF VITAMIN A DEFICIENCY ON WORM MIGRATION AND EARLY STAGE OF WORM DEVELOPMENT

Vitamin A Status	No. of animals	Challenge		Larvae (L_4) in brain		P value
		Route	No. of larvae	Total	Challenging dose (%)	
A^-	5	Subcutaneous	150	59.6 ± 4.3[a]	39.7 ± 2.9	>0.05
A^+	3	Subcutaneous	150	63.3 ± 5.9	42.2 ± 3.9	
A^-	6	Oral	350	83.7 ± 2.3	23.9 ± 0.6	<0.01
A^+	4	Oral	350	65.0 ± 2.5	18.6 ± 0.7	

[a] Values indicate mean ± SE of the mean.

for 5 more days (from T_6 to T_{10}) as described for the experiment detailed in Table I. Infected A^+ and A^- rats were then transferred to one more 18 day/10 day standard cycle and followed with 8 more days of *ad libitum* feeding on 2 μg retinoic acid/g diet, thus allowing the parasite to complete its development judging from the appearance of L_1 in the feces. It should be emphasized at this point that under this condition an effective protective immunity would have developed following an initial challenge in pellet-fed adult rats (14). Both groups of animals were then similarly rechallenged orally with 350 L_3 6 days after retinyl palmitate rehabilitation, which was initiated 42 days after the first (sublethal) oral challenge. They were then recycled on the standard supplementation–deprivation regimen until the time of death. In essence therefore the first (sublethal) challenge took place while the animals were in either the A^+ or A^- state, whereas the second (lethal) challenge was initiated and allowed to proceed after the A^- animals had fully recovered from the previous deficiency state. The results presented in Table III show that there was a significant difference ($P < 0.05$) in the worm recovery between the two groups of animals. Although the dose of L_3 used in the second challenge was lethal for both groups of animals, the A^+ group survived longer than the A^- group (Table III). In that the two groups of animals differed in vitamin A status primarily during the early phase of the initial infection, these data suggest that animals challenged while vitamin A sufficient developed a higher degree of protective immunity to reinfection than those that received their first challenge whilst vitamin A deficient.

Discussion. The two major findings reported in this study, namely a reduced nonspecific local resistance at the site of entry (Tables I and II) and a reduced specific immunity to reinfection by *A. cantonensis* in vitamin A-deficient rats (Table III), strongly support the concept that vitamin A deficiency predisposes the hosts to infections, particularly the ones involving mucosal surfaces (1). Although increased incidence and severity in parasitic infections are known to be associated with vitamin A deficiency in many species of animals and man (1), it is difficult to rule out the effect of secondary inanition. Therefore, the altered host–parasite relationship reported earlier in the literature may not be attributable to vitamin A deficiency alone because other specific nutritional deficiencies are known to have adverse effect on the host immune response (17). Because the animal model used in the present study has as its main advantage the absence of secondary nutritional imbalances, our findings represent the effect of a truly uncomplicated case of vitamin A deficiency. These results therefore not only support the conclusion reached by other investigators but also give more definite evidence regarding a relationship between vitamin A deficiency and infection.

The results presented in Tables I and II show that the increased susceptibility of vitamin A-deficient rats to infection by *A. cantonensis* is partly attributable to a reduced tissue barrier at the site of entry. However, when the tissue barrier was bypassed and a similar number of larvae was deposited directly into the tissue, as in the case of inducing infection by a subcutaneous route, deficient animals still developed a more severe infection (Table I). It is unlikely that the increased susceptibility in the latter situation is due to changes in the tissues of A^- rats which would in turn allow the worms to thrive more successfully in these deficient hosts. The data showing that the development of parasite from L_3 to L_4 stages was unaffected by vi-

TABLE III. EFFECT OF VITAMIN A DEFICIENCY ON THE INDUCTION OF PROTECTIVE IMMUNITY

Vitamin A status			Survival period		Worm recovery		
1st challenge[a]	2nd challenge[b]	No. of animals	Days	*P* value	Total	%	*P* value
A^-	A^+	4	30.0 ± 0.40[c]	>0.05	145.8 ± 6.4	41.6 ± 1.8	<0.05
A^+	A^+	13	34.3 ± 2.29		90.1 ± 13.4	25.7 ± 3.8	

[a] 50 L_3 orally on T_6.
[b] 350 L_3 orally 6 days after retinyl palmitate rehabilitation.
[c] Values indicate mean ± SE of the mean.

tamin A deficiency (Table II) provide indirect evidence that argues in favor of the above contention.

The remaining possibility that could have made these vitamin A-deficient rats more susceptible to infection is a direct effect of vitamin A deficiency on the host immune response. The results presented in Table III strongly suggest that a protective immunity that usually develops following a sublethal infection with infective larvae (14–16) depends on their vitamin A status. Animals challenged while they were in a deficiency state develop a lower degree of protective immunity to a subsequent lethal challenge, as revealed from shorter survival period and a higher worm burden in A^- animals. These findings show that vitamin A deficiency occurring during a critical period, even only for a brief interval, could adversely affect the development of a protective immunity in these animals.

It is not possible to associate any particular immune defect(s) that makes these A^- rats more susceptible to infection by *A. cantonensis* since we do not yet fully understand the immune aberrations that exist in our animal model. We have shown previously that the complement system in these animals is not depressed (13). However, preliminary data indicate that both the humoral and cellular responses in deficient rats may be adversely affected (unpublished). It remains to be investigated if these systemic changes are primarily responsible for a lower degree of protective immunity that develops following an initial challenge with this parasite. A defective local immune response that occurs in A^- rats (unpublished data) may act synergistically with the impaired tissue integrity, thus making these vitamin A-deficient animals highly susceptible to rechallenge with this same parasite. It is apparent from the above discussion that a more detailed investigation along this line is needed if we are to fully understand the role of vitamin A in host defense, particularly against infections occurring at the body surfaces.

Summary. The effect of vitamin A deficiency on susceptibility to infection by *A. cantonensis* was studied using rats reared by a procedure enabling the synchronous induction of vitamin A deficiency and the stringent control of both dietary protein and energy input. Vitamin A-deficient (A^-) rats were more susceptible to infection by third-stage larvae than (A^+) controls, as revealed from mortality rate, survival period, and/or worm recovery. Evidence was presented to show that more larvae were able to penetrate the intestinal mucosa of A^- rats than that of A^+ controls, a finding that is consistent with the fact that one of the functions of vitamin A is to maintain the morphological and functional integrity of the mucosa. Moreover, following a primary infection A^- rats developed a lower degree of protective immunity than A^+ control. Animals that were initially exposed to the parasite during the A^+ state had a longer survival period and a lower worm burden than the group that was deficient in vitamin A during the early period of initial infection.

1. Scrimshaw, N. S., Taylor, C. E., and Gordon, J. E., WHO Monograph Ser. **57** (1968).
2. Krishnan, S., Krishnan, A. D., Mustafa, A. S., Talwar, G. P., and Ramalingaswami, V., J. Nutr. **106,** 784 (1976).
3. Newbern, P. M., and Suphakarn, V., Cancer **40,** 2553 (1977).
4. Nauss, K. M., Mark, D. A., and Suskind, R., Fed. Proc. **38,** 485 (1978).
5. Bang, B. G., Bang, F. B., and Foard, M. A., Amer. J. Path. **68,** 147 (1972).
6. Bang, B. G., Foard, M. A., and Bang, F. B., Proc. Soc. Exp. Biol. Med. **143,** 1140 (1973).
7. Krishnan, S., Bhuyan, U. N., Talwar, G. P., and Ramalingaswami, V., Immunology **27,** 383 (1974).
8. Wolbach, S. B., *in* "The Vitamins" (W. H. Sebrell and R. S. Harris, eds.), p. 106. Academic Press, New York (1954).
9. Olson, J. A., Israel J. Med. Sci. **8,** 1170 (1972).
10. Lamb, A. J., Apiwantanaporn, P., and Olson, J. A., J. Nutr. **104,** 1140 (1974).
11. Alicata, J. E., and Jindrak, K., *in* "Angiostrongylosis in the Pacific and Southeast Asia," p 1. Thomas, Springfield, Ill. (1970).
12. Sirisinha, S., Techasoponmani, R., Dharmkrong-at, A., and Uahkowitchai, V., J. Sci. Soc. Thailand **3,** 157 (1977).
13. Madjid, B., Sirisinha, S., and Lamb, A. J., Proc. Soc. Exp. Biol. Med. **158,** 92 (1978).
14. Lunghdara, R., M.Sc. thesis, Mahidol University, Bangkok, Thailand (1974).
15. Lee, S. H., Proc. Helminth. Soc. Wash. **36,** 95 (1969).
16. Heyneman, D., and Lim, B. L., Med. J. Malaya **20,** 162 (1965).
17. Suskind, R. M., *in* "Malnutrition and the Immune Response." Raven Press, New York (1977).

Received March 19, 1979. P.S.E.B.M. 1979, Vol. 161.