Vitamin A deficiency and Newcastle disease virus infection in chickens: a model for the study of measles infection in vitamin A deficient children

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Vitamin A deficiency and Newcastle disease virus infection in chickens: a model for the study of measles infection in vitamin A-deficient children

Proefschrift
ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
op gezag van de rector magnificus,
dr. H. C. van der Plas,
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STELLINGEN

1. Er bestaat een interactie tussen Vitamine A-status en Newcastle disease virus-infectie.
(dit proefschrift)

2. Het verkrijgen van marginaal vitamine A-deficiënte kuikens die gedurende langere tijd in een ogenschijnlijk gezonde conditie dienen te blijven, lijkt simpel, maar is het niet.
(dit proefschrift).

3. In het toekomstig vitamine A-onderzoek dient het effect van marginale vitamine A-deficiëntie op het functioneren van het afweersysteem nader onderzocht te worden.
(Sommer et al. (1983) Lancet ii: 585-588: dit proefschrift)

4. Een lentogene stam van Newcastle disease virus kan wel degelijk pathogeen zijn in vitamine A-deficiënte kuikens.
(dit proefschrift)

5. Vanwege het veelvuldig voorkomen van eiwit- en energietekorten in vitamine A-deficiënte proefdieren worden veel waargenomen effecten ten onrechte toegeschreven aan vitamine A-deficiëntie per sé.

6. Het gebruik van proefdieren bij het uittesten van 'biological response modifiers' met een mogelijke therapeutische werking bij de mens is slechts van beperkte waarde.

7. Het gebruik van cytokines in combinatie met cytostatica lijkt een veelbelovende manier om tumoren te bestrijden.

8. Bacteriën zijn, in tegenstelling tot virussen, eerder een zegen dan een vloek voor de mensheid.

9. De wetenschap dat 'human growth hormone' een sterk anabool effect bezit, goedkoop geproduceerd kan worden met biotechnologische technieken en bovendien (nog) niet kan worden aangetoond tijdens dopingcontroles zal voor veel topsporters eerder een vloek dan een zegen zijn.

10. Hoe dodelijker een virus des te geringer is de overlevingskans van dat virus.

11. Het niet mogen vermelden van de term vitamine C als antioxidant op verpakkingen, in tegenstelling tot haar naam ascorbinezuur, is een vorm van bedrog.

12. De naam van de studierichting "voeding van de mens" aan de Landbouwuniversiteit geeft geen enkel inzicht in de aard van de kennis van een hierin afgestudeerde ingenieur.

13. De afbeelding van proefdieren op de voorkant van menig proefschrift doet het ergste vermoeden.

14. Uit het feit dat stellingen altijd op een los vel papier aan proefschriften moeten worden toegevoegd, mag niet geconcludeerd worden dat ze van geen enkele waarde zijn bij een promotie.

Proefschrift S.R. Sijtsma.
Vitamin A deficiency and Newcastle disease virus infection in chickens: a model for the study of measles infection in vitamin A-deficient children.
Wageningen, 8 september 1989.

Aan mijn ouders

Aan Annie

CONTENTS

VOORWOORD

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CHAPTER 1

General introduction

MALNUTRITION AND INFECTION

Millions of people die each year because of the combined effects of malnutrition and infectious disease (1,2). This is of particular relevance in developing countries where malnutrition and infection often co-exist, especially in infants and weanling children. The estimated daily rate of death from infection and malnutrition is 40,000 and this far exceeds the number of lives lost from heart disease, cancer, trauma or any other cause (3). Metabolic, hormonal and physiological responses to infection all combine to accelerate the utilization of body nutrients. In addition, the combination of multiple host responses to infection produces substantial direct and indirect losses of nitrogen and many other essential nutrients from the body. Food intake is lowered and the absorption of nutrients from the intestinal tract is reduced (4). Malnutrition can also alter the nature and degree of response, and thus the susceptibility of humans and animals to a number of infections (1,2). The interaction of nutrition and infectious disease can be synergistic thus leading to a progressively more severe state of disease with numerous complications. This progression has been described as a "vicious circle" or "downhill spiral" which eventually leads to death (1,3,4). Both malnutrition and infection, alone or in combination, can initiate important secondary effects on body defense mechanisms, either nonspecific or of the immune system itself. Virtually each form of malnutrition has an adverse effect on some generalized aspect of host resistance (5-7) and produces specific effects on many of the recognized nonimmunological and immunological mechanisms of host defense (8). Different infectious diseases also exert specific effects on the immune system itself (4,9).

VITAMIN A DEFICIENCY AND MALNUTRITION

Vitamin A deficiency is one of the three major micronutrient deficiencies, together with iodine deficiency and nutritional anemia, affecting significant

proportions of certain population cohorts (10). The occurrence of vitamin A deficiency has been documented extensively. The causal factors may relate to the host, the diet, and the environment (11,12). Vitamin A deficiency is confined to the lower socioeconomic levels of the society (13). It results from the consumption of diets with low levels of (pro)vitamin A or other nutrients important for absorption of this vitamin from the intestine, and is usually accompanied by infection and infestation in people who are generally poor and uneducated (10). Young children constitute the most vulnerable group. At this stage of life, children are growing quickly and have high physiological requirements for vitamin A (14). Boys are more susceptible than girls (14,15) but this difference is possibly explained by cultural factors rather than by intrinsic sex-linked factors (13). Moreover, availability of vegetables and fruit which contain provitamin A (carotenoids) is often seasonal and vitamin A status varies accordingly (14). It should be stressed that vitamin A deficiency usually does not occur as an isolated problem but is almost invariably accompanied by protein-energy malnutrition (10,16) and sometimes by other micronutrient deficiencies (17-20). The relationship between cause and effect is unclear. For example, on the one hand, hypoproteinemia impairs the absorption of vitamin A and carotenoids and the synthesis of the retinol carrier proteins, retinol-binding protein and transthyretin (21-23). On the other hand, vitamin A deficiency can lead to loss of appetite with concomitant protein-energy malnutrition (20).

SYSTEMIC MANIFESTATIONS OF VITAMIN A DEFICIENCY

Vitamin A deficiency can result in xerophthalmia of increasing severity. Xerophthalmia, the term used for all ocular manifestations of impaired vitamin A metabolism (24), is the most obvious and dramatic manifestation of vitamin A deficiency (25-28). The eye lesions are classified by the World Health Organization (12) as "mild xerophthalmia" (night blindness, conjunctival xerosis and Bitot's spots) and as "active xerophthalmia" (night blindness, conjunctival xerosis, Bitot's spots, corneal xerosis and corneal ulceration/ keratomalacia). Apart from lesions resulting from its role in the visual cycle (29,30), other systemic changes thought to result from vitamin A deficiency are less well understood. Most of the systemic manifestations have been investigated in laboratory animals. It has been shown that the earliest clinical signs of vitamin A deficiency are an elevation of cerebrospinal fluid

pressure (31,32), followed by reduced food intake, inhibition of growth (13,32-34), and by evidence that cells of epithelial and mesenchymal origin are not maintaining their normal pattern of differentiation (32,35,36). In vitamin A deficiency, surface epithelia such as those of the respiratory, gastrointestinal and genitourinary tracts undergo keratinizing metaplasia (32,35,37). Besides this epithelia-damaging effect which impairs host defense against invading agents, vitamin A deficiency also affects other forms of nonspecific resistance and the immune system itself. Several studies indicate an impairment of both humoral and cell-mediated immune response (38-40). Vitamin A is also necessary for reproduction and fetal development (32,41). Recently, interest has been revived in the relationship between vitamin A and cancer. It may well be that carotenoids are more important than retinol in protecting against cancer (42).

VITAMIN A DEFICIENCY, MORBIDITY AND MORTALITY

Vitamin A deficiency is associated with increased morbidity and mortality (1,27,43). It has been reported that 50-80% of children with blindness associated with vitamin A deficiency are dead within several months of the blinding episode (13). Moreover, a longitudinal study, carried out in Indonesia, revealed that even mild signs of vitamin A deficiency in preschool age children were associated with a two- to three-fold increase in diarrhea and respiratory disease and a four-fold increase in mortality (27). Similar observations were reported for Indian children (44). In an intervention community-based trial in another ethnically distinct population in Indonesia, childhood mortality was 34% lower in preschool children supplemented with large, oral doses of vitamin A (45). Similar results were reported in a field trial in India with vitamin A fortification of monosodium glutamate (46). These studies indicate that children may suffer physiologically important consequences of vitamin A deficiency even before they reach the stage of exhibiting clinical xerophthalmia. In laboratory animals it has been found that the interactions between bacteria, rickettsiae, viruses, protozoa and helminths on the one hand and vitamin A deficiency on the other hand are synergistic (1).

INTERACTION BETWEEN VITAMIN A STATUS AND MEASLES INFECTION

There would appear to be a specific relationship between vitamin A status and measles infection (23,47,48). Evidence is mounting that measles is an important risk factor in the development of severe vitamin A deficiency in Africa and some countries in Asia (13,49-53) and that vitamin A status at the time of measles infection is critical to the outcome of the disease (13). However, the interaction between vitamin A deficiency and measles infection is poorly understood because of the concomitant actions of protein-energy malnutrition and secondary infection.

Measles is an acute viral infection, and many of the symptoms and signs arise from the changes it creates in epithelial tissues (54). Unvaccinated children below the age of 5 years are most susceptible to measles infection and the attack rate is as high as 95-100%. Although only one natural, wild strain of virus is known to exist in the world (55,56), the epidemiological pattern and severity of disease vary from country to country (57). In developing countries, a severe form of measles has been reported for many years (54,57). Measles kills 2 million children each year, accounting for more than half of the deaths attributable to the 6 target diseases for the WHO Expanded Programme on Immunization (47,48). In regions where the disease is most severe, community studies consistently show case-fatility rates over 1% (48) while those in hospitals show rates between 5 and 25% (57). Death is associated with serious complications such as diarrhea, pneumonia, protein-energy malnutrition and eye lesions (58,59). However, in Western countries measles with such severe consequences virtually disappeared prior to the widespread use of antibiotics or vaccination (57). The etiology of the severe forms of measles remains unknown and a lot of controversial data have been published. One theory states that the outcome of the illness is dictated by the condition of the individual prior to infection: young age and a malnourished state are regarded as important predisposing factors. Although some authors have shown that very young children are more likely to die (58,60-62), others have also shown an increased risk of death in measles-infected adults over the age of 55 years (63). The impact of premorbid nutritional status on measles severity as measured by anthropometry is also controversial: data from studies in Kenya and in rural West Africa supported such a theory (64,65) but data from a study in Bangladesh found measles mortality unrelated to the prior nutritional state (66). Another theory to account for the severity of measles infection proposes that the synergism

between primary and secondary infections is important (66-70). Since patients with measles infection show a depressed immune response (71-73), they might be more susceptible to secondary infection. However, measles virus itself is also known to infect and damage the intestinal epithelium (74-77), resulting in a substantial loss of protein from the gut (78). This effect persists for a long period (77,79). Malnutrition may also contribute to reduced host defense (1,4). Hence, nutritional factors could play an important role in prediposing an individual to severe measles but the evidence to date is not convincing. It has been suggested that the immune response may be more depressed in a child with measles and malnutrition than with either condition alone (80-84).

Post-measles eye lesions (or post-measles blindness when loss of vision is complete) are another complication associated with severe measles (85). Vitamin A deficiency has been indicated by some authors as the primary cause with intercurrent measles as a precipitating factor (86,87). Other authors have attributed the lesions to a consequence of measles keratitis (85). Moreover, some studies also indicate a possible role of herpes simplex virus infection (88) and the harmful use of traditional eye medicine (89). Recently, from a hospital study in Tanzania, it was concluded that the primary cause of post-measles eye lesions could be attributed to vitamin A deficiency (50%), measles keratitis (12.5%), herpes simplex infection (20.8%) and traditional medicines (16.7%) (90).

It is thought that measles depletes vitamin A reserves by markedly increasing utilization and by reducing dietary intake and absorption. Marginal vitamin A stores in the liver of malnourished children are rapidly exhausted. In Thailand, one-third of children with measles had serum vitamin A concentrations below 0.35 μmol/l (10 μg/dl). This level could be regarded as deficient (91) and is accompanied by a very high risk of developing corneal ulcers (92). In Indonesia, children who had measles during the preceding 4 weeks were 11 times more likely to develop corneal xerophthalmia than children who had not had measles (13). By depleting vitamin A, measles can precipitate rapid deterioration of the cornea and blindness. The mechanisms probably differ from the direct invasion of the cornea by either measles or herpes simplex viruses (50,93).

Mortality associated specifically with measles may also be greatly reduced by supplying adequate vitamin A. A clinical trial in Tanzania of children admitted to hospital with measles has looked at the effects of large oral doses of vitamin A on mortality (94). Children given 200,000 IU vitamin A on 2 successive days were less likely to die than children given routine treatment.

Mortality was almost twice as high in the control group (13%) as in the supplemented group (7%), the greatest difference being in children under the age of 2 years.

THE NEED FOR AN ANIMAL MODEL

A well controlled experimental study to examine the possible interaction between vitamin A deficiency and measles infection is very difficult for three reasons. Firstly, it is impossible to infect vitamin A-deficient children with measles virus for ethical and practical considerations. Secondly, it is impossible to study the effect of vitamin A deficiency per se in children as this is almost invariably accompanied by protein-energy malnutrition. Thirdly, infection with measles virus produces characteristic signs of disease only in humans and some other primates (95,96) while infection with the wild strains of the virus in nonprimates does not produce a satisfactory model (97,98). For these reasons, it is inevitable that an animal model with another virus with similar properties and a suitable host will have to be used to gain further insight into the interaction between vitamin A deficiency and measles infection.

Virus diseases have not been widely used in development of animal models to study the effect of nutritional imbalances on the outcome of infection or the effect of infection on nutritional aspects because of difficulties with viral propagation, quantitation, and microbiological safety (3). However, studies with a nutritional component have been carried out in mouse, rat, guinea pig, hamster, chicken and monkey models to study influenza virus (99), hemorrhagic fever viruses (100), canine hepatitis virus (5), herpes simplex viruses (101,102), Newcastle disease virus (NDV) (103-105) and several different arboviruses (99,106).

A NEWCASTLE DISEASE VIRUS - VITAMIN A DEFICIENCY - CHICKEN MODEL

Measles virus belongs to the family Paramyxoviridae and is classified in the genus Morbillivirus (Table 1) (97,107). Models based on viruses of the genus Morbillivirus, such as distemper which affects dogs and other members of the canine and mustelids families, rinderpest which affects cattle, and "peste des petits ruminants" which affects sheep and goats, would appear to be the

most suitable but cannot be used readily because of practical and ethical reasons. A suitable alternative would be a model based on NDV infection in chickens (108). NDV belongs to the family Paramyxoviridae and is classified in the genus Paramyxovirus. Although both measles virus and NDV are certainly not identical and are not related antigenically (109-111), they do have many features in common. Both are single-stranded RNA viruses with a linear viral genome known to be complementary in base sequence to monocistronic messenger RNA species. That is, the genomic RNA is not messenger RNA; instead, a complementary positive strand is described (112). Their virions are enveloped and pleomorphic of shape, and they possess helical, elongated nucleocapsids (97,107). The most important difference is the presence of neuraminidase activity in NDV and the absence in measles virus (97,107). Apart from the similarity in morphology and construction, both viruses also exert a pronounced effect on epithelial tissues and both are immunosuppressive (105,107,113-119). NDV has long been recognized as a suitable model for the investigation of virus pathogenicity, especially for viruses from the family Paramyxoviridae (120).

TABLE 1. Viruses of the family Paramyxoviridae.

Family	Genus	Species
Paramyxoviridae	Paramyxovirus	Newcastle disease virus Mumps Parainfluenza 1-4
	Morbillivirus	Measles Distemper Rinderpest Peste des petits ruminants
	Pneumovirus	Respiratory syncitial viruses Pneumonia

After Kingsbury et al. (97) and Sellers (107).

Chickens represent a species of unique value for modeling many nutritional deficiencies, especially those involving micronutrients such as vitamin A (3). Vitamin A metabolism in chickens is similar to that in humans (121-129). The rapid growth of young chickens makes it possible to control study groups well since inhibition of growth indicates that the birds are being subjected to

abnormal stress. In fact, the relationship between vitamin A deficiency and NDV infection in chickens was studied as early as 1961 by Squibb & Veros (103) and in 1973 by Bang et al. (105).

NEWCASTLE DISEASE VIRUS

NDV, a prototype Paramyxovirus, is an enveloped, negative-stranded RNA virus (130-132). The membrane of the virus particle contains three virus-coded proteins, the hemagglutinin-neuraminidase (HN) glycoprotein, the fusion (F) glycoprotein, and the unglycosylated membrane (M) protein (130,131,133). The HN and F glycoproteins form the spike structures on the virion surface and play crucial roles in the initiation of infection (130,131). The HN glycoprotein is responsible for the attachment of the virus to the surface of infected cells (130) while the F glycoprotein mediates the fusion of the viral and cellular membrane brought in close proximity by the interaction of the HN protein and a cellular receptor (134,135). Besides cell fusion and virus penetration, the F protein is involved in hemolysis (136). The HN protein possesses a hemagglutinating activity (136) which may reflect the binding of the molecule to the cellular receptor (130). The HN protein also has neuraminidase activity (135,136) which is thought to play a role in the release of the virus from the surfaces of infected cells (130). However, hemagglutinating activity of the protein is distinct from neuraminidase activity (137). Both HN and F proteins are important for induction of immunity against infection (138).

The sequence of events following introduction of NDV into the chicken is as follows: multiplication at the site of introduction and liberation of virus into the blood stream; a second cycle of multiplication in visceral organs; and than a second release into the blood stream (139). Signs of disease and release of virus into the environment are associated with the second release of virus into the blood stream and the course of the disease is determined by defense mechanisms that come into play at this time (139). Clinical manifestations of viral infections in general reflect damage to particular tissues during the process of intracellular virus replication and release, and during the subsequent inflammatory and immune responses initiated by the appearance of viral (or host) antigens on affected cells. In the case of NDV and other viruses from the family Paramyxoviridae, cell damage is also inflicted through a toxin-like action of the virion itself (140-142).

There is a wide range of strains of NDV which differ markedly in virulence for their natural host, the chicken. Strains of NDV are classified as lentogenic (almost avirulent), mesogenic (mildly virulent) or velogenic (highly virulent) based on a battery of tests used to designate strains of NDV (139,143). Chickens infected with velogenic strains develop severe disease, exhibit one or more type of lesions and often die. Lentogenic strains, such as the La Sota strain, normally produce only a mild or inapparent disease in chickens and thus have been extensively used as vaccines (139). However, when presented to chickens in small-particle aerosols, lentogenic strains can induce severe respiratory reactions (139).

AIMS OF THE STUDY

There are strong indications based on clinical and epidemiological studies discussed earlier in this chapter, that an interaction between vitamin A status and measles infection exists (13,47-53). However, the role of vitamin A deficiency per se in this interaction remains unclear and support for the existence of such an interaction from animal models is not strong. Moreover, it is not known whether this interaction is specific for measles virus or whether the relationship is more general. In this thesis an animal-virus model is presented, in which the La Sota strain of NDV is used instead of measles virus in chickens varying in vitamin A status.

The aim of using this model, in which extreme vitamin A deficiency, protein-energy malnutrition and secondary infection are all absent, is to answer the following questions concerning the relationship between vitamin A status and infection with NDV in chickens:

1. Is there an interaction between vitamin A status and viral infection in a model using NDV as infective agent and the chicken as experimental animal?

2. If there is an interaction between vitamin A status and NDV infection, is the interaction specific for NDV and what are the mechanisms involved?

3. What is the effect of marginal vitamin A deficiency and NDV infection on various aspects of host resistance such as nonspecific mechanisms, systemic immunity and mucosal immunity?

Results of the experiments with this model may contribute to a better understanding of the interaction in humans between vitamin A status and infection, especially for viruses such as measles.

OUTLINE OF THE THESIS

This thesis is divided into 11 chapters. In this chapter (Chapter 1), the current knowledge of the relationship between vitamin A deficiency and infectious diseases has been summarized followed by a description of the animal model used and the aims of the thesis. In Chapter 2, the method for producing marginally vitamin A-deficient chickens in a healthy condition without concomitant protein-energy malnutrition is described. In Chapter 3, the interaction between vitamin A status and Newcastle disease virus infection is described. Chapter 4 is a sequel to Chapter 3 and deals with the mechanism by which NDV infection can lower plasma retinol concentration. For this purpose, retinol and the retinol carrier proteins (retinol-binding protein and transthyretin) were measured throughout the disease period in plasma and liver. The specificity of NDV in the interaction process was determined on the basis of an experiment described in Chapter 5, in which NDV was replaced by either infectious bronchitis virus (family Coronaviridae) or reovirus (family Reoviridae). In Chapter 6, changes induced by vitamin A deficiency and NDV infection in lymphoid organs and blood lymphocytes as measured by methods including flow cytometry are described. Chapter 7 deals with the effect of vitamin A deficiency on cytotoxic T lymphocyte activity directed to NDV as an important part of the cell-mediated immune response to viral infection. In Chapter 8, the consequences of vitamin A deficiency on systemic humoral immune responses to NDV are described, together with the combined effect of vitamin A deficiency and NDV infection on humoral immune responses to other antigens. In Chapter 9, the effect of vitamin A deficiency on nonspecific host defense is investigated by measuring the phagocytic and metabolic activity of macrophages from chickens infected with NDV. In Chapter 10, the effect of vitamin A deficiency and NDV infection on some aspects of mucosal immunity are discussed. Chapter 11 comprises a general discussion.

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CHAPTER 2

Production of chickens with marginal vitamin A deficiency

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ABSTRACT

Marginally vitamin A-deficient day-old chickens capable of maintaining a healthy condition for at least 6 wk were produced using a two-generation model. In this model, hens fed diets with a limited vitamin A content were used to obtain day-old chickens which were marginally deficient in vitamin A. Only hens with a narrow range of plasma retinol values (0.60-0.85 μmol/l) were satisfactory for this purpose. Above this range, the day-old chickens were not marginally vitamin A-deficient. Below this range, egg production and hatchability were affected to some extent depending on the degree of vitamin A deficiency. Even when egg production and hatchability remained at a high level in such birds, the day-old chickens produced were not sufficiently strong to survive the first weeks of life. The advantages of the two-generation model for producing marginally vitamin A-deficient chickens are the increased uniformity and predictability of the chickens with respect to body weight, general health and vitamin A status. However, it does take about 3 months to produce such chickens.

INTRODUCTION

Animal models have often been used in vitamin A research (1,2). However, many problems can arise in producing vitamin A deficiency in animals (3,4). It can take a long time to produce deficient animals and often large variations in vitamin A status can be observed within the same diet group. In addition, diets free of vitamin A can produce sudden and uncontrolable vitamin A deficiency with consequent loss of appetite and concomitant protein-energy malnutrition. Signs of vitamin A deficiency are sometimes irreversible and, together with increased sensitivity to infection, can lead to death. A few models have been described, in which many of these problems have been overcome (5-9). One system which can be used is a two-generation model, in which parent animals are made marginally deficient in vitamin A (5,7,9). Their progeny are also marginally vitamin A-deficient and can be used in studies requiring young animals. Vitamin A deficiency in chickens has been described many times and

vitamin A-deficient hens have often been obtained (6,10-17). However, the production of marginally vitamin A-deficient day-old chickens using a two-generation model is not very easy: the level of vitamin A in the feed of the laying hens must be sufficiently high to enable hens to produce sufficient eggs, for the embryo to hatch and for the day-old chickens to have sufficient reserves for the first weeks of life (11).

The aim of the present study was twofold. Firstly, we wanted to produce day-old chickens marginally deficient in vitamin A for a long period without health problems. Secondly, we wanted to test the advantages of such a two-generation model over a one-generation model based on day-old chickens with an adequate vitamin A status. Vitamin A-deficient chickens produced by the two-generation model have been used in studies on the relationship between vitamin A deficiency and Newcastle disease virus infection (18,19).

MATERIALS AND METHODS

Animals, diets and experimental design

Two independent experiments were carried out under completely different conditions. In the first experiment, White Leghorn laying hens (strain Lohmann Selected Leghorn), aged approximately 18 wk, were obtained from a commercial breeder (Verbeek, Barneveld, the Netherlands). The birds had been vaccinated according to the recommendations of the "Stichting Gezondheidszorg voor Dieren" (the Netherlands) (20). On arrival, the hens were housed individually in a room controlled for temperature (20°C), relative humidity (40-50%) and light-dark cycles (16 h of dimmed light and 8 h of darkness/d). A commercial diet (Opfokvoer 2, Rijnzate, Wageningen, the Netherlands) was fed for 1 wk. After this period, the birds were allocated randomly in five dietary groups, each of 12 hens. The first 3 groups (P-0, P-300 and P-600) were fed a purified diet marginally deficient in vitamin A (0, 300 or 600 retinol equivalents (RE)/kg, respectively) (Table 1). The fourth group (P-1500) was fed a purified diet with adequate vitamin A (1500 RE/kg) and the fifth group (N-1500) was fed a diet based on nonpurified components with the same amount of vitamin A (Table 1). The latter group was added in order to test for possible differences between purified and nonpurified diets. Water and diets were provided ad libitum and the hens had free access to oyster shell grit. The laying hens were studied for a total period of 19 wk during which time feed

TABLE 1. Composition of the purified and nonpurified diets[1].

Purified diet			Nonpurified diet	
Ingredient	Hens	Chickens	Ingredient	Hens
	g/kg			g/kg
Maize starch	280	280	White maize	340
Dextrose	245	286	Tapioca 65%	200
Soy isolate[2]	180	210	Sorghum	160
Cellulose	91	65	Soy flour 50%	140
$CaCO_3$[3]	55.5	5	$CaCO_3$[3]	51.5
Molasses	50	50	Animal meal	25
$CaHPO_4.2H_2O$	25	30	Fish meal	25
Soy oil	25	25	Coconut oil	15
Coconut oil	15	15	$CaHPO_4.2H_2O$	11
$KHCO_3$	15	15	Soy oil	10
Vitamin and mineral premix[4]	10	10	Vitamin and mineral premix[5]	10
NaCl	4	4	$KHCO_3$	6
DL-Methionine	2.5	3	NaCl	2.5
MgO	2	2	DL-Methionine	2
			L-Lysine.HCl	2

1 Diets were manufactured in pelleted form following the guidelines of the National Research Council (21) by the Institute of Animal Nutrition and Physiology (IGMB-TNO, Wageningen, the Netherlands). The metabolizable energy of the purified hen and chicken diets, and the nonpurified hen diet was 3000, 3200 and 2975 kcal/kg feed, respectively.

2 Soy isolate: Purina Protein 500 E, Ralston Purina, St Louis, MO, containing 880 g protein/kg isolate.

3 The laying hens had free access to oyster shell grit.

4 The vitamin and mineral premix (10 g/kg purified diet) was prepared with dextrose and contained (mg/kg diet): thiamin, 2.5; riboflavin, 5.5; pantothenic acid, 15.0; nicotinic acid, 50.0; pyridoxine, 5.0; biotin, hens: 0.10, chickens: 0.15; folic acid, hens: 0.45, chickens: 0.75; choline chloride, hens: 1000, chickens: 1850; cyanocobalamin, 0.015; inositol, 100; p-aminobenzoic acid, 50.0; cholecalciferol, 3000 IU; all-rac-α-tocopherol, 30.0; menadione, 5.0; L-ascorbic acid, 50.0; $FeSO_4.7H_2O$, 400; MnO_2, 150; $CuSO_4.5H_2O$, 100; $ZnSO_4.H_2O$, 200; $Na_2SeO_3.5H_2O$, 0.3; KI, 5; ethoxyquin, 100. Vitamin A was added as retinyl acetate and retinyl palmitate, Rovimix A 500 (522,000 IU/g, F. Hoffmann-La Roche, Basel, Switzerland), hens: 0, 300, 600 or 1500 retinol equivalents/kg diet, chickens: 0, 120 or 1200 retinol equivalents/kg diet.

5 The vitamin and mineral premix (10 g/kg nonpurified diet) was prepared with soy flour and contained (mg/kg diet): riboflavin, 5.5; pantothenic acid, 15.0; nicotinic acid, 30.0; pyridoxine, 2.0; biotin, 0.05; folic acid, 0.1; choline chloride, 500; cyanocobalamin, 0.015; cholecalciferol, 3000 IU; all-rac-α-tocopherol, 30.0; menadione, 5.0; L-ascorbic acid, 50.0; $FeSO_4.7H_2O$, 400; MnO_2, 150; $CuSO_4.5H_2O$, 100; $ZnSO_4.H_2O$, 200; $Na_2SeO_3.5H_2O$, 0.3; KI, 2; ethoxyquin, 100. Vitamin A was added as retinyl acetate and retinyl palmitate, Rovimix A 500 (522,000 IU/g, F. Hoffmann-La Roche, Basel, Switzerland), 1500 retinol equivalents/kg diet.

consumption, body weight, egg production and plasma retinol concentration were measured. Hatchability and health status of day-old chickens were determined three times during the experiment. Therefore, the laying hens were artificially inseminated three times in each of the three periods and their eggs collected for 11 d starting in wk 8, 13 and 18, respectively. At the conclusion of each period, all eggs were weighed and placed simultaneously in incubators at the Spelderholt Centre for Poultry Research and Extension (Beekbergen, the Netherlands).

In the second experiment, different methods for producing marginally vitamin A-deficient chickens were compared. Female day-old chickens, progeny of marginally vitamin A-deficient hens were allocated to one of two groups, depending on the vitamin A status of their mothers. The first and second group (C1-120 and C2-120, respectively) comprised chickens derived from hens with plasma retinol levels between 0.35-0.50 μmol/l and 0.60-0.85 μmol/l, respectively. Allocation to the two groups was made after the experiment when it was obvious that plasma retinol level in hens was an important criterion for survival of their progeny. Both groups were fed a purified diet limited in vitamin A (120 RE/kg) (Table 1). In addition, female day-old chickens (strain Lohmann Selected Leghorn) were obtained from a commercial hatchery (Verbeek, Barneveld). These chickens were allocated to a further three groups. One of those groups (C3-120) was also fed a purified diet limited in vitamin A (120 RE/kg); another (C4-0) a purified diet free of vitamin A; while a third group (C5-0/120) was also fed the purified diet free of vitamin A for 2 wk and fed the diet limited in vitamin A (120 RE/kg) afterwards. The day-old chickens, 12 birds/group and 1 group/cage, were housed in an air-filtered room maintained under reduced pressure, controlled for temperature (decreasing from 32^{o}C during the first day to 20^{o}C after 2 wk), relative humidity (40-50%) and light-dark cycles (24 h dimmed light/d during the first 2 d, and 10 h of dimmed light and 14 h of darkness/d from the third day). The day-old chickens were studied for a period of 6 wk during which health status and plasma retinol levels were determined.

All diets were manufactured in pelleted form according the recommendations of the National Research Council (20) by the Institute of Animal Nutrition and Physiology (IGMB-TNO, Wageningen, the Netherlands).

Vitamin A status

In the first experiment, retinol levels were determined in plasma from laying hens collected at wk 0, 5, 8, 11, 13, 15, 17 and 19, and in plasma from

their day-old progeny. In the second experiment, plasma retinol analyses were carried out in plasma from chickens collected at the age of 0, 2, 3.5-4 and 5-6 wk. A reversed-phase high performance liquid chromatography (HPLC) method modified from that of Driskell et al. (22) was used with retinyl acetate as internal standard. The results obtained were checked against pooled control samples. The HPLC system comprised a Constametric III Metering pump, a Constametric Spectromonitor D variable wavelength detector (both LDC/Milton Roy, Riviera Beach, FL), an injection valve with a 50-μl injection loop (Type 7010, Rheodyne, Cotati, CA), and a computing integrator (Model SP 4100, Spectra-Physics, Santa Clara, CA) using a C-18 10 ODS reversed-phase column (240 x 4.6 mm) and a guard column (75 x 2.1 mm) (both from Chrompack, Middelburg, the Netherlands). The column was eluted isocratically at a flow rate of 1.5 ml/min using a mobile phase consisting of methanol:water, 96:4 v/v. Absorbance was measured at 325 nm.

Vitamin A content of diets

In order to determine the content and stability of vitamin A in diets, analyses were carried out at 1 and 10 wk after manufacturing by F. Hoffmann-La Roche & Co, Ltd (Basel, Switzerland). The method used was that of Manz and Philipp (23). Briefly, after homogenization and saponification of the diets in a solution (3:1 v/v) of ethanol:KOH (50% wt/v in water), the retinol (vitamin A alcohol) released was totally extracted with ethyl ether. The extract was dried under reduced pressure and the residue dissolved in n-hexane. Determination of the retinol content was carried out by HPLC with separation of isomers using a 5 ODS LiChrosorb Si 60 column (125 x 4 mm) (Merck, Darmstadt, West Germany) and n-hexane with 2% isopropanol as mobile phase. Measurements were carried out against an external vitamin A standard which had undergone the same procedure as the sample. The carotene content of the diets was calculated on the basis of feed analysis tables (21,24,25).

Statistical analysis

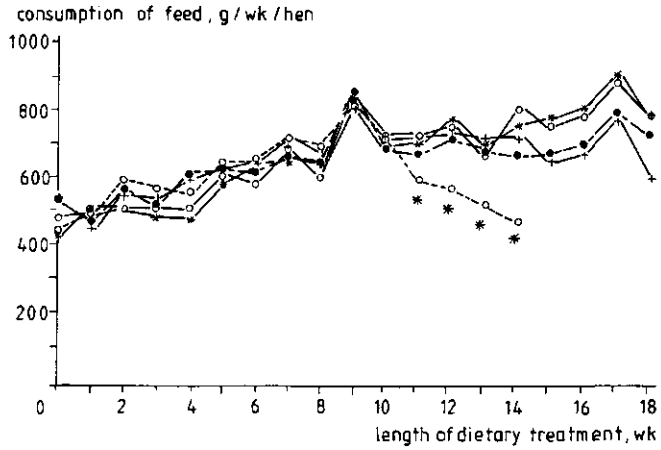
All statistical comparisons among treatment groups were performed by one-way analysis of variance after testing for normality. Differences between group means were evaluated by Tukey's range test. All procedures were based on the principles outlined by Snedecor and Cochran (26) and were performed using a VAX-8600 computer system with a SPSS-X software package (SPSS, Chicago, IL) (27).

RESULTS

Health status of laying hens (experiment 1)

In wk 14, two hens in the P-0 group died suddenly. Although their body weight was normal, feed consumption had declined. Postmortem examination revealed some morphological signs of vitamin A deficiency: white plaques in the oesophagus and pale kidneys. Since the condition of the remaining chickens in the P-0 group also deteriorated as indicated by a rapid decline in feed consumption and poor feathering, it was decided for ethical reasons to remove these birds from the experiment. In wk 18, one hen in the P-300 group died suddenly. However, body weight, feed consumption and egg production were normal and postmortem examination did not reveal characteristic signs of vitamin A deficiency. Some other chickens from the P-300 group showed poor feathering from wk 16 to the end of the experiment. All other hens appeared to be in a healthy condition and overt clinical signs of vitamin A deficiency, such as granular eyelids, nutritional roup (xerophthalmia) or white cheesy deposits under the eyelids (25) were not observed.

FIGURE 1. Body weight of laying hens fed diets varying in vitamin A content. Each point represents the mean for 10-12 hens. SEM varied from 2 to 5%. (○·○), P-0 ; (+), P-300; (○–○), P-600; (●), P-1500; and (✱), N-1500 group. Significant differences were not found among diet groups.



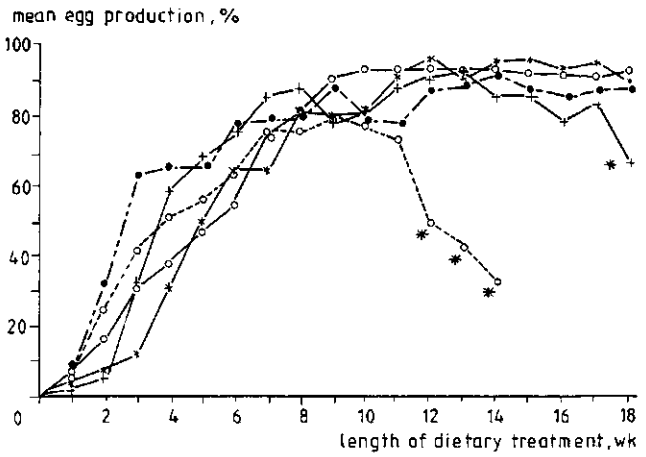


FIGURE 2. Feed consumption of laying hens fed diets varying in vitamin A content. Each point represents the mean for 10–12 hens. SEM varied from 3 to 17% and was higher at the beginning than at the end of the experiment. (○·○), P-0 ; (+), P-300; (○–○), P-600; (●), P-1500; and (✱), N-1500 group. * Significantly different from the control group (P-1500) at $P < 0.05$.

FIGURE 3. Egg production of laying hens fed diets varying in vitamin A content. Each point represents the mean for 10–12 hens. (○·○), P-0 ; (+), P-300; (○–○), P-600; (●), P-1500; and (✱), N-1500 group. * Significantly different from the control group (P-1500) at $P < 0.05$.

Body weight, feed consumption and egg production of hens

Significant differences in body weight among the groups could not be observed throughout the experiment (Fig. 1). From wk 11-12, feed consumption and egg production in the P-0 group were significantly lower than in the P-1500 group (Fig. 2 and 3, respectively). Chickens fed a purified diet adequate in vitamin A and counterparts fed a nonpurified diet with the same vitamin A level were similar with respect to body weight, feed consumption and egg production.

TABLE 2. Hatchability of eggs layed by hens fed diets varying in vitamin A content.

Period [1]		Group				
		P-0	P-300	P-600	P-1500	N-1500
		Proportion of eggs layed				
1	Total [2]	100 (86)	100 (101)	100 (98)	100 (79)	100 (115)
	Fertile eggs	94.2	98.2	94.9	98.7	93.0
	Embryos surviving to 8 days	90.7	93.1	89.8	92.4	91.3
	Embryos surviving to 17 days	89.5	93.1	87.8	91.1	90.4
	Live chickens	89.5	93.1	82.7	90.6	87.0
2	Total	100 (54)	100 (101)	100 (95)	100 (110)	100 (103)
	Fertile eggs	96.3	97.0	98.9	100	99.0
	Embryos surviving to 8 days	66.7	95.0	95.8	100	99.0
	Embryos surviving to 17 days	61.7	94.1	95.8	100	99.0
	Live chickens	44.4	92.1	94.7	95.5	98.1
3	Total	- [3]	100 (72)	100 (99)	100 (93)	100 (93)
	Fertile eggs	-	97.2	99.0	97.9	100
	Embryos surviving to 8 days	-	95.8	96.0	96.8	97.8
	Embryos surviving to 17 days	-	94.4	96.0	96.8	97.8
	Live chickens	-	81.9	92.9	94.6	93.5

1 Eggs collected over 11 days were hatched in three successive periods starting in wk 8, 13 and 18.
2 The number of eggs incubated are shown between parentheses. Numbers exclude eggs that were cracked or contained double yolks.
3 Hens were removed before the third egg collection period.

Hatchability

Hatchability was not affected in the first period (Table 2) and the day-old chickens produced were in a healthy condition. However, in the second period, hatchability was poor in the P-0 group. Many embryos died during the first 8 days of incubation, while some other embryos were too weak to break out of the shell. Most of the surviving day-old chickens were ataxic and

showed other deficiency signs such as 'star-gazing', nakedness and hematomas. In the third period, hatchability tended to be lower in the P-300 group than in the other groups. Although most of these day-old chickens were in a healthy condition, some of them also showed the signs of deficiency described earlier. Differences in weight of eggs or body weight of day-old chickens could not be observed among the diet groups in all periods measured (data not shown). Weight of eggs and body weight of day-old chickens increased slightly from the first to the third period.

Plasma retinol concentration in hens

At the start of the experiment, mean plasma retinol levels of the 18-wk-old hens ranged from 2.4 to 3.0 μmol/l (Fig. 4). These levels decreased in all groups, including the P-1500 and N-1500 group, throughout the experiment. The rate of decrease in plasma retinol levels was negatively related to the level of vitamin A in the diet. From wk 11, the P-0 group had plasma retinol levels which could be considered as marginally deficient. Similar results were found for the P-300 and P-600 group from wk 13 and wk 19, respectively. Plasma retinol levels did not decrease below 0.3 μmol/l and remained stable at that level in the P-300 group from wk 15 to wk 19. In both the P-1500 and N-1500 group, plasma retinol levels did not decrease below 2.2 μmol/l.

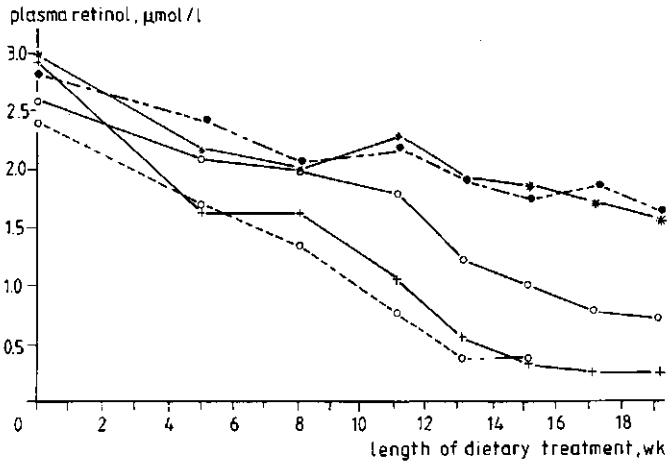


FIGURE 4. Plasma retinol concentration of laying hens fed diets varying in vitamin A content. Each point represents the mean for 10-12 hens. SEM varied from 1 to 10%. (○·○), P-0 ; (+), P-300; (○–○), P-600; (●), P-1500; and (✳), N-1500 group.

Plasma retinol concentration in day-old chickens

After the first hatching period, plasma retinol concentrations were similar in all groups (Table 3). However, plasma retinol levels in the progeny of the P-300 group were significantly lower than in the progeny of all other groups after the second period. Similar results were obtained after the third period for the progeny of the P-300 and P-600 group. Plasma retinol concentrations in the progeny of the P-1500 and N-1500 group were stable throughout the experiment.

TABLE 3. Plasma retinol concentration in day-old chickens derived as progeny from hens fed diets varying in vitamin A content.

Group	Retinol concentration[1]		
	Period 1	Period 2	Period 3
		µmol/l	
P-0	0.86 ± 0.04 a	ND	ND[2]
P-300	0.97 ± 0.17 a	0.11 ± 0.02 a	0.07 ± 0.01 a
P-600	1.05 ± 0.07 a	0.89 ± 0.09 b	0.24 ± 0.02 b
P-1500	1.01 ± 0.06 a	1.05 ± 0.11 b	1.04 ± 0.10 c
N-1500	0.99 ± 0.11 a	1.14 ± 0.08 b	0.96 ± 0.07 c

1 **Values are means ± SEM for the progeny of 6 hens/group. Plasma samples from all day-old chickens from 1 hen were pooled. Means within a column not sharing a common superscript letter are significantly different at P < 0.05 (Tukey).**

2 **ND: not determined**

Health status and vitamin A status in chickens (experiment 2)

In chickens, progeny of marginally vitamin A-deficient laying hens (plasma retinol levels between 0.60-0.85 µmol/l), plasma retinol concentrations remained rather stable at a level that could be considered as marginally deficient (Fig. 5). Marginal vitamin A levels could also be obtained when chickens, progeny of normal hens, were fed a diet without vitamin A during the first 2 wk and with 120 RE/kg afterwards. However, the variation in plasma retinol levels within this group was much higher than within the group which comprised progeny of marginally vitamin A-deficient hens. In fact, at 3.5 wk of age, of the 12 chickens 3 had plasma retinol levels less than 0.35 µmol/l and 4 had levels more than 0.70 µmol/l. Chickens, progeny of marginally vitamin A-deficient hens (plasma retinol levels between 0.35-0.50 µmol/l) did not remain healthy for more than a few days. Gain in body weight was extremely

low and after 2 wk only 5 out of 12 birds were still alive. Similar findings were observed in the progeny of normal hens fed a diet without vitamin A. In the third week, 4 of these birds died. Chickens which were progeny of normal hens and fed a diet with 120 RE/kg were not marginally deficient in vitamin A after 5 wk.

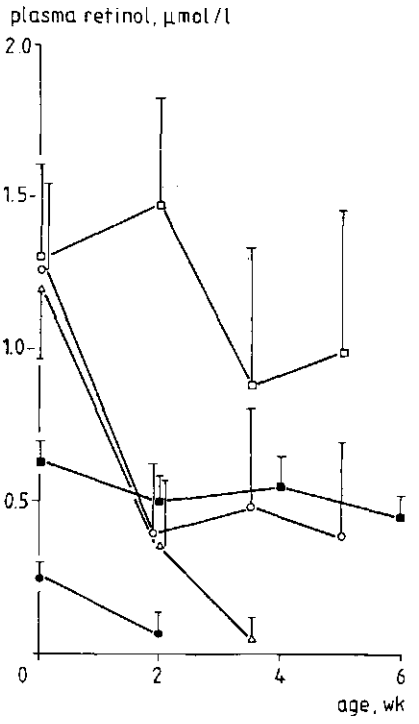


FIGURE 5. Effect of different methods for producing marginal vitamin A deficiency on plasma retinol concentration in chickens. Values are means with the vertical bars representing one SD. (●), C1-120; (■), C2-120; (□), C3-120; (Δ), C4-0; (○), C5-0/120 group.

Vitamin A content of diets

Analysis of the diets 1 wk after manufacturing revealed that all diets contained more vitamin A than added (Table 4). This could not be the result of bad mixing of the vitamin and mineral premix through the diets, because at least 5 samples were taken from each diet before pooling them to 1 sample for analysis. After 10 wk of storage, the content of vitamin A had decreased in

TABLE 4. Calculated and analyzed vitamin A content of diets[1].

Group	Retinol content			Carotene content
	Calculated	Analyzed Period after manufacturing		Calculated
		1 wk	10 wk	
		μg Retinol equivalents/kg		
P-0	0	0	0	0
P-300	300	420	240	0
P-600	600	720	570	0
P-1500	1500	1830	1790	0
N-1500	1500	2040	1620	60

[1] **Calculation of the retinol and carotene content was based on the amount of vitamin A added to the diet and on feedstuffs analysis tables (20,23,24), respectively. All of the carotene present was assumed to be ß-carotene and the values were divided by 6 to convert to retinol equivalents.**

all diet groups but the decrease was relatively most pronounced in the P-300 group. The results of the vitamin A analyses in diets should be read with care, as the detection limit of this method for a reliable and reproducable analysis was about 300 RE/kg. The calculated carotene content of purified diets was negligible and that of the N-1500 diet ranged between 30-90 RE/kg. This was based on feedstuffs analysis tables used (20,23,24) and is related to the carotene content of white maize and soy flour.

DISCUSSION

The aim of the present study was to develop a method for producing chickens which remained not only marginally vitamin A-deficient but also healthy. Since a two-generation model was used for this purpose, the advantages and disadvantages compared with other methods have been investigated.

The first signs of vitamin A deficiency, such as loss of appetite, decreased egg production and ruffled feathers, appeared after 11 wk in hens completely deprived of vitamin A when plasma retinol levels were about 0.75 μmol/l. This is in accordance with earlier reports that a period ranging from 2 to 5 months is necessary to induce similar signs of vitamin A deficiency in hens (11-17). Other clinical signs of vitamin A deficiency, such as weakness,

general inactivity, granular eyelids, nutritional roup (xerophthalmia), watery and sore eyes and white cheesy deposits under the eyelids (12,14), could not be observed. Sudden death, which was observed in three hens fed a diet deprived or deficient in vitamin A, has also been described as a sign of vitamin A deficiency (13) and it might be the result of lower resistance to infections (1-4). In two of these birds, postmortem examination revealed overt morphological signs of vitamin A deficiency, such as white plaques in the oesophagus and pale kidneys.

Hatchability was seriously affected when eggs from vitamin A-deficient hens were collected after 13 wk on the experimental diets. Many embryos died during the first 8 d of incubation. Some of the embryos that survived 17 d of incubation were too weak to break out of the shell or were too weak to survive the first day. Impaired hatchability has often been described as a sign of vitamin A deficiency (12,14,29). Egg production and hatchability were optimal in hens fed diets with more than 600 RE/kg throughout the experiment. Other investigators have reported threshold values for maximal egg production and hatchability between 900-1400 RE/kg diet (30-32).

Although vitamin A content in liver is the most reliable estimate of vitamin A body storage (33-35), plasma retinol concentration do reflect liver stores after moderate to severe depletion. It is also a good indicator of circulating vitamin A available to extrahepatic tissues (33-34). Thus plasma retinol concentrations were used as an indicator of vitamin A status in our experiment. After approximately 8-11 wk on the experimental diets, plasma retinol concentration in hens reflected the levels of vitamin A in the diets. This indicated that vitamin A stores in liver were moderately to severely depleted by that time. Plasma retinol levels in the day-old chickens correlated with the vitamin A intake of their mothers, provided that these hens were fed vitamin A-deprived or vitamin A-deficient diets for at least 13 wk. Similar correlations have been reported previously (12,14,36).

Day-old chickens, progeny of hens with plasma retinol levels between 0.60 and 0.85 μmol/l, remained marginally deficient for at least 6 wk without faltering body weight gain or health problems (37). However, for progeny of hens with plasma retinol levels between 0.35 and 0.50 μmol/l, the situation was different; although the chickens were apparently healthy when born, they were not strong enough to survive the first weeks of life. Thus the results from these experiments indicate that plasma retinol levels of hens should be between 0.60 and 0.85 μmol/l in order to obtain marginally vitamin A-deficient day-old chickens which remain marginally deficient for at least 6 wk without

health problems. Above this range, chickens will not be marginally deficient; below this range, although hatching will be normal, the day-old chickens will be too weak to survive.

A comparison between methods for producing marginally vitamin A-deficient chickens shows that the two-generation model produces the best results. Marginal vitamin A deficiency can be induced in commercially obtained day-old chickens within a few weeks and these birds can be kept marginally deficient for at least another few weeks by feeding them a diet free of added vitamin A during the first 2 wk and deficient in vitamin A (120 RE/kg) thereafter. However, the variation in plasma retinol level between birds is unacceptably high. In the studies reported here, some of the birds were deficient after 2 wk, while others had normal values after 6 wk. When commercially obtained chickens were fed a diet completely devoid of vitamin A, plasma retinol levels were almost negligible after 3 wk. In addition, when such day-old chickens were fed a diet deficient in vitamin A (120 RE/kg), plasma retinol levels were still far above deficiency levels after 6 wk. Previous reports have shown that day-old chickens, derived from hens with an adequate intake of vitamin A and receiving a diet completely devoid of vitamin A, had marginally deficient levels of vitamin A from the third week (38) and showed signs of deficiency from the sixth week (14,38).

The nonpurified diet adequate in vitamin A produced comparable results as the purified diet with the same amount of vitamin A with respect to body weight, feed consumption, egg production, plasma retinol concentration and hatchability. However, it is difficult to control the carotene content of nonpurified diets. It is probably better to use purified diets for producing marginally vitamin A-deficient chickens. Storage of the diets for a period of 10 wk produced a reduction in vitamin A content, especially in the diet with 300 RE/kg. Reduced vitamin A levels in diets after storage have been described earlier by Fullerton et al. (39). They reported that the vitamin A content of purified diets was more sensitive to oxidation than that of natural diets. Therefore, it is recommended that new diets should be manufactured every month. It should also be noted that the vitamin A preparation used should be capable of being evenly dispersed throughout the feed. As high levels of vitamin A are usually added to animal feeds, the amount of vitamin A in each particle of the preparation is relatively high. Thus at low levels of vitamin A, the preparation is not distributed evenly through the feed. Preparations of vitamin A should not be ground prior to addition to feeds as this destroys the

oxidative protection provided by the vitamin E which is also incorporated in the granules.

In conclusion, the results from the present paper indicate that it is possible to produce marginally vitamin A-deficient day-old chickens that are sufficiently healthy to survive and grow normally for at least 6 wk after hatching. The advantages of the two-generation model in producing marginally vitamin A-deficient chickens over one-generation models are lower variation in vitamin A status within a treatment group and more stable and controlable vitamin A status in such birds. A disadvantage is the long period before day-old chickens are available. It should also be noted that the range of plasma retinol values in hens which allows the production of satisfactory chickens is very narrow. We have found that the marginally vitamin A-deficient chickens obtained are particularly suitable as starting material for studying the interaction between vitamin A status and Newcastle disease virus infection.

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CHAPTER 3

The interaction between vitamin A status and Newcastle disease virus infection in chickens

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ABSTRACT

Newcastle disease virus (NDV) infection in chickens differing in vitamin A status has been selected as a model to examine the interrelationship between marginal vitamin A deficiency and the severity of consequences of measles infection in humans. Day-old chickens with limited vitamin A reserves, the progeny of marginally vitamin A-deficient hens, were fed purified diets containing either marginal (120 retinol equivalents/kg diet, ad libitum) or adequate (1200 retinol equivalents/kg diet, ad libitum or pair-fed) levels of vitamin A for a period of 10 wk. At 4 wk of age, half of the chickens in each group were infected intraocularly with the lentogenic, i.e. mildly pathogenic, La Sota strain of NDV. Within 1 wk of infection, plasma retinol levels in the infected, marginally vitamin A-deficient chickens showed a significant and persistent decrease compared to their noninfected counterparts fed the same diet. Moreover, infection with NDV resulted in increased rates of morbidity in the marginally vitamin A-deficient chickens compared with nondeficient chickens. The results of this study indicate that pre-existing marginal vitamin A status increases the severity of disease following NDV infection, and that infection with NDV reduces marginal plasma vitamin A levels to levels which can be regarded as deficient.

INTRODUCTION

The interaction between infection and malnutrition is well recognized (1,2). Malnutrition is associated with increased susceptibility, severity and duration of infection, particularly in measles, and this is a serious problem in many developing countries (3-5). Vitamin A deficiency is probably an important determinant of the severity of the consequences of measles infection, although clinical studies are always complicated by the presence of protein-energy malnutrition and other nutrient deficiencies (6-9). Moreover, recent epidemiological studies have revealed that even marginal vitamin A deficiency is associated with increased morbidity and mortality (10,11). On

the other hand, measles, and probably other infections, seems to be an important risk factor in the development of severe vitamin A deficiency (7,8,12). However, a well-controlled experimental study to examine the interrelationship between vitamin A deficiency and measles infection is difficult, because infection with measles virus produces characteristic disease signs only in some primates (13-15) and infection with wild strains of the virus in nonprimates does not produce a satisfactory model (15,16).

In this paper we report an experiment in which Newcastle disease virus (NDV) infection in chickens varying in vitamin A status has been used as a model to examine the interrelationship between vitamin A deficiency and severity of consequences of measles infection in humans. Vitamin A metabolism in chickens is basically similar to that in humans (17-19). NDV, like measles virus, belongs to the family of Paramyxoviridae: the former to the genus Paramyxovirus, and the latter to the genus Morbillivirus (15). Although the viruses are certainly not identical, they have several features in common; both viruses have a pronounced effect on epithelial tissues and both are immunosuppressive (20-25).

The aim of this experiment is to test, in a model that does not exhibit protein-energy malnutrition or secondary infection, whether inadequate vitamin A nutriture increases the severity of infection and whether infection reduces vitamin A status, especially when vitamin A status is marginal.

MATERIALS AND METHODS

Animals and diets

Female day-old White Leghorn chickens (strain Lohmann Selected Leghorn) with limited vitamin A reserves were obtained as progeny of marginally vitamin A-deficient laying hens fed a marginally vitamin A-deficient diet (300 retinol equivalents (RE)/kg feed) for a period of 3 mo. The chickens were fed purified diets ad libitum containing either adequate (1200 RE/kg feed) or marginal amounts of vitamin A (120 RE/kg feed) (Table 1). In order to ensure that the intake of the marginally vitamin A-deficient chickens differed only insofar as vitamin A is concerned, a pair-fed control group was also included. At 4 wk of age, half of the chickens in each of the three groups were infected with the lentogenic La Sota strain of NDV. In this way six experimental groups were formed: noninfected (n=56) and infected (n=56) groups fed a marginally vitamin A-deficient diet [A-(adlib)I- and A-(adlib)I+, respectively], noninfected

TABLE 1. Composition of the purified diets[1].

Ingredient	Amount	
	Hens	Chickens
	g/kg	
Soy isolate[2]	180	210
DL-Methionine	2.5	3
Maize starch	280	280
Dextrose	245	286
Molasses	50	50
Coconut oil	15	15
Soy oil	25	25
Cellulose	91	65
Vitamin and mineral premix[3]	10	10
$KHCO_3$	15	15
NaCl	4	4
MgO	2	2
$CaCO_3$[4]	55.5	5
$CaHPO_4.2H_2O$	25	30

1 Diets were manufactured in pelleted form following the guidelines of the National Research Council (26) by the Institute of Animal Nutrition and Physiology (IGMB-TNO), Wageningen, the Netherlands. The metabolizable energy of the hen and chicken diets was 3000 and 3200 kcal/kg feed, respectively.

2 Soy isolate: Purina Protein 500 E, Ralston Purina, St Louis, MO, containing 880 g protein/kg isolate.

3 The vitamin and mineral premix (10 g/kg diet) was prepared with dextrose and contained (mg/kg diet): thiamin, 2.5; riboflavin, 5.5; pantothenic acid, 15.0; nicotinic acid, 50.0; pyridoxine, 5.0; biotin, hens: 0.10, chickens: 0.15; folic acid, hens: 0.45, chickens: 0.75; choline chloride, hens: 1000, chickens: 1850; cyanocobalamin, 0.015; inositol, 100; p-aminobenzoic acid, 50.0; cholecalciferol, 3000 IU; all-rac-α-tocopherol, 30.0; menadione, 5.0; L-ascorbic acid, 50.0; $FeSO_4.7H_2O$, 400; MnO_2, 150; $CuSO_4.5H_2O$, 100; $ZnSO_4.H_2O$, 200; $Na_2SeO_3.5H_2O$, 0.3; KI, 5; ethoxyquin, 100. Vitamin A was added as retinyl acetate and retinyl palmitate, Rovimix A 500 (522,000 IU/g, F. Hoffmann-La Roche, Basel, Switzerland); hens: 300 retinol equivalents/kg diet, chickens: 120 or 1200 retinol equivalents/kg diet.

4 The laying hens had free access to oyster shell grit.

(n=56) and infected (n=56) pair-fed groups fed a diet with adequate vitamin A [A+(pair)I- and A+(pair)I+, respectively] and noninfected (n=32) and infected (n=32) ad libitum-fed groups fed a diet with adequate vitamin A [A+(adlib)I- and A+(adlib)I+, respectively]. Infected and noninfected chickens were housed separately in air-filtered rooms maintained under reduced pressure, controlled for temperature (decreasing from 32°C during the first day to 20°C after 2 wk), relative humidity (40-50%) and light-dark cycles (24 h dimmed light/d during the first 2 d, and 10 h of dimmed light and 14 h of darkness/d from the third day). The birds, housed eight to a cage, had free access to water and could all eat at the same time without competition. Pair-feeding was carried out by matching the feed consumption in one cage with another, corrected for the number of birds in case of mortality. The chickens were studied for a total period of 10 wk during which time feed consumption, body weight gain, vitamin A status and protein status were measured. Moreover, the birds were observed daily for clinical signs of vitamin A deficiency and/or NDV infection.

Sampling of blood

Blood from a wing vein was collected weekly in heparinized tubes from the same 11-15 chickens/group; after centrifugation, plasma was stored at -20°C.

Experimental NDV infection

One batch of lyophilized La Sota NDV (Delvax, Gist-Brocades, Delft, the Netherlands) was reconstituted in cold phosphate-buffered saline and each bird was inoculated intraocularly with a 10^8 median embryo-infectious dose of the virus. Control birds were inoculated with phosphate-buffered saline. In order to confirm the exposure to the viral infection, hemagglutination-inhibition (HI) antibody titers to NDV were measured 0, 16 and 37 d after inoculation according to the method of De Jong (27) using the ß-procedure (constant-virus diluted-serum).

Check for secondary infection

In order to check for secondary infection (bacterial or other viral infections), plasma fibrinogen levels were measured. Fibrinogen in chickens is a strong, positive acute phase protein, the level of which provides an accurate indication of general infection (28). Plasma fibrinogen levels were measured immediately prior to NDV infection (0 d after inoculation) and during the acute and postacute phase of the infection (7 and 28 d after inoculation,

respectively) by rocket immunoelectrophoresis essentially as described by Laurell (29). Briefly, diluted antiserum was mixed with melted agarose (1% wt/v) in tricine buffer (0.025 M, pH 8.6) containing sodium EDTA (0.001 M) and allowed to gel. Diluted plasma samples were added, and after electrophoresis and staining, precipitation rockets were measured. Purified chicken fibrinogen was a gift of Dr. G. Beuving (Spelderholt Centre of Poultry Research and Extension, Beekbergen, the Netherlands). Antiserum against fibrinogen was raised in rabbits and assayed for specificity and titer by immunodiffusion using the method of Ouchterlony (30). Stock solutions of purified fibrinogen were used as standards, in which total fibrinogen concentrations were analyzed according to the method of Lowry et al. (31) and standardized against bovine serum albumin. In addition, pooled samples of serum were used as internal controls. Plasma fibrinogen concentration is expressed in μmol/l based on a molecular weight of chicken fibrinogen of 320,000 (32).

Protein status

Plasma albumin levels were measured as an indicator of protein status immediately prior to NDV infection (0 d after inoculation) and during and subsequent to the acute phase of the infection (7 and 28 d after inoculation, respectively). Plasma albumin levels were determined by the use of the bromcresol green reaction method described by Gustafsson (33). Stock solutions of purified protein were used as standards, in which total albumin concentrations were analyzed according to the method of Lowry et al. (31) and standardized against bovine serum albumin. In addition, pooled samples of serum were used as internal controls. Plasma albumin concentration is expressed in μmol/l based on a molecular weight of chicken albumin of 65,000 (34).

Vitamin A status

Retinol levels were determined in plasma collected at 0, 2, 4, 5, 6, 8 and 10 wk of age. A reversed-phase high performance liquid chromatography (HPLC) method modified from that of Driskell et al. (35) was used with retinyl acetate as internal standard. The results obtained were checked against pooled control samples. The HPLC system comprised a Constametric III Metering pump, a Constametric Spectromonitor D variable wavelength detector (both LDC/Milton Roy, Riviera Beach, FL), an injection valve with a 50-μl injection loop (Type 7010, Rheodyne, Cotati, CA), and a computing integrator (Model SP 4100, Spectra-Physics, Santa Clara, CA) using a C-18 10 ODS reversed-phase column

(240 x 4.6 mm) and a guard column (75 x 2.1 mm) (both from Chrompack, Middelburg, the Netherlands). The column was eluted isocratically at a flow rate of 1.5 ml/min using a mobile phase consisting of methanol:water, 96:4 v/v. Absorbance was measured at 325 nm.

Statistical analysis

The influence of vitamin A nutriture, NDV infection and the interaction between these two factors on plasma proteins and retinol concentrations were evaluated after testing for normality by two-way analysis of variance (ANOVA) and/or analysis of variance of changes between preinfection and postinfection values. Differences between group means in feed consumption, body weight gain, plasma proteins and retinol concentrations were evaluated with Tukey-honestly significant difference statistics. Chi-square analysis in a log-linear model was used to evaluate differences in morbidity and mortality among groups. All procedures were based on the principles outlined by Snedecor and Cochran (36) and were performed using a VAX-8600 computer system with a SPSS-X software package (SPSS, Chicago, IL) (37).

RESULTS

Feed consumption and body weight gain

The effect of vitamin A nutriture on feed consumption was significant from wk 6 to the end of the experiment (Fig. 1). Both infected and noninfected chickens fed the marginally vitamin A-deficient diet had significantly lower feed consumption than their counterparts fed the control diet ad libitum. The effect of NDV infection on feed consumption was most pronounced between 7 and 14 d after virus administration. Feed consumption in infected chickens fed the marginally vitamin A-deficient diet, and also in infected chickens fed the control diet ad libitum, was significantly lower than that in noninfected chickens fed the same diets. After this period, infection did not seem to have an effect on feed consumption, except for a tendency for lower intake in wk 9 of chickens fed the control diet ad libitum compared with that of noninfected birds fed the same diet.

The effect of inadequate vitamin A nutriture on body weight gain was significant during the second and third week after virus administration (Fig. 2). In this period, body weight gain was significantly lower in chickens fed the marginally vitamin A-deficient diet than in ad libitum-fed controls.

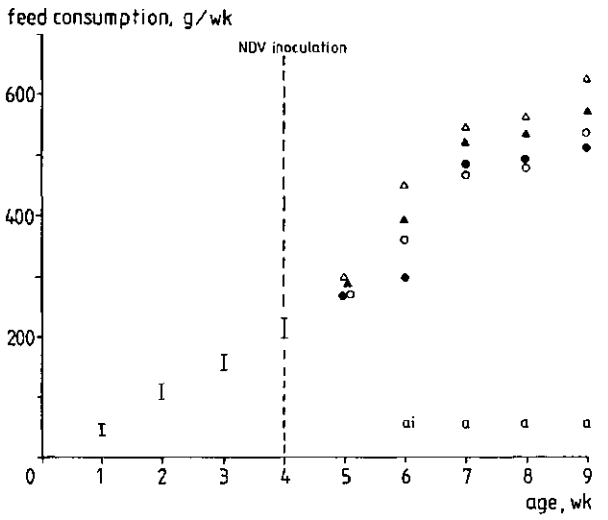

FIGURE 1. Effect of Newcastle disease virus infection on feed consumption in chickens fed diets with marginal or adequate amounts of vitamin A. (○), A-(adlib)I-; (●), A-(adlib)I+; (△), A+(adlib)I-; (▲), A+(adlib)I+. For simplicity, only the range of the lowest and highest mean values from wk 1 to wk 4 are shown. Significant effects of vitamin A nutriture and NDV infection are shown at $P < 0.05$ (a and i, respectively) (ANOVA).

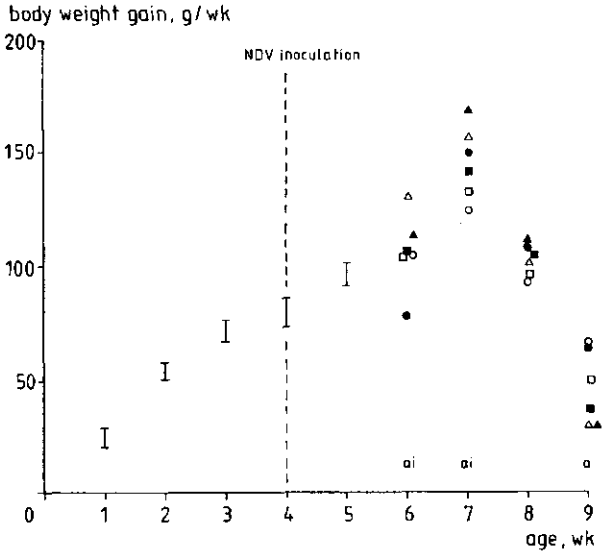

FIGURE 2. Effect of Newcastle disease virus infection on body weight gain in chickens fed diets with marginal or adequate amounts of vitamin A. (○), A-(adlib)I-; (●), A-(adlib)I+; (□), A+(pair)I-; (■), A+(pair)I+; (△), A+(adlib)I-; (▲), A+(adlib)I+. For simplicity, only the range of the lowest and highest mean values from wk 1 to wk 5 are shown. Significant effects of vitamin A nutriture and NDV infection are shown at $P < 0.05$ (a and i, respectively) (ANOVA).

However, comparison of noninfected chickens fed the marginally vitamin A-deficient diet with chickens pair-fed the control diet did not reveal any differences. Body weight gain was significantly lowered by NDV infection during the second week after virus administration in the chickens fed the marginally vitamin A-deficient diet and their counterparts fed the control diet ad libitum. However, in the third week after virus administration body weight gain was significantly higher in infected chickens than in noninfected chickens. Apparently, infected birds showed catch-up growth following the acute phase of disease in comparison with noninfected counterparts fed the same diets. In wk 9, chickens fed the marginally vitamin A-deficient diet had significantly higher body weight gain than their counterparts fed the control diet. Body weight continued to increase in all of the experimental groups.

Infection

On the day of inoculation with the virus, no maternal HI antibodies against NDV could be detected. Furthermore, throughout the experiment, all noninoculated chickens had HI antibody titers ≤ 4, which could be considered as noninfected (27). In contrast, all inoculated chickens had HI antibody titers of at least 6 at both 16 and 37 d after administration of the virus. Mean titer on d 16 for inoculated groups was 8.3, and on d 37, 7.3. This indicates exposure to the virus of the infected chickens.

TABLE 2. Effect of Newcastle disease virus infection on plasma fibrinogen concentration in chickens.

Group[1]	Fibrinogen concentration[2,4]			Change in fibrinogen concentration[3,4]	
	Day 0	Day 7	Day 28	Day 0-7	Day 0-28
			μmol/l		
A-(adlib)I-	7.79 ± 0.61[a]	8.23 ± 0.44	8.66 ± 0.61	0.44 ± 0.77[a]	0.87 ± 0.64[a]
A-(adlib)I+	7.61 ± 0.53[a]	10.59 ± 1.14	9.28 ± 0.79	2.98 ± 1.18[b]	1.67 ± 0.87[a]
A+(pair)I-	8.05 ± 0.70[a]	8.84 ± 0.44	8.66 ± 0.43	0.79 ± 0.56[a]	0.61 ± 0.70[a]
A+(pair)I+	7.79 ± 0.44[a]	11.38 ± 0.96	8.92 ± 0.53	3.59 ± 1.13[b]	1.13 ± 0.97[a]
A+(adlib)I-	8.14 ± 0.53[a]	9.28 ± 0.79	9.53 ± 0.72	1.14 ± 0.79[a]	1.39 ± 0.72[a]
A+(adlib)I+	8.23 ± 0.78[a]	11.95 ± 1.49	9.10 ± 0.51	3.72 ± 1.58[b]	0.87 ± 0.84[a]

1 From the time of hatching, chickens were fed purified diets containing marginal or adequate levels of vitamin A (see Table 1) and were inoculated with NDV at 4 wk of age (Day 0).

2 Values are means ± SEM of 8 chickens/group.

3 Values are means of differences between two time points ± SE of differences of 8 chickens/group.

4 Analysis of variance revealed a significant ($P < 0.001$) effect of infection at Day 0-7. Means within a column not sharing a common superscript letter are significantly different at $P < 0.05$ (Tukey).

Plasma fibrinogen levels, which can be used as an indicator for the presence of secondary infection, are shown in Table 2. There was a significant effect of NDV infection during the acute phase of disease, 7 d after virus inoculation. There was no significant effect of infection on fibrinogen levels 28 d after NDV inoculation.

Clinical signs of disease

The effect of NDV infection on morbidity and mortality is illustrated in Figure 3. Since period of incubation, intensity and duration of virus involvement were highly variable within groups, it was difficult to quantitate clinical signs. However, chickens were considered ill when they had signs of general weakness (closed eyes, ruffled feathers, inactivity) or respiratory problems (characteristic "gasping" and "sniveling") for at least two

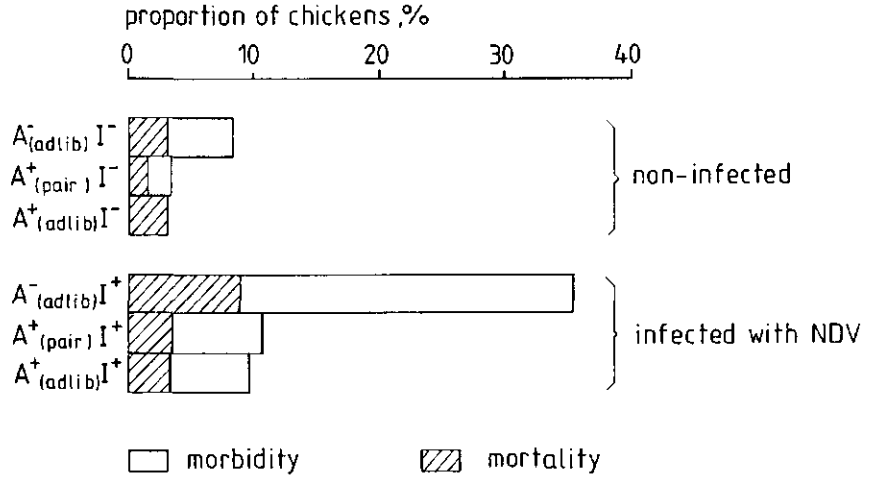

FIGURE 3. Effect of Newcastle disease virus infection on morbidity and mortality in chickens fed diets with marginal or adequate amounts of vitamin A. Morbidity is defined as the appearance within 14 d of infection of clinical signs of disease such as respiratory problems and general weakness. Mortality is defined as death within 14 d of infection. Chi-square analysis in a log-linear model revealed the following effects (since the pair-fed and ad libitum-fed control groups did not differ with respect to morbidity and mortality, the results from these groups were considered to be from one group): Morbidity : vitamin A nutriture (P = 0.001), infection (P = 0.001); Mortality : vitamin A nutriture (P = 0.188), infection (P = 0.319).

consecutive days. Within 14 d of virus inoculation, there was a significant effect of vitamin A nutriture and NDV infection on morbidity, with a strong synergistic component. The infected chickens fed the marginally vitamin A-deficient diet showed a higher proportion (33.9%) of birds with clinical signs of disease compared with all other groups (3.2 - 10.6%). Almost all respiratory problems were observed in the former group. In addition, morbidity in infected chickens fed the marginally vitamin A-deficient diet was manifested by more signs of disease. Respiratory problems were not observed in any of the noninfected birds. Duration of the clinical signs of infection was not related to vitamin A status. Although mortality rate was nearly 10% in the infected chickens fed the marginally vitamin A-deficient diet and less than 4% in the other groups, chi-square analysis did not reveal significant differences. Overt clinical signs of vitamin A deficiency (38), such as granular eyelids, nutritional roup (xerophthalmia) or white cheesy deposits under the eyelids, were not seen.

TABLE 3. Effect of Newcastle disease virus infection on plasma albumin concentration in chickens.

Group[1]	Albumin concentration[2,4]			Change in albumin concentration[3,4]	
	Day 0	Day 7	Day 28	Day 0-7	Day 0-28
			μmol/l		
A-(adlib)I-	532 ± 7[a]	525 ± 7	529 ± 8	- 7 ± 5[ab]	- 3 ± 6[ab]
A-(adlib)I+	550 ± 15[a]	505 ± 7	521 ± 17	-45 ± 14[a]	-29 ± 17[a]
A+(pair)I-	527 ± 13[a]	533 ± 18	547 ± 11	6 ± 9[b]	20 ± 6[b]
A+(pair)I+	530 ± 6[a]	522 ± 6	540 ± 6	- 8 ± 5[ab]	10 ± 4[ab]
A+(adlib)I-	550 ± 10[a]	558 ± 20	569 ± 17	8 ± 14[b]	19 ± 10[b]
A+(adlib)I+	543 ± 19[a]	546 ± 13	562 ± 20	3 ± 6[b]	19 ± 2[b]

1 From the time of hatching, chickens were fed purified diets containing marginal or adequate levels of vitamin A (see Table 1) and were inoculated with NDV at 4 wk of age (Day 0).
2 Values are means ± SEM of 8 chickens/group.
3 Values are means of differences between two time points ± SE of differences of 8 chickens/group.
4 Analysis of variance revealed a significant ($P < 0.01$) effect of vitamin A nutriture at Day 0-7 and Day 0-28, and a significant ($P < 0.05$) effect of infection at Day 0-7. Means within a column not sharing a common superscript letter are significantly different at $P < 0.05$ (Tukey).

Protein status

Prior to infection, no differences in plasma albumin levels were found among the treatment groups (Table 3). However, analysis of variance of changes

between preinfection and postinfection values showed a significant main effect of vitamin A nutriture during the acute and postacute phases of disease and a significant main effect of NDV infection during the acute phase. Although infection did not have an effect in the pair-fed and ad libitum-fed control groups, chickens fed the marginally vitamin A-deficient diet showed a tendency for lower plasma albumin concentrations 7 and 28 d after virus inoculation than noninfected birds fed the same diet.

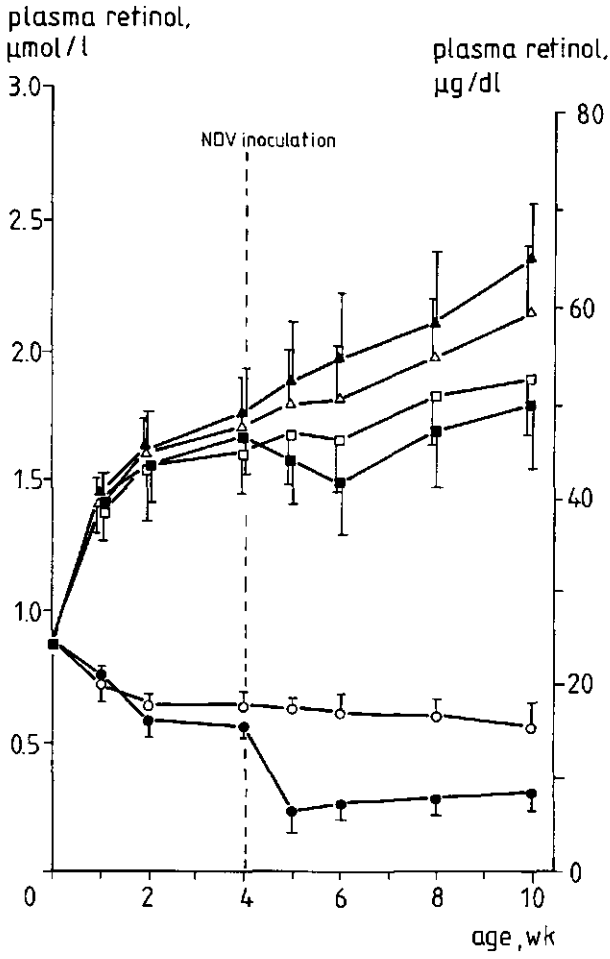

FIGURE 4. Effect of Newcastle disease virus infection on plasma retinol concentration in chickens fed from hatching diets containing marginal or adequate amounts of vitamin A. Values are means with the vertical bars representing one SD (n=11-15). (○), A-(adlib)I-; (●), A-(adlib)I+; (□), A+(pair)I-; (■), A+(pair)I+; (△), A+(adlib)I-; (▲), A+(adlib)I+.

Vitamin A status

Plasma retinol concentrations are shown in Figure 4. Plasma retinol levels of day-old chickens were 0.86 μmol/l (24.5 μg/dl), indicating limited vitamin A reserves. Within 1 wk of infection, and continuing until the end of the experiment, plasma retinol levels in chickens fed marginally vitamin A-deficient diets were significantly lower ($P < 0.001$) than levels in noninfected chickens fed the same diet. Plasma retinol levels decreased during the acute phase of disease to 0.23 μmol/l (6.6 μg/dl), which is considered as deficient (39). Even 6 wk after inoculation with virus, plasma retinol levels had still not returned to the levels of the noninfected birds fed the same marginally vitamin A-deficient diet [0.29 μmol/l (8.4 μg/dl) compared with 0.53 μmol/l (15.2 μg/dl), respectively]. After 1 wk of consuming the experimental diets, the plasma retinol levels in the pair-fed and ad libitum-fed control groups rose sharply to approximately 1.4 μmol/l (40 μg/dl). The level increased only slightly throughout the experiment in the noninfected and infected pair-fed groups [1.83 μmol/l (52.4 μg/dl) and 1.73 μmol/l (49.5 μg/dl) at 10 wk of age, respectively] with a small, although significant ($P < 0.05$) drop in the pair-fed control group after the infection. Plasma retinol levels increased somewhat more in the noninfected and infected chickens fed the control diet ad libitum [2.07 μmol/l (59.1 μg/dl) and 2.18 μmol/l (62.4 μg/dl), respectively at 10 wk of age]. Infection did not appear to influence plasma retinol concentration in these birds.

DISCUSSION

Since clinical and epidemiological studies have revealed an association between vitamin A deficiency and susceptibility to infection (6-9), animal models have been used to elucidate the underlying mechanisms. Experiments in which vitamin A-deficient birds were challenged with parasites (40,41), bacteria (42,43) and viruses (20,21,44-47) have yielded useful, although sometimes conflicting, information. Most experimental models described in the literature have used severely vitamin A-deficient animals, in which it is difficult to separate the effects of vitamin A deficiency per se from those of more generalized protein-energy malnutrition. In addition, experiments involving challenge with infectious diseases, especially viral infections, have many problems such as standardization, detection of virus, microbiological safety and manifestation of infectious processes that do not

resemble those seen in humans. Susceptibility of severely vitamin A-deficient animals to secondary infections can further complicate the interpretation of results. In the present study, NDV infection in chickens varying in vitamin A status has been used as a model to obtain a better understanding of the underlying mechanisms of importance in the interrelationship between marginal vitamin A deficiency and severity of consequences of measles infection in humans. In this model, a large dose of the La Sota strain of NDV has been used. The lentogenic La Sota strain is relatively mild and only provokes minor respiratory problems, diarrhea and some general signs of disease in chickens in a healthy condition (48,49). Moreover, the virus can be used without danger to laboratory personnel and exposure to the virus can easily be confirmed by measuring hemagglutination-inhibition antibody titers. Furthermore, by using pair-fed controls it is possible to correct for a decrease in feed consumption and reduced body weight gain. Growth depression and loss of appetite are two of the earliest signs of vitamin A deficiency reported in chickens (38) and other animals (47,50,51). Although in the present study, marginal vitamin A intake influenced feed consumption, vitamin A status per se did not affect body weight gain when compared with the pair-fed control group. Moreover, the result of both inadequate vitamin A nutriture and NDV inoculation during the acute phase of the infection has been a reduction in feed consumption and a concomitant reduction in body weight gain but the latter was much more pronounced than in the pair-fed controls. However, neither the chickens fed the marginally vitamin A-deficient nor those pair-fed the control diet could be considered malnourished, since their mean body weights were not more than 8% less than the ad libitum-fed control group and all chickens continued to increase in body weight throughout the experiment. Body weight gain in chickens fed the marginally vitamin A-deficient diet was even higher than in the control groups at 9 wk of age. This could be the result of catch-up growth following the period of growth retardation. Moreover, plasma albumin levels remained stable in all birds except for the small decrease in infected chickens fed the marginally vitamin A-deficient diet during the acute and postacute phase. Secondary infections did not play a role as measured by analysis of the acute phase protein, fibrinogen, in plasma.

The results of this study indicate that pre-existing marginal vitamin A status increases the severity of disease following NDV infection. This observation is in agreement with the findings of Squibb and Veros (44) and of Nauss et al. (46) working with NDV-chicken and herpes simplex-rat models, respectively. They also found that viral infections were more severe in

vitamin A-deficient animals. However, in these studies extremely vitamin A-deficient animals were used. Generally, more attention has been focused on the effects of severe rather than marginal vitamin A deficiency on specific (20,25,45,51) and nonspecific (42,43) host defense mechanisms. An alteration in function of these defense mechanisms can explain the increased morbidity after challenge with NDV.

Another important finding was that infection with NDV reduced premorbid marginal plasma vitamin A levels during the acute phase to levels which could be regarded as deficient (39). In addition, these depressed levels continued for at least 1 mo after the disappearance of the clinical disease signs and after restoration of plasma albumin levels. Although Squibb and Veros (44) were not able to detect an effect of virus infection on vitamin A reserves, clinical studies in humans (7,8) demonstrated the same phenomenon for measles infection in humans. The depression in vitamin A levels produced by infections that destroy epithelial tissue are probably the result of reduced absorption of vitamin A during acute infection (6), increased loss of vitamin A into the gut or urine (52), increased vitamin A requirement for the regeneration of damaged tissues (53), impaired mobilization of retinol in the blood circulation (54,55), or a combination of several or all of these factors.

In conclusion, the results of the present study indicate that there is an interaction between vitamin A status and NDV infection, without interference of protein-energy malnutrition. On the one hand, a viral infection produces more morbidity in pre-existing marginal vitamin A deficiency and, on the other hand, infection reduces plasma retinol levels. However, there is an interaction between two factors, in which it is difficult to separate cause and effect. Additional studies are necessary to investigate the mechanisms by which NDV infection in marginally vitamin A-deficient birds can reduce vitamin A status and to investigate the consequences of infection-induced vitamin A deficiency. Particular emphasis should be paid to the effects on host defense mechanisms. The NDV-chicken model would appear to be particularly suitable for this purpose. Results of these experiments may contribute to a better understanding of the interrelationship in humans between vitamin A status and infection, especially for infection with viruses such as measles.

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CHAPTER 4

Effect of Newcastle disease virus infection on vitamin A metabolism in chickens

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ABSTRACT

The effect of Newcastle disease virus (NDV, La Sota strain) infection on vitamin A metabolism was investigated in chickens maintained on normal or marginal vitamin A intake. NDV, a virus of the Paramyxoviridae family that primarily affects epithelial tissue, was administered at 4 wk of age. Plasma levels of retinol, retinol-binding protein and, to a lesser extent, transthyretin were found to be significantly lower during both the acute and postacute phases of infection in chickens fed a diet marginally deficient in vitamin A compared to noninfected birds fed the same diet, while vitamin A content in liver was unaffected. However, in chickens fed adequate vitamin A, NDV infection did not influence the parameters measured. Levels of retinol-binding protein in liver were significantly increased by inadequate vitamin A nutriture but infection partly reduced this increase. The results suggest that the reduced vitamin A status in marginally vitamin A-deficient chickens infected with NDV can be attributed to a combination of a direct effect of the virus on retinol-binding protein metabolism in liver and an increased rate of utilization and catabolism of retinol and retinol-binding protein by extrahepatic tissues.

INTRODUCTION

It is well established that vitamin A metabolism is affected in many disease states (1). Although insufficient intake of vitamin A is the primary cause of poor vitamin A status, depletion of vitamin A reserves can also result from diseases which impair absorption (2-5), impair hepatic mobilization (6,7), increase the rate of utilization in tissues (6,8,9) or alter catabolism in the kidney (10,11). However, relatively little is known about the effect of viral infections on vitamin A metabolism, especially when the premorbid vitamin A status is marginal.

This report is a sequel to a previous report in which we demonstrated an interaction between vitamin A status and Newcastle disease virus (NDV, La Sota

strain) infection in a chicken model when differences in feed intake and absence of secondary infections were controlled (12). NDV belongs to the family Paramyxoviridae and the primary effect of the La Sota strain is to invade and damage epithelial tissues (13-17). In our previous report (12), we showed that NDV infection produced greater rates of morbidity in chickens fed a marginally vitamin A-deficient diet than in chickens fed a diet adequate in vitamin A. In addition, plasma retinol levels in infected chickens fed the vitamin A-deficient diet were significantly lower than in noninfected chickens fed the same diet. The aim of the present report is to present data which could help to explain the mechanism of the latter phenomenon. Therefore, levels of retinol and retinol carrier proteins, retinol-binding protein (RBP) and transthyretin (formerly refered to as prealbumin) in plasma and liver were determined immediately prior to and during the acute and postacute phases of infection.

MATERIALS AND METHODS

Animals and experimental design

Chickens in this experiment were selected at random from the birds in which plasma retinol concentrations were measured, and a detailed description of the animals, housing conditions, diets, administration and detection of the viral infection, and experimental design can be found in the previous paper (12). In brief, female day-old White Leghorn chickens (strain Lohmann Selected Leghorn) with limited vitamin A reserves, progeny of marginally vitamin A-deficient laying hens (Wageningen Agricultural University, the Netherlands), were fed purified diets ad libitum containing either adequate (1200 retinol equivalents (RE)/kg feed) or marginal amounts of vitamin A (120 RE/kg feed). In order to ensure that the intake of the marginally vitamin A-deficient chickens differed only with respect to vitamin A, a pair-fed control group was also used. At 4 wk of age, half of the chickens in each of the three groups were infected intraocularly with 10^8 EID_{50} of the lentogenic La Sota strain of NDV. In this way six experimental groups were formed: noninfected (n=56) and infected (n=56) groups fed a diet marginally deficient in vitamin A [A-(adlib)I- and A-(adlib)I+, respectively]; noninfected (n=56) and infected (n=56) pair-fed control groups fed a diet with adequate vitamin A [A+(pair)I- and A+(pair)I+, respectively]; and noninfected (n=32) and infected (n=32) ad libitum-fed control groups [A+(adlib)I- and A+(adlib)I+, respectively].

Plasma retinol, RBP and transthyretin were measured in 8 chickens/group immediately prior to NDV infection (0 d after inoculation) and during the acute and postacute phases of the infection (7 and 28 d after inoculation, respectively). The degree of saturation of plasma RBP with retinol was calculated from the molar ratio, assuming all retinol in plasma was bound to RBP. Vitamin A and RBP content in liver were determined in 8 chickens/group just prior to NDV infection (2 d before inoculation) and just after the acute phase of infection (9 d after inoculation).

Sampling of blood

Blood from a wing vein was collected in heparinized tubes from the same 8 chickens/group; after centrifugation, plasma was separated and stored at $-20^{\circ}C$.

Preparation of liver homogenates

Livers were collected from six 1-d-old chickens and from 8 chickens/group 2 d before and 9 d after inoculation of the virus. Livers were excised, rinsed in phosphate-buffered saline and blotted dry. Liver homogenates were prepared by homogenizing samples of freshly isolated liver in a mixer (Sorvall Omni-Mixer, Sorvall, Newtown, CO) cooled on ice. Part of the homogenate was immediately frozen at $-20^{\circ}C$ for liver vitamin A determination and the remainder was further homogenized with three volumes (wt/v) of a 0.15 M KCl solution in a Potter-Elvehjem homogenizer. The resultant homogenate was centrifuged at 2000 x g for 30 min at $4^{\circ}C$. After removal of the fatty upper layer, the supernatants obtained were frozen at $-20^{\circ}C$ until determination of RBP and the sediment was discarded as it did not contain measurable amounts of RBP. Plasma, not cellular, RBP was measured.

Vitamin A analyses

Plasma retinol content was determined by a reversed-phase high performance liquid chromatography (HPLC) method modified from that of Driskell et al. (18) as described previously (12). Hepatic vitamin A content was determined by a HPLC method modified from that of Brubacher, Müller-Mulot and Southgate (19), in which retinyl esters in homogenized liver were first saponified. Recovery of vitamin A after saponification and extraction in liver samples was determined with the spiking method and exceeded 90%.

Protein determinations

Plasma RBP and transthyretin were determined by rocket immunoelectrophoresis essentially as described by Laurell (20). Briefly, diluted antisera were mixed with melted agarose (1% wt/v) in tricine buffer (0.025 M, pH 8.6) and allowed to gel. Diluted plasma samples were added, and after electrophoresis and staining, precipitation rockets were measured. Chicken RBP and transthyretin were purified according to the method of Abe, Muto and Hosoya (21). Antisera against these proteins were raised in rabbits and assayed for specificity and titer by immunodiffusion using the method of Ouchterlony (22). Stock solutions of purified proteins were used as standards, in which total RBP and transthyretin concentrations were determined by measuring absorbance at 280 nm assuming molar extinction coefficients of 19.2 and 22.8, respectively (21). Plasma protein concentrations are expressed in μmol/l based on molecular weights of chicken RBP and transthyretin of 24,000 and 54,000, respectively (21).

Supernatants prepared from liver homogenates were used directly in the rocket immunoelectrophoresis assay for chicken RBP.

Statistical analysis

The influence of vitamin A nutriture, NDV infection and the interaction between these two factors on concentrations of proteins and retinol in plasma and liver were evaluated after testing for normality by two-way analysis of variance (ANOVA) and/or analysis of variance of changes between preinfection and postinfection values. Differences between group means were evaluated with Tukey-honestly significant difference statistics. All procedures were based on the principles outlined by Snedecor and Cochran (23) and were performed using a VAX-8600 computer system with a SPSS-X software package (SPSS, Chicago, IL) (24).

RESULTS

The effect of NDV infection in chickens fed either marginal or adequate amounts of vitamin A on feed consumption, body weight gain, plasma albumin levels and general health were reported in a previous paper, as were data on virus exposure and absence of secondary infections (12).

Plasma retinol concentration

Analysis of variance showed a significant effect of vitamin A nutriture immediately prior to NDV inoculation (Table 1). In addition, analysis of variance of changes between preinfection values and values measured during the acute or postacute phases of disease, 7 and 28 d after virus inoculation, respectively, demonstrated a significant effect of vitamin A nutriture, NDV infection and the interaction of these factors on plasma retinol levels.

TABLE 1. Effect of infection with Newcastle disease virus on plasma retinol concentration in chickens.

Group[1]	Retinol concentration[2,4]			Change in retinol concentration[3,4]	
	Day 0	Day 7	Day 28	Day 0-7	Day 0-28
			μmol/l		
A-(adlib)I-	0.60 ± 0.01[a]	0.59 ± 0.01	0.54 ± 0.02	-0.01 ± 0.01[c]	-0.06 ± 0.02[b]
A-(adlib)I+	0.56 ± 0.01[a]	0.24 ± 0.02	0.28 ± 0.02	-0.32 ± 0.02[a]	-0.28 ± 0.02[a]
A+(pair)I-	1.56 ± 0.04[b]	1.62 ± 0.05	1.75 ± 0.05	0.06 ± 0.02[d]	0.19 ± 0.04[d]
A+(pair)I+	1.61 ± 0.04[b]	1.51 ± 0.04	1.65 ± 0.06	-0.10 ± 0.02[b]	0.04 ± 0.05[c]
A+(adlib)I-	1.67 ± 0.06[b]	1.77 ± 0.06	1.93 ± 0.07	0.10 ± 0.01[d]	0.26 ± 0.04[d]
A+(adlib)I+	1.71 ± 0.05[b]	1.84 ± 0.07	2.04 ± 0.08	0.13 ± 0.02[d]	0.33 ± 0.04[e]

1 Chickens were fed from hatching purified diets containing marginal or adequate amounts of vitamin A and were inoculated with NDV at 4 wk of age (Day 0).
2 Values are means ± SEM for 8 chickens/group.
3 Values are means of differences between two time points ± SE of differences for 8 chickens/group.
4 Analysis of variance revealed the following effects: Day 0: vitamin A nutriture, $P < 0.001$; Day 0-7 and Day 0-28: vitamin A nutriture, $P < 0.001$; NDV infection, $P < 0.001$; and vitamin A nutriture x NDV infection, $P < 0.001$. Means within a column not sharing a common superscript letter are significantly different at $P < 0.05$ (Tukey).

Since the chickens used in this experiment were selected at random from the birds for which plasma retinol concentrations have been described earlier (12), the results were similar. Briefly, within 1 wk of infection, plasma retinol levels in those chickens fed the marginally vitamin A-deficient diet were significantly lower than in noninfected chickens fed the same diet. Plasma retinol levels decreased from marginal (0.56 μmol/l or 16.0 μg/dl) to deficient levels (0.24 μmol/l or 6.9 μg/dl). Even 28 d after virus inoculation, plasma retinol levels were still significantly lower than in noninfected birds fed the same diet marginally deficient in vitamin A (0.28 μmol/l or 8.0 μg/dl and 0.54 μmol/l or 15.4 μg/dl, respectively). NDV

infection had the same effect, although less pronounced, in the pair-fed control group compared with noninfected chickens fed the same diet. However, infection did not affect plasma retinol levels in the control birds fed ad libitum.

Liver vitamin A content

The content of total vitamin A in liver of chickens just prior to infection and just after the acute phase of the infection is shown in Table 2.

TABLE 2. Effect of infection with Newcastle disease virus on liver vitamin A and liver retinol-binding protein (RBP) concentrations in chickens.

Group[1]	Liver vitamin A[2,3]		Liver RBP[2]	
	Day -2	Day 9	Day -2	Day 9
	nmol/g		nmol/g	
A-(adlib)I-	13 ± 2 [a]	8 ± 2 [a]	3.22 ± 0.24 [a]	3.58 ± 0.26 [a]
A-(adlib)I+	12 ± 2 [a]	9 ± 1 [a]	3.42 ± 0.31 [a]	2.67 ± 0.43 [b]
A+(pair)I-	99 ± 12 [b]	106 ± 7 [b]	1.33 ± 0.20 [b]	1.46 ± 0.15 [c]
A+(pair)I+	95 ± 10 [b]	124 ± 16 [b]	1.43 ± 0.13 [b]	1.38 ± 0.20 [c]

1 Chickens were fed from hatching purified diets containing marginal or adequate amounts of vitamin A and were inoculated with NDV at 4 wk of age (Day 0).

2 Values are means ± SEM for 8 chickens/group. Analysis of variance revealed the following effects: liver vitamin A, Day -2 and Day 9: vitamin A nutriture, $P < 0.001$; liver RBP, Day -2: vitamin A nutriture, $P < 0.001$; liver RBP, Day 9: vitamin A nutriture, $P < 0.001$, and NDV infection, $P < 0.05$. Means within a column not sharing a common superscript letter are significantly different at $P < 0.05$ (Tukey).

3 Total liver vitamin A concentration expressed as nmol retinol/g liver was determined after saponification of retinyl esters to retinol. Total liver vitamin A concentration of day-old chickens (n=6) was 26 ± 3 nmol RE/g.

Liver vitamin A content was significantly lower in chickens fed the marginally vitamin A-deficient diet than in those pair-fed the control diet, both 2 d before and 9 d after inoculation with virus. Although there was a tendency for higher levels of vitamin A in the infected pair-fed group than in noninfected chickens fed the same diet, infection did not significantly influence liver vitamin A levels in chickens fed diets with adequate or marginal amounts of vitamin A. Relative liver weight was not significantly different among the groups.

Plasma RBP concentration

Vitamin A nutriture significantly affected plasma RBP levels prior to virus inoculation (Table 3). In addition, analysis of variance of changes

TABLE 3. Effect of infection with Newcastle disease virus on plasma retinol-binding protein (RBP) concentration in chickens.

Group[1]	RBP concentration[2,4]			Change in RBP concentration[3,4]	
	Day 0	Day 7	Day 28	Day 0–7	Day 0–28
			μmol/l		
A–(adlib)I–	1.43 ± 0.11[a]	1.42 ± 0.16	1.32 ± 0.11	–0.01 ± 0.11[a]	–0.11 ± 0.08[ab]
A–(adlib)I+	1.60 ± 0.12[a]	0.80 ± 0.11	1.15 ± 0.13	–0.80 ± 0.08[b]	–0.45 ± 0.12[a]
A+(pair)I–	2.09 ± 0.14[b]	1.90 ± 0.14	2.01 ± 0.15	–0.19 ± 0.07[a]	–0.08 ± 0.13[ab]
A+(pair)I+	1.86 ± 0.16[ab]	1.65 ± 0.14	1.92 ± 0.09	–0.21 ± 0.06[a]	0.06 ± 0.12[ab]
A+(adlib)I–	2.13 ± 0.16[b]	2.10 ± 0.16	2.14 ± 0.12	–0.03 ± 0.12[a]	0.01 ± 0.16[ab]
A+(adlib)I+	2.01 ± 0.15[ab]	2.11 ± 0.11	2.36 ± 0.17	0.10 ± 0.08[a]	0.35 ± 0.17[b]

1 Chickens were fed from hatching purified diets containing marginal or adequate amounts of vitamin A and were inoculated with NDV at 4 wk of age (Day 0).
2 Values are means ± SEM for 8 chickens/group.
3 Values are means of differences between two time points ± SE of differences for 8 chickens/group.
4 Analysis of variance revealed the following effects: Day 0: vitamin A nutriture, $P < 0.001$; Day 0–7: vitamin A nutriture, $P < 0.001$; NDV infection, $P < 0.001$; and vitamin A nutriture x NDV infection, $P < 0.001$; Day 0–28: vitamin A nutriture, $P < 0.001$; and vitamin A nutriture x NDV infection, $P < 0.05$. Means within a column not sharing a common superscript letter are significantly different at $P < 0.05$ (Tukey).

in plasma RBP concentration between preinfection values and values measured during the acute phase of disease revealed a significant effect of vitamin A nutriture and NDV infection and a significant interaction between these factors. Chickens fed the diet marginally deficient in vitamin A had lower plasma RBP concentrations than chickens fed the control diet. Moreover, plasma RBP concentrations declined markedly in chickens fed the marginally vitamin A-deficient diet during the acute phase of disease when compared with the preinfection values, while levels in noninfected chickens fed the same diet remained the same. During the postacute phase, plasma RBP levels were still lower than before infection. In contrast, infection did not influence RBP levels in chickens fed the control diet.

The degree of saturation of plasma RBP with retinol is shown in Table 4. Analysis of variance revealed a significant effect of vitamin A nutriture on the degree of saturation prior to virus administration, and analysis of

variance of changes between preinfection values and values measured during the postacute phase of disease revealed a significant effect of NDV infection. Chickens fed the marginally vitamin A-deficient diet had lower values (23.9 - 43.8%) than control birds fed the diet with adequate vitamin A (74.6 - 91.7%).

TABLE 4. Effect of infection with Newcastle disease virus on plasma retinol-binding protein (RBP) saturation with retinol in chickens.

Group	RBP saturation[1,2,4]			Change in RBP saturation[1,3,4]	
	Day 0	Day 7	Day 28	Day 0-7	Day 0-28
			(%)		
A-(adlib)I-	43.5 ± 3.1[a]	43.2 ± 2.9	43.8 ± 2.4	-0.3 ± 1.7[a]	0.3 ± 1.5[b]
A-(adlib)I+	33.9 ± 2.1[a]	29.2 ± 3.4	23.9 ± 2.2	-4.7 ± 2.6[a]	-10.0 ± 2.7[a]
A+(pair)I-	74.6 ± 3.9[b]	85.7 ± 3.1	87.9 ± 2.6	11.1 ± 2.2[b]	13.3 ± 2.7[c]
A+(pair)I+	87.3 ± 2.7[b]	91.7 ± 3.6	84.9 ± 4.2	4.4 ± 2.5[ab]	-2.4 ± 1.9[ab]
A+(adlib)I-	78.0 ± 2.9[b]	82.9 ± 3.2	90.0 ± 3.7	4.9 ± 5.1[ab]	12.0 ± 2.8[c]
A+(adlib)I+	84.8 ± 3.6[b]	86.5 ± 5.1	86.6 ± 3.5	1.7 ± 2.3[ab]	1.8 ± 1.9[b]

1 For calculating the proportion of retinol-binding protein saturated with retinol, all of the retinol in plasma was assumed to be bound to RBP.
2 Values are means ± SEM for 8 chickens/group.
3 Values are means of differences between two time points ± SE of differences for 8 chickens/group.
4 Analysis of variance revealed the following effects: Day 0: vitamin A nutriture, $P < 0.001$; Day 0-28: NDV infection, $P < 0.05$. Means within a column not sharing a common superscript letter are significantly different at $P < 0.05$ (Tukey).

This indicated that most of the circulating RBP in chickens fed the marginally vitamin A-deficient diet was present as apo-RBP, while in chickens fed diets with adequate vitamin A most was present as holo-RBP. Twenty-eight days after inoculation, the degree of saturation of plasma RBP with retinol remained stable in noninfected chickens fed a diet marginally deficient in vitamin A but was lower in infected chickens fed the same diet during the experimental period.

Liver RBP concentration

The level of RBP in liver was elevated in chickens with inadequate vitamin A intake compared with their counterparts pair-fed a vitamin A-adequate diet (Table 2). However, NDV infection partly counteracted this increase in birds fed inadequate vitamin A but did not affect RBP levels in livers of chickens fed an adequate amount of vitamin A.

Plasma transthyretin concentration

Analysis of variance of changes in plasma transthyretin levels between preinfection values and values measured during the acute and postacute phases of disease showed a significant effect of vitamin A nutriture and the interaction of vitamin A nutriture and infection (Table 5). Prior to infection, no differences among the treatment groups were found. Infection did not influence plasma transthyretin levels in the same way. On the one hand, transthyretin levels were significantly lower during the acute phase of disease in infected chickens fed a marginally vitamin A-deficient diet than in noninfected birds fed the same diet. On the other hand, the opposite was found 28 d after infection in chickens pair-fed the control diet.

TABLE 5. Effect of infection with Newcastle disease virus on plasma transthyretin concentration in chickens.

Group[1]	transthyretin concentration[2,4]			Change in transthyretin concentration[3,4]	
	Day 0	Day 7	Day 28	Day 0-7	Day 0-28
			μmol/l		
A-(adlib)I-	5.36 ± 0.16[a]	5.53 ± 0.20	5.58 ± 0.24	0.17 ± 0.09[bc]	0.22 ± 0.13[a]
A-(adlib)I+	5.49 ± 0.21[a]	5.04 ± 0.18	5.32 ± 0.19	-0.45 ± 0.06[a]	-0.17 ± 0.05[a]
A+(pair)I-	5.92 ± 0.15[a]	6.09 ± 0.15	6.03 ± 0.24	0.17 ± 0.17[bc]	0.11 ± 0.19[a]
A+(pair)I+	5.38 ± 0.23[a]	5.74 ± 0.23	5.80 ± 0.22	0.36 ± 0.18[c]	0.42 ± 0.12[b]
A+(adlib)I-	6.13 ± 0.25[a]	5.95 ± 0.24	6.29 ± 0.22	-0.18 ± 0.06[a]	0.16 ± 0.09[a]
A+(adlib)I+	5.66 ± 0.20[a]	5.69 ± 0.19	5.91 ± 0.23	0.03 ± 0.10[ab]	0.25 ± 0.20[ab]

1 Chickens were fed from hatching purified diets containing marginal or adequate amounts of vitamin A and were inoculated with NDV at 4 wk of age (Day 0).
2 Values are means ± SEM for 8 chickens/group.
3 Values are means of differences between two time points ± SE of differences for 8 chickens/group.
4 Analysis of variance revealed the following effects: Day 0-7: vitamin A nutriture, $P < 0.01$; and vitamin A nutriture x NDV infection, $P < 0.001$; Day 0-28: vitamin A nutriture, $P < 0.05$; and vitamin A nutriture x NDV infection, $P < 0.01$. Means within a column not sharing a common superscript letter are significantly different at $P < 0.05$ (Tukey).

DISCUSSION

Although much is known about the metabolism of vitamin A, little attention has been focused on the effect of infectious diseases in these processes. Since infections are extremely common and frequently associated with inadequate vitamin A status in developing countries (4,5,9,25-29), it is of

great importance to carry out studies in order to be able to understand the underlying mechanisms. However, clinical studies are limited by ethical considerations, while conclusions from animal studies have often been based on experiments using animals not only with extreme vitamin A deficiency but also with general malnutrition, resulting in very poor general health. In this and a previous paper (12), we describe an animal model with which we have demonstrated that there is an interaction between vitamin A status and NDV infection in chickens. Using this model, we can study the effects of a single virus infection superimposed on marginal vitamin A status without interference from protein-energy malnutrition or secondary infections. The La Sota strain of NDV is relatively mild and produces only very limited respiratory and other problems (13-15), although severity strongly depends on initial vitamin A status (12).

After intestinal absorption, dietary vitamin A is transported as retinyl esters in chylomicrons to the liver, which is the main organ for storage of vitamin A (30,31). Retinol is mobilized from the liver and transported to peripheral target tissues as a retinol-RBP complex (holo-RBP). Normally, this complex circulates with transthyretin, thus avoiding renal catabolism and glomerular filtration, both in humans (10,32) and chickens (21,33-35).

Our results indicate that inadequate vitamin A nutriture can reduce plasma RBP levels and can increase the content of RBP in liver. The secretion of RBP by the liver would appear to be controlled in part by the availability of retinol for the formation of the holo-RBP complex at the level of the liver cell as reported by other investigators (36-38). Moreover, NDV infection further reduced plasma RBP and consequently plasma retinol levels in birds fed the diet marginally deficient in vitamin A. Since the content of RBP in liver was significantly lower in these chickens than in the noninfected birds fed the same diet, while vitamin A content in liver was unaffected, NDV infection would appear to impair RBP metabolism in liver. Since the level of RBP in liver from vitamin A-deficient chickens infected with NDV was still higher than that in chickens fed adequate amounts of vitamin A, an impairment of RBP metabolism in liver would probably not only concern a possible inhibition of RBP synthesis but also an increased rate of catabolism in and/or release from the liver. This is probably not the case for transthyretin and albumin. Plasma levels of transthyretin and, as described earlier (12), albumin were only relatively less affected by NDV inoculation in vitamin A-deficient chickens during both the acute and the postacute phases of the infection. Therefore, in spite of differences in rates of synthesis of these proteins (39), it would

appear that NDV infection has a more specific effect on the inhibition of RBP metabolism in liver. Moreover, it is not likely that synthesis of RBP was impaired by lack of substrate as a consequence of protein malnutrition because of the relative lack of effect on the synthesis of the other proteins. In addition, it has been demonstrated in monkeys that infection does not affect the rate of synthesis of albumin in liver (40).

Lowered plasma retinol and RBP concentrations following infection can also result from an increased rate of utilization or catabolism by tissues relative to the rate of hepatic mobilization (9). It is assumed that more retinol is required for regeneration of damage to extrahepatic tissues (1), while shortly after retinol is delivered to its target cell, the free RBP remaining is removed from the circulation by glomerular filtration (32,41). Moreover, a rapid fall in plasma RBP levels might result from markedly accelerated catabolism of RBP in other peripheral tissues which was also demonstrated for other proteins after infection (40,42). Such a hypothesis is supported by the differences in plasma RBP and retinol levels, hepatic RBP levels and the proportion of saturation of RBP with retinol that were observed between infected and noninfected chickens fed the same marginally vitamin A-deficient diet. However, it is not supported by the finding that hepatic vitamin A levels following infection were not changed nor by the observation that the decrease in plasma RBP levels was about five times higher than that of transthyretin and, as described previously, albumin (12). Although the chickens were in a febrile condition during the acute phase of the infection, there is another reason why increased tissue utilization or catabolism alone cannot satisfactorily explain the observed results. The concentrations of both retinol and its carrier protein, RBP, in plasma of vitamin A-deficient chickens continued to be reduced significantly during the postacute phase of the infection. Since in this period neither clinical signs of NDV infection, secondary infection and diarrhea, nor differences in growth rate between noninfected and infected birds fed the same diet marginally deficient in vitamin A could be observed, there was no reason to assume a higher rate of tissue utilization.

An alternative explanation for the decreased concentration of RBP in plasma of infected, marginally vitamin A-deficient chickens could be an increased rate of excretion of uncomplexed RBP as a result of decreased formation of the RBP-transthyretin complex. RBP in such a complex is not readily excreted by kidney glomeruli. However, inhibition of the complex formation is not a result of the unavailability of transthyretin, since the

levels of transthyretin in marginally vitamin A-deficient chickens fall only 8% 7 d after infection compared with a drop of 50% for RBP. Perhaps reduced complex formation could arise from reduced affinity between the available RBP and transthyretin. It is known that apo-RBP has a lower affinity for transthyretin than does holo-RBP (6,41). After infection, most of the RBP in plasma of the marginally vitamin A-deficient chickens was apo-RBP, not holo-RBP. However, this probably just reflects the shortage of newly released holo-RBP from the liver. Thus, although interference with RBP-transthyretin complex formation may play a role, it is very difficult from the data available to contradict the hypothesis that the reduced levels of RBP in plasma observed after NDV infection of marginally vitamin A-deficient chickens is due to an effect of both impaired RBP metabolism in the liver and increased rate of utilization and catabolism of retinol and RBP.

Although the La Sota strain of NDV can affect epithelia in the intestinal tract (13-15) and, as a possible consequence, impair absorption of vitamin A, histological examination in this laboratory (unpublished data) could not demonstrate serious pathological changes.

In conclusion, our results indicate that infection with the La Sota strain of NDV did not appear to influence plasma concentrations of RBP, transthyretin and, as described earlier (12), albumin in chickens fed adequate amounts of vitamin A but did influence these parameters in chickens fed a marginally vitamin A-deficient diet. However, we are dealing with an interaction between two factors in which it is difficult to separate cause and effect. Since the appearance and severity of clinical signs is dependent on vitamin A status prior to infection, comparison of changes in retinol or retinol-carrier protein concentrations during infection between chickens fed adequate or marginal amounts of vitamin A could be influenced by the extent of illness. Nevertheless, the results suggest a strong effect of infection on vitamin A metabolism when individuals are in a premorbid, marginally vitamin A-deficient condition, and this effect even remains during the postacute phase of the infection. In our model, the most plausible explanation is a combination of a direct effect of NDV on RBP metabolism in liver, and an increased rate of utilization and catabolism of retinol and RBP by extrahepatic tissues.

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CHAPTER 5

Effect of epithelia-damaging virus infections on vitamin A metabolism in chickens

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ABSTRACT

The effect of infection with infectious bronchitis virus (IBV, M41 strain) and reovirus (RV, 1637 strain) on vitamin A metabolism was investigated in chickens with a normal or marginal intake of vitamin A. The rationale of the study was to test the hypothesis that Newcastle disease virus (NDV) infection specifically lowers vitamin A status, as observed previously. At the age of 4 wk, chickens were infected with either IBV or RV which affect primarily the respiratory and intestinal tracts, respectively. Both viruses lowered plasma retinol levels significantly. The effect was more pronounced in chickens fed a diet marginally deficient in vitamin A than in those fed a diet adequate in vitamin A. Levels of retinol-binding protein, transthyretin and albumin in RV-infected chickens were also significantly lower than in noninfected chickens fed the same diets, while in chickens infected with IBV, there was no effect. These results suggest that the reduced vitamin A status in IBV-infected chickens could be attributed to increased rate of utilization by tissues. In RV infection, this mechanism could play a role but impaired absorption of nutrients including vitamin A and direct loss of nutrients via the intestinal tract could also be important. As NDV affects vitamin A metabolism partly via other mechanisms, it was concluded that NDV acts to some extent specifically in lowering plasma retinol levels.

INTRODUCTION

Vitamin A metabolism is affected in many disease states (1). Recently, we demonstrated in chickens (2) an interaction between vitamin A status and infection with the La Sota strain of Newcastle disease virus (NDV). NDV infection produced higher morbidity in chickens fed a diet marginally deficient in vitamin A than in chickens fed a diet adequate in vitamin A. In addition, plasma retinol levels in infected birds fed a marginally vitamin A-deficient diet were significantly lower than those in noninfected chickens fed the same diet. The reduction in plasma vitamin A levels was probably due to increased utilization of vitamin A by tissues combined with a direct effect

of NDV on retinol-binding protein (RBP) metabolism in the liver rather than impaired absorption from the gut (3). The aim of the present study is to present data which provide an insight into the specificity of the effect of NDV on vitamin A metabolism. As NDV belongs to the family Paramyxoviridae (4) and primarily affects epithelial tissue (5,6), two other viruses which also invade and damage epithelial tissue were used and studied in a similar model. Infectious bronchitis virus (IBV, M41 strain) belongs to the family Coronaviridae and predominantly affects the respiratory tract (7,8) and reovirus (RV, I637 strain) belongs to the family Reoviridae and predominantly affects the intestinal tract (9,10).

MATERIALS AND METHODS

Animals, diets and experimental design

Female day-old White Leghorn chickens (strain Lohmann Selected Leghorn) with limited vitamin A reserves were obtained as progeny of laying hens fed a marginally vitamin A-deficient diet (300 retinol equivalents (RE)/kg diet) for a period of 3 mo. The chickens were fed purified diets ad libitum containing either adequate (1200 RE/kg feed) or marginal amounts of vitamin A (120 RE/kg feed). Diets were manufactured according to the recommendations of the National Research Council (11) by the Institute of Animal Nutrition and Physiology (IGMB-TNO, Wageningen, the Netherlands) as described in detail previously (2). At 4 wk of age on Day 0, one third of the chickens in each group were infected with either IBV or RV, while the remainder were mock-infected. In this way, 6 experimental groups were formed: noninfected, IBV-infected or RV-infected groups fed a diet marginally deficient in vitamin A [A-I- (n=21), A-I+IBV (n=23) and A-I+RV (n=21), respectively], and noninfected, IBV-infected or RV-infected groups fed a diet with adequate vitamin A [A+I- (n=20), A+I+IBV (n=23) and A+I+RV (n=21), respectively]. Birds were housed from hatching until the age of 23 d in air-filtered rooms and after this period in air-filtered isolators, maintained at a negative pressure (Poultry Health Institute, Doorn, the Netherlands), controlled for temperature (decreasing from 32^{o}C during the first day to 20^{o}C after 2 wk), relative humidity (40-50%) and light-dark cycles (24 h dimmed light/d during the first 2 d, and 10 h of dimmed (air-filtered room) or red (isolators) light and 14 h of darkness/d from the third day). Each group of the chickens was housed in a separate isolator and all chickens had free access to water and feed: there

were sufficient feed troughs to allow all to eat at the same time without competition.

The birds were observed regularly for clinical signs of vitamin A deficiency, IBV or RV infection. Gross postmortem examination with special attention to the trachea in IBV-infected chickens and to the digestive tract in RV-infected ones was carried out at Day 6 and Day 14. Plasma retinol, RBP, transthyretin (formerly refered to as prealbumin) and albumin were measured in 8 birds/group immediately prior to and during infection (Day 0, Day 6 and Day 14). The degree of saturation of plasma RBP with retinol was calculated from the molar ratio, assuming all retinol in plasma to be bound to RBP. Vitamin A and RBP content in liver were determined in 5 birds/group just prior to and after inoculation (Day 0 and Day 14).

Experimental infection

Birds were inoculated intraocularly and intranasally (0.1 ml) with either a $10^{4.7}$ median embryo-infectious dose (EID_{50}) of IBV virus (M41 strain, 8th chicken embryo passage) or inoculated orally and intraocularly with 10^{5} EID_{50} of RV virus (I637 strain, 3rd chicken embryo passage, isolated at the Poultry Health Institute, Doorn, the Netherlands). Control birds were inoculated with a suspension of sterile allantoic fluid and diluted with phosphate-buffered saline (PBS) to the same degree as the virus suspension. In order to confirm the exposure to the viral infections, hemagglutination-inhibition (HI) antibody titers to IBV and antibody titers to RV were measured on Day 0 and Day 14. HI antibody titers to IBV were determined according to the method of Alexander and Chettle (12) using pretreatment of virus with phospholipase-C. Antibody titers to RV were determined following an enzyme-linked immunosorbent method using an assay kit (IDEXX, Mainz, West Germany) (13). Alkaline phosphatase (ALP) activity in plasma was determined following RV infection by the colorimetric method of Bessey, Lowry and Brock (14) using an assay kit (Sigma Chemical Co., St Louis, MO).

Retinol and protein analysis in plasma and liver

Sampling of blood, preparation of liver homogenates, and determination of the level of retinol, RBP, transthyretin and albumin in plasma and/or liver, have been described in detail previously (2,3). In brief, retinol content of plasma and of liver after saponification of retinyl esters was determined by methods using reversed-phase high performance liquid chromatography (HPLC) modified from that of Driskell et al. (15) and Brubacher, Müller-Mulot and

Southgate (16), respectively. Plasma RBP and transthyretin levels were determined by rocket immunoelectrophoresis essentially as described by Laurell (17), as was the determination of RBP in liver. Both chicken proteins were purified according to the method of Abe, Muto and Hosoya (18). Plasma albumin levels were determined by use of the bromcresol green reaction method as described by Gustafsson (19).

Statistical analysis

The influence of vitamin A nutriture, infection and the interaction between these two factors on concentrations of proteins and retinol in plasma and liver were evaluated after testing for normality by two-way analysis of variance (ANOVA) and/or analysis of variance of changes between preinfection and postinfection values. Differences between group means were evaluated with Tukey-honestly significant difference statistics. All procedures were based on the principles outlined by Snedecor and Cochran (20) and were performed using a VAX-8600 computer system with a SPSS-X software package (SPSS, Chicago, IL) (21).

RESULTS

Clinical observation

From Day 4, chickens infected with IBV had more or less watering eyes, swollen conjunctivae and respiratory problems, such as sneezing, gasping, tracheal rales and wet nostrils. In addition, loss of appetite resulted in significantly lower body weight gain in IBV-infected chickens from Day 0 to Day 14 than in noninfected counterparts fed the same diets (Table 1). Although two vitamin A-deficient chickens died during the acute phase of disease, overt differences in clinical signs of disease due to vitamin A status could not be demonstrated.

Chickens infected with RV excreted from Day 6 more or less watery, sometimes mucoid faeces with the same color as normal faeces. In addition, some of the RV-infected chickens fed the marginally vitamin A-deficient diet showed poor feathering. None of the birds died during the infection but body weight gain was significantly lower from Day 0 to Day 14 than that of noninfected counterparts fed the same diets (Table 1).

TABLE 1. Effect of vitamin A status and/or infection with either infectious bronchitis virus (IBV) or reovirus (RV) on body weight (gain) in chickens.

Group[1]	Body weight[2,4]		Body weight gain[3,4]
	Day 0	Day 14	Day 0-14
		g	
A-I-	248 ± 8 ^{a}	425 ± 11 ^{ab}	177 ± 5 ^{ab}
A+I-	259 ± 8 ^{a}	450 ± 13 ^{b}	191 ± 7 ^{a}
A-I+IBV	247 ± 7 ^{a}	391 ± 14 ^{a}	144 ± 8 ^{cd}
A+I+IBV	254 ± 8 ^{a}	410 ± 15 ^{ab}	156 ± 8 ^{bc}
A-I+RV	255 ± 7 ^{a}	373 ± 15 ^{a}	118 ± 10 ^{d}
A+I+RV	263 ± 9 ^{a}	390 ± 15 ^{a}	127 ± 8 ^{d}

1 Chickens were fed from hatching purified diets containing marginal or adequate amounts of vitamin A and were inoculated with IBV or RV at 4 wk of age (Day 0).
2 Values are means ± SEM for 8 chickens/group.
3 Values are means of differences between two time points ± SE of differences for 8 chickens/group.
4 Means within a column not sharing a common superscript letter are significantly different at $P < 0.05$ (Tukey).

Overt clinical signs of vitamin A deficiency such as granular eyelids or white cheesy deposits under the eyelids (22) were not observed. However, body weights were lower, although not significantly, in vitamin A-deficient chickens than in counterparts fed the control diet either or not infected (Table 1).

Postmortem examination

Macroscopical tracheal lesions varying from slight reddening to highly red tracheal mucosa and the presence of much mucus in the lumen were observed in 8 out of 8 vitamin A-deficient birds and in 5 out of 8 birds fed the control diet at Day 6 after IBV inoculation. Hyperaemia of the tracheal mucosa was more severe in vitamin A-deficient birds. At Day 14 after IBV inoculation, tracheal lesions were found in 6 out of 8 chickens fed a marginally vitamin A-deficient diet and in 3 out of 10 birds fed the control diet.

Postmortem examination of RV-infected chickens did not reveal obvious pathological changes in the digestive tract.

Serological confirmation of infection

Maternal antibodies to IBV or RV could not be detected just prior to virus inoculation (Table 2). At Day 14 after inoculation, HI antibody titers to IBV and antibody titers to RV were significantly increased without differences due

TABLE 2. Serological confirmation of infection with infectious bronchitis virus (IBV) or reovirus (RV) in chickens.

Group[1]	IBV		RV			
	HI antibody titer[2]		Antibody titer[2]		Alkaline phosphatase[2]	
	Day 0	Day 14	Day 0	Day 14	Day 6	Day 14
	$\log_2$		$\log_{10}$		U/l	
A-I-	< 3	< 3	0	0	191 ± 21 [a]	190 ± 20 [b]
A+I-	< 3	< 3	0	0	202 ± 22 [a]	211 ± 21 [b]
A-I+IBV	< 3	8.3 ± 1.0	ND [3]	ND	ND	ND
A+I+IBV	< 3	8.7 ± 0.9	ND	ND	ND	ND
A-I+RV	ND	ND	0	3	575 ± 87 [b]	356 ± 35 [b]
A+I+RV	ND	ND	0	3	655 ± 80 [b]	336 ± 36 [b]

1 Chickens were fed from hatching purified diets containing marginal or adequate amounts of vitamin A and were inoculated with IBV or RV at 4 wk of age (Day 0).

2 Values are means ± SEM for 8 chickens/group.

3 Means within a column not sharing a common superscript letter are significantly different at $P < 0.05$ (Tukey).
ND = Not determined.

to vitamin A status. Antibodies were present in all birds infected and in none of those not infected. In addition, ALP activity in plasma was significantly higher in RV-infected chickens than in noninfected counterparts fed the same diet (Table 2). Increased ALP activity was more pronounced at Day 6 than at Day 14 after RV inoculation. However, 2 out of 8 vitamin A-deficient and 1 out of 8 birds fed the control diet did not show increased ALP activity although they did have antibodies to RV.

Plasma retinol concentration

Chickens fed a diet marginally deficient in vitamin A had significantly lower levels of plasma retinol than counterparts fed a diet adequate in vitamin A prior to virus inoculation (Table 3). During the experimental period, plasma retinol values decreased in noninfected vitamin A-deficient chickens and increased in noninfected counterparts fed the control diet. Chickens infected with IBV or RV had significantly lower plasma retinol levels

than their noninfected counterparts fed the same diets at Day 6 after IBV inoculation and at Day 14 after RV inoculation. In vitamin A-deficient chickens infected with RV, plasma retinol levels even decreased from marginally deficient to levels which are regarded as deficient (< 0.35 μmol/l) (23). Although absolute changes in comparison with preinfection values were almost the same irrespective of vitamin A nutriture, relative changes were much greater in vitamin A-deficient chickens.

TABLE 3. Effect of infection with infectious bronchitis virus (IBV) or reovirus (RV) on plasma retinol concentration in chickens.

Group[1]	Retinol concentration[2,4]			Change in retinol concentration[3,4]	
	Day 0	Day 6	Day 14	Day 0–6	Day 0–14
			μmol/l		
A–I–	0.50 ± 0.02[a]	0.48 ± 0.02	0.47 ± 0.03	−0.02 ± 0.02[ab]	−0.03 ± 0.01[ab]
A+I–	1.49 ± 0.06[b]	1.52 ± 0.05	1.55 ± 0.05	0.03 ± 0.01[b]	0.06 ± 0.02[a]
A–I+IBV	0.52 ± 0.03[a]	0.42 ± 0.03	0.47 ± 0.03	−0.10 ± 0.02[c]	−0.05 ± 0.01[ab]
A+I+IBV	1.51 ± 0.05[b]	1.45 ± 0.05	1.52 ± 0.05	−0.06 ± 0.03[ac]	0.01 ± 0.01[a]
A–I+RV	0.52 ± 0.02[a]	0.46 ± 0.02	0.32 ± 0.03	−0.06 ± 0.02[ac]	−0.20 ± 0.03[c]
A+I+RV	1.52 ± 0.05[b]	1.46 ± 0.05	1.39 ± 0.07	−0.06 ± 0.01[ac]	−0.13 ± 0.06[bc]

1 Chickens were fed from hatching purified diets containing marginal or adequate amounts of vitamin A and were inoculated with IBV or RV at 4 wk of age (Day 0).
2 Values are means ± SEM for 8 chickens/group.
3 Values are means of differences between two time points ± SE of differences for 8 chickens/group.
4 Means within a column not sharing a common superscript letter are significantly different at $P < 0.05$ (Tukey).

Liver vitamin A content

In chickens fed a marginally vitamin A-deficient diet, liver vitamin A content was significantly lower than in counterparts fed a diet adequate in vitamin A (Table 4). Both infection with IBV and RV resulted in lower vitamin A levels in liver at Day 14 after inoculation in comparison with noninfected counterparts fed the same diets, although this effect was not statistically significant in vitamin A-deficient chickens infected with IBV. In vitamin A-deficient chickens infected with RV, vitamin A content in liver was even reduced to nondetectable levels.

TABLE 4. Effect of infection with infectious bronchitis virus (IBV) or reovirus (RV) on liver vitamin A and liver retinol-binding protein (RBP) concentrations in chickens.

Group[1]	Liver vitamin A[2]		Liver RBP[2]	
	Day 0	Day 14	Day 0	Day 14
	nmol/g		nmol/g	
A-I-	15 ± 3 [a]	14 ± 2 [a]	3.62 ± 0.28 [a]	3.80 ± 0.32 [a]
A+I-	117 ± 14 [b]	156 ± 11 [b]	1.28 ± 0.15 [b]	1.42 ± 0.12 [b]
A-I+IBV	18 ± 3 [a]	6 ± 1 [a]	3.32 ± 0.30 [a]	3.62 ± 0.33 [a]
A+I+IBV	112 ± 7 [b]	96 ± 5 [c]	1.48 ± 0.13 [b]	1.38 ± 0.12 [b]
A-I+RV	14 ± 2 [a]	0[3] [d]	3.42 ± 0.28 [a]	3.84 ± 0.27 [a]
A+I+RV	130 ± 11 [b]	82 ± 9 [c]	1.46 ± 0.11 [b]	1.28 ± 0.15 [b]

1 Chickens were fed from hatching purified diets containing marginal or adequate amounts of vitamin A and were inoculated with IBV or RV at 4 wk of age (Day 0). Total liver vitamin A concentration expressed as nmol retinol/g liver was determined after saponification of retinyl esters to retinol.

2 Values are means ± SEM for 5 chickens/group. Means within a column not sharing a common superscript letter are significantly different at $P < 0.05$ (Tukey).

3 Not detectable.

TABLE 5. Effect of infection with infectious bronchitis virus (IBV) or reovirus (RV) on plasma retinol-binding protein (RBP) concentration in chickens.

Group[1]	RBP concentration[2,4]			Change in RBP concentration[3,4]	
	Day 0	Day 6	Day 14	Day 0-6	Day 0-14
			μmol/l		
A-I-	1.45 ± 0.10[a]	1.44 ± 0.09	1.40 ± 0.09	-0.01 ± 0.01[ab]	-0.05 ± 0.01[ab]
A+I-	2.01 ± 0.12[b]	2.08 ± 0.13	2.00 ± 0.12	0.07 ± 0.03[a]	-0.01 ± 0.01[a]
A-I+IBV	1.48 ± 0.10[a]	1.48 ± 0.11	1.41 ± 0.10	0.00 ± 0.04[ab]	-0.07 ± 0.04[ab]
A+I+IBV	2.08 ± 0.15[b]	2.06 ± 0.15	2.08 ± 0.15	-0.02 ± 0.04[ab]	0.00 ± 0.05[a]
A-I+RV	1.47 ± 0.10[a]	1.36 ± 0.12	1.13 ± 0.10	-0.11 ± 0.05[ab]	-0.34 ± 0.07[c]
A+I+RV	1.98 ± 0.12[b]	1.83 ± 0.11	1.73 ± 0.10	-0.15 ± 0.08[b]	-0.25 ± 0.09[bc]

1 Chickens were fed from hatching purified diets containing marginal or adequate amounts of vitamin A and were inoculated with IBV or RV at 4 wk of age (Day 0).

2 Values are means ± SEM for 8 chickens/group.

3 Values are means of differences between two time points ± SE of differences for 8 chickens/group.

4 Means within a column not sharing a common superscript letter are significantly different at $P < 0.05$ (Tukey).

Plasma retinol-binding protein concentration

Plasma RBP concentrations were significantly lower in vitamin A-deficient chickens than in counterparts fed the control diet (Table 5). Chickens infected with RV had significantly lower plasma RBP levels than noninfected counterparts fed the same diets at Day 14 after RV inoculation. At that time point the decrease in plasma RBP levels tended to be more pronounced in vitamin A-deficient chickens than in chickens fed a diet with adequate vitamin A. Plasma RBP levels were not affected by infection with IBV.

Vitamin A-deficient chickens had a significantly lower degree of saturation of plasma RBP with retinol (29.1 to 35.7%) than counterparts fed the control diet (72.6 to 82.2%) (Table 6). In addition, the degree of saturation tended to be lower in vitamin A-deficient chickens infected with IBV and RV than in noninfected chickens fed the same diet at Day 6 and Day 14, respectively. This indicated that more of the circulating RBP in these birds was present as apo-RBP than in their noninfected counterparts fed the same marginally vitamin A-deficient diet.

TABLE 6. Effect of infection with infectious bronchitis virus (IBV) or reovirus (RV) on plasma retinol-binding protein (RBP) saturation with retinol in chickens.

Group	RBP saturation[1,2,4]			Change in RBP saturation[1,3,4]	
	Day 0	Day 6	Day 14	Day 0-6	Day 0-14
			(%)		
A-I-	35.1 ± 2.0[a]	34.1 ± 2.1	34.1 ± 2.1	-0.9 ± 0.9[ab]	-1.0 ± 1.3[a]
A+I-	75.2 ± 1.9[b]	73.9 ± 2.8	78.4 ± 3.0	-1.3 ± 1.3[ab]	3.2 ± 0.9[a]
A-I+IBV	35.5 ± 1.0[a]	29.1 ± 1.1	33.7 ± 1.2	-6.4 ± 1.0[a]	-1.8 ± 1.2[a]
A+I+IBV	74.4 ± 4.1[b]	72.6 ± 4.4	75.1 ± 3.9	-1.8 ± 1.9[ab]	0.7 ± 1.0[a]
A-I+RV	35.7 ± 1.4[a]	34.9 ± 2.1	29.6 ± 3.2	-0.8 ± 1.5[ab]	-6.1 ± 3.1[a]
A+I+RV	77.8 ± 2.3[b]	81.3 ± 4.6	82.2 ± 5.9	3.5 ± 4.4[b]	4.4 ± 5.6[a]

1 For calculating the proportion of retinol-binding protein saturated with retinol, all of the retinol in plasma was assumed to be bound to RBP.
2 Values are means ± SEM for 8 chickens/group.
3 Values are means of differences between two time points ± SE of differences for 8 chickens/group.
4 Means within a column not sharing a common superscript letter are significantly different at $P < 0.05$ (Tukey).

Liver RBP concentration

In vitamin A-deficient chickens, the level of RBP in liver was significantly higher than in their counterparts fed the control diet (Table

4). Neither IBV nor RV infection affected the level of RBP in liver at Day 14 after inoculation.

TABLE 7. Effect of infection with infectious bronchitis virus (IBV) or reovirus (RV) on plasma transthyretin concentration in chickens.

Group[1]	Transthyretin concentration[2,4]			Change in transthyretin concentration[3,4]	
	Day 0	Day 6	Day 14	Day 0–6	Day 0–14
			μmol/l		
A–I–	5.60 ± 0.16[a]	5.64 ± 0.16	5.75 ± 0.15	0.04 ± 0.05[a]	0.15 ± 0.03[a]
A+I–	5.74 ± 0.14[a]	5.83 ± 0.18	5.84 ± 0.20	0.09 ± 0.09[a]	0.10 ± 0.10[a]
A–I+IBV	5.34 ± 0.16[a]	5.26 ± 0.19	5.39 ± 0.15	−0.08 ± 0.10[a]	0.05 ± 0.15[a]
A+I+IBV	5.60 ± 0.18[a]	5.54 ± 0.20	5.53 ± 0.16	−0.06 ± 0.09[a]	−0.07 ± 0.07[a]
A–I+RV	5.49 ± 0.17[a]	5.36 ± 0.17	4.75 ± 0.25	−0.13 ± 0.06[a]	−0.74 ± 0.10[b]
A+I+RV	5.86 ± 0.14[a]	5.71 ± 0.18	5.15 ± 0.26	−0.15 ± 0.06[a]	−0.71 ± 0.25[b]

1 Chickens were fed from hatching purified diets containing marginal or adequate amounts of vitamin A and were inoculated with IBV or RV at 4 wk of age (Day 0).
2 Values are means ± SEM for 8 chickens/group.
3 Values are means of differences between two time points ± SE of differences for 8 chickens/group.
4 Means within a column not sharing a common superscript letter are significantly different at $P < 0.05$ (Tukey).

TABLE 8. Effect of infection with infectious bronchitis virus (IBV) or reovirus (RV) on plasma albumin concentration in chickens.

Group[1]	Albumin concentration[2,4]			Change in albumin concentration[3,4]	
	Day 0	Day 6	Day 14	Day 0–6	Day 0–14
			μmol/l		
A–I–	541 ± 12[a]	537 ± 12	528 ± 13	− 4 ± 1[a]	−13 ± 3[a]
A+I–	548 ± 13[a]	545 ± 12	550 ± 12	− 3 ± 2[a]	2 ± 1[a]
A–I+IBV	542 ± 10[a]	530 ± 11	529 ± 15	−12 ± 5[a]	−13 ± 4[a]
A+I+IBV	553 ± 14[a]	545 ± 16	544 ± 15	− 8 ± 4[a]	− 9 ± 6[a]
A–I+RV	542 ± 12[a]	529 ± 12	492 ± 18	−13 ± 2[a]	−50 ± 9[b]
A+I+RV	546 ± 14[a]	535 ± 14	497 ± 22	−11 ± 3[a]	−49 ± 9[b]

1 Chickens were fed from hatching purified diets containing marginal or adequate amounts of vitamin A and were inoculated with IBV or RV at 4 wk of age (Day 0).
2 Values are means ± SEM for 8 chickens/group.
3 Values are means of differences between two time points ± SE of differences for 8 chickens/group.
4 Means within a column not sharing a common superscript letter are significantly different at $P < 0.05$ (Tukey).

Plasma transthyretin concentration

Vitamin A nutriture did not affect plasma transthyretin levels prior to infection (Table 7). However, chickens infected with RV had significantly lower levels of plasma transthyretin at Day 14 after virus inoculation than noninfected counterparts fed the same diets.

Plasma albumin concentration

Albumin levels in plasma parallelled that of transthyretin (Table 8). Chickens infected with RV had significantly lower levels of plasma albumin than noninfected counterparts fed the same diets at Day 14 after RV inoculation, while inadequate vitamin A intake did not affect plasma albumin concentrations.

DISCUSSION

In the present study, the influence of infection with either IBV or RV on vitamin A metabolism was investigated in chickens varying in vitamin A status. The rationale for this study was to compare the results with those obtained earlier (2,3) from experiments using a similar model but with NDV infection in an attempt to gain a better insight into the specificity of NDV in reducing vitamin A status.

IBV infection resulted in acute respiratory disease of chickens with similar clinical signs as described previously (24). However, the infection was more serious in vitamin A-deficient chickens. These chickens showed a higher frequency and severity of tracheal lesions at postmortem examination. Although gross postmortem examination of the intestinal tract of RV-infected chickens did not reveal pathological characteristics, RV infection resulted in higher plasma ALP activity and decreased body weight gain. Similar observations were described earlier (25,26) and it was demonstrated (25) that increased ALP activity in plasma could be attributed mainly to one of the ALP isoenzymes which was most likely of intestinal origin. Increased plasma ALP activity together with the diarrhea observed, demonstrated that RV affected the intestinal tract of the chickens studied. In contrast to the situation with IBV infection, vitamin A-deficient chickens infected with RV did not appear to have more severe signs of disease than their counterparts fed adequate amounts of vitamin A. In experiments with other strains of RV associated with the so called 'malabsorption syndrome', it was demonstrated

that normal levels of vitamin A in the diet can inhibit the uptake of other nutrients, while low levels of vitamin A in the diet did not result in deficiency of other nutrients and severe signs of malnutrition (27).

In our noninfected vitamin A-deficient chickens, low levels of retinol in plasma and liver were paralleled by low levels of RBP in plasma and high levels of RBP in liver, similar to the results observed previously (3). It has been demonstrated in experiments with rats that RBP release from the liver is controlled in part by the availability of retinol, while RBP synthesis is unaffected by this factor (28,29).

In both IBV- and RV-infected chickens, levels of retinol in plasma were decreased in a similar way to that found earlier following NDV inoculation (2,3). In vitamin A-deficient chickens infected with RV, retinol levels even decreased from marginally deficient to levels which are regarded as deficient (23). However, the mechanism by which these viruses can lower vitamin A status appeared to be, at least partially, different.

In IBV-infected chickens, levels of RBP, transthyretin and albumin in plasma and levels of RBP in liver were not affected, while body weights were only slightly lower in comparison with noninfected counterparts fed the same diets. This indicated that these birds had a normal absorption of nutrients in the intestinal tract, did not suffer from protein-energy malnutrition and that there was normal synthesis in and release from the liver of RBP, transthyretin and albumin in comparison with noninfected birds fed the same diets. Therefore, the results indicate that lowering of plasma retinol levels following IBV infection is mainly the result of an increased rate of utilization of retinol relative to the rate of hepatic mobilization, as this vitamin is required for regeneration of damaged tissues (1,30). Further support comes from the lower levels of vitamin A in liver following IBV infection and the tendency for a lower proportion of holo-RBP in plasma in vitamin A-deficient chickens infected with IBV during the acute phase of disease compared with the situation in noninfected counterparts fed the same diet.

In RV-infected chickens, body weight gain, levels of retinol, RBP, transthyretin and albumin in plasma, and vitamin A content in liver were significantly lower during the acute phase of disease than that in noninfected counterparts fed the same diets. This indicates that a number of factors played a role. These factors include an impaired absorption of nutrients including vitamin A, a markedly accelerated catabolism or utilization in tissues of the plasma components measured, and/or a direct loss of plasma

proteins, vitamin A and other (micro)nutrients via the intestinal tract, all of which lead to mild undernutrition. Impaired synthesis of the retinol-carrier proteins in liver resulting from a shortage of amino acids, which is a normal feature in protein deficiency (31), would not appear to be likely as the content of RBP in liver was not affected by RV infection. In addition, it has been demonstrated in monkeys that infection did not affect the rate of synthesis of albumin in liver (32).

Plasma retinol levels were decreased relatively more with respect to preinfection levels in chickens fed a diet marginally deficient in vitamin A than in counterparts fed a diet adequate in vitamin A in both IBV, RV and, as described previously (2,3), NDV infection. This demonstrates that a primary infection with epithelia-damaging viruses affects vitamin A status much more when this is already in a marginal instead of a normal condition. As vitamin A plays an important role in host resistance against invading pathogens (33,34) this might have some serious implications.

Infection with the three viruses (NDV, IBV and RV) reduces plasma retinol levels and also produces signs of disease. However, it is difficult to attribute such signs of decrease to the effects of the virus on the plasma retinol levels per se or to the pathogenic effect of the virus. It may well be that the relative contribution of the two effects is different for the three viruses.

In conclusion, although infection with IBV and RV, and as was demonstrated earlier with NDV (2,3), can affect vitamin A metabolism, the mechanism appears to be, at least partially, different. The results from the previous (3) and present study suggest that in RV infection levels of retinol, RBP, transthyretin and albumin in plasma, and levels of vitamin A in liver are lowered by impaired absorption, increased catabolism of proteins or utilization of vitamin A, and/or directly induced (micro)nutrient-losing enteropathy, that in IBV infection levels of retinol in plasma and in liver are lowered by increased rate of utilization in tissues, and that in NDV infection levels of retinol and RBP in plasma are lowered to a very marked extent without concomitant reduction of vitamin A stores in liver, by increased rate of utilization and catabolism in extrahepatic tissues, possibly aggravated by a direct effect of virus on RBP metabolism in liver. Therefore, from the result obtained it can be concluded that, to some extent, NDV acts specifically in lowering plasma retinol levels.

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CHAPTER 6

Changes in lymphoid organs and blood lymphocytes induced by vitamin A deficiency and Newcastle disease virus infection

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ABSTRACT

The effect of vitamin A deficiency in the presence or absence of Newcastle disease virus infection (NDV, La Sota strain) on weight of lymphoid organs and on the number and type of circulating white blood cells (WBC) was investigated in chickens. Day-old chickens with limited vitamin A reserves were fed purified diets containing either marginal (ad libitum) or adequate (pair-fed) levels of vitamin A and at 21-28 d of age, half of the chickens in each group were infected with NDV. Absolute and relative weights of bursa of Fabricius were significantly lower in noninfected vitamin A-deficient birds than in counterparts fed adequate vitamin A while weight of thymus, spleen and liver were not different between these groups. Absolute and relative weights of bursa and thymus in vitamin A-deficient chickens infected with NDV were significantly lower than in infected counterparts fed adequate vitamin A. Relative weight of spleen was significantly higher in infected chickens than in noninfected counterparts, irrespective to vitamin A status. Both vitamin A deficiency and NDV infection resulted in leukopenia, while the lowest number of WBC were observed in vitamin A-deficient chickens during the acute phase of NDV. Subsequent to leukopenia due to NDV infection, a marked leukocytosis was observed in control chickens and to a lesser extent in vitamin A-deficient birds. Changes in the number of WBC could be attributed to changes in the number of peripheral blood lymphocytes. Vitamin A deficiency significantly lowered the number of B cells and to a lesser extent the number of T cells. Moreover, NDV tended to affect the number of T cells. The results from the present study indicate that vitamin A deficiency affects lymphoid cell systems and that this is aggravated by concomitant NDV infection.

INTRODUCTION

Vitamin A deficiency is associated with increased susceptibility, severity and duration of infection (1,2). These changes can be attributed to a detrimental effect of vitamin A deficiency on the immune system, sometimes aggravated by immunosuppressive effects of the infectious agent (3,4). Bang et al. (5,6) reported that vitamin A deficiency affected lymphoid cell systems in

chickens and, in combination with Newcastle disease virus (NDV) infection, led to a rapid destruction of these tissues. This combined effect was synergistic in nature and was much more severe than both factors individually. Recently, we have developed a model, in which chickens differing in vitamin A status are infected with a less virulent strain of NDV (7). It has been demonstrated with this model, in which protein-energy malnutrition (PEM) and secondary infection are absent, that chickens fed a diet marginally deficient in vitamin A are indeed more susceptible to the mildly pathogenic La Sota strain of NDV and that these birds show more severe signs of disease than counterparts fed a diet adequate in vitamin A (7). Although Bang et al. (6) reported detrimental effects on the thymus and bursa of Fabricius, they did not investigate the effect on the number and type of circulating white blood cells (WBC). Therefore, the aim of the present studies was twofold. Firstly, the studies were designed to investigate the effect of vitamin A deficiency either alone or in combination with La Sota NDV infection on weight of lymphoid organs in order to confirm the results of Bang et al. (6) in our model. Secondly, the effect on the number of circulating WBC, differentiated in cell types from both the lymphoid and myeloid lineage was investigated.

MATERIALS AND METHODS

Animals, diets and experimental design

Data were collected from two separate experiments using a similar animal model as described earlier (7). Briefly, female day-old White Leghorn chickens (strain Lohmann Selected Leghorn) with limited vitamin A reserves, progeny of marginally vitamin A-deficient laying hens (Wageningen Agricultural University, the Netherlands) were fed purified diets, manufactured according to the recommendations of the National Research Council (8), containing either marginal (120 retinol equivalents (RE)/kg feed, ad libitum-fed) or adequate amounts of vitamin A (1200 RE/kg feed, pair-fed). At the age of 28 d (experiment 1) or 21 d (experiment 2), half of the chickens in both groups were infected intraocularly with a 10^8 median embryo-infectious dose of the lentogenic La Sota strain of NDV (Delvax, Gist-Brocades, Delft, the Netherlands) while the remainder were mock-infected with phosphate-buffered saline. In this way four experimental groups were formed: noninfected and infected groups fed a diet marginally deficient in vitamin A (A-I- and A-I+, respectively), and noninfected and infected pair-fed control groups fed a diet

adequate in vitamin A (A+I- and A+I+, respectively). In the first experiment, body weight and weights of lymphoid organs and liver of 10 birds/group were measured 9 d after inoculation, just following the acute phase of disease. Measurement of liver weight served as a control. In addition, differential blood cell counts and determination of total protein content in plasma were carried out 5 and 11 d after inoculation in 12 birds/group. In the second experiment, the number of circulating lymphocytes, differentiated in B and T cells were measured in 5 birds/group 9 d after inoculation.

Hemagglutination-inhibition (HI) antibodies to NDV

In order to confirm the exposure to the viral infection, HI antibody titers to NDV were measured immediately prior to and 14 d after inoculation according to the method of De Jong (9) using the ß-procedure (constant-virus diluted-serum).

Vitamin A status

Retinol levels were determined in plasma collected just prior to and 21 d after virus inoculation. A reversed-phase high performance liquid chromatography (HPLC) method modified from that of Driskell et al. (10) and described in detail previously (7) was used with retinyl acetate as internal standard. The results obtained were checked against pooled samples which served as a control.

Isolation of organs

Chickens were weighed and killed by CO_2 asphyxiation. Liver, bursa of Fabricius, thymus and spleen were excised, cleaned, blotted dry and weighed.

Differential blood cell counts

Heparinized blood taken from the wing vein was stained using the method described by Natt and Herrick (11) for counting the total number of erythrocytes and WBC in a Bürker counting chamber, while differential WBC counts were carried out after blood smears were treated with Giemsa stain.

Isolation of peripheral blood lymphocytes (PBL)

PBL were obtained by diluting (1:1 v/v) blood with RPMI-1640 buffered with $NaHCO_3$ (pH 7.4), supplemented with bovine serum albumin (1% wt/v, Sigma Chemical Co., St Louis, MO), penicillin (100 IU/ml; Serva, Heidelberg, West Germany), streptomycin (100 µg/ml; Serva) and L-glutamine (2 mM; Merck,

Darmstadt, West Germany) followed by centrifugation (20 min, 400 x g, 20°C) over a ficoll-metrizoate gradient (Lymphoprep, 1.077 g/ml, Nyegaard & Co. AS, Oslo, Norway). PBL were washed twice with RPMI-1640 (10 min, 400 x g, 4°C) and resuspended in this medium to approximately 10^6 cells/ml.

Flow cytometry

Lymphocyte populations were characterized by flow cytometry using a mouse monoclonal antibody specific reacting with chicken Ig light chain (CVI-ChIg-47.5, 1:8000, a gift of Dr. G. Koch, Central Veterinary Institute, Lelystad, the Netherlands). PBL were incubated (45 min on ice) with this monoclonal antibody. After washing twice with RPMI-1640 (10 min, 400 x g, 4°C), cells were incubated (45 min on ice) with polyclonal, FITC-conjugated rabbit antibodies to mouse Ig (1:400, heavy chain specific, Dakopatts, Glostrup, Denmark). Excess antibody was removed by washing with RPMI-1640. Viability was determined using propidium iodide as a second fluorescence marker. A Facstar flow cytometer (Becton Dickinson, Palo Alto, CA) equipped with a 5-W argon laser provided incident light at 488 nm. With log amplification and optimal gain settings, fluorescent cells [surface Ig-positive (sIg-positive) cells: B cells] and non-fluorescent cells with the same forward and side scatter (sIg-negative cells: predominantly T cells) were counted and expressed as a proportion of the number of viable PBL. This was converted to an absolute number by multiplying with the total number of PBL.

Statistical analysis

The influence of vitamin A nutriture, NDV infection and the interaction between these two factors on blood cell counts, levels of retinol and total protein in plasma, HI antibody titers, body and organ weights, and the number of circulating B and T lymphocytes, were evaluated after testing for normality by two-way analysis of variance. Differences between group means were evaluated with Tukey-honestly significant difference statistics. All procedures were based on the principles outlined by Snedecor and Cochran (12) and were performed using a VAX-8600 computer system with a SPSS-X software package (SPSS, Chicago, IL) (13).

RESULTS

General health

During the acute phase of NDV infection, clinical signs of disease such as general weakness and respiratory problems, were mainly limited to vitamin A-deficient birds. Chickens infected in the second experiment showed more and severe signs of disease than those infected in the first experiment. Overt clinical signs of vitamin A deficiency, such as granular eyelids or white cheesy deposits under the eyelids, were not observed in either experiment.

Vitamin A status

Prior to NDV inoculation, plasma retinol levels in chickens fed a diet inadequate in vitamin A were significantly lower than those in pair-fed control birds in both experiments and these vitamin A levels could be considered as marginally deficient (14) (Table 1).

TABLE 1. Effect of vitamin A intake and/or infection with Newcastle disease virus (NDV) on plasma retinol concentration in chickens.

Group	Retinol concentration Days after NDV inoculation			
	Experiment 1		Experiment 2	
	0	21	0	9
	μmol/l			
A-I-	0.46 ± 0.06^{a}	0.43 ± 0.08^{a}	0.48 ± 0.07^{a}	0.44 ± 0.08^{a}
A-I+	0.49 ± 0.07^{a}	0.27 ± 0.06^{b}	0.44 ± 0.07^{a}	0.25 ± 0.05^{b}
A+I-	1.73 ± 0.18^{b}	1.76 ± 0.29^{c}	1.68 ± 0.33^{b}	1.84 ± 0.42^{c}
A+I+	1.66 ± 0.20^{b}	1.72 ± 0.31^{c}	1.77 ± 0.30^{b}	1.82 ± 0.35^{c}

Chickens were inoculated with NDV at the age of 28 and 21 d in experiment 1 and 2, respectively. Values are means ± SD for 11-12 and 5 birds/group in experiment 1 and 2, respectively. Means within a column not sharing a common superscript letter are significantly different at $P < 0.05$ (Tukey).

After NDV inoculation, plasma retinol concentrations in infected chickens fed a diet marginally deficient in vitamin A were reduced to levels which could be considered as deficient (14), whereas the levels in the other groups were comparable with preinfection values.

Hemagglutination-inhibition (HI) antibody titers to NDV

In both experiments, maternal HI antibodies to NDV could not be detected just prior to NDV inoculation (HI titer $\leq$ 4). At 14 d after NDV inoculation, antibodies were present in all chickens infected with NDV and in none of the noninfected birds. HI antibody titers (mean $\pm$ SD) from all infected chickens were 8.6 $\pm$ 1.9 and 9.2 $\pm$ 1.7 in the first and second experiment, respectively, and there was no effect of vitamin A status.

Body and organ weights

Both vitamin A deficiency and NDV infection tended to result in lower body weights 9 d after virus inoculation (Table 2). Relative and absolute weights of the bursa of Fabricius were significantly lower in noninfected and infected chickens deficient in vitamin A than in noninfected counterparts fed the control diet. In addition, relative and absolute weights of the bursa of Fabricius and thymus were also significantly lower in vitamin A-deficient birds infected with NDV than in infected counterparts fed the control diet.

TABLE 2. Effect of vitamin A nutriture and Newcastle disease virus (NDV) infection on body, liver and lymphoid organ weights.

Group	Body weight	Organ weight				Relative organ weight			
		Liver	Bursa	Thymus	Spleen	Liver	Bursa	Thymus	Spleen
	g	g				percentage of body weight			
A-I-	332±47^{a}	9.66±1.14^{a}	1.46±0.22^{ab}	1.55±0.21^{ab}	0.81±0.21^{a}	2.91±0.32^{a}	0.44±0.04^{ab}	0.47±0.07^{a}	0.24±0.04^{a}
A-I+	305±40^{a}	8.48±0.92^{a}	1.24±0.16^{a}	1.31±0.26^{a}	0.90±0.22^{a}	2.78±0.25^{a}	0.41±0.06^{a}	0.43±0.06^{a}	0.29±0.05^{bc}
A+I-	351±44^{a}	9.90±1.08^{a}	1.84±0.23^{c}	1.75±0.47^{b}	0.89±0.23^{a}	2.82±0.31^{a}	0.53±0.05^{c}	0.49±0.08^{ab}	0.25±0.04^{ac}
A+I+	332±42^{a}	9.76±1.13^{a}	1.67±0.19^{bc}	1.85±0.27^{b}	1.06±0.22^{a}	2.94±0.34^{a}	0.51±0.07^{bc}	0.56±0.09^{b}	0.32±0.04^{b}

Body and organ weights were measured 9 d after NDV inoculation at the age of 37 d. Values are means $\pm$ SD of 10 chickens/group. Means within a column not sharing a common superscript letter are significantly different at $P < 0.05$ (Tukey).

NDV infection resulted in a significantly higher relative weight of spleen, irrespective of the vitamin A status. Weight of liver, which served as a control measurement, was affected neither by vitamin A deficiency nor NDV infection.

Number of circulating blood cells

The number of circulating WBC was significantly lower in noninfected vitamin A-deficient chickens than in noninfected counterparts fed a diet adequate in vitamin A throughout the experiment (Table 3).

TABLE 3. Effect of vitamin A nutriture and Newcastle disease virus (NDV) infection on hematological parameters.

Days after infection	Parameter	Group			
		A-I-	A-I+	A+I-	A+I+
5	Total WBC (x 10^7/ml)	2.2 ± 0.3^a	1.8 ± 0.4^b	2.5 ± 0.3^c	2.1 ± 0.3^a
	Lymphocytes (x 10^7/ml)	1.3 ± 0.3^a	1.0 ± 0.1^b	1.7 ± 0.1^c	1.3 ± 0.1^a
	Heterophils (x 10^7/ml)	0.7 ± 0.1^a	0.6 ± 0.1^a	0.6 ± 0.1^a	0.7 ± 0.1^a
	Lymphocytes (%)	61.1 ± 4.7^a	56.2 ± 5.0^b	69.7 ± 4.2^c	61.8 ± 3.5^a
	Heterophils (%)	30.1 ± 4.5^a	35.1 ± 4.2^b	22.8 ± 3.5^c	31.2 ± 3.1^{ab}
	Basophils (%)	1.3 ± 0.9^a	1.3 ± 1.0^a	1.5 ± 1.4^a	1.4 ± 1.2^a
	Eosinophils (%)	1.9 ± 0.8^a	1.8 ± 1.0^a	1.8 ± 1.1^a	1.5 ± 1.2^a
	Monocytes (%)	5.6 ± 1.7^a	5.6 ± 1.7^a	4.2 ± 1.8^a	4.1 ± 2.4^a
	Erythrocytes (x 10^9/ml)	2.4 ± 0.4^a	2.3 ± 0.5^a	2.4 ± 0.3^a	2.5 ± 0.4^a
	Total protein (g/dl)	5.0 ± 0.3^a	4.6 ± 0.3^a	5.0 ± 0.2^a	5.0 ± 0.2^a
11	Total WBC (x 10^7/ml)	2.0 ± 0.2^a	2.4 ± 0.3^b	2.5 ± 0.3^b	3.0 ± 0.4^c
	Lymphocytes (x 10^7/ml)	1.2 ± 0.1^a	1.5 ± 0.1^b	1.8 ± 0.1^c	2.1 ± 0.1^d
	Heterophils (x 10^7/ml)	0.6 ± 0.1^a	0.7 ± 0.1^a	0.5 ± 0.1^a	0.7 ± 0.2^a
	Lymphocytes (%)	61.4 ± 5.6^a	62.0 ± 5.1^a	72.1 ± 3.8^b	69.4 ± 4.1^b
	Heterophils (%)	29.9 ± 5.0^a	30.0 ± 4.2^a	19.8 ± 3.2^b	22.1 ± 3.7^b
	Basophils (%)	1.4 ± 1.0^a	1.1 ± 0.9^a	1.6 ± 1.5^a	1.2 ± 1.1^a
	Eosinophils (%)	2.1 ± 1.4^a	1.8 ± 0.9^a	1.7 ± 1.2^a	2.3 ± 1.9^a
	Monocytes (%)	5.2 ± 1.9^a	5.3 ± 2.2^a	4.8 ± 2.2^a	5.0 ± 2.5^a
	Erythrocytes (x 10^9/ml)	2.5 ± 0.5^a	2.4 ± 0.6^a	2.3 ± 0.4^a	2.5 ± 0.3^a
	Total protein (g/dl)	5.0 ± 0.4^a	4.9 ± 0.3^a	5.0 ± 0.3^a	5.1 ± 0.2^a

Values are means ± SD for 10-12 chickens/group. Means within a row not sharing a common superscript letter are significantly different at $P < 0.05$ (Tukey).

In NDV-infected birds, the number of circulating WBC was significantly lower during the acute phase of disease (5 d after NDV inoculation) than in noninfected counterparts fed the same diets, while the number was significantly higher after the acute phase of disease had passed (11 d after inoculation). The lowest number of circulating WBC was observed in vitamin A-deficient chickens infected with NDV during the acute phase of disease.

Changes in the number of WBC could be attributed completely to changes in the number of PBL and not in the number of heterophils. Therefore, a more detailed description of the number of PBL throughout the disease period is provided in Figure 1. A significant reduction in the number of PBL was found 3 and 5 d after NDV inoculation. Although this infection-induced lymphopenia was most pronounced in chickens fed the control diet, vitamin A-deficient birds showed the lowest number of PBL. After this period, the number of PBL in infected chickens increased to normal and to significantly elevated levels at 7 and 11 d postinoculation, respectively. However, this increase was less pronounced in vitamin A-deficient chickens. The number of circulating PBL in infected birds returned to levels comparible to those observed in noninfected counterparts fed the same diets 21 d postinoculation. The proportion of basophils, eosinophils and monocytes were unaffected throughout the experiment. In addition, vitamin A deficiency and NDV infection did not affect the number of circulating erythrocytes. Total protein content in plasma tended to be lower in vitamin A-deficient chickens infected with NDV than in the other birds during the acute phase of disease.

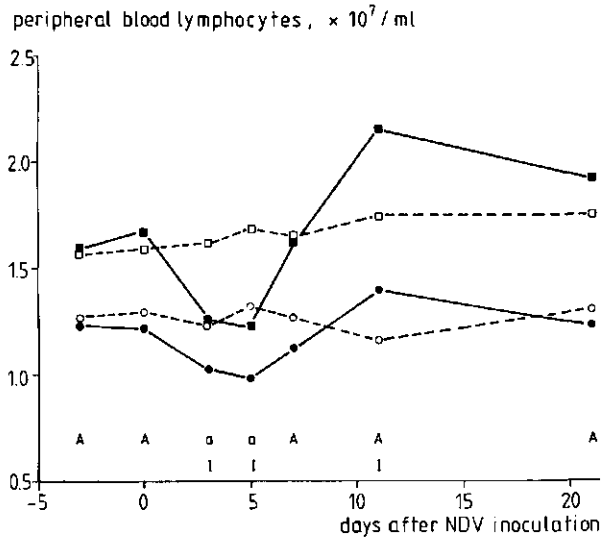

FIGURE 1. Effect of vitamin A deficiency and Newcastle disease virus (NDV) infection on the number of circulating peripheral blood lymphocytes in chickens. (○), A-I-, n=11-12, SD ranged from 11-25%; (●), A-I+, n=10-12, SD ranged from 10-26%; (□), A+I-, n=12, SD ranged from 9-24%; (■), A+I+, n=12, SD ranged from 7-35%. Significant effects of vitamin A nutriture and/or NDV infection are shown at $P < 0.01$ (A and I, respectively) and at $P < 0.05$ (a).

B and T lymphocytes

Fluorescence intensity, forward angle light scatter (correlating to cell size) (15) and side light scatter (correlating to cytoplasmic granularity) (15) profiles clearly revealed a group of sIg-positive cells (B cells) and sIg-negative cells with the same forward and side scatter, probably predominantly T cells (Fig. 2). Both populations together represented about 95% of the total number of lymphocytes. The other PBL probably comprised lymphoblasts and plasma cells. Viability as measured using propidium iodide as a second fluorescence marker exceeded 95% (Fig. 2). The absolute number of PBL for each group in this experiment (Table 4) was comparable with that observed in the same period in the experiment described above (Table 3 and Fig. 1). The number of B cells was significantly lower in vitamin A-deficient chickens than in their counterparts fed the control diet. However, NDV infection did not affect the absolute number of B cells. The number of sIg-negative cells tended to be lower in noninfected, vitamin A-deficient chickens than in their noninfected counterparts fed the control diet. In vitamin A-deficient chickens infected with NDV, the number of sIg-negative cells was even significantly lower than that in infected counterparts fed adequate vitamin A.

TABLE 4. Flow cytometric analysis of the effect of vitamin A deficiency and/or NDV infection on peripheral blood lymphocyte (PBL) subpopulations.

Group	number of PBL		
	sIg+	sIg-	Total
		10^7/ml	
A-I-	0.18 ± 0.03^{a}	1.05 ± 0.35^{a}	1.26 ± 0.39^{a}
A-I+	0.22 ± 0.06^{a}	1.34 ± 0.29^{ab}	1.68 ± 0.36^{bc}
A+I-	0.39 ± 0.07^{b}	1.46 ± 0.18^{ab}	1.94 ± 0.22^{bc}
A+I+	0.31 ± 0.06^{b}	1.92 ± 0.31^{b}	2.38 ± 0.42^{c}

Values are means ± SD for 5 chickens/group. PBL were isolated 9 d after NDV inoculation. The monoclonal antibody, CVI-ChIg-47.5, recognizes immunoglobulins (light chain specific) on cell surfaces. sIg-positive and sIg-negative cells were analyzed by flow cytometry. The total number of PBL was determined light microscopically with a counting chamber. Means within a column not sharing a common superscript letter are significantly different at $P < 0.05$ (Tukey).

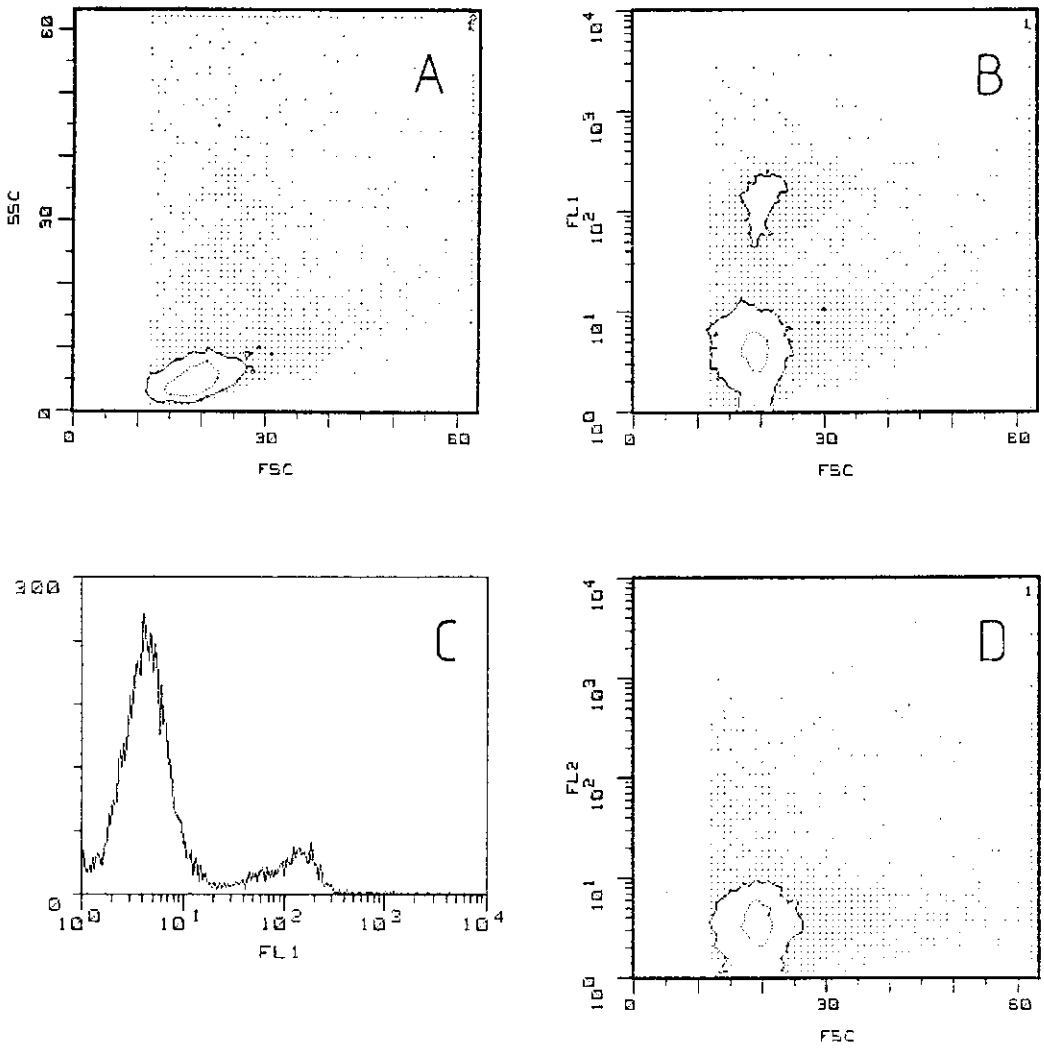

FIGURE 2. (A, B, C): Flow cytometric profiles of purified peripheral blood lymphocytes stained with mouse anti-chicken monoclonal antibody (CVI-ChIg-47.5) followed by FITC-conjugated rabbit anti-mouse Ig. (A) Forward angle light scatter (X-axis) and side light scatter (Y-axis); (B) Forward angle light scatter (X-axis) and fluorescence intensity (Y-axis); (C) Fluorescence intensity (X-axis) and cell number (Y-axis). These profiles show a distinct population of sIg-positive cells (B cells) and sIg-negative cells with similar forward and side scatter properties (predominantly T cells). (D): Viability of purified peripheral blood lymphocytes as measured by flow cytometry using propidium iodide as fluorescence marker; Forward angle light scatter (X-axis) and fluorescence intensity (Y-axis). This profile shows a very small proportion of dead cells.

DISCUSSION

In the present study, the effect of vitamin A status and NDV (La Sota strain) infection on weights of lymphoid organs and on the number and type of circulating WBC in chickens, is described. It is clear from the results reported here that moderate vitamin A deficiency in the absence or presence of infection has only a slight effect on body weight but has a marked detrimental effect on the weight of the bursa of Fabricius. In addition, weight of thymus and spleen in noninfected chickens were found to be unaffected by vitamin A status. Similar results with regard to weight of bursa and spleen in vitamin A-deficient chickens have been described by other authors (16,17). Weight of thymus appears to be affected more when vitamin A deficiency is severe enough to reduce feed intake and thus precipitates PEM; vitamin A deficiency-induced PEM has been shown to reduce weight of thymus in chickens (5,17) and rats (18), whereas weight of thymus was found to be normal in vitamin A-deficient chickens (16,17) and rats (19,20) without PEM. This is in accordance with our observation showing the lowest absolute and relative weights of thymus in vitamin A-deficient chickens infected with NDV, which also tended to grow more slowly. Infection with NDV resulted in a significant increase in relative weight of spleen, irrespective of vitamin A status. Beard and Hanson (21) attributed an increase in weight of spleen to swelling. This was also observed regularly in other parenchymatous organs after NDV infection (21). Swelling might be the result of the liberation of endogenous permeability factors with as a consequence oedema by leakage of blood (22) and because of the formation of marginal centres.

In our experiment, changes in weight of lymphoid organs were accompanied by changes in the number of circulating WBC. Both vitamin A deficiency and NDV infection resulted in a significant leukopenia at least during the acute phase of infection, while the combination of these factors resulted in the lowest number of WBC. Since the number of erythrocytes and total protein content in plasma were unaffected, leukopenia would not appear to be the result of a dilution of blood. In contrast to the number of PBL, the number of polymorphonuclear granulocytes and monocytes was unaffected by vitamin A deficiency and by NDV infection. Therefore, leukopenia could be attributed entirely to decreased numbers of PBL. Lymphopenia induced by vitamin A deficiency might be attributed to impaired development of primary lymphoid organs with consequent impaired proliferation and differentiation of lymphoid cells (5,6,17,23) and by changes in homing patterns (24,25). Lymphopenia is

also a normal feature during the acute phase of NDV infection (22,26). NDV-induced lymphopenia could be explained, at least partially, by direct virus-induced cytolysis resulting from virus contact with cells (27), by the ability of the La Sota strain of NDV to multiply inside chicken lymphocytes with as a consequence host defense reactions against these cells (27), and by NDV-induced change in migration patterns (28). Although Bang et al. (6) did not measure the number of circulating lymphocytes, they reported that, while vitamin A deficiency alone produced only moderate loss of lymphocytes from thymus and bursa in 3-wk-old chickens, infection of vitamin A-deficient chickens with a virulent strain of NDV caused substantial or even total loss of lymphocytes from the thymus and bursa in addition to rapid loss of body weight. The increase in the number of circulating PBL following the period of NDV-induced lymphopenia might be attributed to a change in migration pattern (28) rather than increased production of new lymphocytes (22). Both the lymphopenic effect and the subsequent increase in the number of PBL due to NDV infection were less pronounced in vitamin A-deficient chickens than in their counterparts pair-fed the control diet. It might suggest that in vitamin A deficiency the normal response to infection has been impaired.

Vitamin A deficiency significantly lowered the number of circulating B cells and tended to lower the number of T cells. Although we are not aware of similar measurements in PBL, an increased number of B cells in spleen and lymph node has been observed in vitamin A-deficient mice (29), while a decreased number of B cells has been found in spleens of vitamin A-deficient rats which also suffered from PEM (23). NDV infection did not affect the number of B cells but tended to increase the number of circulating T cells at the moment of measurement. This might suggest that lymphocytosis following the period of lymphopenia was mainly the result of an increase in the number of T cells. It should be stressed that the number of B and T cells were only measured 9 d after inoculation during NDV-induced lymphocytosis. Preliminary results have shown that NDV-induced lymphopenia could be attributed to both a lower number of circulating B and T cells (unpublished results).

The number of circulating B and T cells in each group was, to some extent, related to the weights of the bursa and thymus. The higher the number of circulating B or T cells, the higher the relative weight of the bursa or thymus, respectively.

In conclusion, the most striking result from the present study was the strong lymphopenic effect of vitamin A deficiency per se and this effect was even more pronounced in combination with NDV infection. Although we did not

observe such a marked effects on lymphoid organs as have been described by Bang et al. with a more virulent virus (5,6), it is clear from the results presented here that vitamin A deficiency, especially in the presence of NDV infection, has a strong effect on lymphoid cell systems and this may have serious consequences for host defense mechanisms.

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CHAPTER 7

Vitamin A deficiency impairs cytotoxic T lymphocyte activity in Newcastle disease virus-infected chickens

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ABSTRACT

The effect of vitamin A deficiency on cytotoxic T lymphocyte (CTL) activity was investigated in chickens with or without Newcastle disease virus infection (NDV, La Sota strain). Day-old chickens with limited vitamin A reserves were fed purified diets containing either marginal (ad libitum) or adequate (pair-fed) levels of vitamin A and at 3 wk of age, half of the chickens in each group were infected with NDV. Cytotoxic activity was investigated in an assay system with either peripheral blood lymphocytes (PBL) or nonadherent splenocytes as effector cells and adherent splenocytes from the same animal as target cells, 7 d after primary inoculation during the acute phase of disease and 1 d after secondary inoculation. CTL activity was confirmed by observation of typical characteristics such as nonadherence, antigen-specificity, histocompatibility-restriction, rapid killing and dose-dependency. In addition, NK and K cell involvement were ruled out by specificity and Fc receptor blocking experiments, respectively. Cytotoxic activity could only be demonstrated in nonadherent splenocytes after primary inoculation. However, vitamin A deficiency resulted in significantly reduced CTL activity at all effector/target cell ratios tested. In addition, CTL activity could only be demonstrated in PBL from chickens fed the control diet after reinfection, possibly demonstrating a diminished pool of CTL in vitamin A deficiency. The results of this study indicate that vitamin A deficiency impairs CTL activity, a part of the cell-mediated defense system, and this can have important implications for recovery from viral infection.

INTRODUCTION

The relationship between vitamin A deficiency and infection is well recognized. Vitamin A deficiency is associated with increased susceptibility, severity and duration of infection (1,2). In order to investigate the mechanisms by which vitamin A deficiency can influence the disease process after infection with viruses belonging to the family Paramyxoviridae, we have developed a model in which chickens differing in vitamin A status are infected with the lentogenic, i.e. mildly pathogenic, La Sota strain of Newcastle

disease virus (NDV) (3). Using this model we can study the effects of a single virus infection superimposed on marginal vitamin A status without interference from protein-energy malnutrition or secondary infection. In previous experiments we have demonstrated that chickens fed a diet marginally deficient in vitamin A were indeed more susceptible to the La Sota strain of NDV and these birds showed more severe signs of disease than counterparts fed a diet adequate in vitamin A (3).

As cell-mediated immune responses are very important in recovery from viral infections, vitamin A deficiency could exert its effect by inhibiting such responses. Impairment of cell-mediated immune response in vitamin A deficiency is well documented (4,5) but most attention has been focused on mitogen-stimulated lymphocyte proliferation responses. Thus impairment of activity of particular lymphocyte subpopulations important in resistance against microbial invaders would not be recognized. In the case of NDV infection, specific cytotoxic T lymphocytes (CTL) would appear to be very important in cell-mediated immune response (6,7).

The present studies were designed to investigate the effect of vitamin A deficiency on CTL activity in chickens infected with the La Sota strain of NDV during the acute phase of infection and 1 d after reinfection.

MATERIALS AND METHODS

Animals, diets and experimental design

Female day-old White Leghorn chickens (strain Lohmann Selected Leghorn) with limited vitamin A reserves were obtained as progeny of marginally vitamin A-deficient laying hens (Wageningen Agricultural University) maintained on a marginally vitamin A-deficient diet (300 retinol equivalents or RE/kg diet) for a period of 5 months. The chickens were fed purified diets containing either marginal amounts of vitamin A (120 RE/kg feed) or adequate amounts (1200 RE/kg feed). Diets were manufactured according to the recommendations of the National Research Council (8) and described in detail previously (3). In order to ensure that the intake of the marginally vitamin A-deficient chickens differed only as far as vitamin A was concerned, control birds were pair-fed. At the age of 21 d, half of the chickens in both groups were infected with the lentogenic La Sota strain of NDV. In this way four experimental groups were formed: noninfected and infected groups fed a marginally vitamin A-deficient diet (A-I- and A-I+, respectively), and noninfected and infected pair-fed

groups fed a diet with adequate vitamin A (A+I- and A+I+, respectively). Reinfection with NDV was carried out at the age of 31 d. Infected and noninfected chickens were housed separately in air-filtered rooms maintained under reduced pressure, controlled for temperature (decreasing from 32°C during the first day to 20°C after 2 wk), relative humidity (40-50%) and light-dark cycles (24 h dimmed light/d during the first 2 d, and 10 h dimmed light and 14 h darkness/d from the third day). The chickens were studied for a total period of 32 d during which body weight, vitamin A status and hemagglutination-inhibition (HI) antibody titers to NDV were measured. The birds were observed daily for clinical signs of vitamin A deficiency and/or NDV infection. Cytotoxic activity was measured 7 d after primary virus inoculation, and 1 d after reinfection with NVD.

Experimental NDV infection

One batch of lyophilized La Sota NDV (Delvax, Gist-Brocades, Delft, the Netherlands) was reconstituted in cold phosphate-buffered saline (PBS) and each bird was inoculated intraocularly with a 10^8 median embryo-infectious dose of the virus. Reinfection was carried out with the same virus strain and dose in the same way. Control birds were inoculated with PBS. In order to confirm the exposure to viral infection, HI antibody titers to NDV were measured just prior to and 7 or 10 d after primary inoculation according to the method of De Jong (9) using the ß-procedure (constant-virus diluted-serum). Chickens were not vaccinated against any other diseases.

Vitamin A status

Retinol levels were determined in plasma collected just prior to virus inoculation and at days when cytotoxic activity was measured. A reversed-phase high performance liquid chromatography (HPLC) method modified from that of Driskell et al. (10) was used with retinyl acetate as internal standard. The results obtained were checked against pooled control samples.

Cell suspensions

Spleen cells were obtained by removing the capsule and teasing spleen tissue through a nylon gauze. The cells, kept in siliconized tubes, were centrifuged (20 min, 400 x g, 20°C) over a ficoll-metrizoate gradient (Nycodenz, 1.083 g/ml, Nyegaard & Co AS, Oslo, Norway). PBL were obtained by diluting (1:1 v/v) heparinized blood taken from the wing vein with PBS followed by centrifugation over a ficoll-metrizoate gradient (Lymphoprep,

1.077 g/ml, Nyegaard & Co AS). All cells, either isolated from blood or spleen, were washed twice with PBS (10 min, 400 x g, 4°C) and resuspended in macrophage medium (11) supplemented with newborn bovine serum (10% v/v) to a concentration of 2.0 x 10^7 cells/ml.

Cytotoxic cell assay

The method used was essentially that described by Müllbacher et al. (12) as modified for chicken cytotoxic cell activity by Chubb et al. (13) but some slight modifications were introduced. Nonadherent spleen cells, predominantly lymphocytes, were separated from adherent cells by incubating (3 h, 41°C, 5% CO_2) 10 ml of the cell suspension in plastic tubes. The nonadherent spleen cell and PBL suspensions which did not contain granulocytes or macrophages/monocytes as determined by Natt and Herrick staining (14), were used as sources of effector cells.

In order to obtain target cells for the cytotoxicity test, 1 ml of the cell suspension containing 1 x 10^6 cells was added to 24-well plates (1.6 cm diameter, Costar, Cambridge, MA) and incubated (3 h, 41°C, 5% CO_2). Nonadherent cells were removed and counted to give a rough estimate of remaining adherent cells (usually 20-30%). Adherent cells were washed twice with PBS. Activation of target cells was carried out by incubating (2 h, 41°C, 5% CO_2) the monolayers with a La Sota NDV suspension (1 ml, 15 μg lyophilized virus/ml macrophage medium, Delvax, Gist-Brocades). The cells were washed twice with PBS and then incubated (30 min, 41°C, 5% CO_2) with neutral red (1 ml, 0.04% wt/v in macrophage medium, Sigma Chemical Co., St Louis, MO) followed by further washing with PBS.

The adherent target cell cultures were challenged (5 h, 41°C, 5% CO_2) with autologous or heterologous nonadherent lymphocytes (1 ml, effector/target cell ratios of 1:1, 10:1 and 50:1 for effector cells from spleen and an effector/target cell ratio of 5:1 for PBL) or with medium alone. The nonadherent cells were removed by gently rinsing twice with PBS. The remaining adherent cells were lysed to release the neutral red by adding acetic acid (0.75 ml, 0.05 M in 50% v/v ethanol). Subsequently, the optical density of the neutral red was measured spectrophotometrically with test and reference wavelengths at 540 nm and 630 nm, respectively. The difference was used as a measure (D) of the number of cells remaining which took up neutral red and were not lysed by the effector cells. Results, expressed as the proportion of target cells lysed, were calculated by use of the formula:

$$\text{Lysis (\%)} = \frac{DC - DA}{DC} \times 100$$

where DC is the control measurement (virus activated cultures with heterologous lymphocytes) and DA is the assay measurement (virus activated cultures with autologous lymphocytes). Heterologous effector cells were obtained by pooling lymphocytes from all other chickens in the same experimental group. The optical density of the control cultures was at least 0.15 but reached mostly values between 0.25 and 0.35. Results were based on mean values for triplicate cultures.

Confirmation of cytotoxic T lymphocyte (CTL) involvement

In order to distinguish cytotoxic activity mediated by natural killer (NK) cells from that mediated by CTL, target cells were infected in vitro with infectious bronchitis virus (IBV; live attenuated strain H120, 1 ml per well, 15 μg lyophilized virus/ml in macrophage medium, Delvax, Gist-Brocades) instead of NDV and used in the same assay together with in vivo NDV-primed effector cells. In addition, in order to distinguish cytotoxic activity mediated by killer (K) cells from that mediated by CTL, binding of specific antibody on Fc receptors was blocked by adding an excess of purified chicken IgG (10 μl per well, 10 mg/ml, Sigma Chemical Co.) to the assay system.

Statistical analysis

The data were expressed as the mean $\pm$ SD. The influence of vitamin A deficiency and NDV infection on body weight and vitamin A status were evaluated after testing for normality by two-way analysis of variance (ANOVA) and differences between group means were tested with Tukey-honestly significant difference statistics. The effect of vitamin A deficiency on cytotoxic activity in infected chickens was evaluated with Student's t-test statistics. All statistical procedures were based on the principles outlined by Snedecor and Cochran (15) and were performed using a VAX-8600 computer system with a SPSS-X software package (SPSS, Chicago, IL) (16).

RESULTS

General health

Prior to NDV infection, body weight was not affected by vitamin A status (Table 1). However, body weight tended to be lower in birds 11 d after NDV inoculation compared with noninfected controls. This tendency for lower body weight following infection was most pronounced in chickens fed a diet marginally deficient in vitamin A.

TABLE 1. Effect of Newcastle disease virus (NDV) infection on body weight in chickens fed diets with marginal or adequate amounts of vitamin A.

Group	Body weight		
	Day 21	Day 28	Day 32
		g	
A-I-	171 ± 13 (8)	243 ± 22 (8)	286 ± 31 (5)
A-I+	172 ± 21 (12)	229 ± 26 (12)	261 ± 15 (5)
A+I-	180 ± 16 (8)	254 ± 22 (8)	301 ± 29 (5)
A+I+	181 ± 19 (12)	245 ± 25 (12)	285 ± 29 (5)

Values are means ± SD with the number of birds in parentheses. At Day 21, chickens were primed by inoculation with the La Sota strain of NDV; at Day 28, chickens were used for the first cytotoxic activity experiment; at Day 31, chickens reinfected with NDV at Day 31 were used for the second experiment. Analysis of variance did not reveal significant effects.

During the acute phase of NDV infection, clinical signs of disease such as general weakness and respiratory problems were mainly limited to birds fed inadequate amounts of vitamin A. Although overt clinical signs of vitamin A deficiency were not seen at the time of carrying out the cytotoxic activity experiments, many chickens died due to vitamin A deficiency during the first 2 wk of their lives.

Vitamin A status

At the moment of NDV inoculation, plasma retinol levels of vitamin A-deficient chickens were significantly lower than of counterparts pair-fed the control diet and these levels could be considered as marginal deficient (17) (Table 2). At 7 and 11 d after NDV inoculation plasma retinol concentrations were reduced further in NDV-infected chickens fed a marginally vitamin A-deficient diet, while these levels remained stable in noninfected counterparts fed the same diet.

TABLE 2. Effect of vitamin A intake and/or infection with Newcastle disease virus (NDV) on plasma retinol concentration in chickens.

Group	Retinol concentration		
	Day 21	Day 28	Day 32
		μmol/l	
A-I-	0.48 ± 0.07[a]	0.48 ± 0.09[a]	0.44 ± 0.08[a]
A-I+	0.45 ± 0.07[a]	0.25 ± 0.07[b]	0.27 ± 0.05[b]
A+I-	1.67 ± 0.34[b]	1.79 ± 0.38[c]	1.82 ± 0.44[c]
A+I+	1.76 ± 0.29[b]	1.70 ± 0.30[c]	1.85 ± 0.36[c]

Values are means ± SD for 8-12 (Day 21 and Day 28) or 5 (Day 31) birds/group. At Day 21, chickens were primed by inoculation with the La Sota strain of NDV. Means within a column not sharing a common superscript letter are significantly different at $P < 0.05$ (Tukey).

Hemagglutination-inhibition (HI) antibody titers to NDV

Maternal HI antibodies to NDV could not be detected at the moment of NDV inoculation (HI titer ≤ 4). Although 7 d post inoculation HI antibodies to NDV could not be demonstrated, 10 d after primary NDV inoculation, HI antibodies were present in all birds infected with NDV and in none of the noninfected birds. Vitamin A status did not influence HI antibody response (HI titers, mean ± SD: 7.4 ± 1.6 and 7.3 ± 1.5 for the A-I+ and A+I+ groups, respectively).

Cytotoxic T cell activity

Seven days after primary NDV inoculation, cytotoxic activitity was observed only in spleen cell cultures isolated from NDV-infected chickens (Fig. 1). However, cytotoxic activity of the nonadherent spleen cells was significantly lower at all effector/target cell ratios (E:T) in cultures from birds fed a diet marginally deficient in vitamin A than in cultures from pair-fed counterparts.

Cytotoxic activity was negligible (cytotoxic activity < 3%) in PBL either isolated from infected or noninfected birds at an effector/target cell ratio of 5:1.

Reinfection with NDV 10 d after primary virus inoculation resulted in cytotoxic activity 1 d post reinfection in either nonadherent spleen cells (E:T = 10:1) and PBL (E:T = 5:1) from chickens fed a diet adequate in vitamin A (Fig. 2). However, cytotoxic activity was significantly lower in cultures

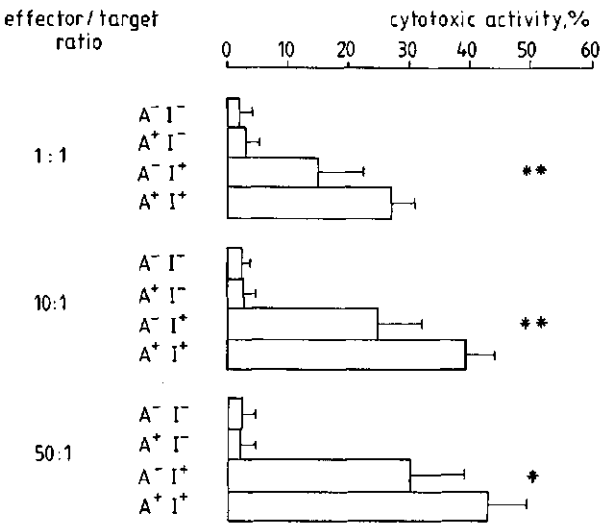

FIGURE 1. Effect of vitamin A deficiency on cytotoxic activity of in vivo NDV-primed nonadherent spleen (effector) cells on in vitro NDV-infected adherent spleen (target) cells. Values are means with the horizontal bars representing one SD. A-I-, (n=3); A-I+, (n=7); A+I- (n=3); A+I+, (n=7). * Significantly different from A+I+ at $P < 0.05$; ** Significantly different from A+I+ at $P < 0.01$ (Student's t-test).

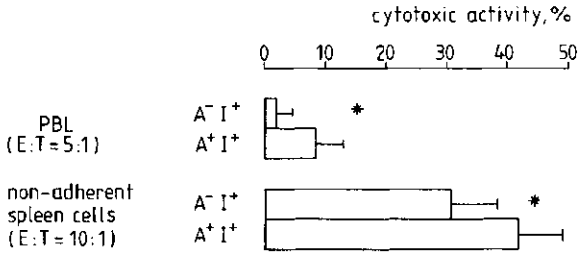

FIGURE 2. Effect of vitamin A deficiency on cytotoxic activity of in vivo NDV-reinfected PBL and nonadherent spleen cells (effector cells) on in vitro NDV-infected spleen (target) cells. Values are means of 5 chickens per group with the horizontal bars representing one SD.
* Significantly different from A+I+ at $P < 0.05$ (Student's t-test).

with nonadherent spleen cells and almost negligible in cultures with PBL from birds fed inadequate amounts of vitamin A. In the latter group cytotoxic activity of PBL could only be demonstrated in 2 of the 5 birds. In both groups, cytotoxic activity of nonadherent spleen cells was higher after reinfection than 7 d after primary inoculation.

Confirmation of cytotoxic T lymphocyte (CTL) involvement

Cytotoxic activity could not be demonstrated when nonadherent spleen cells from one chicken, shown to be cytotoxic for target cells from itself, had been used to challenge target cells from another, heterologous chicken (Table 3).

Nonadherent spleen cells from chickens primed and reinfected in vivo with NDV did not show cytotoxic activity towards autologous adherent spleen cells when they were not virally infected or when they were incubated with IBV in vitro at an effector/target cell ratio of 10:1 (Table 3).

Blocking of possible Fc receptors on cytotoxic cells with an excess of nonspecific chicken IgG did not result in diminished cytotoxic activity (Table 3).

TABLE 3. Confirmation of cytotoxic T lymphocyte involvement.

Effector cell Fc blocking	Target cell infection (in vitro)	Target cell source	Cytotoxic activity (%)
-	NDV	autologous	42.0 ± 6.9
-	-	autologous	2.4 ± 0.6
-	IBV	autologous	3.2 ± 1.8
Chicken IgG	NDV	autologous	36.4 ± 8.2
-	NDV	heterologous	2.2 ± 1.3

Experiments were carried out with nonadherent (effector) and adherent (target) spleen cells from chickens fed a diet adequate in vitamin A after in vivo priming and reinfection with NDV. Values are means of the same 5 chickens ± SD. Effector/target cell ratio was 10:1.

DISCUSSION

Cytotoxic lymphocytes play an important role in recovery from viral infections (18). In the present study, we have investigated the effect of vitamin A deficiency on the activity of CTL in NDV-infected chickens. Neutral red was introduced as an indicator in cytotoxicity assays to replace ^{51}Cr by Müllbacher et al. (12) for studies with allogeneic lymphocytes in inbred

strains of mice. However, in our experiments the chickens were not inbred. Therefore, the modification of Chubb et al. (13), in which both target and effector cells came from the same animal was used to overcome histoincompatibility.

Cytotoxic activity in our experiments was probably mediated by CTL because the results observed met the principle characteristics of CTL, such as nonadherence and antigen-specificity (19), MHC-restriction (20), and rapid killing and dose-dependency (21). Cytotoxic activity was probably not mediated by NK cells or K cells as in vivo NDV-primed and reinfected effector cells were not able to lyse noninfected or in vitro IBV-infected autologous target cells, while blocking of Fc receptors with an excess of chicken IgG did not result in diminished cytotoxic activity. As granulocytes and monocytes play an important role in natural cytotoxicity and antibody-dependent cellular cytotoxicity in chickens (22), the absence of these cells in our effector cell suspensions further indicated that the activity was confined to CTL. Moreover, the La Sota strain of NDV is a poor inducer of interferon (23), which is important for the activation of NK cells (24).

Lymphocytes isolated from the spleen of in vivo NDV-infected chickens 7 d following inoculation, showed pronounced CTL activity to in vitro NDV-infected target cells as has been demonstrated previously (6,7). However, this T cell-mediated cytotoxic activity was markedly reduced by vitamin A deficiency for all effector/target cell ratios tested. The results obtained could not be attributed to protein-energy malnutrition, since body weights were not affected. Possible mechanisms could be a shift in the proportion of various lymphocyte subpopulations to lower numbers of CTL, reduced activation of CTL (for example by T helper cells or T helper cell-derived lymphokines) or disturbed recognition of virally infected target cells by CTL. Although there is little information on the effect of vitamin A deficiency on CTL response to cells infected with virus, more is known about the role of retinoids on rejection of skin and tumor transplants. CTL appeared to be involved in rejection of both types of transplant. Skin from male C57BL/6 mice grafted onto syngeneic female mice were rejected significantly faster when the transplanted mice were given retinyl palmitate (25) or retinyl acetate (26). However, results from these experiments might be complicated by a toxic action of the retinoids. Injection of retinoic acid for 6 d prior to tumor challenge strongly stimulated induction of CTL (27).

Although CTL activity could not be demonstrated after primary NDV inoculation in PBL of chickens irrespective of their vitamin A status, a small

but detectable cytotoxic response was observed in PBL from birds fed a diet adequate in vitamin A one day after reinfection. In contrast, PBL from chickens fed a diet marginally deficient in vitamin A did not show such a CTL response. These results suggest that there is a pool of active CTL in the spleen of normal birds which can disseminate via the circulatory system to the affected site after reinfection with the same virus. Similar findings have been reported recently by Chubb et al. (13) in IBV-infected chickens. However, our results indicate that in vitamin A deficiency, this pool is depleted markedly. As a consequence, CTL may not be available for dissemination. This hypothesis is supported by the studies of Bang et al. (28,29) who found a reduced number of lymphocytes in spleens of vitamin A-deficient chickens. This effect was even more pronounced when their vitamin A-deficient chickens were infected with NDV.

In conclusion, the results of the present study indicate that vitamin A deficiency can diminish CTL activity directed towards NDV-infected target cells. As a consequence, killing of virus-infected cells may be delayed. This would explain, at least partially, the increased morbidity observed in NDV-infected chickens fed a diet marginally deficient in vitamin A (3).

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CHAPTER 8

Effect of vitamin A deficiency on the systemic humoral immune response in Newcastle disease virus-infected chickens

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ABSTRACT

The effect of vitamin A deficiency on the systemic humoral immune response was investigated in chickens infected with the La Sota strain of Newcastle disease virus (NDV). Day-old chickens with limited vitamin A reserves were fed diets containing either marginal (ad libitum) or adequate (ad libitum and pair-fed) levels of vitamin A and at 4 wk of age, half of the chickens in each group were infected with NDV. Primary and secondary immunization with antigens differing in thymus dependency (sheep red blood cells (SRBC) and bovine serum albumin (BSA) as thymus-dependent antigens and Brucella abortus (BA) as a more or less thymus-independent antigen) was carried out during and after the acute phase of disease. Vitamin A deficiency did not affect the hemagglutination-inhibition antibody response to NDV. Vitamin A deficiency and NDV infection did not affect the kinetics of the primary and secondary immune response to the three antigens. However, the level of the primary and secondary IgG response to BSA and of the primary IgG response to SRBC and BA was reduced, while the secondary IgM response to SRBC and BA was slightly elevated by vitamin A deficiency. NDV infection reduced primary IgM and IgG responses to SRBC and BSA but not to BA, indicating a defect in T-helper cell function. The combination of vitamin A deficiency and NDV infection resulted in the lowest IgG titers to thymus-dependent antigens. As most pathogens are of the latter type, the risk for secondary infection would appear to be increased in vitamin A deficiency and NDV infection.

INTRODUCTION

The relationship between vitamin A deficiency and infection is well recognized. Vitamin A deficiency is associated with increased frequency, susceptibility and severity of infectious diseases (1-3). However, underlying mechanisms are difficult to explore since clinical studies are limited by ethical considerations, while conclusions from animal studies have often been based on experiments using animals with extreme vitamin A deficiency or deficiency of other nutrients or energy resulting in poor health of the experimental animals. In previous papers (4,5) we described an animal model

with which we have demonstrated the relationship in chickens between vitamin A status and consequences of infection with the La Sota strain of Newcastle disease virus (NDV). Vitamin A deficiency increased morbidity in chickens infected with NDV. NDV belongs to the family Paramyxoviridae as does measles virus (6). In developing countries, measles is one of the most life-threatening infectious diseases (7,8), the degree of severity of which has been very often associated with vitamin A deficiency (9,10). Although viruses classified in the family Paramyxoviridae are certainly not identical, they do have many characteristics in common. For instance, NDV and measles virus both have a pronounced effect on epithelial tissues and both are immunosuppressive (11-18). Using our model we can study the effects of a single virus infection superimposed on marginal vitamin A status without interference from protein-energy malnutrition (PEM) or secondary infections. In contrast to laboratory conditions, primary infections under field conditions in vitamin A-deficient animals and humans are mostly accompanied by secondary infections which can aggravate the disease process. In the human situation this phenomenon can be observed often in children with poor vitamin A status during and just after measles infection (19). As the humoral immune system is one of the most important specific defense mechanisms against microbial invaders (20), it is extremely important to know more about the combined effect of vitamin A deficiency and primary infection with viruses belonging to the family Paramyxoviridae on specific host defense. Impairment of the humoral immune system in experimental animals by vitamin A deficiency is well documented. Reduced specific antibody responses to antigens have been found in vitamin A-deficient rabbits (21), chickens (22), swine (23), rats (24,25) and mice (26,27), while normal and elevated specific responses have been observed in vitamin A-deficient chickens (28) and mice (26), respectively. Moreover, elevated levels of nonspecific immunoglobulin in serum were found in vitamin A-deficient rabbits (21), swine (23) and mice (26). However, except for two studies with mice (26,27), the influence of vitamin A deficiency on the kinetics of the humoral immune response and the level of antibody production differentiated in classes has not been investigated. In addition, little attention has been focused on the combined effect of vitamin A deficiency and a primary viral infection on the humoral immune response to other antigens.

In this paper, we report an experiment in which not only humoral immune responses to NDV infection in chickens differing in vitamin A status were investigated but also the combined effect of a low vitamin A intake and NDV

infection on the humoral immune response to other antigens. Therefore, chickens were immunized during the acute phase of NDV infection with one of three antigens differing in thymus dependency: sheep red blood cells (SRBC) and bovine serum albumin (BSA) as thymus-dependent (29,30) and Brucella abortus (BA) as a more or less thymus-independent antigen (30,31). The effects were evaluated by examining the kinetics of the primary and secondary antibody response to these antigens and by examining the level of antibody production differentiated in immunoglobulin classes or 2-mercaptoethanol (2ME)-resistance.

MATERIALS AND METHODS

Animals, diets and experimental design

Chickens were selected at random from birds used in the studies of which details of the animals, housing conditions, diets, administration of NDV infection and experimental design have been described earlier (4). In brief, female day-old White Leghorn chickens (strain Lohmann Selected Leghorn) with limited vitamin A reserves, progeny of marginally vitamin A-deficient laying hens (Wageningen Agricultural University), were fed purified diets ad libitum containing either adequate (1200 retinol equivalents or RE/kg feed) or marginal amounts of vitamin A (120 RE/kg feed). In order to ensure that the intake of the marginally vitamin A-deficient chickens differed only insofar as vitamin A is concerned, a pair-fed control group was also included. At 4 wk of age, half of the chickens in each of the three groups were infected intraocularly with a 10^8 median embryo-infectious dose of the lentogenic La Sota strain of NDV (Delvax, Gist-Brocades, Delft, the Netherlands). In this way, six experimental groups were formed: noninfected and infected groups fed a diet marginally deficient in vitamin A [A-(adlib)I- and A-(adlib)I+, respectively]; noninfected and infected pair-fed groups fed a diet adequate in vitamin A [A+(pair)I- and A+(pair)I+, respectively]; and noninfected and infected ad libitum-fed groups fed a diet adequate in vitamin A [A+(adlib)I- and A+(adlib)I+, respectively]. Chickens were not vaccinated against any disease apart from NDV during the experimental period.

Primary and secondary immunization

Primary and secondary immunization with either SRBC, BA or BSA were carried out at 4 and 25 d after inoculation with NDV, respectively. Chickens

were injected intramuscularly with 1.0 ml packed SRBC (about 2.6×10^{10} cells). SRBC from six Texel sheep were suspended in physiological saline (0.9% wt/v NaCl) containing heparin (10 IU/ml) and washed three times in physiological saline. The dose was injected in two equal portions, one into the muscle of each thigh. Immunization with BA (5×10^{10} whole heat-killed bacteria/ml, Central Veterinary Institute, Lelystad, the Netherlands) was carried out by injecting 0.1 ml of the suspension into the wing vein. Immunization with BSA (Sigma Chemical Co., St Louis, MO) was carried out by injecting (1.0 ml per kg of body weight) a sterile solution of BSA (4% wt/v in phosphate-buffered saline (PBS)) in the wing vein.

Sampling of blood

Blood from a wing vein was collected in heparinized tubes from the same 16 chickens/group at each time point for the determination of plasma retinol and of hemagglutination-inhibition (HI) antibodies, and from the same 16 to 20 birds/group immunized with one of three antigens at each time point for the analyses of specific antibody. After centrifugation, plasma was separated and stored at $-20^{\circ}C$.

Vitamin A analyses

Plasma retinol content was determined by a reversed-phase high performance liquid chromatography (HPLC) method modified from that of Driskell et al. (32) as described previously (4).

Immunoglobulin analyses

Hemagglutination-inhibition antibody titers to NDV were measured 0, 4, 7, 11, 16 and 37 d after inoculation according to the method of De Jong (33) using the ß-procedure (constant-virus diluted-serum). Antigen-specific globulins to SRBC, BA and BSA were measured 0, 3, 5, 7 and 12 d after primary and secondary immunization. Total and 2ME-resistant antibodies to SRBC and BA were determined in a hemagglutination assay according to the method of Van der Zijpp and Leenstra (34) and in an agglutination assay slightly modified from that of Toivanen and Toivanen (35), respectively. As 2ME destroys the activity of the IgM component, 2ME-resistant antibodies are predominantly of the IgG class. Titers were expressed as the $\log_2$ of the reciprocal of the highest dilutions giving visible agglutination. Antibodies of the IgM and IgG classes against BSA were determined in an enzyme-linked immunosorbent assay (ELISA). Micro-wells of flat-bottomed 96-well polyvinyl plates (Omnilabo, Breda, the

Netherlands) were coated with BSA (0.1 ml, 40 μg/ml, 24 h, 4°C) and washed twice with a Tween-20 solution (0.05% v/v in water). A dilute solution of Tween-20 in PBS (0.05% v/v, 0.1 ml) was pipetted into each well, diluted plasma (0.1 ml) was added and the plates were incubated (1 h, 37°C). Unbound immunoglobulin was removed by extensive washing with aqueous Tween-20 solution. Peroxidase-conjugated rabbit antibody against chicken IgG (0.1 ml, dilution 1/4000, Cooper Biomedical, Malvern, PA) or goat antibody against chicken IgM (0.1 ml, dilution 1/1000, Cooper Biomedical) were added to the wells. The plates were incubated (1 h, 37°C) and extensively washed with Tween-20 solution to remove unbound rabbit or goat immunoglobulins. Substrate (0.1 ml, 6 mg 3-3'-5-5'tetramethylbenzidine/ml dimethylsulfoxide in 60 ml 0.015 M sodium acetate buffer, pH 5.6 containing 2.5 μM hydrogen peroxide) was added and incubated for 10 min at room temperature. The reaction was stopped by addition of H_2SO_4 (0.05 ml, 4 N) after which absorbance (450 nm) was determined with an automatic ELISA reader (Titertek Multiscan, Flow Laboratories, Finland). Antibody titers were calculated using a curve-fitting programme based on an algorithm for least square estimation of nonlinear parameters.

Statistical analysis

The effect of vitamin A nutriture, NDV infection and interaction between these two factors on plasma retinol, HI antibody titers to NDV and on antigen-specific antibody titers were evaluated after testing for normality by two-way analysis of variance (ANOVA). Differences between plasma retinol group means were evaluated with Tukey-honestly significant difference statistics. All procedures were based on the principles outlined by Snedecor and Cochran (36), and were performed using a VAX-8600 computer system with a SPSS-X software package (SPSS, Chicago, IL) (37).

RESULTS

General health

The effect of NDV infection in chickens fed either marginal or adequate amounts of vitamin A on feed consumption, body weight gain and clinical signs of disease were reported in detail in a previous paper, as were data on absence of secondary infections (4). In brief, during the acute phase of NDV infection slightly lower feed consumption, body weight gain and plasma albumin

levels were observed, which were most pronounced in chickens fed a diet marginally deficient in vitamin A. However, this did not result in PEM. Moreover, infection with NDV resulted in increased rates of morbidity in chickens fed a diet inadequate in vitamin A compared with control-fed counterparts. Clinical signs of vitamin A deficiency were not observed. Normal levels of the acute phase protein fibrinogen demonstrated the absence of secondary infection.

Plasma retinol concentration

Immediately prior to primary and secondary immunization, plasma retinol concentrations of chickens fed a diet inadequate in vitamin A were significantly lower than those of pair-fed and ad libitum-fed control birds, and these vitamin A levels could be regarded as marginally deficient (Table 1). As described earlier (4), NDV infection further reduced plasma retinol concentrations in chickens fed a marginally vitamin A-deficient diet to levels which could be regarded as deficient.

TABLE 1. Plasma retinol concentration in chickens at the time of primary and secondary immunization.

Group[1]	Retinol concentration[2]	
	Primary immunization	Secondary immunization
	μmol/l	
A-(adlib)I-	0.61 ± 0.07^{a}	0.57 ± 0.07^{a}
A-(adlib)I+	0.56 ± 0.08^{a}	0.27 ± 0.07^{b}
A+(pair)I-	1.56 ± 0.15^{b}	1.76 ± 0.19^{c}
A+(pair)I+	1.62 ± 0.16^{b}	1.65 ± 0.23^{c}
A+(adlib)I-	1.66 ± 0.20^{b}	1.92 ± 0.23^{d}
A+(adlib)I+	1.70 ± 0.17^{b}	2.04 ± 0.27^{d}

1 Chickens were fed from hatching purified diets containing marginal or adequate amounts of vitamin A and were inoculated with NDV at 4 wk of age. Primary and secondary immunization were carried out at 4 and 25 d after virus inoculation, respectively.

2 Values are means ± SD for 11-15 chickens/group. Analysis of variance revealed a vitamin A nutriture effect of $P < 0.001$ at the time of primary infection, and a vitamin A nutriture effect of $P < 0.001$, a NDV infection effect of $P < 0.001$ and an interaction effect of these factors of $P < 0.05$ at the time of secondary immunization (ANOVA). Means within a column not sharing a common superscript letter are significantly different at $P < 0.05$ (Tukey).

Hemagglutination-inhibition (HI) antibody titers to NDV

The kinetics and level of the HI antibody response to NDV were not affected by vitamin A status (Table 2). Maternal HI antibodies could not be detected at the moment of NDV inoculation. The increase in HI antibody titers was observed after 11 d, while high HI antibody titers were still present at 37 d postinoculation. HI antibodies were present in all the birds infected with NDV and in none of the noninfected birds.

TABLE 2. Hemagglutination-inhibition (HI) antibody titers to Newcastle disease virus (NDV) in chickens either or not exposed to NDV infection.

Group[1]	HI antibody titers[2] Days after NDV inoculation					
	0	4	7	11	16	37
				$\log_2$		
A-(adlib)I-	≤ 4			≤ 4	≤ 4	≤ 4
A-(adlib)I+	≤ 4			6.6 ± 1.5	8.3 ± 1.8	7.4 ± 1.6
A+(pair)I-	≤ 4			≤ 4	≤ 4	≤ 4
A+(pair)I+	≤ 4			6.3 ± 1.3	8.3 ± 1.7	7.2 ± 1.4
A+(adlib)I-	≤ 4			≤ 4	≤ 4	≤ 4
A+(adlib)I+	≤ 4			6.4 ± 2.0	8.3 ± 2.0	7.3 ± 1.6

[1] From the time of hatching, chickens were fed purified diets containing marginal or adequate levels of vitamin A and were inoculated with NDV at 4 wk of age.

[2] Values are means ± SD of the same 9-16 chickens/group at each time point. HI antibody titers ≤ 4 indicate nonexposure to NDV. Significant differences were not found among the exposed groups.

Antibody responses to antigens differing in thymus dependency

As the mean antibody titers in response to SRBC, BA and BSA immunization of the pair-fed and ad libitum-fed control chickens were not significantly different from each other in either the SRBC, BA and BSA experiment, only the results from the pair-fed chickens are presented (Fig. 1-6).

Primary immune response

Although vitamin A deficiency did not affect the kinetics and level of the primary total antibody response to SRBC, 2ME-resistant antibody titers were reduced significantly at 7 and 12 d postimmunization (Fig. 1). NDV infection reduced the level of both total and 2ME-resistant antibody response to SRBC significantly at 5 and 7 d postimmunization.

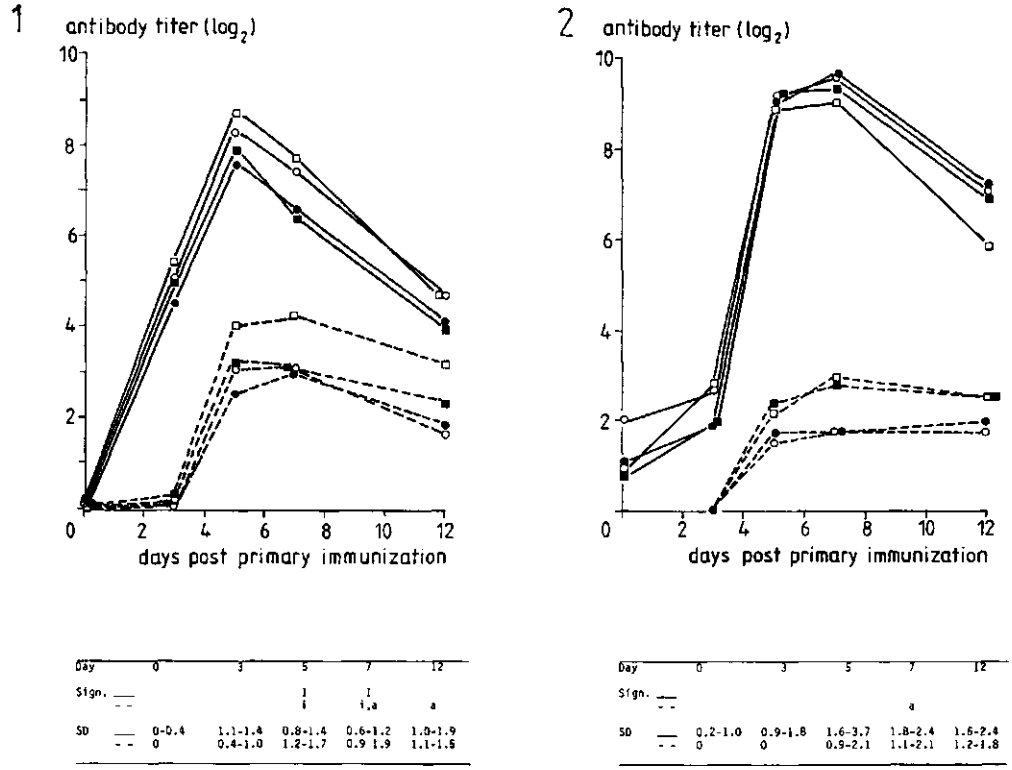

FIGURE 1. Kinetics of the primary total (——) and 2ME-resistant (- -) antibody response to sheep red blood cells (SRBC) in chickens fed diets with adequate or marginal amounts of vitamin A either noninfected or infected with Newcastle disease virus (NDV). Each point represents the mean of 16 to 20 birds. (○), A-(adlib)I-; (●), A-(adlib)I+; (□), A+(pair)I- ; (■), A+(pair)I+. Ranges of SD are given for each time point. Significant effects of vitamin A nutriture and NDV infection are shown at $P < 0.05$ (a and i, respectively) and at $P < 0.01$ (I) (ANOVA).

FIGURE 2. Kinetics of the primary total (——) and 2ME-resistant (- -) antibody response to heat-killed Brucella abortus (BA) in chickens fed diets with adequate or marginal amounts of vitamin A either noninfected or infected with Newcastle disease virus (NDV). Each point represents the mean of 16 to 20 birds. (○), A-(adlib)I-; (●), A-(adlib)I+; (□), A+(pair)I- ; (■), A+(pair)I+. Ranges of SD are given for each time point. Significant effects of vitamin A nutriture are shown at $P < 0.05$ (a) (ANOVA).

Although the level of total antibody produced in response to BA immunization was not affected by vitamin A deficiency, 2ME-resistant antibody titers were significantly reduced at 7 d postimmunization (Fig. 2). NDV infection did not affect the kinetics or level of total and 2ME-resistant

antibody response to BA.

Chickens infected with NDV showed a significant decrease in IgG and IgM response to BSA at 7 d and at 3 and 5 d postimmunization, respectively (Fig. 3). Chickens fed diets marginally deficient in vitamin A showed significantly lower IgG antibody titers than their control counterparts fed diets adequate in vitamin A at 7 d postimmunization. The kinetics and level of the IgM response to BSA were not influenced by vitamin A deficiency.

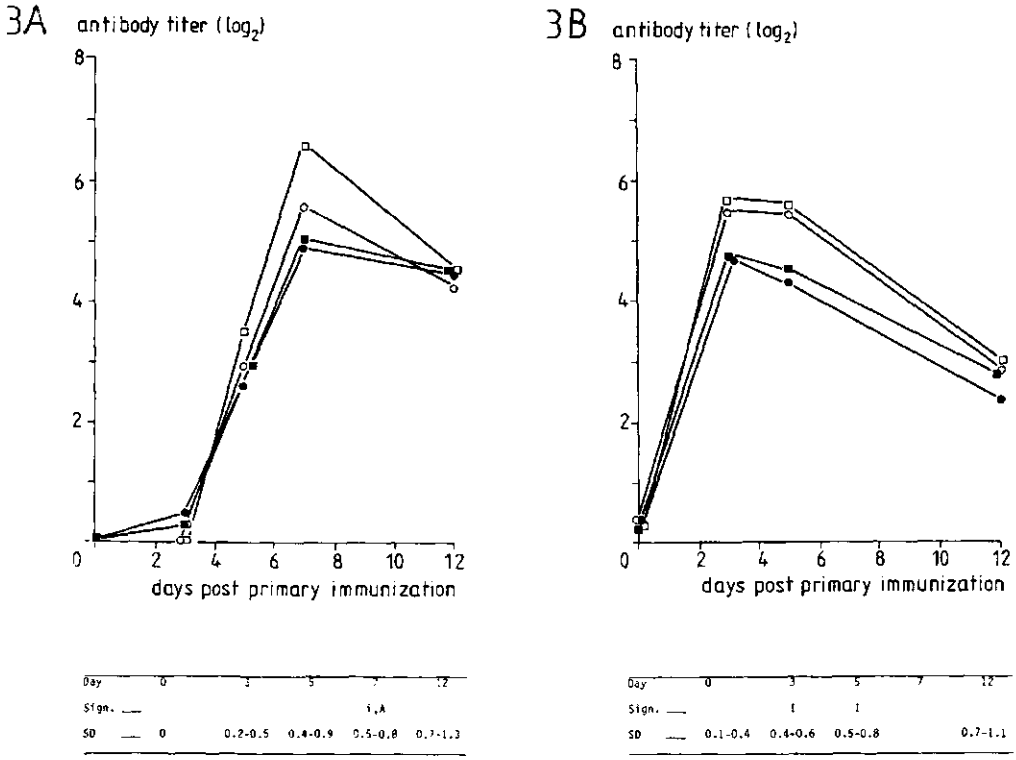

FIGURE 3. Kinetics of the primary IgG (A) and IgM (B) response to bovine serum albumin (BSA) in chickens fed diets with adequate or marginal amounts of vitamin A either noninfected or infected with Newcastle disease virus (NDV). Each point represents the mean of 16 to 20 birds. (○), A-(adlib)I-; (○), A-(adlib)I+; (□), A+(pair)I-; (■), A+(pair)I+. Ranges of SD are given for each time point. Significant effects of vitamin A nutriture and NDV infection are shown at $P < 0.05$ (a) and at $P < 0.01$ (A and I, respectively) (ANOVA). IgM response at 7 d postimmunization was not determined.

Secondary immune response

Chickens fed diets marginally deficient in vitamin A had significantly higher total antibody titers to SRBC and BA than their control counterparts at 5 d and at 3, 5, 7 and 12 d after secondary immunization, respectively (Fig. 4 and 5). As 2ME-resistant antibody responses to SRBC and BA were not affected

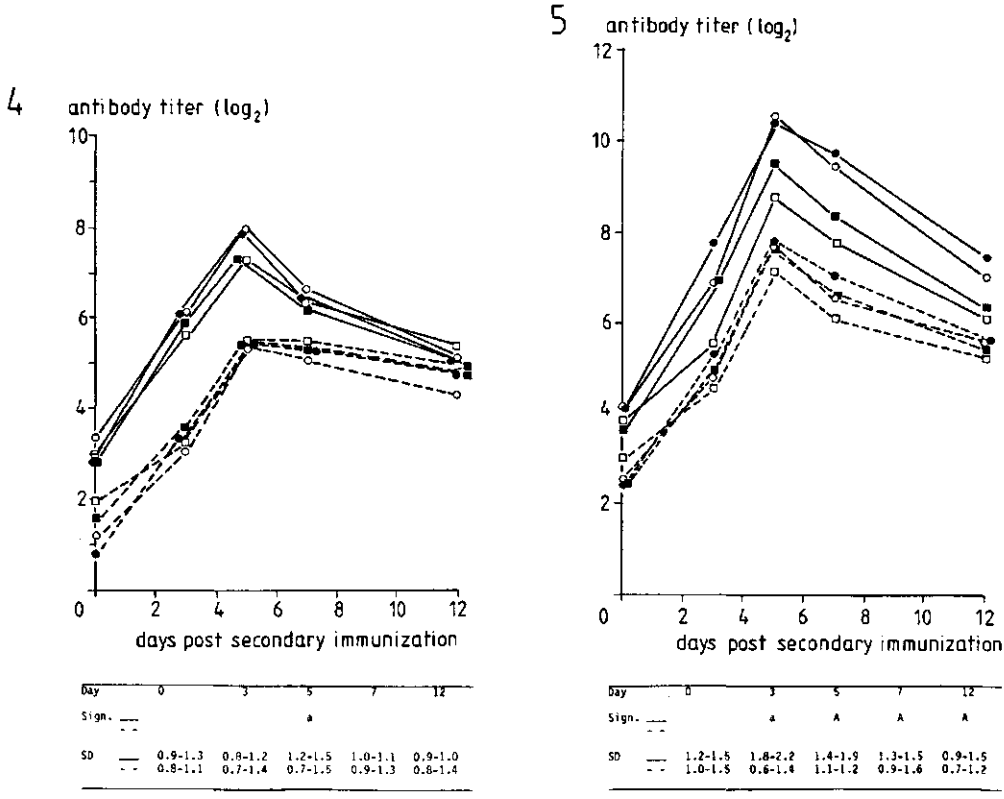

FIGURE 4. Kinetics of the secondary total (——) and 2ME-resistant (– –) antibody response to sheep red blood cells (SRBC) in chickens fed diets with adequate or marginal amounts of vitamin A either noninfected or infected with Newcastle disease virus (NDV). Each point represents the mean of 16 to 20 birds. (○), A-(adlib)I-; (●), A-(adlib)I+; (□), A+(pair)I-; (■), A+(pair)I+. Ranges of SD are given for each time point. Significant effects of vitamin A nutriture are shown at $P < 0.05$ (a) (ANOVA).

FIGURE 5. Kinetics of the secondary total (——) and 2ME-resistant (– –) antibody response to heat-killed Brucella abortus (BA) in chickens fed diets with adequate or marginal amounts of vitamin A either noninfected or infected with Newcastle disease virus. Each point represents the mean of 16 to 20 birds. (○), A-(adlib)I-; (●), A-(adlib)I+; (□), A+(pair)I-; (■), A+(pair)I+. Ranges of SD are given for each time point. Significant effects of vitamin A nutriture are shown at $P < 0.05$ (a) and at $P < 0.01$ (A) (ANOVA).

by vitamin A deficiency, the increase in level of total antibody was predominantly of the IgM class. NDV infection did not affect the kinetics and the level of the secondary total and 2ME-resistant antibody responses to SRBC and BA.

Vitamin A deficiency changed the level of the secondary antibody response to BSA in a slightly different way (Fig. 6). In contrast to IgM responses

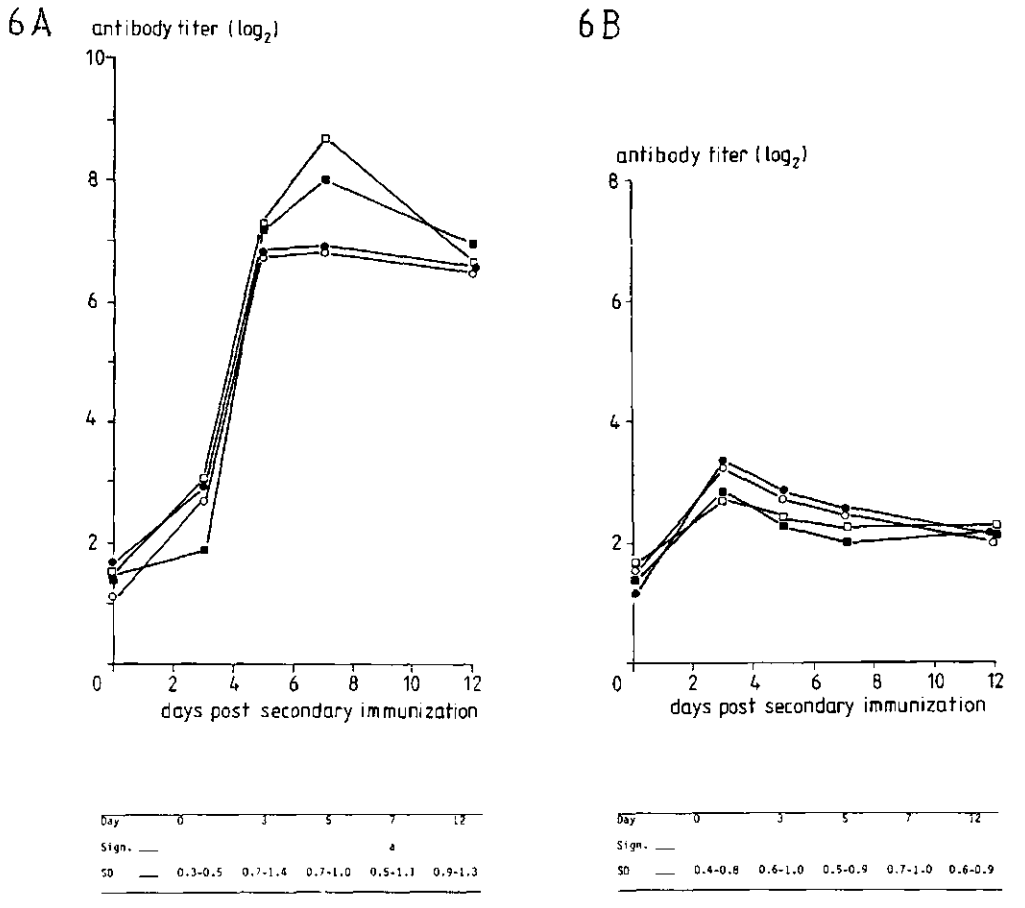

FIGURE 6. Kinetics of the secondary IgG (A) and IgM (B) response to bovine serum albumin (BSA) in chickens fed diets with adequate or marginal amounts of vitamin A either noninfected or infected with Newcastle disease virus. Each point represents the mean of 16 to 20 birds. (○), A-(adlib)I-; (●), A-(adlib)I+; (□), A+(pair)I-; (■), A+(pair)I+. Ranges of SD are given for each time point. Significant effects of vitamin A nutriture are shown at $P < 0.05$ (a).

which tended to increase, IgG response to BSA in vitamin A-deficient chickens was significantly decreased at 7 d after secondary immunization. The kinetics and level of IgM and IgG responses were not affected by NDV infection.

DISCUSSION

In this study, the primary antibody response to NDV infection in chickens fed a normal or marginally vitamin A-deficient diet is described. In addition, data are also presented on the primary and secondary antibody response to thymus-dependent (SRBC and BSA) and thymus-independent (BA) antigens in the same chickens immunized and challenged with these antigens during the acute and postacute phase of the NDV infection, respectively.

Vitamin A deficiency and NDV infection resulted in slightly lower feed consumption but this did not lead to PEM. As demonstrated in earlier experiments (4), body weight gain and plasma albumin levels are only slightly affected in this model. Thus, although PEM is known to markedly affect the humoral immune response (38), any effects on antibody titers observed in this experiment cannot be attributed to PEM. This is supported by the finding that the antibody response in pair-fed and ad libitum-fed control birds were similar.

The antigens used differ in the way in which their antibody response is regulated (29-31). BSA is a small nonsymmetric soluble protein without identical epitopes, while SRBC is a large multi-determinant antigen with identical epitopes. Although both antigens need T-helper cell cooperation for B cell stimulation, BSA is more dependent. BA is a nonmitogenic antigen with a highly repeating epitope which can stimulate B cells more or less without help from T cells.

The kinetics and level of the primary and secondary immune response to these three antigens in the noninfected pair-fed control group are comparible with results from other authors (34,39,40). Neither vitamin A deficiency nor NDV infection affected the kinetics of the primary or secondary response to these antigens. However, the level of the primary IgG responses to all antigens, either measured directly or as 2ME-resistant antibody, was reduced significantly by vitamin A deficiency. This reduction was equal irrespective of the measure of thymus dependency of the antigen used. Therefore, impairment of T-helper cell function by vitamin A deficiency as was suggested earlier (27) could not explain entirely the results observed. In addition to the role

of T-helper cells or of T-helper cell-derived lymphokines in activation, proliferation and maturation of B cells, antibody responses are controlled by binding of antigen to membrane immunoglobulin receptors, by processing and presenting of antigen by macrophages, and by the action of monokines (30,41). The results from the BA immunization suggest that vitamin A deficiency might also affect one or more of these factors.

The level of IgG in response to secondary immunization with BSA, the most thymus-dependent antigen used, was lower in chickens fed a diet marginally deficient in vitamin A than in control-fed counterparts. On the other hand, the antibody titers to the other antigens were either normal (2ME-resistant titers) or even higher (total titers) after secondary immunization. Increased levels of IgM to the two particulate antigens, as deduced from higher total antibody levels and normal levels of 2ME-resistant antibody, could be the result of impaired feedback (42) or of delayed processing of the particles.

Vitamin A deficiency did not impair the HI antibody response to NDV, which was predominantly of the IgG class (43), indicating that the priming of antibody production was not affected by vitamin A status. This was surprising as there were reduced levels of IgG in response to the three antigens used but the apparent anomaly could be the result of two contrasting events. Vitamin A deficiency might reduce both HI antibody response to NDV and IgG response to the other antigens in the same way. However, the immunizing antigen dose could be increased because NDV replication is faster in chickens fed a marginally vitamin A-deficient diet as a result of reduced cellular defense (44).

NDV infection reduced the level of total and 2ME-resistant antibody in the primary response to SRBC, and the level of the IgM and IgG primary response to BSA. However, the primary response to BA was unaffected. These results indicate that NDV impairs T-helper cell function, as suggested earlier (15). In addition, impairment of the humoral immune response by NDV might explain partially the secondary infection often observed following vaccination with the La Sota strain of NDV (45).

The combination of vitamin A deficiency and NDV infection tended to be additional and not synergistic with respect to IgG or 2ME-resistant responses to primary immunization with the thymus-dependent antigens BSA and SRBC, respectively. Although the influence of NDV infection in chickens fed a marginally vitamin A-deficient diet was less than in their counterparts fed adequate vitamin A, the combination of these two factors resulted in the lowest IgG titers.

In conclusion, our results indicate that both vitamin A deficiency and the presence of NDV infection can impair the level but not the kinetics of the humoral immune response to three antigens differing in thymus-dependency. However, the mechanisms involved appear to be different, at least partially. We postulate that NDV infection suppresses T-helper cell function, whereas vitamin A deficiency impairs T-helper cell function and possibly also another factor necessary for induction of antibody production. The combination of both factors did not result in synergistic but rather in small additional effects, in which vitamin A deficiency during the acute phase of NDV infection resulted in the lowest antibody titers to thymus-dependent antigens.

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CHAPTER 9

Effect of vitamin A deficiency on the activity of macrophages in Newcastle disease virus-infected chickens

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ABSTRACT

The effect of vitamin A deficiency on the activity of peritoneal macrophages (PM) was investigated in noninfected and Newcastle disease virus (NDV)-infected chickens. Day-old chickens with limited vitamin A reserves were fed diets containing either marginal (120 retinol equivalents (RE)/kg) or adequate (1200 RE/kg) levels of vitamin A. At 4 wk of age, half of the chickens in each group were infected with the La Sota strain of NDV and 11 or 12 d later, PM were isolated. These were used for counting the uptake of fluorescein isothiocyanate-labeled yeast cells as an indicator of phagocytic activity and for measuring the reduction of nitroblue tetrazolium (NBT) which provides an estimate of oxygen-dependent killing of microorganisms. Vitamin A deficiency impaired NBT reduction and to a lesser extent phagocytosis in both infected and noninfected chickens. Infection with NDV increased phagocytosis and NBT reduction in both vitamin A-deficient chickens and their counterparts fed adequate vitamin A. In general, this effect was more pronounced in the latter group.

INTRODUCTION

The relationship between vitamin A deficiency and infection is well recognized. Vitamin A deficiency is associated with increased susceptibility, severity and duration of infection (1-3). In order to investigate the mechanisms by which vitamin A deficiency can influence the disease process following infection with viruses from the family Paramyxoviridae, we have developed a model with chickens differing in vitamin A status and the lentogenic, i.e. mildly pathogenic, La Sota strain of Newcastle disease virus (NDV) as a source of infection (4). In previous experiments we have demonstrated that chickens fed a diet marginally deficient in vitamin A were indeed more susceptible to the La Sota strain of NDV and that these birds showed more severe signs of disease than counterparts fed a diet adequate in

vitamin A (4,5). Moreover, vitamin A deficiency seriously affected cytotoxic T lymphocyte activity directed to NDV (6) which might be harmful to recovery from viral infection (7).

Apart from cytotoxic T lymphocytes, macrophages are also considered as important ultimate effectors of antiviral cell mediated immunity (8,9). Macrophages do not only constitute an immediate barrier to virus penetration and dissemination in the body but T cell-activated macrophages in particular are hyperactive and show increased microbicidal capacity (8,9). This increased microbicidal capacity is at least partially nonspecific since activated macrophages arising in response to infection with one microorganism also show increased activity against other microorganisms (9).

Although relatively much is known about the effect of vitamin A deficiency on cell-mediated immunity and nonspecific resistance (10,11), only limited information is available on its effect on macrophage activity, especially T cell-activated macrophage activity.

The aim of the present study was to test whether vitamin A deficiency impairs the activity of unstimulated macrophages and macrophages isolated from chickens infected with NDV as this virus is a good inducer of T cell-mediated macrophage activity (12,13). Since cell-mediated immune responses appear some days after initiation of primary infection, peritoneal macrophages (PM) were harvested during the postacute phase of disease. Activity of PM was determined by counting the uptake of fluorescein isothiocyanate (FITC)-labeled yeast cells as a measure for phagocytosis and by the nitroblue tetrazolium (NBT) reduction assay as a measure of oxygen-dependent killing of microorganisms.

MATERIALS AND METHODS

Animals, diets and experimental design

A detailed description of the animals, housing conditions, diets and experimental design can be found in a previous paper (4). In brief, female day-old White Leghorn chickens (strain Lohmann Selected Leghorn) obtained as progeny of marginally vitamin A-deficient laying hens were fed purified diets ad libitum containing either adequate (1200 retinol equivalents (RE)/kg feed) or marginal (120 RE/kg feed) amounts of vitamin A. Diets were manufactured according to the recommendations of the National Research Council (14). The chickens were divided equally over two separate air-filtered rooms maintained

under reduced pressure, controlled for temperature, relative humidity and light-dark cycles. At 28 d of age, all chickens in one room were infected with the lentogenic La Sota strain of NDV. In this way four experimental groups were formed: noninfected and infected groups fed a diet marginally deficient in vitamin A (A-I- and A-I+, respectively), and noninfected and infected groups fed adequate vitamin A (A+I- and A+I+, respectively). The uptake of FITC-labeled yeast cells and NBT reduction were measured at 39 or 40 d of age in the same 7 or 8 birds/group selected at random.

Assessment of vitamin A status

Plasma retinol concentrations were measured immediately prior to virus inoculation and at the moment of macrophage isolation. A reversed-phase high performance liquid chromatography (HPLC) method modified from that of Driskell et al. (15) was used with retinyl acetate as internal standard. The results obtained were checked against samples of pooled control sera.

Experimental infection

The La Sota strain of Newcastle Disease Virus (Delvax, Gist-Brocades, Delft, the Netherlands) was administered intraocularly with a 10^8 median embryo-infectious dose per bird at 28 d of age. Control birds were inoculated with phosphate-buffered saline (PBS). In order to confirm the exposure to the viral infection, hemagglutination-inhibition (HI) antibody titers to NDV were measured 9 d after inoculation according to the method of De Jong (16) using the ß-procedure (constant-virus diluted-serum).

Peritoneal macrophage (PM) harvest

The macrophage harvest was divided over two consecutive days, 11 and 12 d after NDV inoculation, with all four experimental groups being represented equally. One day before isolation, chickens were injected intraperitoneally with sterile Freund's incomplete adjuvant (2 ml, 10% v/v in sterile PBS, Difco, Detroit, MI), to obtain adequate numbers of macrophages for in vitro testing. PM were harvested with 25 ml cold PBS containing 10 IU heparin/ml. The cells were washed twice in PBS (10 min, 400 x g, $4^{o}C$) and resuspended in RPMI-1640 medium supplemented with streptomycin (100 µg/ml, Serva, Heidelberg, West Germany), penicillin (100 IU/ml, Serva), L-glutamine (2 mM, Merck, Darmstadt, West Germany), sodium citrate (5% wt/v) and pooled chicken plasma (10% v/v) (refered to as RPMI-1640^{+}) to a concentration of 1.0×10^6 cells/ml.

Preparation of FITC-labeled yeast cells

The method used to label and opsonize yeast cells was based on that of Winter and Buschmann (17). Briefly, 1 g of active baker's yeast (Saccharomyces cerevisiae) was killed by boiling for 30 min in 100 ml distilled water, then washed twice (10 min, 400 x g, 4°C) with distilled water. FITC (Isomer I, 1 ml, 2% wt/v in PBS, Sigma Chemical Co., St Louis, MO) was added to the yeast pellet and incubated (1 h, 41°C). The yeast cells were washed with PBS (10 min, 400 x g, 4°C) until the supernatant after centrifugation was found to be uncontaminated by free fluorescent dye. FITC-labeled cells were opsonized with pooled chicken plasma (2 ml, 1 h, 41°C) followed by further washing in PBS. Labeled yeast cells were resuspended in RPMI-1640$^+$ medium to a concentration of 5.4 x 10^7 cells/ml and stored in small aliquots at -20°C.

FITC-labeled yeast cell ingestion assay

Isolated peritoneal cells (0.5 ml, 1.0 x 10^6 cells/ml) were plated in duplicate on glass cover slips (18 x 18 mm, Chance Propper, Smethwick, Warley, U.K.), each placed in a separate culture dish (35 x 10 mm, Falcon, Becton Dickinson Labware, Oxnard, CA). After incubation (1 h, 41°C, 5% CO_2), plated cells were carefully rinsed with PBS to remove nonadherent cells. From the adherent cells about 90% were macrophages as was established with Natt and Herrick staining (18). The adherent macrophage monolayer was incubated (1 h, 41°C) with FITC-labeled yeast cells (1.3 ml, 5.4 x 10^7 cells/ml). Adherent cells were washed with PBS to remove non-cell-associated yeast cells and examined immediately by fluorescence microscopy (x 400 magnification, phase-contrast, inverted fluorescence microscope, Type IM, Zeiss, West Germany). The mean number of ingested yeast cells per macrophage in at least 100 macrophages per sample was calculated including and excluding nonphagocytic macrophages.

NBT reduction assay

The NBT reduction assay was performed according to the method of Pick, Charon and Mizel (19) as modified for increased sensitivity by Rook et al. (20) with slight modifications. Briefly, isolated peritoneal cells (1.0 x 10^6 cells/ml) were purified by passage through a ficoll-metrizoate gradient (Nycodenz, 1.070 g/ml, Nyegaard & Co AS, Oslo, Norway) by centrifugation (20 min, 400 x g, 20°C). The suspension obtained was washed twice (10 min, 400 x g, 4°C) with PBS and resuspended in RPMI-1640$^+$ to a concentration of 1.0 x 10^6 cells/ml. These cells revealed morphological and functional characteristics of

macrophages as was confirmed after treatment with Natt and Herrick stain (18) and adherent properties, respectively. The macrophage suspension (0.10 ml) was added to individual wells of 96-well flat-bottom microtiter plates (Omnilabo, Breda, the Netherlands). From each sample, 16 wells were filled for the NBT reduction assay and on a separate plate, 8 wells for protein determination. Cells were left to adhere (1 h, 41°C, 5% CO_2) and nonadherent cells were removed by gently washing with PBS. Protein concentrations were determined according to the method of Lowry et al. (21) adjusted for microtiter plates. The mean protein content of 8 wells/sample was considered to be representative of the particular experiment. In addition to the 16 wells with macrophages, another 16 wells/sample were used as blanks in the NBT reduction assay. RPMI-1640$^+$ (0.10 ml) and NBT (0.05 ml, 1 mg/ml in PBS, Grade III, Sigma Chemical Co.) were added to all cells. Macrophages and blanks in 8 of the 16 wells were stimulated by adding zymosan A (0.01 ml, 10 mg/ml in PBS, prepared from Saccharomyces cerevisiae, Sigma Chemical Co.) which was opsonized with pooled chicken plasma according to the method of Winter and Buschmann (17). PBS (0.10 ml) was added to the other 8 wells. Following incubation (1 h, 41°C, 5% CO_2), the reaction was stopped by adding methanol (0.075 ml, 70% v/v in PBS) and washed twice with the same solution (10 min, 900 x g, 4°C). The wells were prepared for measurement by fixing the cells in absolute methanol (0.15 ml) and, after discarding the supernatant, the formazan deposits were resuspended in dimethylsulfoxide (0.10 ml). In order to increase the intensity of the color, KOH (0.01 ml, 1 N) was added. The absorbance was measured at 690 nm with an eight-channel photometer (Titertek Multiscan, Flow Laboratories, Finland).

Statistical analysis

After testing for normality, the influence of vitamin A intake, NDV infection and interaction between these two factors were tested by two-way analysis of variance. Differences between group means were evaluated with Tukey-honestly significant difference statistics. Pearson correlation coefficients were calculated between the phagocytosis of yeast cells and the NBT reduction activity. All procedures were based on the principles outlined by Snedecor and Cochran (22) and were performed using a VAX-8600 computer with a SPSS-X software package (SPSS, Chicago, IL) (23).

RESULTS

General health

Body weight gain and feed consumption were slightly reduced by vitamin A deficiency alone and somewhat more, although not significantly more, in combination with NDV infection. At the time of macrophage harvest, neither clinical signs of NDV infection and vitamin A deficiency nor perceptible signs of secondary bacterial infections were observed.

Vitamin A status

Prior to virus inoculation, plasma retinol levels were significantly lower in chickens fed diets inadequate in vitamin A than in their counterparts fed adequate vitamin A (Table 1).

TABLE 1. Effect of vitamin A intake and NDV infection on plasma retinol concentration.

Group	Retinol concentration	
	Day 0	Day 11-12
	μmol/l	
A-I-	0.29 ± 0.02^{a}	0.29 ± 0.01^{a}
A-I+	0.37 ± 0.02^{b}	0.24 ± 0.02^{b}
A+I-	2.41 ± 0.06^{c}	2.44 ± 0.06^{c}
A+I+	2.35 ± 0.07^{c}	2.27 ± 0.07^{c}

Values are means ± SEM for 6 to 8 birds/group. Plasma retinol concentrations were measured prior to NDV inoculation and at the time of macrophage harvest at the age of 28 d and 39-40 d, respectively. Means within a column not sharing a common superscript letter are significantly different at $P < 0.05$ (Tukey).

Although chickens of both vitamin A-deficient groups were chosen at random and treated similarly prior to infection, the A-I+ group had a significantly higher plasma retinol level than the A-I- group. At the time of macrophage isolation, plasma retinol concentrations had been reduced further in NDV-infected chickens fed a diet inadequate in vitamin A to levels which could be regarded as deficient (24).

HI antibody titers to NDV

HI antibodies to NDV were present in all infected chickens 9 d after

inoculation, indicating exposure to the virus (log_2 HI titer; mean 9.3, SEM 0.2). In addition, HI antibodies to NDV were absent in all noninfected birds (log_2 HI titer; mean 4.6, SEM 0.2). Significant differences were not found between the infected groups.

Phagocytic activity of peritoneal macrophages

Although the mean number of nonphagocytic PM ranged from 15 to 24%, significant differences among the groups were not found because of the large variation within the groups (Fig. 1). For statistical analysis, similar

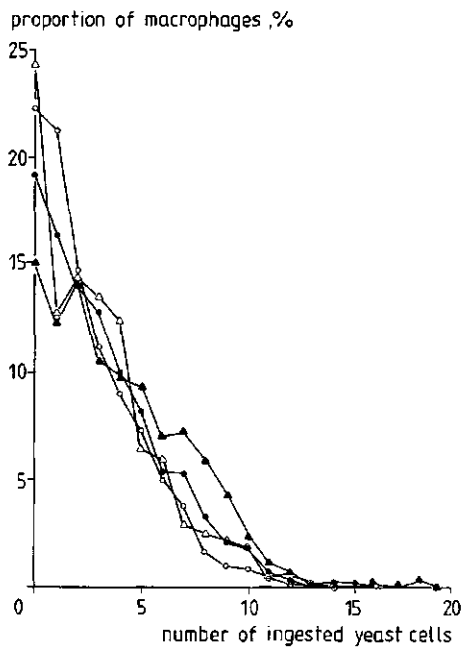

FIGURE 1. Effect of vitamin A intake and NDV infection on the phagocytic capacity of peritoneal macrophages. Nonphagocytic macrophages were included in calculation. Values are means for 6 to 8 chickens/group. (○), A-I-; (●), A-I+; (△), A+I-; (▲), A+I+.

results were obtained when the mean number of phagocytized yeast cells were calculated including or excluding nonphagocytic PM. Vitamin A-deficient birds, either noninfected or NDV-infected, tended to have fewer phagocytosed yeast

cells per PM than their noninfected or infected counterparts fed adequate vitamin A, respectively (Table 2).

TABLE 2. Effect of vitamin A intake and infection with NDV on the phagocytic activity of chicken peritoneal macrophages (PM).

Group	Phagocytosed yeast cells per PM	
	Including nonphagocytic PM	*Excluding* nonphagocytic PM
A-I-	2.64 ± 0.30^{a}	3.38 ± 0.32^{a}
A-I+	3.15 ± 0.32^{ab}	3.85 ± 0.26^{ab}
A+I-	2.87 ± 0.39^{a}	3.70 ± 0.27^{a}
A+I+	3.86 ± 0.17^{b}	4.55 ± 0.16^{b}

Results are expressed as mean ± SEM for 6 to 8 birds/group. Means within a column not sharing a common superscript letter are significantly different at $P < 0.05$ (Tukey).

This was borne out by a shift in distribution pattern towards a higher proportion of macrophages phagocytosing only a few yeast cells (Fig. 1). Infected chickens fed adequate vitamin A showed a significantly higher mean number of ingested FITC-labeled yeast cells per PM than their noninfected counterparts fed the same diet. A similar tendency, although not significant, was observed in infected, vitamin A-deficient birds when compared with their noninfected counterparts fed the same diet. These differences were also reflected in a shift in distribution pattern; infected chickens appeared to have a higher proportion of PM phagocytosing a large number of yeast cells compared with their noninfected counterparts (Fig. 1).

NBT reduction by peritoneal macrophages

Although an equal number of PM had been added to all wells, the amount of protein in wells with PM derived from chickens fed a diet inadequate in vitamin A tended to be lower after incubation and washing procedures than that in wells with PM derived from counterparts fed adequate vitamin A (Table 3). To correct for this protein difference, NBT absorbance values were expressed on a protein basis. However, the pattern of results remained the same when absorbance was expressed on the basis of the number of macrophages or macrophage protein. Both with and without zymosan A stimulation, NBT reduction was significantly lower in macrophages derived from noninfected, vitamin

A-deficient chickens than when they were derived from noninfected chickens fed the control diet. PM derived from NDV-infected birds tended to have a higher NBT reduction activity than PM from their noninfected counterparts fed the same diets.

TABLE 3. Effect of vitamin A intake and NDV infection on NBT reduction by chicken peritoneal macrophages (PM).

Group	PM protein content	NBT reduction	
		without zymosan A	with zymosan A
	μg/well	absorbance/mg protein	
A-I-	5.32 ± 0.15[a]	7.00 ± 1.21[a]	20.86 ± 1.79[a]
A-I+	5.30 ± 0.11[a]	13.11 ± 1.62[b]	26.18 ± 2.17[ab]
A+I-	5.63 ± 0.11[a]	11.59 ± 1.09[b]	32.92 ± 1.59[b]
A+I+	5.61 ± 0.12[a]	14.88 ± 2.13[b]	41.81 ± 3.60[c]

Values are means ± SEM for 7 to 8 birds/group. Protein content was determined after plating, incubating and washing of 1.0 x 10^5 peritoneal macrophages/well. Means within a column not sharing a common superscript letter are significantly different at $P < 0.05$ (Tukey).

DISCUSSION

In the present study, the effect of vitamin A deficiency on phagocytosis and NBT reduction activity of peritoneal macrophages in noninfected and NDV-infected chickens is described. Such macrophages do not only constitute an immediate barrier to virus penetration and dissemination in the body but T cell-activated macrophages in particular become hyperactive and show increased microbicidal capacity (8,9).

The tendency for reduced phagocytosis of FITC-labeled yeast particles in noninfected vitamin A-deficient chickens when compared with their noninfected vitamin A-sufficient counterparts and the even more pronounced differences in phagocytosis due to vitamin A status after activation with NDV indicated that vitamin A deficiency can impair functioning of PM. This may not only be the case during the postacute phase of disease when T cell mediated activation of macrophages is present but also as early as in the first few days after infection. Increased phagocytosis of yeast cells by macrophages isolated from chickens infected with NDV also illustrated the nonspecific character of this type of resistance. Reduced phagocytosis of yeast cells could not be attributed to an increased number of nonphagocytic macrophages but rather to a

decreased number of yeast cells ingested by each phagocytic macrophage. Since vitamin A is necessary in glycoprotein synthesis of membrane receptors (25,26), it might be possible that reduced uptake of yeast particles was the result of disturbed attachment of the opsonized particles to the macrophage surface. In studies with rats and mice, it has been demonstrated that vitamin A stimulates clearance from the circulation by phagocytes after challenge with different types of bacteria and fungi (27,28).

NBT is reduced by reactive oxygen radicals which are generated during the process often refered to as "respiratory burst" (19,20,29). Therefore, reduction of NBT is a measure of intracellular bactericidal activity of macrophages. Vitamin A deficiency depressed the NBT reduction activity significantly in noninfected chickens both in the presence and absence of zymosan A. Moreover, when PM were isolated from chickens infected with NDV followed by a second stimulation with zymosan A in vitro, differences in NBT reduction activity due to vitamin A status were also significant. In contrast, significant differences due to vitamin A status in PM isolated from birds infected with NDV were not observed without additional stimulation with zymosan A. Zymosan A particles prepared from yeast cells were used to stimulate the respiratory burst in vitro. As a consequence, differences in NBT reduction could be partially attributed to the decrease in uptake described earlier. As mentioned before, even without stimulation of the respiratory burst in vitro, differences were still significant in noninfected birds.

Phagocytosis of yeast cells and NBT reduction activity after zymosan A stimulation were correlated significantly ($r = 0.68$, $P < 0.05$) in PM isolated from noninfected chickens fed a diet adequate in vitamin A. However, PM isolated from noninfected, vitamin A-deficient birds did not show such a correlation ($r = 0.23$, NS). Thus it would appear that vitamin A is necessary in the process of oxygen radical formation per se, in addition to the less pronounced need for vitamin A in the uptake of particles.

Activation of PM as found in our experiment 11 and 12 d after virus inoculation indicated an indirect effect of NDV on these cells possibly mediated by T cell-derived lymphokines. It appeared that this NDV-induced activation of macrophages to phagocytose yeast cells was more pronounced in vitamin A-sufficient birds than in vitamin A-deficient counterparts. Similar results were observed in NBT reduction activity after additional stimulation with zymosan A in vitro but not without this additional stimulation. Therefore, it is difficult to draw conclusions concerning the effect of vitamin A deficiency on T cell activation of macrophages. However, a disturbed

lymphokine synthesis, an impaired release of lymphokines from T cells or an impaired lymphokine-receptor binding due to vitamin A deficiency cannot be excluded.

Activation of macrophages by NDV is in disagreement with the results of Ficken et al. (30). They reported depression of phagocytosis of complexes with sheep erythrocyte-IgG and with erythrocyte-complement and reduced intracellular killing of E. coli in respiratory macrophages from turkeys infected with the La Sota strain of NDV. However, von Bülow and Klasen (12) reported activation of bone marrow macrophages expressed by morphological transformation and increased metabolism of cells after infection with a lentogenic strain of NDV in vitro. Moreover, in their experiments, virus-induced activation was not associated with the production of detectable viral antigens in intact transformed cells. Therefore, they suggested that the mechanism of activation resembled lymphokine-induced macrophage transformation (13). The discrepancy between the results discussed could perhaps be attributed to differences in origin of macrophages, the mode of administration of virus and to differences in the time of isolation from the organ.

In addition to activation of macrophages by T cell-derived lymphokines, macrophage-derived monokines or macrophages themselves are also essential prerequisites for initiation and amplification of many immune responses (31). It is interesting to speculate that impairment of humoral and cell-mediated immune responses often seen in vitamin A deficiency (10,11) could be the consequence of such disturbed macrophage function.

In conclusion, our results indicate that vitamin A deficiency impairs functioning of PM both in noninfected and NDV-infected birds. As NBT reduction is more affected than phagocytosis, it appears that oxygen-dependent killing mechanisms in particular rather than attachment and ingestion of particles in PM are impaired in vitamin A deficiency. Infection with NDV increases phagocytosis and NBT reduction in both vitamin A-deficient chickens and their counterparts fed adequate vitamin A. In general, this effect appears to be more pronounced in the latter group. Therefore, vitamin A deficiency impairs functioning of PM both during the early and later stages of infection, and this may have serious consequences for nonspecific host resistance.

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CHAPTER 10

Vitamin A deficiency and Newcastle disease virus infection lower biliary secretion of immunoglobulin A in chickens

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ABSTRACT

The effect of vitamin A deficiency either alone or in combination with Newcastle disease virus (NDV, La Sota strain) infection on tissue distribution of plasma cells and on the levels of immunoglobulins in bile and plasma were investigated. Day-old chickens with limited vitamin A reserves were fed diets ad libitum containing either marginal or adequate levels of vitamin A. At 4 wk of age, half of the chickens in each group were infected with NDV. Vitamin A-deficient birds had significantly lower levels of IgA in the bile and this effect was even more pronounced in deficient chickens infected with NDV, whereas no differences in IgM levels could be found. However, the number of IgA- or IgM-containing plasma cells in mucosal tissues was not affected by vitamin A deficiency and only slightly increased by NDV infection which demonstrated that neither class-switching nor homing of plasma cells is influenced by vitamin A deficiency or NDV infection. These results, together with the slightly increased levels of IgA in plasma found in vitamin A-deficient chickens, suggest that the hepatobiliary transport of IgA has been impaired by vitamin A deficiency. This effect was even more pronounced when those chickens were infected with NDV. In addition, disturbed synthesis of IgA in plasma cells or its subsequent release or both cannot be excluded.

INTRODUCTION

Vitamin A deficiency is associated with increased vulnerability to infection and defects in both natural and acquired defense mechanisms (1,2). The invading infectious agent itself can also affect host resistance (3). The effects of vitamin A deficiency and infection are synergistic; together they can lead to more severe disease processes than each factor alone (1) and the synergism appears, at least in part, to be due to concomitant secondary infection (4,5). This relationship has been observed in vitamin A-deficient chickens infected with Newcastle disease virus (NDV) (6-8): vitamin A deficiency appeared to affect lymphoid cell systems in chickens (7) and, in

combination with NDV infection, to cause substantial loss of lymphocytes from the thymus and bursa of Fabricius as well as rapid loss of body weight (8). Recently we have shown in a model, in which protein-energy malnutrition and secondary infection do not develop, that the number of circulating lymphocytes was markedly depressed in vitamin A-deficient birds during the acute phase of disease after infection with a less virulent strain of NDV (9,10). Moreover, the systemic humoral and cell-mediated immune responses of such vitamin A-deficient chickens infected with NDV were also impaired (11,12). Since many infectious agents including NDV invade the host via mucosal surfaces, mucosal immunity plays an important role in host resistance (13). The systemic and mucosal immune systems are very dependent on the differentiation, distribution and migration of lymphocytes (13). As these processes in systemic lymphoid organs can be affected both by vitamin A deficiency (4,14) and NDV infection (15,16), it could also be expected that similar changes would occur in mucosal-associated lymphoid cells and tissues. Such changes could reduce the relatively high levels of 'background' immunoglobulin, especially secretory IgA (17), at the mucosal surfaces which normally prevent microbial penetration. Recently, several studies have indicated that vitamin A deficiency might affect mucosal immunity (18-23) but knowledge on the subject is still limited.

In the present paper, the effect of vitamin A deficiency either alone or in combination with NDV infection on the distribution of plasma cells in tissues (particularly mucosal tissues) and on levels of IgA and IgM in bile and plasma is reported.

MATERIALS AND METHODS

Animals, diets and experimental design

Data were collected from two separate experiments using a similar animal model described in detail earlier (9). In brief, female day-old White Leghorn chickens (strain Lohmann Selected Leghorn) obtained as progeny of marginally vitamin A-deficient laying hens were fed purified diets ad libitum containing either adequate (1200 retinol equivalents (RE)/kg feed) or marginal (120 RE/kg feed) amounts of vitamin A. Diets were manufactured according to the recommendations of the National Research Council (24) by the Institute of Animal Nutrition and Physiology (IGMB-TNO, Wageningen, the Netherlands). The chickens were divided equally between two separate air-filtered rooms

maintained under reduced pressure, controlled for temperature, relative humidity and light-dark cycles. At the age of 28 d (experiment 1) or 23 d (experiment 2), all chickens in one room were inoculated intraocularly with a 10^8 median embryo-infectious dose per bird of the lentogenic La Sota strain of Newcastle disease virus (Delvax, Gist-Brocades, Delft, the Netherlands). Control birds in the other room were inoculated with phosphate-buffered saline (PBS). In this way, four experimental groups were formed: noninfected and infected groups fed a diet marginally deficient in vitamin A (A-I- and A-I+, respectively), and noninfected and infected groups fed a diet adequate in vitamin A (A+I- and A+I+, respectively). Except for the exposure to NDV, the chickens were not vaccinated against any diseases. At the age of 34 d (experiment 1) or 29 d (experiment 2), the birds were killed and blood, bile (in experiment 2 only) and tissues were collected.

Sampling and preparation of blood, bile and tissues

Blood from the wing vein was collected in heparinized tubes and, after centrifugation, plasma was stored at -20°C. Afterwards, the birds were anaesthetized by intraperitoneal injection with Nembutal (C.E.V.A., Paris, France) and weighed. In order to remove free erythrocytes, from the tissues, birds were perfused with Ringer solution (0.15 M NaCl, 5.6 mM KCl and 2.25 mM $CaCl_2$) until the liver appeared pale. A second perfusion with acid formol (4% v/v formaldehyde and 2% v/v acetic acid, pH 2.5) was used to fix the tissues in situ. Harderian gland, oesophagus, jejunum, caecal tonsil, caecum, spleen, bursa of Fabricius, bone marrow (experiment 1) and jejunum (experiment 2) were dissected out and postfixed for 24 h in acid formol. Tissues were rinsed for a further 24 h in destilled water, dehydrated, vacuum-embedded in Paraplast Plus (Sherwood, U.K.) and serially sectioned at 5 μm. Sections were mounted on poly-L-lysine (MW: 350,000; Sigma Chemical Co., St Louis, MO) coated slides and examined immunohistochemically. In experiment 2, bile was collected from the gall-bladder and stored at -20°C.

Assessment of vitamin A status

Retinol concentration was measured in plasma collected immediately before the birds were killed. A reversed-phase high performance liquid chromatography (HPLC) method modified from that of Driskell et al. (25) was used with retinyl acetate as internal standard. The results obtained were checked against samples of pooled control sera.

Antibodies

Details of the antibodies used are listed in Table 1. Polyclonal antibodies were used for determination of the concentration of IgM and IgA in plasma and bile, and monoclonal antibodies were used for immunohistochemistry.

TABLE 1. Description of antibodies used.

Antibody	Code	Source	Specification (Reference)
Goat anti-chicken IgA	-	Miles Laboratories Ltd., Stoke Poges, Slough, U.K.	Polyclonal IgA purified from the gall bladder
Goat anti-chicken IgM	-	Cooper Biomedical Inc., Malvern, PA	Polyclonal
Mouse anti-chicken IgA	CVI-ChIgA-46.1	G. Koch, Central Veterinary Institute, Lelystad, the Netherlands 2x	Monoclonal, IgG1
Mouse anti-chicken IgG	CVI-ChIgG-47.3		Monoclonal, IgG2a
Mouse anti-chicken IgM	HIS-C12	F.G.M. Kroese, Dept of Histology University of Groningen, Groningen, the Netherlands	Monoclonal, IgG1 (26)

Determination of IgA, IgM and total protein in body fluids

The concentration of IgA and IgM in bile and plasma was determined by a radial immunodiffusion assay (27). Purified biliary IgA (Miles Laboratories Ltd.) and serum IgM (Cooper, Biomedical Inc.) were used as standard assuming a molecular extinction coefficient of 14 (28). All samples were tested in duplicate. Protein concentrations were determined according to the method of Lowry et al. (29) adjusted for microtiter plates using bovine serum albumin (BSA) as standard.

Immunohistochemistry

The immunohistochemical method was based on the principles outlined by Brandtzaeg (30). After deparaffination and elimination of endogenic peroxidase activity using H_2O_2 in methanol (0.3% v/v), slides were treated with a solution of BSA-containing PBS (0.2% wt/v) to avoid nonspecific binding of antibodies. After rinsing with PBS, slides were incubated for 75 min with mouse anti-chicken IgM, IgG or IgA monoclonal antibodies, rinsed again with PBS, and subsequently incubated for 60 min with polyclonal goat anti-mouse peroxidase conjugate (Dakopatts, Glostrup, Denmark). Slides were rinsed with

PBS and finally with Tris-HCl buffer (0.05 M, pH 7.6) before staining for peroxidase activity using DAB (3,3'-diaminobenzidinetetrahydrochloride, Sigma Chemical Co.) and counterstaining with haematoxylin. The following controls were carried out and found negative: firstly, omitting one or both incubation steps with the antibodies; and secondly, liquid-phase preabsorption of the monoclonal antibodies with IgA, IgG or IgM in diluted antiserum (10–20 nmol/ml).

Plasma cell countings

The number of DAB-positive cells was assessed by counting 20 fields (3.2 mm^2 in total) randomly chosen in the caecal tonsil, spleen, bone marrow, bursa of Fabricius and Harderian gland, and in the lamina propria of the oesophagus, caecum and jejunum (not in top of the villi).

Statistical analysis

Since the number of plasma cells showed a large variation between birds within the same treatment group, results were expressed in relative terms. Differences between group means of the concentration of retinol and immunoglobulin in plasma, and the immunoglobulin content in bile were evaluated with Tukey-honestly significant difference statistics. All procedures were based on the principles outlined by Snedecor and Cochran (31) and were performed using a VAX-8600 computer with a SPSS-X software package (SPSS, Chicago, IL) (32).

RESULTS

General health

Body weight was significantly lower in NDV-infected chickens fed a diet inadequate in vitamin A than in noninfected counterparts fed a diet adequate in vitamin A (Table 2). However, vitamin A-deficient chickens infected with NDV were still growing rapidly and their body weights had not plateaued. In both experiments, infection with NDV produced mild signs of disease such as tracheal 'rales', diarrhea and general inactivity, predominantly in vitamin A-deficient chickens. Clinical signs of vitamin A deficiency, such as xerophthalmia and ruffled feathers (33) were not observed.

TABLE 2. Effect of vitamin A nutriture and NDV infection on body weight and plasma retinol concentration.

Group	Body weight		Retinol concentration	
	Exp. 1	Exp. 2	Exp. 1	Exp.2
	g		µmol/l	
A-I-	339 ± 12^{ab}	307 ± 12^{ab}	0.50 ± 0.03^{a}	0.48 ± 0.04^{a}
A-I+	316 ± 9^{a}	285 ± 8^{a}	0.28 ± 0.03^{b}	0.31 ± 0.05^{a}
A+I-	367 ± 12^{b}	342 ± 17^{b}	1.78 ± 0.05^{c}	2.37 ± 0.19^{b}
A+I+	341 ± 11^{ab}	312 ± 7^{ab}	1.67 ± 0.04^{c}	2.00 ± 0.15^{b}

Values are means ± SEM for 6-8 (experiment 1) or 8-12 (experiment 2) chickens/group. Means within a column not sharing a common superscript letter are significantly different at P < 0.05 (Tukey).

Vitamin A status

Plasma retinol concentration was significantly lower in chickens fed a diet inadequate in vitamin A than in chickens fed a diet adequate in vitamin A (Table 2). Moreover, NDV infection lowered (significantly in experiment 1) plasma retinol concentration in chickens with a marginal vitamin A intake when compared to their noninfected counterparts fed the same diet. NDV infection also tended to reduce plasma retinol concentration in chickens fed adequate vitamin A in both experiments.

IgA and IgM concentration in bile and plasma

The most striking effect of both vitamin A deficiency and NDV infection was found in the concentration of IgA in the bile (Fig. 1). Noninfected, vitamin A-deficient chickens had a significantly lower IgA concentration in bile than noninfected birds fed adequate vitamin A and this was even more pronounced when vitamin A-deficient chickens were infected with NDV. NDV infection also tended to lower biliary IgA levels in birds fed a diet adequate in vitamin A when compared with noninfected counterparts fed the same diet. Differences in biliary concentration of IgA could not be attributed to concomitant dilution of bile as was established by measurement of total protein content in bile. In fact, biliary protein content was even higher in vitamin A-deficient birds. IgM concentration in bile tended to be higher in noninfected, vitamin A-deficient birds than in their vitamin A-sufficient counterparts. Although total protein content in plasma of vitamin A-deficient chickens infected with NDV was significantly lower than in their noninfected, vitamin A-sufficient counterparts, plasma levels of IgA and IgM were not or

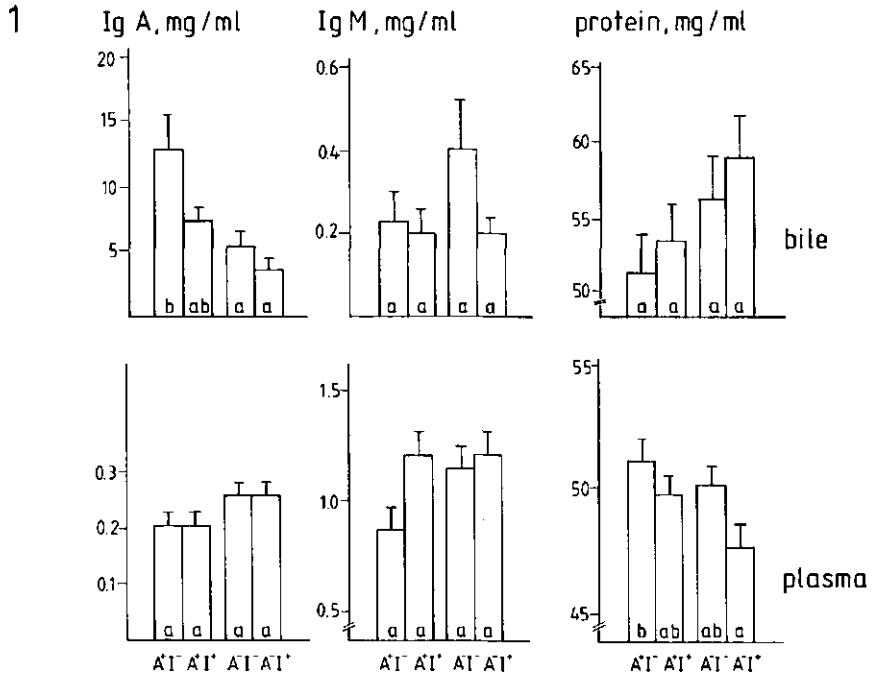
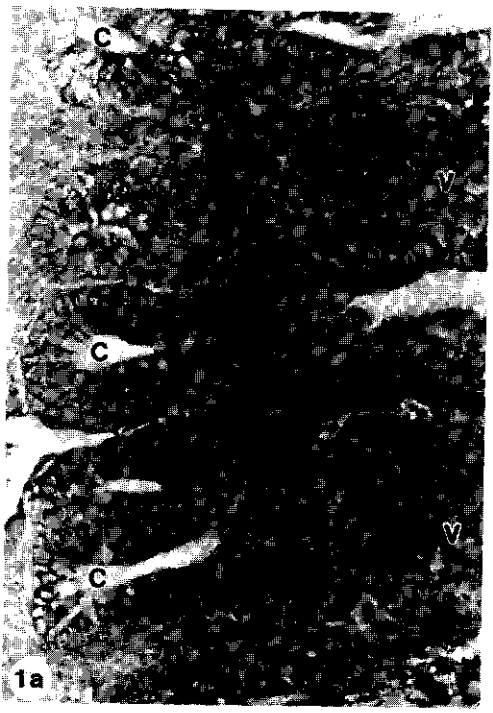


FIGURE 1. Effect of vitamin A deficiency and NDV infection on IgA, IgM and total protein concentration in bile and plasma. Values are means ± SEM for 8–12 chickens/group. Bars not sharing a common letter are significantly different at $P < 0.05$ (Tukey).

FIGURE 2. Plasma cells (arrows) in jejunum of a noninfected chicken fed adequate vitamin A. x300. (1a) CVI-ChIgA-46.1 recognizes many IgA-containing plasma cells which were situated in the lamina propria from the crypts (C) into the villi (V); (1b) HIS-C12 recognizes IgM-containing plasma cells which were predominantly situated in the lamina propria at the upperpart of the crypts.

FIGURE 3. Plasma cells in spleen of a noninfected chicken fed adequate vitamin A. x400. (2a) CVI-ChIgA-46.1-immunoreactive IgA-containing plasma cells which are scarcely present; (2b) HIS-C12-immunoreactive IgM-containing plasma cells which are abundantly present; (2c) CVI-ChIgG-47.3-immunoreactive IgG-containing plasma cells.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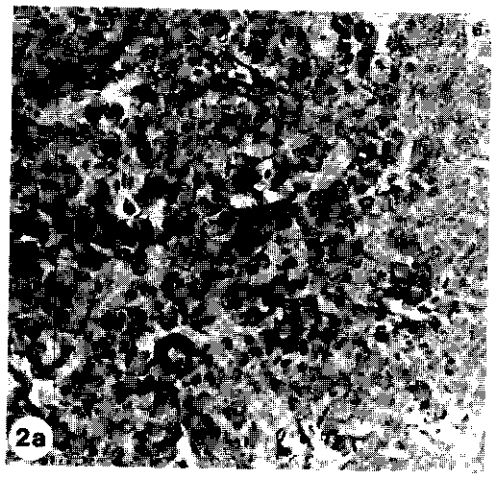
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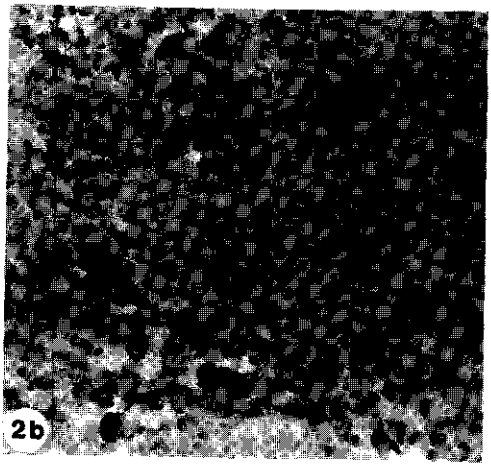
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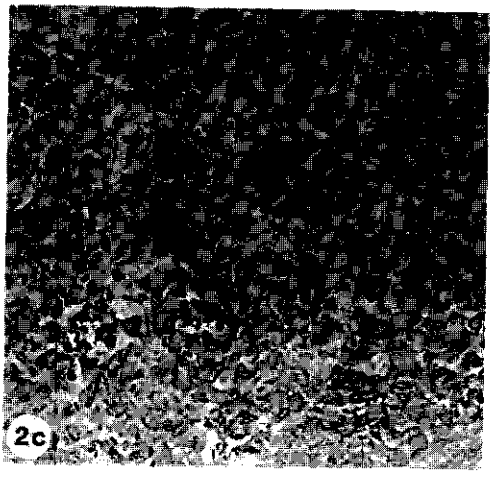
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hardly affected by either vitamin A deficiency or NDV infection. Since the IgA purified from bile is dimeric and dimeric IgA contributes only partially to the total concentration of IgA in plasma, concentrations of IgA in plasma expressed in absolute terms should be interpretated with care.

Distribution of plasma cells in tissues

The immunocytochemical method used appeared to be suitable for detection of IgA-, IgM- and IgG-containing plasma cells in chicken tissues (Fig. 2 and 3). A comparison of the number of plasma cells between various tissues of birds fed adequate vitamin A without infection showed that IgA-containing plasma cells were predominant in jejunum and caecum, and IgM-containing plasma cells in the spleen and bursa of Fabricius (Table 3). IgG-containing plasma cells did not dominate above IgM- or IgA-containing plasma cells in the tissues investigated. In addition, IgA-containing plasma cells were abundant but not predominant in the caecal tonsil and rarely observed in the Harderian gland, oesophagus, spleen, bursa of Fabricius and bone marrow. IgM-containing plasma cells were abundant in the caecal tonsil and caecum but rarely observed in the Harderian gland, oesophagus, jejunum and bone marrow. IgG-containing plasma cells were abundant in the caecal tonsil and spleen but rather scarce

TABLE 3. Effect of vitamin A deficiency and NDV infection on tissue distribution of IgM-, IgG- and IgA-containing plasma cells.

Tissue	Relative number of plasma cells[1]											
	IgM				IgG				IgA			
	A-I-	A-I+	A+I-	A+I+	A-I-	A-I+	A+I-	A+I+	A-I-	A-I+	A+I-	A+I+
Experiment 1												
Harderian gland	-/+	++	-/+	++++	-	-/+	-	+	-/+	++	-/+	+++
Oesophagus	-	++	-	+++	-	-/+	-	-/+	-	+	-	+
Jejunum	++	++	+	++	-	-	-	+	+++	++++	++++	++++
Caecal tonsil	++++	++++	+++	++++	+++	+++	+++	+++	+++	++++	+++	++++
Caecum	++++	++++	+++	++++	+	+	+	+	++++	+++++	+++++	+++++
Spleen	+++++	+++++	+++++	+++++	+++	+++	+++	+++	+	+++	+	+++
Bursa of Fabricius	+++	++	+++	+++	+	+	+	+	+	+	+	+
Bone marrow	-	++	-	++++	-	-	-	-	-/+	+	-/+	++
Experiment 2												
Jejunum	++	++	++	++	ND[2]	ND	ND	ND	+++	++++	+++	++++

1 Relative number of plasma cells for 6-8 (experiment 1) and 5 (experiment 2) chickens/group: -, 0 cells/mm^2; -/+, 0-2 cells/mm^2; +, 3-7 cells/mm^2; ++, 8-20 cells/mm^2; +++, 21-40 cells/mm^2; ++++, 41-120 cells/mm^2; +++++, > 120 cells/mm^2.

2 ND: Not determined.

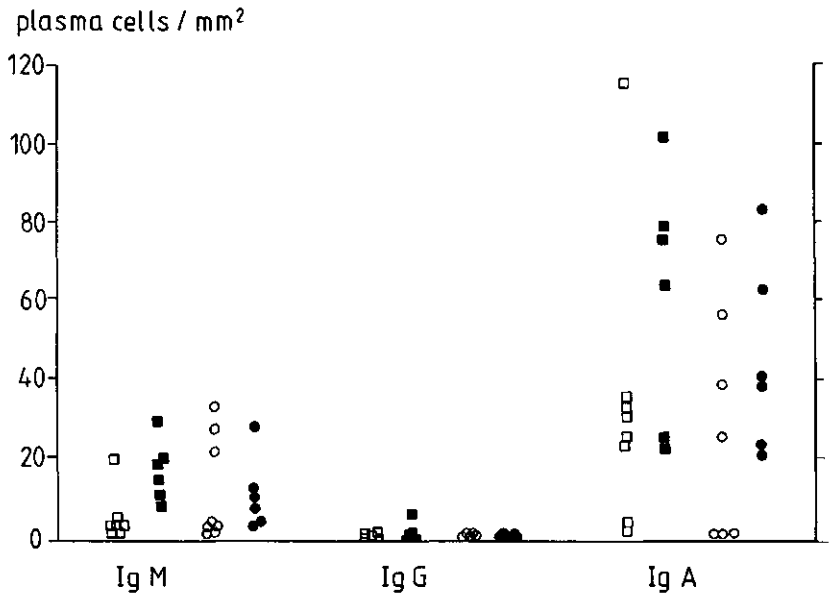

FIGURE 4. Effect of vitamin A deficiency and NDV infection on IgM-, IgG- and IgA-containing plasma cells in jejunum as an example for the large within-group variation observed. Each symbol represent the number of plasma cells per mm^2 tissue in one chicken. (□), A+I-; (■), A+I+; (○), A-I-; (●), A-I+ group.

in the other tissues investigated. Significant differences in the number of plasma cells between treatment groups could not be demonstrated which may be due partly to the large within-group variation. This variation is shown for the jejunum in Figure 4 but was similar in other tissues. Nevertheless, NDV infection tended to increase the number of IgM-containing plasma cells, especially in the Harderian gland, oesophagus and bone marrow and to increase the number of IgA-containing plasma cells in the Harderian gland, spleen and bone marrow. Infection did not affect the number of IgG-containing plasma cells in the tissues investigated.

In experiment 2, similar distribution patterns as in experiment 1 were observed in the jejunum.

DISCUSSION

The results of the present study show that vitamin A deficiency lowered the concentration of IgA in bile and this effect was even more pronounced in deficient chickens infected with NDV. However, the number of IgA-containing plasma cells in mucosal tissues was not affected to any extent by vitamin A deficiency and only slightly by NDV infection. These observations can be explained by disturbed synthesis of IgA in intestinal plasma cells or its subsequent release or both and/or impaired hepatobiliary transport of IgA. The small increase in plasma IgA levels in plasma in vitamin A-deficient chickens also further suggests that the hepatobiliary transport of IgA is impaired by vitamin A deficiency. Similar results have been described in noninfected, vitamin A-deficient rats by Puengtomwatanakul and Sirisinha (21). They reported not only reduced levels of total biliary IgA but also reduced antibody response to sheep red blood cells and Brucella abortus after multiple injections of these antigens in Peyer's patches. Both in chickens and in rats, the hepatobiliary transport of IgA plays a significant biological role in mucosal immunity (13,34). Biliary IgA contributes approximately 90% of the total amount of this immunoglobulin in intestinal secretions in the rat (35) but this proportion could be even higher in chickens, since the concentration of IgA in bile is even higher in this species (36-38). In other species such as humans, dimeric IgA is predominantly transported from mucosa to intestinal lumen as part of a complex with secretory component (SC) which is abundantly present as the extramembranous part of the receptor for polymeric immunoglobulin on the surface of intestinal epithelial cells. In rats and chickens, SC would appear to be present mainly on the surface of the hepatocytes and hence dimeric IgA is transported from intestine to liver and subsequently complexed with SC and released into bile (13,39). When the above factors are taken into account, vitamin A deficiency and/or NDV infection might interfere with the synthesis of SC, with the amount of this glycoprotein present on the surface of hepatocytes and with the IgA-binding capacity of SC. From vitamin A deficiency it is known that it can affect glycoprotein differentiation and synthesis (40,41). Support for impaired synthesis and/or membrane incorporation of SC comes from a study of Sirisinha et al. (18), in which a slight decrease in intensity of immunofluorescent staining for SC in the intestinal cells of vitamin A-deficient rats has been observed. NDV can affect glycoproteins as well by its neuraminidase activity (42).

The possibility that NDV affected the concentration of IgA in bile indirectly by lowering the plasma retinol concentration would not appear to be likely as NDV also tended to affect biliary IgA content in chickens fed adequate vitamin A.

Because plasma IgA levels were only slightly increased in vitamin A-deficient chickens (both infected and noninfected) but not affected in infected chickens fed adequate vitamin A, the impairment of hepatobiliary IgA transport cannot explain adequately the lower IgA levels seen in these groups. It may well be that the synthesis of IgA in and the release of IgA from intestinal plasma cells are also disturbed. However, in vitamin A-deficient mice, immunoglobulin secretion rates per secreting cell have been reported to be unaffected (43) and in our experiments IgM levels in bile like in plasma are not disturbed as well. We are not aware of similar observations concerning the effect of NDV infection on the synthesis of IgA in or its subsequent release from plasma cells.

Impaired mucosal immunity and biliary transport of IgA have also been observed in rats with severe PEM (44,45). Although body weight and plasma protein content in our vitamin A-deficient chickens infected with NDV were significantly lower than in noninfected counterparts fed adequate vitamin A, they were not suffering from PEM. These chickens were still growing rapidly and had not reached a weight plateau. Moreover, total protein content in bile was even slightly higher in those birds.

Plasma cell distribution, both in systemic and in mucosal lymphoid tissues, was not affected by vitamin A deficiency and only slightly affected by NDV infection. The most marked effect was an increase of the number of IgM-containing plasma cells in the Harderian gland, oesophagus and bone marrow following infection. This is not surprising as the Harderian gland and oesophagus are directly exposed to NDV, while the former is an important secondary lymphoid organ involved in the local immune response (46). An increasing number of plasma cells in the bone marrow following infection indicate a systemic response. More or less similar distribution patterns of plasma cells in noninfected chickens have been reported earlier (47,48). An exception should be made for the absence of IgG-containing plasma cells in the lamina propria of the jejunum in our study, while the presence of these cells in intestine have been reported by others (47).

In contrast to our results, impaired homing and functioning of lymphoid cells in mucosal-associated lymphoid tissues have been reported by a number of groups. McDermott et al. (19) found impaired intestinal localization of

mesenteric lymphoblasts associated with vitamin A deficiency and PEM in rats as a result of an altered distribution of glycoproteins on the lymphocyte membrane. Takagi and Nakano (20) observed a reduced lymphocyte-trapping in local lymph nodes of vitamin A-deficient rats as a result of a defect in receptor glycoproteins on the high-endothelial-venules. Finally, Majumder et al. (22,23) observed a reduced number of Peyer's patches in ilea from vitamin A-deficient guinea pigs, while lymphocytes isolated from this tissue showed decreased response following mitogenic stimulation in vitro. Moreover, they found decreased numbers of rosette-forming and immunoglobulin-bearing cells in gut-associated lymphoid tissues. This discrepancy between the results might be attributed to the degree of vitamin A deficiency (and concomitant PEM) and to differences in species.

IgM levels were also measured in bile in order to check for defects in class-switching (49). Although biliary IgM levels were higher in vitamin A-deficient birds, the total level of IgM in comparison with that of IgA was almost negligible. Therefore, it can be concluded that a reduction in biliary IgA levels is not compensated by IgM indicating normal class-switching.

In conclusion, the susceptibility of vitamin A-deficient chickens to infection via the intestinal mucosa might at least partially be the result of a defect in the hepatobiliary transport of IgA. This effect is more pronounced when vitamin A-deficient chickens are infected with NDV. However, disturbed synthesis of IgA in plasma cells or its subsequent release or both cannot be excluded. Reduced levels of immunoglobulin in the intestine, together with the effect of vitamin A deficiency on integrity of epithelia (41), would allow microorganisms to colonize and penetrate mucosal membranes more easily.

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CHAPTER 11

General discussion

INTRODUCTION

In the work presented in this thesis, the relationship between vitamin A deficiency and infection with Newcastle disease virus (NDV) in chickens has been investigated as a model for the study of the relationship between vitamin A deficiency and measles virus infection in humans. In this chapter the results will be discussed with special emphasis on the development of the model, the interaction which has been demonstrated to exist between vitamin A status and NDV infection, the role of vitamin A deficiency per se as opposed to protein-energy malnutrition, the mechanism by which vitamin A status is lowered after infection, the specificity of NDV infection in this interaction, the consequences of vitamin A deficiency on host resistance to NDV infection and the combined effect of both factors on defense of the host to secondary infection, and the extent to which the model can be extrapolated to vitamin A deficiency-associated severe measles infection in humans.

DEVELOPMENT OF THE MODEL

In many experimental models extreme vitamin A-deficient animals have been used to study the effects of vitamin A deficiency. As a consequence, results have been confounded by concomitant protein-energy malnutrition and unintentional, sometimes unobserved infections (1-3). In the studies described in this thesis, the vitamin A deficiency induced was less extreme. In Chapter 2, a method was described for producing marginally vitamin A-deficient chickens capable of remaining healthy for an extended period, without showing clinical signs of vitamin A deficiency. This method is based on the use of two generations of chickens. Laying hens fed a diet with limited vitamin A content for a period of approximately 3 months were used to obtain day-old chickens marginally deficient in vitamin A. Only hens with a narrow range of plasma retinol concentrations were satisfactory for this purpose. Above this range, the day-old chickens were not marginally vitamin A-deficient. Below this

range, egg production and hatchability were affected to a varying extent depending on the degree of vitamin A deficiency. Even when egg production and hatchability remained high in such birds, the day-old chickens were not strong enough to survive the first weeks of life. The advantages of the two-generation model for producing marginally vitamin A-deficient chickens are the increased uniformity and predictability of the chickens with respect to body weight, general health and vitamin A status. Increased uniformity appeared to be very important for the biochemical and immunological tests with these birds. However, it does take about 3 months to produce such chickens. Two-generation models have been used earlier in studies with vitamin A-deficient rats and mice (4,5).

At the age of 4 wk, half of the chickens were infected with the lentogenic, i.e. mildly pathogenic, La Sota strain of NDV. The choice of this strain of NDV was partly based on the fact that experience had shown that mortality within vitamin A-deficient chickens was about 10% after inoculation of a high dose of the virus. Mesogenic or velogenic strains which are more pathogenic, would certainly kill more of these chickens before parameters of interest could be measured. Additional reasons for not using more pathogenic strains are based on the safety of laboratory personnel, possible problems with cross-infection of the control groups and the unacceptably high risk of spread of the virus outside the laboratory. Nevertheless, even with the La Sota strain of NDV, the greatest possible precautions were taken to prevent spread of virus. Although we did not use isolators, birds were housed in rooms provided with filtered air under reduced pressure, and all materials coming in and out were disinfected or destroyed. Exposure to the virus could be confirmed readily by measuring hemagglutination-inhibition antibody titers (6). Experiments with the more pathogenic infectious bronchitis virus and reovirus, such as described in Chapter 5, were carried out in isolators.

Using both pair-fed and ad libitum-fed controls it was possible to correct for a decrease in feed consumption and body weight gain. Although loss of appetite and depression of growth are two of the earliest signs of vitamin A deficiency and NDV infection (7-9), overt protein-energy malnutrition was not seen in any of the experiments described. The limited extent to which plasma albumin concentrations were depressed confirmed the absence of protein-energy malnutrition while the absence of raised plasma concentrations of fibrinogen, a strong acute phase protein in chickens (10), ruled out the presence of secondary infection.

This model, sometimes with slight modifications, has been used in all the experiments described. It should be stressed that many discrepancies between our results and those reported by other investigators can be attributed to the less extreme vitamin A deficiency (i.e. marginal vitamin A deficiency) induced and to the less virulent strain of NDV used in our experiments.

INTERACTION BETWEEN VITAMIN A STATUS AND NDV INFECTION

In Chapter 3, an interaction between vitamin A status and NDV infection was demonstrated. On the one hand, infection with NDV resulted in an increased rate of morbidity in marginally vitamin A-deficient chickens compared with chickens fed adequate vitamin A. On the other hand, within 1 wk of infection, plasma retinol concentrations in the infected, marginally vitamin A-deficient chickens were decreased significantly when compared with that in noninfected counterparts fed the same diet. Plasma retinol concentrations were still significantly lower 6 wk after inoculation. Increased rate of morbidity in vitamin A-deficient chickens following a pathogenic strain of NDV has been observed previously by Squibb and Veros (11) and Bang et al. (12) but they could not demonstrate an effect of NDV infection on vitamin A status as their birds were already extremely vitamin A-deficient. Although several studies have shown increased susceptibility, severity and duration of infection both in vitamin A-deficient humans and animals (1-3) and other studies have demonstrated an effect of infection on vitamin A status (13-15), there are apparently no studies which have proved a direct interaction between vitamin A status and infection in general.

NDV-INDUCED LOWERING OF VITAMIN A STATUS

In Chapter 4, an attempt was made to explain the mechanism by which NDV infection can lower plasma retinol levels in chickens which were marginally vitamin A-deficient prior to inoculation. Although the mechanisms involved were not investigated directly, possible explanations for this phenomenon, based on the results obtained, were proposed. It is suggested that an increased rate of utilization and catabolism of retinol and retinol-binding protein (RBP) by extrahepatic tissues as well as a direct effect of the virus on RBP metabolism in liver could explain the effects observed. The reaction of

the extrahepatic tissues is a general mechanism seen with febrile infections and concerns not only vitamin A but also other nutrients (13,16). Such a hypothesis was supported by the differences observed in plasma retinol and RBP levels, hepatic RBP levels and the proportion of saturation of RBP with retinol between infected and noninfected chickens fed the same diet deficient in vitamin A. However, this hypothesis alone could not satisfactorily explain the results observed. Plasma retinol and RBP levels continued to be reduced significantly while plasma transthyretin and albumin levels were normal during the postacute phase of disease. During this time there were neither clinical signs of disease, secondary infection and diarrhea, nor differences in growth rate between NDV-infected and noninfected birds fed the same diet marginally deficient in vitamin A. Thus, there is no reason to assume that NDV infection increased the rate of utilization or catabolism of vitamin A or RBP by tissues. From the observation that the levels of RBP in liver of vitamin A-deficient chickens infected with NDV were not as high as those in noninfected counterparts fed the same diet, it was concluded that NDV might have a direct effect on RBP metabolism in liver. Support for this hypothesis comes from the observation that NDV and other viruses from the family Paramyxoviridae can act as toxins to hepatic cells (17-19). Moreover, impaired hepatic mobilization of retinol has also been reported in other diseases which affect the liver (20,21). Although other mechanisms, such as impaired absorption (22,23) or altered renal catabolism (24,25), could not be ruled out, such mechanisms would not appear to be significant in our model.

SPECIFICITY OF NDV

In Chapter 5, the specificity of NDV infection in the interaction with vitamin A metabolism was investigated in a similar model to that discussed above, in which NDV was replaced by infectious bronchitis virus (IBV) or reovirus (RV). The interaction observed between vitamin A status and IBV infection was similar. IBV infection produced more severe signs of disease in vitamin A-deficient chickens than in counterparts fed adequate vitamin A. In addition, plasma retinol levels were significantly lower in infected chickens fed a diet inadequate in vitamin A than in noninfected birds fed the same diet. RV infection also affected vitamin A status in premorbid marginally vitamin A-deficient birds but did neither increase susceptibility, severity nor duration of infection. The results from the experiment using IBV suggest

that the interaction between vitamin A status and NDV infection is not specific for NDV. However, it appeared that the mechanism of action by which vitamin A status is affected in NDV infection is, at least partially, specific. RV infection would appear to lower the levels of retinol, RBP, transthyretin and albumin in plasma, and levels of vitamin A in liver by impaired absorption, increased rates of catabolism or utilization of proteins and vitamin A, or directly induced (micro)nutrient-losing enteropathy, or a combination of more than one of these effects. On the other hand, IBV infection would appear to affect levels of retinol in plasma and in liver by increasing the rate of utilization of retinol in tissues. As discussed previously, NDV infection would appear to lower plasma retinol and RBP levels in the absence of a concomitant reduction of vitamin A stores in liver by increasing the rate of utilization by tissues. These processes are possibly aggravated by a direct effect of NDV on RBP metabolism in liver.

HOST RESISTANCE: NONSPECIFIC MECHANISMS

Many viral and other microbial infections start with local invasion of an epithelial surface followed by spread to other tissues. Such spread can be helped by the circulatory system but may be hindered by antiviral substances in body fluids and by phagocytes, either polymorphonuclear or mononuclear (26). A common feature of many of these antiviral mechanisms is that they are nonspecific in nature. Nonspecific host resistance factors, e.g. macrophages, represent the main line of defense during the first few days of primary infection. However, recovery from a fully established infection is probably determined by humoral and cell-mediated immune responses appearing some days after initiation of infection. An intense interaction between B and T lymphocyte-mediated immune responses to virus infection occurs and other factors, such as macrophages, are involved as helpers or mediators (27).

Vitamin A deficiency was shown to impair microbicidal activity and to a lesser extent phagocytosis by peritoneal macrophages in both NDV-infected and noninfected chickens (Chapter 9). Infection with NDV increased phagocytosis and microbicidal activity in both vitamin A-deficient birds and their counterparts fed adequate vitamin A. In general, this effect was more pronounced in the latter group. Thus, it can be concluded that vitamin A deficiency may have serious consequences for functioning of this part of nonspecific host defense. In studies with rats and mice, it has been

demonstrated that vitamin A stimulates clearance of various bacteria and fungi from the circulation by phagocytes (28,29). In addition, it has also been reported that vitamin A deficiency affects other aspects of nonspecific host resistance, such as production of mucus and lysozyme, and integrity of epithelial cells (8,30).

HOST RESISTANCE: SYSTEMIC IMMUNITY

The immune system comprises of a number of organs and several different cell types which have evolved to recognize accurately and specifically non-self antigens on viruses or virus-infected cells prior to their elimination (26,27).

Bang et al. (12) reported that although vitamin A deficiency or NDV infection alone had only moderate effects on lymphoid systems, together they caused substantial or even total loss of lymphocytes from primary lymphoid organs. In contrast to what was found in their model, rapid loss of body weight was not observed in our model. Nevertheless, we could demonstrate marked lymphopenia in the circulation of vitamin A-deficient chickens and this was even more pronounced during the acute phase of NDV infection (Chapter 6). In particular B cells appeared to be affected by vitamin A deficiency. Lymphopenia has been described previously in vitamin A deficiency (12,31-33) and NDV infection (26,34). Subsequent to lymphopenia due to NDV infection, a marked lymphocytosis was observed in control chickens and to a lesser extent in vitamin A-deficient birds. This lymphocytosis that mainly concerns T cells, might be attributed to a change in migration pattern (35) rather than to increased production of new lymphocytes (26,36). Vitamin A deficiency decreased relative and absolute weights of bursa of Fabricius, while relative weight of spleen was increased after NDV infection. Relative and absolute weights of thymus were only affected by vitamin A deficiency in chickens infected with NDV. Similar changes have been reported previously (9,32,36). From the results obtained, it may be concluded that vitamin A deficiency affects lymphoid systems and that this is aggravated by concomitant NDV infection. However, we were not able to demonstrate significant effects on the number of plasma cells in various systemic and mucosal lymphoid organs (Chapter 10).

Vitamin A deficiency resulted in significantly reduced cytotoxic T lymphocyte (CTL) activity to NDV (Chapter 7). This was demonstrated in an

assay system with nonadherent spleen cells as effector cells and adherent spleen cells as target cells at all effector/target ratios tested. In addition, CTL activity could only be demonstrated in peripheral blood lymphocytes from chickens fed the control diet after reinfection, possibly demonstrating a diminished pool of CTL in vitamin A deficiency. As CTL activity plays an important role in the cell-mediated defense to NDV infection (37,38), vitamin A deficiency may have serious consequences for recovery.

Although vitamin A deficiency did not affect the hemagglutination-inhibition antibody response to NDV, both factors either alone or in combination did affect humoral immune responses to selected antigens (Chapter 8). Primary and secondary immunization with antigens differing in thymus dependency was carried out during and after the acute phase of disease, respectively. Sheep red blood cells (SRBC) and bovine serum albumin (BSA) were used as T cell-dependent antigens and Brucella abortus (BA) as a more or less T cell-independent antigen. Vitamin A deficiency and NDV infection did not affect the kinetics of the primary and secondary immune response to the three antigens. However, the level of the primary and secondary IgG response to BSA and of the primary IgG response to SRBC and BA was reduced while the secondary IgM response to SRBC and BA was slightly elevated by vitamin A deficiency. Other studies carried out to investigate the effect of vitamin A deficiency on humoral immune responses revealed some contrasting results. Both reduced (36,39-42) and increased (32,41) specific antibody responses to antigens have been found in vitamin A-deficient animals. Moreover, elevated levels of nonspecific immunoglobulin (IgM) in serum have also been reported (39,41,43). NDV infection reduced primary IgM and IgG responses to T cell-dependent SRBC and BSA but not to T cell-independent BA, indicating a defect in T-helper cell function as has been suggested earlier (44). The combination of vitamin A deficiency and NDV infection resulted in the lowest IgG titers to T cell-dependent antigens. As most pathogens are of the latter type, the risk for secondary infection appears to be increased.

HOST RESISTANCE: MUCOSAL IMMUNITY

Mucosal surfaces are continuously exposed to pathogens. A variety of mechanisms, both immunological and nonimmunological, guard against invading microorganisms. The basis of specific protection at mucosal surfaces is formed by secretory IgA (45). In chickens, the hepatobiliary transport of IgA plays a

significant biological role in mucosal immunity of the gut (46,47). Most of the IgA found in the intestine is produced by plasma cells in mucosal tissues and is transported to the intestine via the gall-bladder (46,47). Vitamin A deficiency lowered the concentration of IgA in bile and this was even more pronounced when birds were also infected with NDV, whereas no differences in IgM levels could be found (Chapter 10). However, the number of IgA- or IgM-containing plasma cells in mucosal tissues was not affected by vitamin A deficiency and only slightly increased by NDV infection which demonstrated that neither class-switching nor homing of plasma cells is influenced by vitamin A deficiency or NDV infection. These observations, together with the slightly increased levels of IgA in plasma found in vitamin A-deficient chickens, suggest that the hepatobiliary transport of IgA has been impaired by vitamin A deficiency and in particular in combination with NDV infection. In addition, disturbed synthesis of IgA in plasma cells or its subsequent release or both cannot be excluded. Similar results have been described recently for noninfected, vitamin A-deficient rats (48). Together with the well documented effects of vitamin A deficiency on epithelial tissues (8), reduced levels of IgA on the surface of the intestinal epithelium would allow microorganisms, such as NDV, to colonize and to penetrate mucosa more easily. Moreover, as NDV infection alone also affected biliary IgA content, it might explain, at least partially, the often observed secondary infection following infection with even mildly pathogenic strains of NDV (9,26).

SIGNIFICANCE OF THE MODEL FOR UNDERSTANDING THE RELATIONSHIP BETWEEN VITAMIN A STATUS AND MEASLES INFECTION

An important observation reported in this thesis is the existence of an interaction between vitamin A status and NDV infection. It appears that premorbid marginal vitamin A deficiency per se without concomitant protein-energy malnutrition can result in a severe form of NDV infection and that even a mildly pathogenic strain of NDV can reduce vitamin A status from marginally deficient to deficient. This interaction results in a vicious circle, in which it is difficult to separate cause and effect but might eventually lead to death. A similar interaction has been proposed for vitamin A status and measles infection in humans. However, the biggest difference between the results observed in our model and that of measles infection in developing countries is that, unlike the situation with children infected with

measles in developing countries, the chickens used in our model do not suffer from protein-energy malnutrition. Measles virus itself has a more marked effect on food consumption and absorption of nutrients in the gastrointestinal tract than the NDV strain used in our model. Nevertheless, our results do indicate that the role of vitamin A deficiency in severe measles infection may be more important than sometimes is suggested.

The work described in this thesis also shows that vitamin A deficiency per se without concomitant protein-energy malnutrition affects host resistance in a multifactorial way. Both nonspecific and specific host defense mechanisms in systemic or mucosal organs and tissues are impaired. A plausible explanation for this impairment is based on the requirement of cells for vitamin A to enable them to synthesize glycoproteins (8,49). Cell receptors and many biological substances such as lymphokines and monokines are comprised of glycoproteins (50). As functioning of host defense is extremely dependent on cell receptor recognition and many biologically active factors (50), it would not be surprising that a lack of vitamin A produces impaired resistance. Impaired host defense mechanisms due to vitamin A deficiency allow NDV to damage host tissues to a greater extent resulting in more severe clinical signs of disease. This can explain why even the La Sota strain of NDV, which is mildly pathogenic under normal conditions, produced more and severe signs of disease in vitamin A-deficient birds. In addition, impaired host defense due to vitamin A deficiency can also explain, at least partially, the more severe form of measles infection seen in developing countries. Apart from its effect on primary infections, secondary infections may become established more readily.

The effect of NDV itself on host defense was not as dramatic as has been reported previously (12). It appears that differences in virulence can account for this. Therefore, we have not been able to demonstrate synergistic effects of vitamin A deficiency and NDV infection on different types of host defense mechanisms. Some of the effects on host defense reported in this thesis which were attributed to NDV, may in fact be the result of a NDV-induced lowering of the vitamin A status. As discussed in Chapter 5, it is not possible to evaluate precisely the contribution that the lowered plasma vitamin A levels make to the severity of disease. However, virus infections do lower immunocompetence and cause damage to epithelial tissues (26). Infection with measles virus is probably more immunosuppressive than that with NDV used in our model.

It has also been shown in this thesis that the interaction between vitamin A status and NDV was not virus specific. However, the mechanism by which vitamin A status is reduced in NDV infection might be partially specific in that RBP metabolism in liver appeared to be affected. This mechanism may also play a role in measles infection as most viruses belonging to the family Paramyxoviridae can affect hepatic cells (17-19). It would also explain why, particularly in measles-infected children in developing countries, such low plasma retinol levels have been observed. As the interaction between vitamin A status and NDV infection did not appear to be virus specific, similar interactions may be of importance in other viral and perhaps also in bacterial and protozoal infections in children in developing countries.

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SUMMARY

Vitamin A deficiency is one of the most important micronutrient deficiencies in developing countries and usually does not occur as an isolated problem but is almost invariably accompanied by protein-energy malnutrition. Xerophthalmia, the term used for all ocular manifestations of impaired vitamin A metabolism, is the most obvious and dramatic manifestation of vitamin A deficiency. However, vitamin A deficiency in even a mild form is associated with increased rates of morbidity and mortality. It is known that vitamin A deficiency affects host defense mechanisms such as integrity of epithelial tissues and functioning of other forms of nonspecific resistance and the immune system itself. There would appear to be a specific relationship between vitamin A status and the severity of measles infection in children in some developing countries. Evidence is mounting that measles is an important risk factor in the development of severe vitamin A deficiency and that vitamin A status at the time of measles infection is critical to the outcome of the disease. However, this possible interaction between vitamin A deficiency and measles infection is poorly understood because of the concomitant actions of protein-energy malnutrition and secondary infection. A well controlled experimental study to examine the interaction between vitamin A deficiency and measles infection is very difficult for three reasons. Firstly, it is impossible to infect vitamin A-deficient children with measles virus for ethical and practical considerations. Secondly, it is impossible to study the effect of vitamin A deficiency per se in children as this is almost always accompanied by protein-energy malnutrition. Thirdly, infection with measles virus produces characteristic signs of disease only in humans and some other primates while infection with the wild strains of the virus in nonprimates does not produce a satisfactory model. For these reasons, it is inevitable that an animal model with another virus with similar properties and a suitable host should be used. Newcastle disease virus (NDV) infection in chickens differing in vitamin A status has been selected as a model to examine the interaction between vitamin A status and the severity of consequences of measles infection in humans. Both viruses belong to the family Paramyxoviridae; measles virus is classified in the genus Morbillivirus and NDV in the genus Paramyxovirus. Although both viruses are certainly not identical, they do have many features in common. Both measles virus and NDV have a pronounced effect on epithelial tissues and both are immunosuppressive. NDV has long been recognized as a suitable model for the investigation of

virus pathogenicity, especially for that of viruses from the family Paramyxoviridae.

The aim of using this model with marginal vitamin A deficiency in the absence of protein-energy malnutrition and secondary infection, was to provide a better insight into the role of vitamin A deficiency per se in the relationship with infection and in particular whether an interaction existed between vitamin A status and NDV infection.

In Chapter 1, the current knowledge of vitamin A deficiency in relation to morbidity and mortality is summarized with special emphasis on measles infection, together with a description of the animal model used and the specific aims of this thesis.

In Chapter 2, a method is described for producing marginally vitamin A-deficient chickens capable of remaining healthy for an extended period, without showing clinical signs of vitamin A deficiency. The principle of this method consisted of working with two generations of chickens, in which laying hens were fed a diet with a limited vitamin A content for a period of approximately 3 months in order to obtain day-old chickens which were marginally deficient in vitamin A.

In Chapter 3, experiments demonstrating an interaction between vitamin A status and NDV infection are presented. On the one hand, infection with NDV resulted in an increased rate of morbidity in marginally vitamin A-deficient chickens when compared with chickens fed adequate amounts of vitamin A. On the other hand, plasma retinol concentrations in chickens which were already marginally vitamin A-deficient prior to infection showed a significant decrease after infection when compared with that in their noninfected counterparts fed the same diet.

In Chapter 4, studies are reported in which an attempt is made to explain by which mechanisms NDV infection can lower plasma retinol levels in chickens which were marginally vitamin A-deficient prior to inoculation. Although it was not investigated directly, a plausible explanation for this phenomenon appeared to be an increased rate of utilization and catabolism of retinol and retinol-binding protein (RBP) by extrahepatic tissues, together with a direct effect of the virus on RBP metabolism in liver.

In Chapter 5, studies on the specificity of NDV infection in the interaction observed are reported using a similar model, in which NDV has been replaced by infectious bronchitis virus (IBV) or reovirus (RV). Since IBV infection resulted in a similar interaction with vitamin A status, it is concluded that the interaction between vitamin A status and NDV infection is

not specific for NDV. However, the mechanism by which NDV can reduce vitamin A status appears to be, at least partly, specific.

In Chapter 6, studies demonstrating that both vitamin A deficiency and NDV infection affect lymphoid organs and circulating lymphocytes are presented. Vitamin A deficiency resulted in marked lymphopenia and this was even more pronounced during the acute phase of NDV infection.

In Chapter 7, results are reported of experiments examining the effect of vitamin A deficiency on cytotoxic T lymphocyte (CTL) activity to NDV as this is one of most important cell-mediated defense mechanisms to viral infection and necessary for recovery from NDV infection. Vitamin A deficiency resulted in significantly reduced CTL activity to NDV. In addition, the results also showed a diminished pool of CTLs in vitamin A deficiency.

In Chapter 8, results of the studies showing that vitamin A deficiency does not affect the hemagglutination-inhibition antibody response to NDV are presented. However, following immunization with selected antigens differing in thymus dependency, the level of the primary and secondary IgG response to bovine serum albumin (BSA) and of the primary IgG response to sheep red blood cells (SRBC) and Brucella abortus (BA) was reduced. However, the secondary IgM response to SRBC and BA was slightly elevated by vitamin A deficiency. NDV infection reduced primary IgM and IgG responses to SRBC and BSA but not to BA when immunization was carried out during the acute phase of disease. This suggests a defect in T-helper cell function. The combination of vitamin A deficiency and NDV infection resulted in the lowest IgG titers to T-cell-dependent antigens.

In Chapter 9, experiments are described which demonstrate that vitamin A deficiency impairs microbicidal activity and to a lesser extent phagocytosis by peritoneal macrophages in both infected and noninfected chickens. Infection with NDV increased phagocytosis and microbicidal activity in both vitamin A-deficient chickens and their counterparts fed adequate vitamin A. In general, this effect was more pronounced in the latter group.

In Chapter 10, investigations on the effect of vitamin A deficiency and NDV infection on mucosal immunity are reported. Vitamin A-deficient chickens had significantly lower levels of IgA in bile and this was even more pronounced in combination with NDV infection. However, the number of IgA-containing plasma cells in mucosal tissues was not affected by vitamin A deficiency and only slightly increased by NDV infection. These results, together with slightly increased levels of IgA in plasma of vitamin A-deficient chickens, suggest that the hepatobiliary transport of IgA has been

impaired in vitamin A deficiency and NDV infection but disturbed synthesis of IgA in plasma cells or its subsequent release or both could not be ruled out.

In Chapter 11, a general discussion with some concluding remarks is presented. The demonstration of the existence of an interaction between vitamin A status and NDV infection is an important observation in this thesis. It appeared that pre-existing marginal vitamin A deficiency per se without concomitant protein-energy malnutrition can result in a severe form of NDV infection and that even a mildly pathogenic strain of NDV can reduce vitamin A status from marginally deficient to deficient. This interaction results in a vicious circle, in which it is difficult to separate cause and effect but might eventually lead to death. A similar interaction has been proposed for vitamin A status and measles infection in humans. However, the biggest difference between the results observed in our model and that of measles infection in developing countries is that, unlike the situation with children infected with measles in developing countries, the chickens used in our model did not suffer from protein-energy malnutrition. In addition, measles virus itself has a more marked effect on food consumption and absorption of nutrients in the gastrointestinal tract, and is more immunosuppressive than the NDV strain used in our model. Nevertheless, our results do indicate that the role of vitamin A deficiency in severe measles infection may be more important than sometimes is suggested. Moreover, the results also show that even marginal vitamin A deficiency can affect host defense mechanisms ranging from nonspecific processes to various aspects of systemic and mucosal immunity. Thus primary infections such as measles become more severe and secondary infections often become established more readily. As marginal vitamin A deficiency is observed in a large part of the population in many developing countries, it may have serious consequences for public health.

SAMENVATTING

Vitamine A-deficiëntie is een van de belangrijkste micronutriënt-deficiënties in ontwikkelingslanden en komt meestal niet als een afzonderlijk probleem voor, maar wordt bijna altijd vergezeld door een eiwit- en energie-tekort. Xeroftalmie, de term die gebruikt wordt voor alle afwijkingen in en aan het oog die het gevolg zijn van een verstoord vitamine A-metabolisme, is de meest duidelijke en aangrijpende uiting van een tekort aan vitamine A. Zelfs een marginale vitamine A-deficiëntie is geassocieerd met een verhoogde morbiditeit en mortaliteit. Van vitamine A-deficiëntie is bekend dat het diverse aspecten van het afweersysteem, zoals de integriteit van de epitheliale weefsels en het functioneren van andere vormen van aspecifieke afweer en van het immuunsysteem, aantast. Een specifieke relatie lijkt te bestaan tussen vitamine A-status en de ernst van mazeleninfectie bij kinderen in sommige ontwikkelingslanden. Steeds meer onderzoek duidt er op dat mazeleninfectie een belangrijke risicofactor is in de ontwikkeling van een ernstige vitamine A-deficiëntie en dat de vitamine A-status op het moment van de infectie van wezenlijk belang is voor het verloop van de ziekte. Over het mechanisme waarmee deze mogelijke interactie tussen de vitamine A-status en mazeleninfectie zou kunnen verlopen, is echter vanwege de bijkomende eiwit- en energietekorten en secundaire infecties weinig bekend. Een goed gecontroleerde studie naar de interactie tussen vitamine A-status en mazeleninfectie is vanwege drie redenen zeer moeilijk. Ten eerste is het ethisch en praktisch gezien onmogelijk kinderen met een vitamine A-deficiëntie te infecteren met het mazelenvirus. Ten tweede is het onmogelijk het effect van vitamine A-deficiëntie per se te bestuderen in kinderen aangezien dit vrijwel altijd vergezeld gaat van een eiwit- en energietekort. Ten derde levert een infectie met het mazelenvirus uitsluitend bij de mens en sommige andere primaten de karakteristieke ziekteverschijnselen op, terwijl een infectie met een in de natuur voorkomend mazelenvirusstam bij niet-primaten geen bevredigend model oplevert. Vanwege deze redenen is het onvermijdelijk dat een diermodel met een ander virus met overeenkomstige eigenschappen en een geschikte gastheer wordt gebruikt. Een model waarin al dan niet vitamine A-deficiënte kuikens met Newcastle disease virus (NDV, in het Nederlands ook wel pseudovogelpestvirus genoemd) worden geïnfecteerd, is geselecteerd om de mogelijke interactie tussen vitamine A-status en ernstige vorm van mazeleninfectie, zoals dat voorkomt bij kinderen, te onderzoeken. Beide virussen behoren tot de familie van de Paramyxoviridae; mazelenvirus is ingedeeld bij het genus Morbillivirus

en NDV bij het genus Paramyxovirus. Ofschoon beide virussen zeker niet identiek zijn, hebben zij toch vele overeenkomstige eigenschappen. Zowel mazelenvirus als NDV hebben een uitgesproken effect op epitheliale weefsels en beiden zijn immunosuppressief. NDV wordt reeds lange tijd gebruikt als model om de pathogeniteit van virussen te onderzoeken, vooral van virussen uit de familie van Paramyxoviridae.

Het doel van het gebruik van dit model met marginale vitamine A-deficiëntie zonder verstorende eiwit- en energietekorten en secundaire infecties was een beter inzicht te krijgen in de rol van vitamine A-deficiëntie per se in de relatie met infectie en meer in het bijzonder om uitsluitsel te krijgen omtrent het bestaan van een interactie tussen vitamine A-status en NDV-infectie.

In hoofdstuk 1 wordt de huidige kennis op het gebied van vitamine A-deficiëntie in relatie tot morbiditeit en mortaliteit samengevat, waarin de nadruk is gelegd op de relatie met mazeleninfectie. Bovendien wordt een beschrijving gegeven van het model en van de indeling van dit proefschrift.

In hoofdstuk 2 wordt een methode beschreven om kuikens te verkrijgen met een marginale vitamine A-deficiëntie die gedurende langere tijd in een ogenschijnlijk gezonde conditie blijven zonder klinische verschijnselen van vitamine A-deficiëntie te vertonen. Het principe van deze methode was gebaseerd op een zogenaamd twee-generatiesysteem, waarin de moederhen gedurende een periode van drie maanden een rantsoen verstrekt krijgt met een onvoldoende hoeveelheid vitamine A, zodat het nageslacht marginaal vitamine A-deficiënt wordt.

In hoofdstuk 3 worden experimenten gepresenteerd die een interactie tussen vitamine A-status en NDV-infectie aantonen. Aan de ene kant leidde NDV-infectie tot verhoogde morbiditeit in marginaal vitamine A-deficiënte kuikens in vergelijking met kuikens gevoerd met een adequate hoeveelheid vitamine A. Aan de andere kant vertoonden kuikens, die marginaal vitamine A-deficiënt waren voor het aanbrengen van de infectie, een afname van de plasma-retinolconcentratie na NDV-infectie, indien vergeleken werd met niet-geïnfecteerde kuikens gevoerd met hetzelfde rantsoen.

In hoofdstuk 4 worden studies beschreven, waarin gepoogd wordt een verklaring te geven volgens welk mechanisme NDV-infectie de plasmaretinolconcentratie kan verlagen in kuikens die reeds marginaal vitamine A-deficiënt waren voor het aanbrengen van de infectie. Ofschoon het niet rechtstreeks is onderzocht, lijken een verhoogd verbruik en afbraak van retinol en retinolbindingseiwit (RBP) door weefsels buiten de lever tezamen met een direct effect van

het virus op het RBP-metabolisme aannemelijke verklaringen voor dit fenomeen.

In hoofdstuk 5 wordt ingegaan op de specificiteit van NDV-infectie in de waargenomen interactie. Daartoe werd een experiment uitgevoerd, waarin het NDV was vervangen door het infectieuze bronchitisvirus (IBV) of reovirus (RV). Aangezien IBV-infectie tot een soortgelijke interactie met vitamine A-status leidde, werd geconcludeerd dat de interactie tussen vitamine A-status en NDV-infectie niet specifiek is. Het mechanisme waarmee NDV de vitamine A-status kan verslechteren lijkt ten dele wel specifiek te zijn.

In hoofdstuk 6 worden studies besproken die aantonen dat zowel vitamine A-deficiëntie als NDV-infectie de lymfoïde organen en de circulerende lymfocyten beïnvloeden. Vitamine A-deficiëntie resulteerde in een opvallende lymfopenie en dit was zelfs nog extremer tijdens de acute fase van NDV-infectie.

In hoofdstuk 7 worden experimenten beschreven, waarin het effect van vitamine A-deficiëntie op de activiteit van cytotoxische T-lymfocyten (CTL) gericht tegen NDV is onderzocht. Het functioneren van CTL behoort tot een van de belangrijkste celgebonden afweermechanismen tegen virale infecties en is noodzakelijk voor het herstel na NDV-infectie. Vitamine A-deficiëntie resulteerde in een significant verlaagde activiteit van CTL tegen NDV. Bovendien wezen de resultaten ook op een verminderde voorraad van CTL ten gevolge van vitamine A-deficiëntie.

In hoofdstuk 8 worden resultaten beschreven die aantonen dat vitamine A-deficiëntie de respons van hemagglutinatie-remmende antilichamen tegen NDV niet beïnvloedt. Echter na immunisatie en reïmmunisatie met een T-cel-afhankelijk (schaperodebloedcellen, SRBC en runderserumalbumine, BSA) of -onafhankelijk (Brucella abortus, BA) antigeen, bleek het niveau van de primaire en secundaire IgG-antilichaamrespons tegen BSA en dat van de primaire IgG-respons tegen SRBC en BA verlaagd te zijn, terwijl de secundaire IgM-respons tegen SRBC en BA in geringe mate was verhoogd door vitamine A-deficiëntie. NDV-infectie verlaagde de primaire IgM- en IgG-respons tegen SRBC en BSA, maar beinvloedde de respons tegen BA niet, hetgeen duidde op een defect in het functioneren van T-helper cellen. Het gezamenlijke effect van vitamine A-deficiëntie en NDV-infectie resulteerde in de laagste IgG-antilichaamtiters tegen T cell-afhankelijke antigenen.

In hoofdstuk 9 worden experimenten beschreven die aantonen dat vitamine A-deficiëntie de microbicide-activiteit en in mindere mate de fagocytose-activiteit van peritoneale macrofagen in zowel geïnfecteerde als niet-geïnfecteerde kuikens verstoort. NDV-infectie verhoogde zowel de

fagocytose- als de microbicide-activiteit in vitamine A-deficiënte kuikens en controledieren. In het algemeen was dit effect groter in de laatstgenoemde groep.

In hoofdstuk 10 wordt het effect van vitamine A-deficiëntie en NDV-infectie op de mucosale afweer beschreven. Vitamine A-deficiënte kuikens hadden significant lagere concentraties IgA in de gal en dit effect was zelfs nog extremer wanneer deze kuikens geinfecteerd waren met NDV. Het aantal IgA-bevattende plasmacellen in mucosale weefsels werd echter niet beïnvloed door vitamine A-deficiëntie en slechts in geringe mate door NDV-infectie. Deze resultaten, tezamen met de licht verhoogde concentratie IgA in plasma van vitamine A-deficiënte kuikens, wijzen op een verstoord hepatobiliair transport van IgA ten gevolge van vitamine A-deficiëntie en NDV-infectie. Een verstoorde synthese van IgA in en/of afgifte van IgA door plasmacellen kon echter niet worden uitgesloten.

In hoofdstuk 11 wordt een algemene discussie met een aantal slotopmerkingen gepresenteerd. Het aantonen van het bestaan van een interactie tussen vitamine A-deficiëntie en NDV-infectie is een van de belangrijkste waarnemingen beschreven in dit proefschrift. Het bleek dat een premorbide marginale vitamine A-deficiëntie per se zonder een bijkomend eiwit- en energietekort kan resulteren in een ernstiger vorm van NDV-infectie en dat zelfs een matig pathogene NDV-stam de vitamine A-status kan verslechteren van marginaal deficiënt tot deficiënt. Deze interactie leidt tot een vicieuze cirkel, waarin het moeilijk is oorzaak en gevolg van elkaar te scheiden, en uiteindelijk kan leiden tot de dood. Een overeenkomstige interactie wordt verondersteld voor te komen tussen vitamine A-status en mazeleninfectie bij de mens. Het grootste verschil tussen de resultaten waargenomen in ons model en de situatie bij kinderen in ontwikkelingslanden die een mazeleninfectie hebben opgelopen is dat bij de kuikens in ons model geen eiwit- en energietekort meespeelt. Bovendien heeft het mazelenvirus zelf een groter effect op de voedselinneming en absorptie van nutriënten in het maagdarmkanaal, en is sterker immunosuppressief dan de NDV-stam zoals dat in ons model is gebruikt. Desalniettemin wijzen de resultaten op het feit dat de rol van vitamine A-deficiëntie in de relatie tot de ernstige vorm van mazeleninfectie misschien belangrijker is dan soms wordt verondersteld. Bovendien wijzen de resultaten op het feit dat zelfs een marginale vitamine A-deficiëntie afweermechanismen kan beïnvloeden, variërend van aspecifieke processen tot diverse aspecten van synthemische en mucosale immuniteit. Daardoor leiden primaire infecties, zoals mazelen, tot ernstiger ziekteverschijnselen, terwijl secundaire infecties

sneller kunnen optreden. Dit kan ernstige gevolgen hebben voor de volksgezondheid in veel ontwikkelingslanden, aangezien een marginale vitamine A-status daar in een groot deel van de populatie voorkomt.

CURRICULUM VITAE

Sake Reinder Sijtsma werd op 16 juli 1961 geboren te Wageningen. Hij behaalde in 1979 het VWO-diploma aan het Christelijk Streek Lyceum te Ede. In september 1979 begon hij met zijn studie 'Voeding van de Mens' aan de Landbouwhogeschool te Wageningen. In juni 1986 haalde hij zijn doctoraaldiploma cum laude met als hoofdvakken Toxicologie (prof. dr. J.H. Koeman) en Voedingsleer (prof. dr. J.G.A.J. Hautvast). Zijn stage vervulde hij bij de Gezondheidsdienst voor Pluimvee te Doorn (prof. dr. M.A.T. Frankenhuis). Vanaf april 1986 was hij als onderzoeksassistent in dienst bij de Landbouwuniversiteit te Wageningen op een gezamenlijk project van de vakgroepen Humane Voeding, Veehouderij en Experimentele Diermorfologie en Celbiologie, alwaar het in dit proefschrift beschreven onderzoek werd verricht. Momenteel is hij werkzaam bij de Nationale Raad voor Landbouwkundig Onderzoek te Wageningen, alwaar hij onderzoek verricht naar 'biological response modifiers' voor veeteeltkundige en veterinaire toepassing.