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Lung Retinyl Ester Is Low in Young Adult Rats Fed a Vitamin A–Deficient Diet after Weaning, despite Neonatal Vitamin A Supplementation and Maintenance of Normal Plasma Retinol[1,,2]

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Abstract

Although it is understood that plasma retinol concentration is not proportional to the concentration of vitamin A stored in liver, plasma retinol still is often used as an indicator of vitamin A status. An aim of vitamin A supplementation strategies is to maintain plasma retinol concentration in a range considered adequate, generally >1.05 μmol/L in humans, with some adjustment for age. In the present study in rats, we addressed the following question: Does lung vitamin A increase postnatally, as is observed in rats fed a vitamin A–adequate diet, if plasma retinol is maintained at ~1 μmol/L by supplementation at neonatal age, but the weaning diet is deficient in vitamin A? We treated rats on postnatal d 6, 7, and 8 with placebo (oil), vitamin A, retinoic acid (RA), and a nutrient-metabolite combination of vitamin A and RA, VARA, after which tissues were analyzed on d 9. Other rats treated identically as neonates were fed a vitamin A–deficient diet from 3–9 wk of age, and in parallel, another group of rats was fed a vitamin A–adequate diet. Although supplementation with vitamin A or VARA elevated liver and lung retinyl esters (RE) on d 9 ($P < 0.0001$), and prevented the fall in plasma retinol to <1 μmol/L by 9 wk of age, when the diet was vitamin A–deficient, lung RE fell to 28% of the concentration present in the lungs of rats fed the vitamin A–adequate diet ($P < 0.0001$). We infer that the lungs depend, at least in part, on the uptake of dietary vitamin A, probably from chylomicrons, to develop RE stores in the postweaning growth period.

Introduction

Vitamin A supplementation is now recognized as an effective means to prevent xerophthalmia and reduce morbidity and mortality in infants and young children (1–5). WHO/UNICEF has recommended that vitamin A supplements be given to infants and young children in at-risk populations as a safeguard against an inadequate dietary intake of vitamin A (6,7). Over the years, the concept has developed that providing vitamin A periodically, typically every 4–6 mo and in the range of 15–30 mg retinol for infants and young children up to 11 mo of age, and 60 mg for children 12 mo and older [see discussion in (8,9)], is an adequate strategy, because no further reduction in morbidity and mortality were observed with 4-mo vs. 6-mo intervals (8). It is believed that with these doses, enough vitamin A is stored in the liver to maintain plasma retinol at a sufficient level [generally considered ≥1.05 μmol/L (30 μg/dL) in children (10,11)], for the time between dosings. An underlying

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premise of this strategy is that as long as plasma retinol remains at or above an accepted level, target tissues that use vitamin A will receive an adequate supply of retinol from the circulation. The relationship between liver vitamin A storage and plasma retinol concentration has been studied for a number of years, being first deduced from analysis of human and animal plasma and liver tissue specimens (12), and later studied in vivo using isotope dilution methods, including some studies in which both plasma retinol and liver retinol were directly sampled (13,14). Additionally, numerous studies of plasma and liver retinol levels have been conducted in rats, in which dietary vitamin Awas also controlled (15–18). Nonetheless, there is still a paucity of information concerning the relationship between dietary vitamin A intake, plasma retinol concentration, and the levels of vitamin A in tissues other than the liver. Particularly, it has not been directly demonstrated that an adequate concentration of plasma retinol alone is sufficient to maintain tissue retinol levels under circumstances in which dietary vitamin A intake is low or absent.

This study of lung retinol levels was part of an experiment on the effects of vitamin A supplementation in the neonatal period on measures of immunocompetence early in life and at young adult age in rats that had been fed a vitamin A–deficient diet after weaning (19). In this study, neonatal-age rats nursed by vitamin A–adequate dams were supplemented with vitamin A alone and with the principal active metabolite of retinol, retinoic acid (RA),[5] as well as the combination of both, VARA, consisting of a 10:1 ratio of vitamin A and RA. This combination was tested because it had been shown previously to synergistically augment lung retinyl ester (RE) formation in neonatal-age rats (20). The lungs are of special interest because vitamin A is important in the development of the lungs, both pre-and postnatally, in humans as well as many animal species, including rats and mice (21). Clinically, low plasma retinol is associated with increased risk of bronchopulmonary dysplasia, and vitamin A may improve outcome (22–24). At birth, tissue retinol levels are low (25). An investigation of the ontogeny of lung and liver vitamin A concentrations in the perinatal period, conducted in rats where the dams were vitamin A adequate and the weanling rats were fed a diet with adequate vitamin A, showed that RE and retinol concentrations in the lungs are higher in fetal rats, fall before birth, and remain low up to weaning (26). The sources of retinoids taken up by the lungs are not well understood. Retinol circulates in plasma bound to retinol-binding protein (RBP), which is maintained at nearly constant levels over a wide range of liver retinol concentrations (12,15), while after meals containing vitamin A, RE, and, to a lesser extent, retinol are present in the circulation in chylomicrons, chylomicron remnants, and other lipoproteins to which some RE may transfer (27).

In this study, we analyzed total retinol concentrations in the liver, plasma, and lungs of rats with and without neonatal vitamin A supplementation and after weaning onto a vitamin A–deficient diet, in comparison to rats fed a vitamin A–adequate diet. The results provide evidence that plasma retinol alone, even ~1 μmol/L, may not be adequate for increasing lung total retinol concentrations in the postweaning period, if the diet is inadequate in vitamin A. From these results, we infer that the direct input of vitamin A from the diet to the lungs may be important, regardless of whether liver vitamin A storage is sufficient to maintain a normal concentration of plasma retinol.

[5]Abbreviations used: RA, retinoic acid; RBP, retinol-binding protein; RE, retinyl ester; VARA, vitamin A combined with RA, 10:1 mol:mol.

Materials and Methods

Animals and diets

All procedures with animals were approved by the Institutional Animal Care and Use Committee of The Pennsylvania State University. The neonates were the female offspring or cross-fostered pups of Sprague-Dawley rat dams that were purchased (Charles River) each with 12–14 female pups, 4–5 d old. The rats were housed in a rodent facility at 22°C with a 12-h light-dark cycle, with free access to food and drinking water. Because the dams had been fed a commercial stock diet by the supplier, they were therefore vitamin A adequate, but they were fed a modified vitamin A–deficient AIN-93G diet (28) after arrival to reduce the transfer of vitamin A in milk to the nursling pups (29) and shorten the onset of vitamin A deficiency. The pups were randomized into 4 groups for supplementation on postnatal d 6, 7, and 8, with 1 of 4 doses: oil, vitamin A, RA, or VARA, which are described in the next section. Tissues from some pups were collected on d 9 for determination of tissue retinol immediately after neonatal treatment. Other pups were treated identically with canola oil as vehicle, vitamin A, RA, or VARA on d 6, 7, and 8. These rats stayed with their dams until they were weaned at 3 wk of age and fed a vitamin A–deficient diet until they were 9 wk old. In parallel, a separate group of rats, referred to as vitamin A–adequate age-matched rats, was treated with vehicle when they were neonates and fed the same diet with an adequate level of vitamin A [4mg of retinol, in the form of retinyl palmitate, per kg of diet, (Research Diets)] to maintain normal vitamin A status during growth. Most of the rats in the 9-wk study were also immunized on d 7 and again 7–10 d before the end of the study with tetanus toxoid, as described previously [see Fig. 1A within reference (19)]. This immunization component of this study has been fully described and, because this procedure is not expected to have had any effect on tissue retinol levels, it will not be considered further. All rats had free access to food and water throughout the study.

Supplementation and tissue collection

Rat pups within litters were divided into the following 4 supplementation groups: oil (placebo control containing vehicle only), vitamin A, RA, and VARA (Fig. 1A). The dosages were selected to resemble a dose of retinol used previously in human studies of vitamin A supplementation (30,31), and a dose of RA used in rat studies lung septation (32); the rationale for each dose has been fully described (33). The vitamin A dose preparation contained 50 nmol of retinyl palmitate per mL and the RA dose preparation contained 5 nmol of all-*trans*-RA per mL, each prepared in oil. The VARA dose preparation contained the same amounts of retinyl palmitate and RA as above and, therefore, the retinol to RA ratio was 10:1 (33). Each pup was weighed daily and administered 0.4 μL/g body weight of the assigned dose, which was delivered orally directly into the mouth by micropipette. One set of rats (n = 4/group) was killed on postnatal d 9 to determine the immediate effects on supplementation on liver, plasma, and lung total retinol levels. The other rats, (n = 6/group), remained with their mothers until 3 wk of age and were then fed the vitamin A–deficient diet until they were 9 wk old. The vitamin A–adequate age-matched reference group was treated with oil as neonate and fed the vitamin A–adequate diet after weaning.

Tissue collection and retinol analysis

At d 9 or wk 9, rats were killed by carbon dioxide inhalation, blood was collected into heparinized syringes for preparation of plasma, and the liver and lungs were dissected, weighed, and then rapidly frozen for storage prior to analysis (34). Plasma total retinol concentration was determined after saponification using a reverse-phase HPLC method with detection at 325 nm and trimethylmethoxyphenol retinol as an internal standard (20). Liver and lung tissue was subjected to total lipid extraction, followed by purification of the lipid extract, and either analysis of individual RE and retinol by HPLC, or after saponification of

the total lipid extract and the quantification of total retinol. The sum of the individual RE constituted ~95% of the total retinol in the liver and lungs (33) and, because the results for total retinol were similar without and after saponification, we analyzed most samples as total retinol after saponification.

Statistical analysis

Data are expressed as means ± SEM. When variances among groups were unequal, the data were transformed (log10) before statistical analysis. Differences among the 8 main groups (4 supplementation groups at 2 ages) were analyzed by 2-way ANOVA, followed by Fisher's protected least significant difference test and least squares means test to compare all groups. The vitamin A–adequate reference group was compared by ANOVA to the other 9-wk-old rats (SuperAnova, Abacus Software).A difference of $P < 0.05$ was considered significant.

Results

Liver vitamin A increased after vitamin A and VARA supplementation in neonatal rats and declined on a vitamin A–deficient diet in young adult rats

Rats were treated on the schedule shown in Fig. 1*A*. In 9-d control rats (Fig. 1*B*), liver retinol was <20 nmol/g, well below the value of 70 nmol/g liver [20 μg retinol/g, which is often referenced as a value below which liver reserves are inadequate in humans (10,11)]. However, this value is typical for the newborns of vitamin A–adequate rat dams (35). Supplementation with vitamin A alone or as a component of VARA increased liver total retinol to nearly 300 nmol/g. Supplementation with RA alone, or as a component of VARA, had no effect on liver retinol, because RA cannot be converted to retinol; these data are in agreement with our previous results (20). When the rats were 9 wk old, having been fed the vitamin A–deficient diet for 6 wk, liver retinol was ≤2 nmol/g in the oil and RA groups. In the groups treated with vitamin A and VARA as neonates, the values were lower than they were immediately after supplementation, but they were still at or slightly above 70 nmol/g. Overall, liver retinol fell in all groups of rats fed the vitamin A–deficient diet ($P < 0.0001$ for 9 d vs. 9 wk), but it was higher in rats that had been supplemented as neonates with vitamin A or VARA, compared with oil or RA ($P < 0.0001$).

Plasma retinol was maintained by neonatal supplementation with vitamin A or VARA in rats fed vitamin A–deficient diet after weaning

Plasma retinol was determined in the same 9-d- and 9-wk-old rats (Fig. 2). Values representative of vitamin A status in children (11) are included in the figure for comparison to the determined plasma retinol concentrations in our rats. At 9 d of age, plasma retinol was >1.0 μmol/L in all groups (Fig. 2*A*). The higher concentrations in the vitamin A and VARA groups at this age are likely the result of some residual unprocessed lipoprotein-associated RE from the last supplement existing in the circulation at this time. At 9 wk of age, after 6 wk on the vitamin A–deficient diet, plasma retinol had fallen in the oil and RA groups whereas, in contrast, plasma retinol was still >1 μmol/L in the groups that received vitamin A and VARA as neonates, similar to same-aged rats that were fed the vitamin A–adequate diet after weaning (Fig. 2*B*).

Retinol concentration in the lungs was not maintained by neonatal-age supplementation with vitamin A or VARA when rats were fed a vitamin A–deficient diet after weaning

Lung total retinol, determined in 9-d-old rats after supplementation with vitamin A, RA, or VARA, showed the same pattern we reported previously for similarly treated neonatal rats (20). Whereas the concentration in the control group was low (~1.5 nmol/g), it was significantly increased by vitamin A and RA, each given alone, while VARA containing the

same amount of vitamin A combined with the same amount of RA as in the single doses produced a strongly synergistic increase (Fig. 3*A*). When lung total retinol concentrations were determined at 9 wk of age, values had fallen below 1 nmol/g in the oil and RA groups (Fig. 3*B*). Lung total retinol was significantly higher in rats that had received vitamin A or VARA, but these values were 72% lower than those of the 9-wk-old rats that had been treated with oil as neonates and fed the vitamin A–adequate diet after weaning.

Discussion

The main observation from this study is that even when vitamin A supplementation is sufficient to maintain plasma retinol in the normal range (~1 μmol/L) in the absence of dietary vitamin A, this may not provide retinol to vitamin A–requiring target tissues, such as the lungs, in an amount or manner that is equivalent to that delivered by a vitamin A–adequate diet. The results of our study confirmed previous work showing that physiological levels of lung and liver retinol are low at birth (36), even though plasma retinol is not necessarily low in the offspring of vitamin A–adequate rat dams (35,37; Fig. 2*A*). Thus, neonates have little in the way of tissue retinol reserves on which to draw, and thus they appear to be highly dependent on obtaining adequate vitamin A from mother's milk, supplements, or, later on, from their postweaning diet, to build tissue reserves. The increase in retinol in the lungs of neonatal rats supplemented with vitamin A alone (Fig. 3*A*), implies that the amount of retinol derived from milk (neonatal diet) may be limiting. The increase in lung retinol, most of which was RE [see (33)], after supplementation with vitamin A and RA alone, and the synergistic increase in lung RE after treatment with VARA, may be due to the higher induction of lecithin:retinol acyltransferase (LRAT) in the lungs compared with the liver, as lung LRAT was previously shown to be increased severalfold above control levels by RA of adult rats (38). However, further studies are needed to determine the mechanism underlying the synergistic effect of VARA in the lungs. In any case, VARA supplementation in the neonatal period was very effective in increasing lung RE rapidly (20,33,39; Fig. 3*A*), and this form of vitamin A supplementation might have utility in the treatment of bronchopulmonary dysplasia and other lung disorders, as we have suggested previously (39).

In our previous studies, after cessation of treatment with VARA, lung RE declined over the next 4 d, but the concentration still remained above that in the control group (20). In this study, nearly 8 wk had elapsed after the last treatment with VARA, and at that time there was no remaining evidence of the synergistic increase in lung total retinol seen at 9 d of age. These results imply that lung retinol had completely turned over in this interval and that only dietary vitamin A after weaning was likely to be a determinant of lung total retinol concentration at a young adult age. However, we believe that the most significant observation from these studies is that in the postweaning period, when dietary vitamin A was lacking, the neonatal supplementation treatments that preserved plasma retinol at a normal level did not prevent a >70% reduction in lung total retinol, as compared with the level in rats that were fed a vitamin A–adequate diet. We infer from these data that vitamin A from diet is necessary in the postweaning period to maintain lung retinol concentrations. A possible model for this effect is illustrated in Fig. 4. We speculate that in rats that received vitamin A, RA, or VARA as neonates, lung total retinol was elevated immediately after treatment (Fig. 4*A*). The doses of vitamin A and VARA are expected to rapidly and transiently increase the RE and retinol contents of chylomicrons, nearly in direct proportion to the amount of vitamin A ingested (40). Newly absorbed vitamin A, either due to its form as RE or to its presence in chylomicrons, might be an important contributor of vitamin A as RE and/or retinol to the lungs. When the diet was deficient in vitamin A, it is likely that only retinol released from storage in liver or other tissues and transported on RBP was able to supply retinol to target tissues. Apparently, dietary input, presumably as chylomicron-

associated RE and retinol, is necessary to prevent the level of vitamin A in the lungs from falling to low values, such as those in our young adult rats treated with oil or RA and fed the vitamin A–deficient diet (Fig. 3*B* and Fig. 4*A*). Clearly, there was some residual effect of neonatal-age supplementation with vitamin A or VARA that could still be seen at the young adult age, as lung total retinol concentrations were severalfold higher in the vitamin A and VARA groups than in the control and RA-treated groups (Fig. 3*B*). But nevertheless, neonatal treatment with vitamin A or VARA was not equivalent to the effect of an adequate postweaning diet (Fig. 3*B*), even though plasma retinol levels were indistinguishable between 9-wk-old rats treated with vitamin A or VARA as neonates and the age-matched group fed a vitamin A–adequate diet (Fig. 2*B*). We hypothesize that the frequent intake of vitamin A-containing foods, through formation of RE- and retinol-containing chylomicrons and possibly other lipoproteins (41,42), provides a second source of vitamin A to the lungs (Fig. 4*B*), in addition to that provided by retinol bound to plasma RBP, and that this provides a substrate for the accumulation of vitamin A reserves in the lungs (and liver), as compared with those which accumulate when plasma retinol bound to RBP is the only form of retinol in the circulation. Further studies are needed to directly test the contribution of chylomicrons to lung vitamin A accumulation.

There are 2 principal limitations to the interpretation of our data. First, there are no normative data for vitamin A in lung tissue to which to compare the values in our study, and no “optimal” level of lung vitamin A storage has been determined. However, we contend that our comparison with rats fed a vitamin A–adequate diet is a fair measure of the “normal” postweaning response of the lungs to vitamin A when it is available after uptake from diet. Secondly, we do not at this time have any evidence that the differences in lung retinol levels (see Fig. 3) are associated with physiological differences. Nonetheless, some data suggests that low lung retinol levels exist in pathophysiological contexts. It has been reported that vitamin A in adult rat lung is reduced by 60% by a 1-wk glucocorticoid treatment (43), and by 30% in the lung tissue of patients with lung cancer compared with levels in those without treatment (44). Given the demonstrated roles that retinoids play in regulating gene expression in the lungs and the extensive maturation of the lungs that occurs postnatally in rodents and humans alike [see (45,46) for reviews], and demonstrations that RA can reverse the impairment of lung septation due to glucocorticoid treatment in rat and mouse models (47,48), it seems plausible that adequate tissue RE and retinol concentrations could have an impact on these processes. Thus, we suggest that it is important to further investigate how dietary vitamin A and different vitamin A supplementation strategies, alone and combined, may affect retinol accumulation in the lungs, and to learn what tissue retinol levels may be “optimal” with respect to lung maturation and physiological functioning, both early and later on in life.

In conclusion, this study provides evidence that the lungs of young rats require a steady input of dietary vitamin A, presumably delivered by chylomicrons, to accumulate and/or maintain total retinol in the lungs during the postnatal period of rapid growth. In the absence of dietary vitamin A, a normal level of plasma retinol alone was not enough to support lung vitamin A storage in this period. These results suggest that high-dose vitamin A supplementation, even if it is adequate for maintaining plasma retinol over a period of time, may not be an optimal strategy for maintaining the health of peripheral tissues such as the lungs. Interestingly, there is increasing interest in using dietary, food-based approaches to provide adequate vitamin A to human populations. Such a strategy was advocated in the 1970s (49), and more recently (50,51). We believe our results add new biochemical evidence that a dietary approach could be very important for the establishment and maintenance of adequate vitamin A reserves in peripheral tissues such as the lungs. A diet-based approach may diversify the molecular forms of retinol in plasma, principally by providing RE formed during the intestinal metabolism of vitamin A and transported by

lipoproteins. At present, little is known of the functionality of chylomicron RE, other than as an efficient means of storing or transporting retinol. A better understanding of its role in providing vitamin A to extrahepatic tissues is necessary to help connect dietary vitamin A intake, plasma retinol levels, tissue vitamin A concentration, and, possibly, health outcomes.

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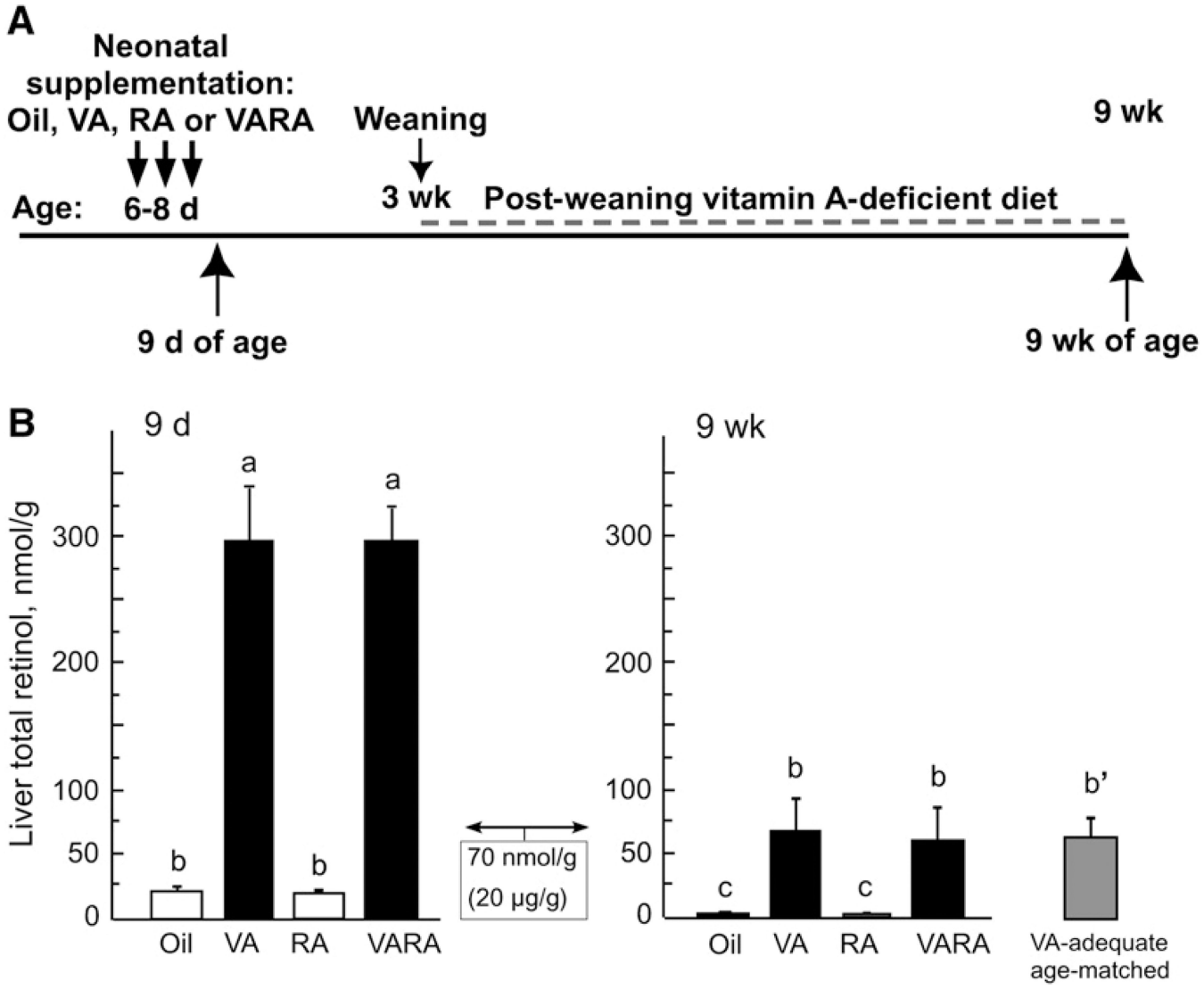

FIGURE 1.

Design of study (*A*) and liver total retinol at 9 d and 9 wk of age (*B*) in rats treated as neonates with oil, vitamin A (VA), RA, or VARA. The box showing 70 nmol/g (20 μg/g) represents a value considered to indicate sufficient liver retinol storage in adult humans (see text). Values are means ± SE, $n = 4$ (9 d) and 6 (9 wk). Means without a common letter differ, $P < 0.0001$. The reference group of 9-wk-old VA–adequate rats marked *b*′ did not differ from the 9-wk groups marked *b*, but was not compared with the 9-d-old rats.

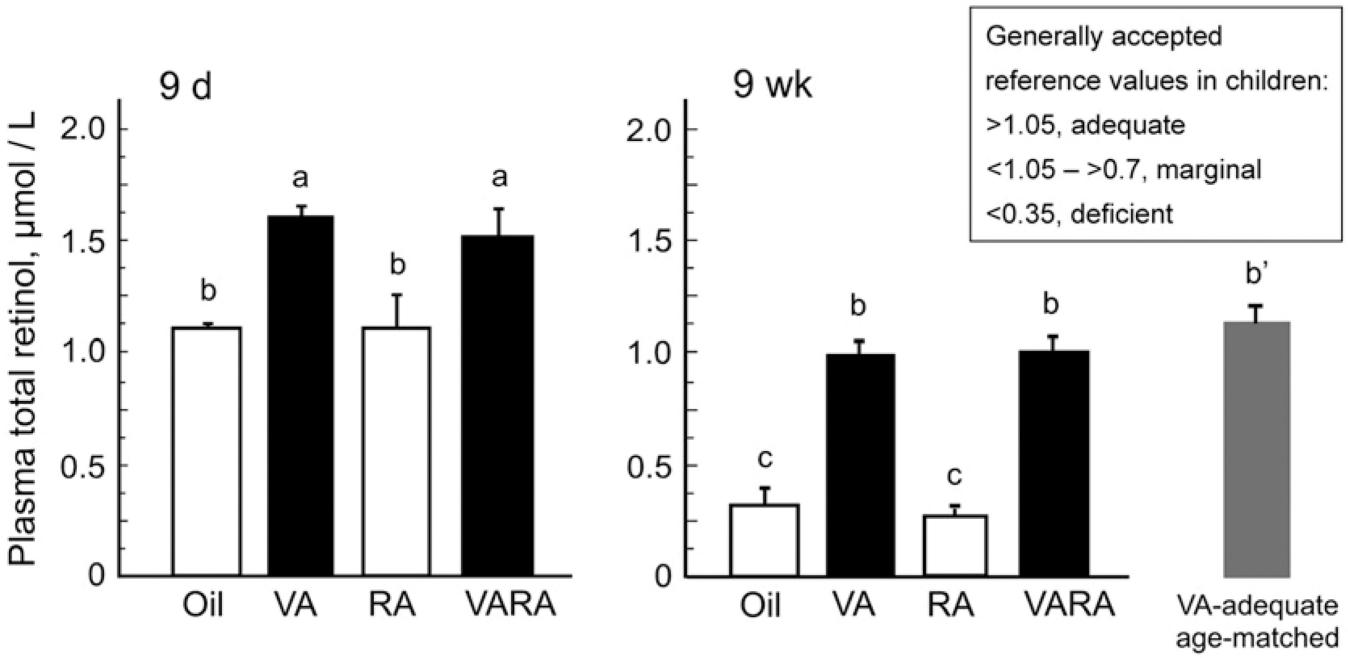

FIGURE 2.

Plasma retinol at 9 d and 9 wk of age in rats treated as neonates with oil, vitamin A (VA), RA, or VARA. At 9 wk the value for a group of same-aged rats fed the vitamin A–adequate diet is also shown. The box shows values that have been used as indicators of vitamin A status in humans (see text). Values are means ± SE, $n = 4$ (9 d) and 6 (9 wk). Means without a common letter differ, $P < 0.05$. The reference group of 9-wk-old VA–adequate rats marked *b*′ did not differ from the 9-wk groups marked *b*, but was not compared with the 9-d-old rats.

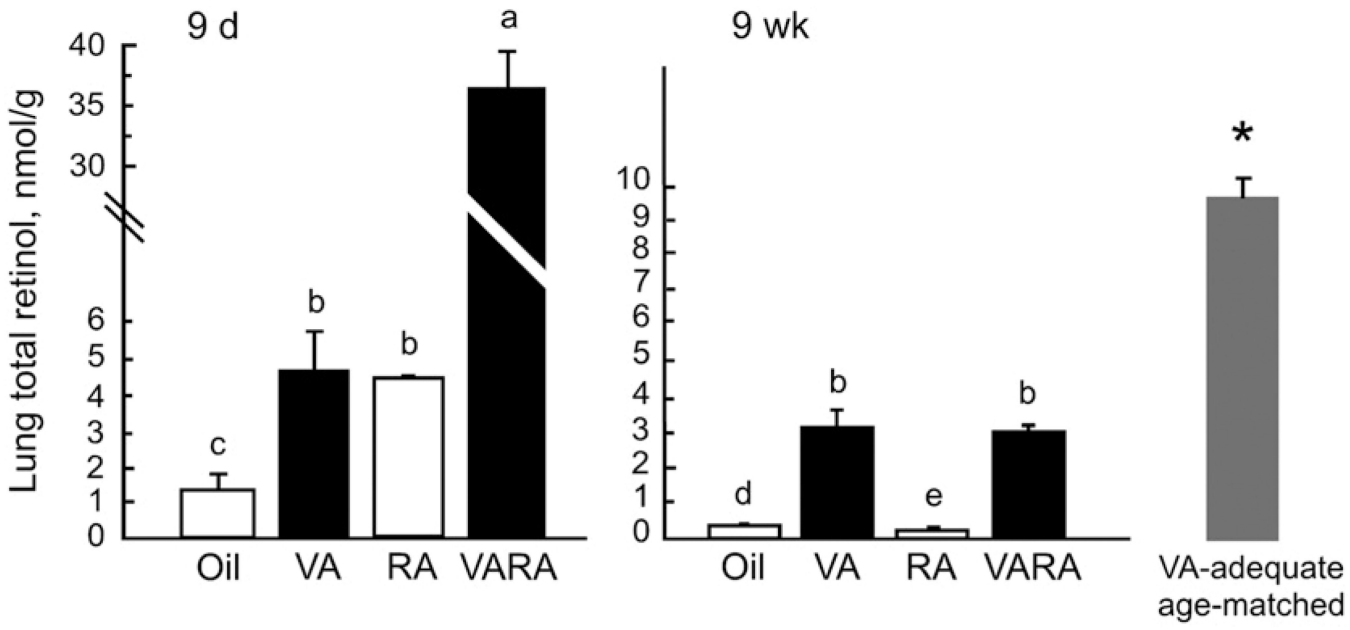

FIGURE 3.

Lung total retinol at 9 d and 9 wk of age in rats treated as neonates with oil, vitamin A (VA), RA, or VARA. At 9 wk the value for a group of same-aged rats fed vitamin A–adequate diet is also shown. No reference values are shown, as none have been established for lung. Values are means ± SE, $n = 4$ (9 d) and 6 (9 wk). Means without a common letter differ, $P < 0.0001$. The reference group of 9-wk-old VA–adequate rats marked * differed from the other 9-wk groups ($P < 0.001$), but was not compared with the 9-d-old rats.

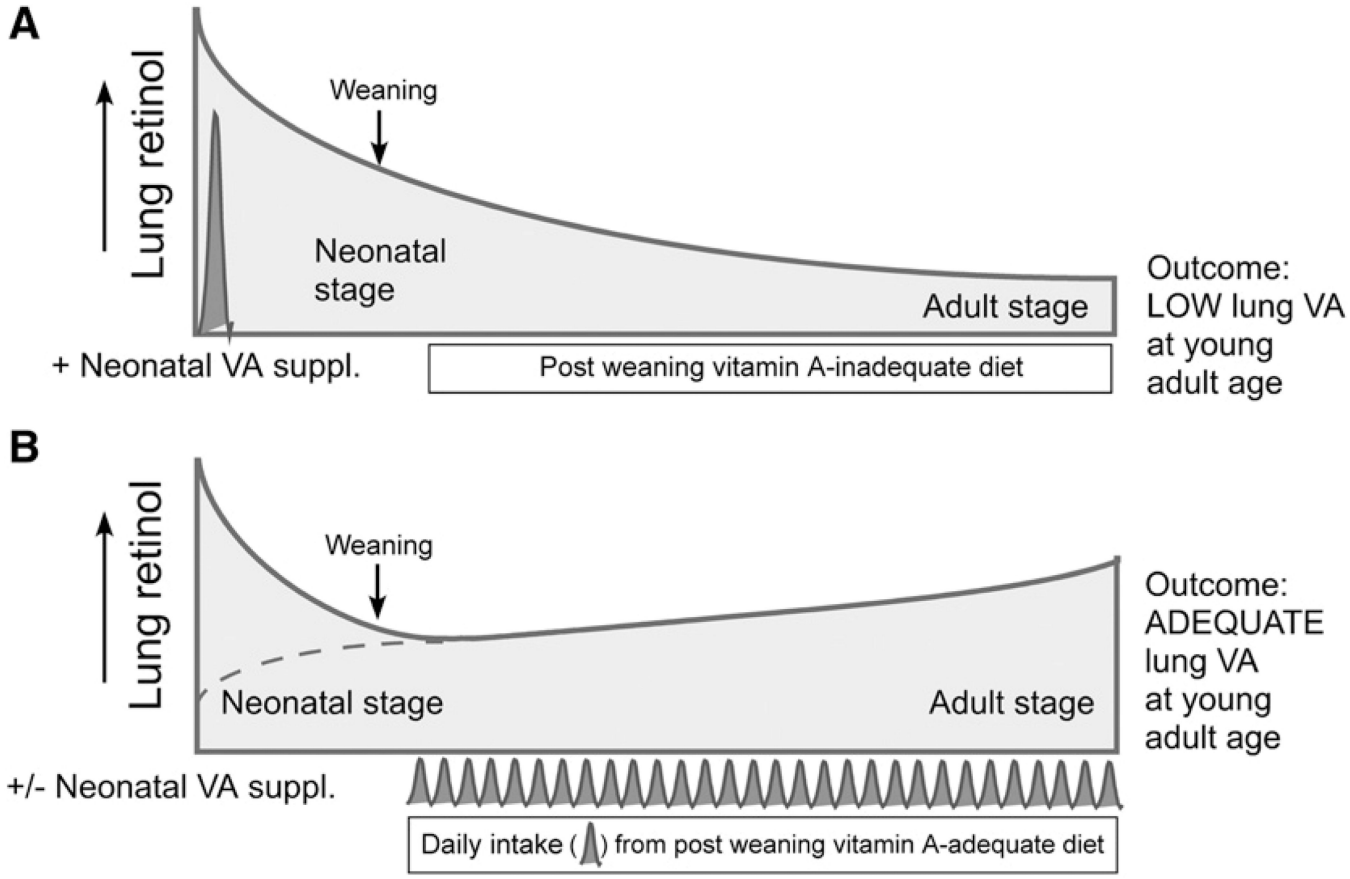

FIGURE 4.

Model of situations and outcomes resulting from neonatal supplementation with vitamin A or VARA, and a postweaning diet either lacking or adequate in vitamin A. (*A*) Supplementation with vitamin A or VARA would be expected to increase lung total retinol in the neonatal period, resulting in somewhat higher levels in the lungs at 9 wk, as compared with supplementation with oil or RA alone (see Fig. 3), but not to the level observed in rats fed vitamin A after weaning. (*B*) Regardless of neonatal vitamin A supplementation (dashed line without vitamin A or VARA; upper solid line with these treatments), the daily input of dietary vitamin A, mostly as RE, supports the gradual accumulation of vitamin A in the lungs, leading to the adult level.