Effect of Retinoic Acid (RA) and Retinyl palmitate (RP) Repletion on Lymphocytes of Vitamin A Deficient Rats*

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SUMMARY The effects of retinoic acid (RA) repletion on the lymphocytes of vitamin A deficient rats were compared with those of retinyl palmitate (RP) repletion. Rats were 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 animals were then put on a diet supplemented with either RA or RP for eight weeks. Changes in the circulating levels of blood lymphocytes were monitored every two weeks. At the end of the repletion period, peripheral blood and splenic lymphocytes were assayed for blast transformation, and the number of antibody-producing cells in the spleen enumerated. The results obtained showed that both RA and RP could effectively correct the immune defects associated with vitamin A deficiency, i.e., lymphopenia, depressed lymphoproliferative response and defective antibody formation.

ASIAN PACIFIC J ALLERG IMMUN 1985; 3:67-72.

The effect of vitamin A deficiency on the immune functions of animals and man, and consequently on their susceptibility to various types of infections, has been amply demonstrated.[1,2] Moreover, such a deficiency state is also known to be associated with an increased incidence of tumours.[3] The lymphoid tissues of vitamin A deficient animals are poorly developed.[4,5] The structural and functional integrity of T cells seems to be more severely affected than that of B cells.[5-7] However, the weight of evidence suggests that in these animals both humoral and cell-mediated immune responses are depressed.[1,8,9] Supplementing the diet with vitamin A or its analogues leads to increased specific and non-specific immunity to infection.[1,2,10-12] Vitamin A and its derivatives are also known to be potent immunological adjuvants.[13-15] Studies on the effects of retinoids *in vitro* have shown that they can alter various immune parameters. For example, RA has been shown to enhance phagocytic capacity,[16] to augment thymocyte and T-cell responses to mitogen stimulation,[17,18] and possibly to alter the immunoregulatory mechanism, resulting in an imbalance of suppressor and helper cell functions.[19] The immune potentiating effects of vitamin A and its analogues also have wide application in the prevention and treatment of several types of cancer.[20-22]

We have developed a novel animal rearing system[23] which permits the rapid and synchronous induction of vitamin A deficiency. We used those animals for our biochemical and immunological studies. This method of rearing rats has several advantages over the conventional means of inducing the deficiency state currently employed by other investigators. For one reason, it eliminates complications associated with secondary inanition which always occurs with the conventional rearing method. In our system, the animals could be made vitamin A deficient within a few days following retinoic acid (RA) withdrawal, thus enabling a precise control of not only the onset of deficiency but also of dietary protein and energy input.

Although RA can substitute for vitamin A in the maintenance of many cellular functions, it is unable

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to restore the visual or reproductive functions of vitamin A deficient animals.[24,25] Other evidence suggests contrary results, i.e. that RA is superior to vitamin A in correcting defects that occur in vitamin A deficient animals, particularly with regard to the differentiation of epithelial cells.[26,27] The cell-mediated immune response of normal mice to infection with *Listeria monocytogenes* can also be enhanced to a higher magnitude with RA.[11] However, in such instances, the possibility of a synergistic interaction between vitamin A reserves in the body and the RA given could not be ruled out as those studies were largely carried out on normal subjects with excess vitamin A reserves.[11,17,18] In the present study, we propose, therefore, to examine the effects of RA repletion on some immune parameters of vitamin A deficient rats and to compare these effects with those of RP repletion.

MATERIALS AND METHODS

Induction of vitamin A deficiency and repletion methods

Weanling male albino rats were fed a vitamin A-free diet *ad libitum* for three weeks until they reached the early weight plateau. Thereafter, the animals were fed a diet supplemented with, and later lacking in, RA as previously described.[23,28] At the end of the fourth supplementation-deprivation cycle, the animals would be depleted of all vitamin A reserves. Those animals selected to be vitamin A sufficent (A^+) controls received a total of 500 μg of RP in 0.4 ml of safflower oil while those selected to be deficient (A^-) received only the safflower oil. Thereafter, both groups were twice daily tube-fed an RA-free diet for eight days. To compare the repletion capacity of RA with that of RP, animals at the end of the fourth deprivation cycle were given for eight weeks either a diet containing RA (5 μg/g diet) *ad libitum* or an RA-free diet *ad libitum* supplemented with 500 μg of RP weekly administered by stomach tube in split doses on two successive days.

Differential white blood cell count

Changes in the circulating levels of different white blood cells taken from blood specimens at appropriate time intervals were studied. These were taken from the ophthalmic venous plexus, using standard haematological techniques.[30-32] The lymphocytes were also classified into small and large subpopulations according to standard criteria.[32]

Immunofluorescent staining of lymphocyte surface immunoglobulins

Immunofluorescent staining of peripheral blood lymphocytes for the presence of surface immunoglobulins was carried out by an indirect technique.[33] Monospecific rabbit anti-rat IgG and IgM sera were obtained commercially (Miles Laboratories, Inc., Elkhart, ID, U.S.A.) and goat anti-rat IgA was a gift from Dr. H. Bazin (University of Louvain, Belgium). Fluorescein conjugated antisera were purchased from Hyland Laboratories, Inc., Costa Mesa, CA, U.S.A.).

Lymphocyte transformation assays

Lymphocyte-rich suspensions were prepared from peripheral blood and spleen by a Ficoll-Hypaque density gradient technique.[34] Blood was obtained from the abdominal aorta and was defibrinated by swirling it gently with siliconised glass beads. A spleen cell suspension was prepared by gently squeezing the spleen through a stainless steel seive. The lymphocyte-rich suspensions were washed in RPMI 1640 medium (GIBCO, Grand Island, NY, U.S.A.) and were finally resuspended and cultured in the same medium supplemented with 5% vitamin A-free rat serum, 2 mM of glutamine and 5×10^{-5} M of mercaptoethanol. The assays were carried out using a microculture technique, for which there were 2×10^5 cells in a total volume of 0.2 ml per well.[35] The cells were cultured in triplicate in the presence or absence of mitogen for 48 hours at 37°C in a 5% CO_2 atmosphere and were pulsed with 0.5 μ Ci of tritiated thymidine (specific activity 2 Ci/mmol, Radiochemical Centre, Amersham, England) 18-24 hours prior to harvesting. The cells were harvested using an automatic cell harvester and the amount of radioactive thymidine incorporated was determined by liquid scintillation counting. Results, expressed as stimulation index, were calculated from mean counts of a triplicate test. The optimal concentration of phytohaemagglutinin-P for rat lymphocytes was 100-200 μg/ml (PHA-P, Difco Lab., Detroit, MI, U.S.A.).

Quantitation of antibody-producing cells.

The procedure for the immunisation of animals with sheep red blood cells (SRBC) and for the quantitation of antibody-producing cells has been described.[37]

Statistical analysis.

The significance of differences between the mean values of different experimental groups was determined by the Student's t test.[38]

RESULTS

Effect of vitamin A deficiency on circulating level of blood lymphocytes

Data presented in Table 1 show clearly that vitamin A deficiency was associated wih lymphopenia. The depressed level of circulating lymphocytes involved both large and small cell subpopulations. No other types of white blood cells, with a possible exception of eosinophils, were particularly affected by the vitamin A deficiency state (data not shown). The depressive effect on circulating lymphocytes was noted within a few days of RA withdrawal and reached a maximum around day 8. Immunofluorescent staining of these circulat-

Table 1 Decreased level of peripheral blood lymphocytes in vitamin A deficient rats.

	No. of		Lymphocytes/mm^3 (mean ± SEM)		
	rats	%	Small	Large	Total
Vitamin A deficient (A^-)†	7	66±5	4,236±743	2,339± 410	6,432±1,081
Vitamin A sufficient (A^+)*	6	85±3	8,446±811	6,201±1,353	14,647±1,490

†Determined eight days after retinoic acid withdrawal (T_8).
*Supplemented with 1,000 μg of retinyl palmitate one and two days prior to the day of retinoic acid withdrawal (T_{-1} and T_{-2}).

Table 2 Effect of RA or RP repletion on the circulating level of blood lymphocytes of vitmain A deficient rats.

Dietary treatment		No. of	Lymphocytes/mm^3 (mean±SEM)			
Retinoids	Duration (wks)	rats	%	Small	Large	Total
A^-	0	12	67±3	4,626±300	2,923±276	7,549± 459
RA repletion	2	12	73±3	6,553±748	3,533±341	10,085±1,000
	4	12	75±2	7,441±700	4,675±367	12,117±1,019
	6	12	77±2	8,158±943	4,951±379	13,110±1,168
A^-	0	8	69±3	4,820±606	3,143±561	7,963±1,007
RP repletion	2	8	83±2	7,970±815	4,011±537	11,981±1,107
	4	8	80±2	6,338±836	3,579±271	9,917±1,073
	6	8	81±2	6,574±876	4,487±345	11,061±1,175

A^- = vitamin A deficient

ing lymphocytes for the presence of surface immunoglobulins showed that the percentage of cells with surface immunoglobulins of A^- animals (22%) was noticeably lower than that of A^+ controls (35%). Such a reduction was noted with all three classes of immunoglobulins, i.e., IgG, IgA and IgM.

Effect of RA and RP repletion on the circulating lymphocyte level of vitamin A deficient rats

In this experiment, blood was taken from all animals on the last day of the fourth supplementation-deprivation cycle (A^- group in Table 2), when their tissues were supposed to be completely depleted of all vitamin A reserves.[28] The circulating level of lymphocytes determined at this time was compared with those determined at the end of weeks 2, 4 and 6 following rearing on appropriated diets. Data presented in Table 2 clearly show that the lymphopenia associated with vitamin A deficiency could be readily corrected following repletion with either RA or RP. After only two weeks of repletion with either supplement, the numbers of lymphocytes, both large and small, continued to increase and were found to be significantly ($P < 0.05$) higher than those taken at the time when vitamin A repletion was started (A^- groups in Table 2). In both groups the proportions of small and large lymphocytes were similar and the ratio did not change significantly throughout the six weeks of observation.

One other interesting observation not shown in the table was the association of eosinopenia with the vitamin A deficiency state. During the deficiency state, the mean number of circulating eosinophils was markedly depressed; in fact they were found only in two of the 20 A^- rats examined. Following repletion of these deficient animals with either RA or RP, the numbers of eosinophils steadily increased and, like the lymphocytes, were significantly higher than the values noted on the day when the repletion was initiated.

Effect of RA and RP repletion on mitogen-induced transformation of lymphocytes of vitamin A deficient rats

Because our preliminary observation showed that lymphocytes from vitamin A deficient rats had a depressed response to PHA stimulation, an experiment was set up in this study to determine whether RA and RP could effectively restore this function. Two months after the vitamin A deficient rats were repleted with RA or RP, their peripheral blood and splenic lymphocytes were tested in a mitogen-induced transformation assay. The results presented in Table 3 show that both the peripheral blood and splenic lymphocytes of RA- and RP-repleted animals were more responsive to PHA stimulation than those of the deficient animals, i.e., lymphocytes obtained at the end of the fourth deprivation phase (A^- groups in Table 3). For both RA and RP groups, a significant increase ($P < 0.05$) was noted for both peripheral blood and splenic lymphocytes. As shown in the table, the values obtained following vitamin A repletion approached those of the pellet-fed controls.

Effect of RA and RP repletion on the number of antibody-producing cells

The results presented in Table 4 showed that RA was as effective as RP in restoring the number of antibody-producing cells in the spleen of vitamin A deficient rats. Two months after vitamin A deficient

Table 3 Effect of RA or RP repletion on lymphoproliferative response of vitamin A deficient rats.

	No. of rats	Source of lymphocytes	PHA stimulation index* (mean ± SEM)
Vitamin A deficient (A^-)	4	Peripheral blood	14±8
RA repletion	13		171±27
RP repletion	6		155±72
Pellet-fed controls	8		190±49
Vitamin A deficient (A^-)	4	Spleen	29± 8
RA repletion	10		112±18
RP repletion	6		71±24
Pellet-fed controls	7		161±48

*Stimulated with phytohaemagglutinin at final concentration of 100 μg/ml.

Table 4 Effect of RA or RP repletion on antibody-producing cells in the spleen of vitamin A deficient rats

	No. of rats	PFC/10^6 spleen cells (mean±SEM)
Vitamin A deficient (A^-)	5	302± 32
RA repletion	10	607±116
RP repletion	10	631±109

PFC = plaque-forming cell.

rats were given either RA or RP, the numbers of antibody-producing cells in the spleen of both treated groups were significantly higher than that of the untreated vitamin A deficient animals. It should be mentioned that the suppressive effect of vitamin A deficiency was not due to a shift of the peak response; as with all three groups of animals shown in the table, the peak response was noted four days after the animals had been immunised with SRBC.

DISCUSSION

Data obtained in the present study indicate that retinoic acid, like its ester counterpart (retinyl palmitate), can effectively correct the functional defects of lymphocytes of vitamin A deficient animals. This study confirms and extends the data previously reported by Mark *et al.*[7] Evidence presented in this study clearly show that RA is just as effective as RP in correcting some of the immune defects associated with vitamin A deficiency state. Other investigators have previously demonstrated that RA may be as active, if not more so, as RP in restoring some other functional defects known to be associated with the vitamin A deficiency state.[26,27,29]

There are many possible explanations of how vitamin A or its analogues could alter the number and functions of circulating lymphocytes. These include an increase in the rate of lymphocyte proliferation and differentiation, a decrease in the rate of lymphocyte destruction and an alteration in the lymphocyte traffic through the various lymphoid organs. Considering the functions of these retinoids on other mammalian tissues,[39] it is not unreasonable to assume that the effect is at least partly associated with enhancing the rate of lymphocyte differentiation and proliferation. However, because of their well-established function in glycoprotein synthesis,[39-42] it is also highly probable that the traffic of these lymphocytes, which is associated in part with the membrane glycoproteins, is also altered. Such a possibility gains support from the work of Bang and associates which shows that in vitamin A deficient chickens the infiltration of small lymphocytes into various submucosal sites is depressed.[4] Also in a more recent report, McDermott *et al*[43] have shown that lymphocytes from vitamin A deficient animals are defective with regard to their migration from the circulation into the intestinal tissue. A rapid recovery of lymphocyte numbers within three days of repletion of vitamin A deficient rats, using either retinoic acid or retinyl acetate, suggests that these lymphocytes may have sequestered in some tissue during the deficiency state.[44] Mark *et al*[7] showed that the membrane glycoproteins extracted from splenic lymphocytes of vitamin A deficient rats are different from those of normal rats. In fact, other lines of evidence also suggest that the effect of vitamin A or its analogues on various immune functions[45-48] is associated at least in part with alteration of membrane glycoproteins.

In conclusion, data presented in the present study as well as those reported previously by other investigators indicate that RA is a least equally as active as RP in correcting some of the known immune defects associated with the vitamin A deficiency state. Such a conclusion implies that RA can substitute for vitamin A or RP in maintaining the integrity of the immune system. This is particularly important in view of the fact that unlike vitamin A and RP, RA is excreted quickly, thus eliminating the chance of its being accumulated to a toxic level. The latter situation is not uncommonly found following the ad-

ministration of an excessively large dose of vitamin A for therapeutic purposes. For this reason, many investigators and clinicians now use RA, either alone or in conjunction with other agents, for the treatment of different types of human tumours. In the future, attempts should be made to search for new vitamin A analogues, more active and more readily tolerated than RA, and if possible, more specific with regard to their action on particular subpopulations of lymphocytes.

ACKNOWLEDGEMENTS

We are grateful to Dr. T.W. Flegel, Department of Microbiology, Faculty of Science, Mahidol University, for his criticisms during the preparation of this manuscript. This work was supported by Grant No. AM 11367 from the National Institutes of Health, U.S.A.

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