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Dietary supplementation with vitamin E modulates avian intestinal immunity

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The effect of dietary vitamin E on immunoglobulin A (IgA) antibody production, which acts as the first line of defence at the intestinal mucosa, has not been evaluated in chickens. In the present study the impact of the inclusion of supplementary levels of vitamin E to the diet, on total and antigen-specific IgA antibody titres, T-cell subsets and Ia+ cells, was assessed. From hatching, chickens received a maize-based diet which was supplemented with either 25, 250, 2500 or 5000 mg dl-α-tocopherol acetate/kg. Primary immunisation with tetanus toxoid (T. toxoid) emulsified in a vegetable oil-in-water adjuvant was administered by the intraperitoneal route at 21 d of age. At 35 d of age all birds received an oral booster vaccination of T. toxoid. Significantly higher total IgA antibody titres were present in the day 42 intestinal scrapings of birds receiving the 5000 mg/kg vitamin E-supplemented diet (VESD) (P=0·05) and a notable increase was observed in birds receiving the 250 mg/kg VESD (P=0·06). At days 21 and 42 total serum IgA antibody titres of birds receiving the 250 mg/kg VESD was significantly higher (P<0·05) than the control birds. Following immunisation with T. toxoid, birds receiving the 250 and the 5000 mg/kg VESD had elevated anti-T. toxoid IgA antibody titres in final day intestinal scrapings, which, for the latter group was statistically significant (P=0·02). Both of these groups also demonstrated increased titres of anti-T. toxoid IgA in the serum at day 42. Birds receiving the 250 mg/kg VESD exhibited a notable increase in the percentage of T-helper cells and Ia+ cells in peripheral blood on day 26. The results illustrate the potential for some levels of dietary vitamin E supplementation to act as an immunomodulator of total and antigen-specific IgA antibody.

Vitamin E: Immunoglobulin A: Avian intestinal immunity: Chickens

The addition of vitamin E to diets at concentrations higher than the requirements for nutritional and reproductive needs (Bendich *et al.* 1986; Tengerdy, 1990) will modulate many facets of the immune system. This includes cell-mediated and humoral immune responses, macrophage function and phagocytosis and improved resistance to disease (Finch & Turner, 1996). Vitamin E influences the development of the immune system in chickens, as a deficiency reduces growth of lymphoid organs (Marsh *et al.* 1986) and the number of circulating lymphocytes (Dietert *et al.* 1983). In contrast, chicks on vitamin E supplemented-diets have an increase in the percentage of CD4+ thymocytes and splenocytes (Erf *et al.* 1998), increased lymphocyte proliferation (Haq *et al.* 1996), and an increase in heterophils:lymphocytes (Boa-Amponsem *et al.* 2000). Supplementary dietary vitamin E generally improved antibody production (Franchini *et al.* 1986; Haq *et al.* 1996; Gore & Qureshi, 1997; Friedman *et al.* 1998; Boa-Amponsem *et al.* 2000), macrophage activity and phagocytic potential (Tengerdy & Brown, 1977; Gore & Qureshi, 1997; Qureshi & Gore, 1997). Furthermore, vitamin E supplementation of poultry diets has improved resistance to disease challenges with *Eimeria tenella* (Colnago *et al.* 1984) and *Escherichia coli* (Heinzerling *et al.* 1974; Tengerdy & Brown, 1977).

Studies in chickens of Newcastle disease virus (Haq *et al.* 1996; Friedman *et al.* 1998), *E. coli* (Heinzerling *et al.* 1974; Tengerdy & Brown, 1977; Friedman *et al.* 1998) and *Eimeria tenella* (Colnago *et al.* 1984) have shown that supplementation of the diet with vitamin E improves antibody (agglutination and immunoglobulin (Ig) G isotype) responses to pathogens which interact at mucosal

Abbreviations: Ig, immunoglobulin; ISS, intestinal scrapings supernatant fraction; MHC II, class II major histocompatibility molecules; PB, peripheral blood; Th, T-helper; T. toxoid, tetanus toxoid; VESD, vitamin E-supplemented diet.

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surfaces. Local mucosal production of IgA antibody plays an important role in protecting mucosal surfaces from pathogens (Muir *et al.* 2000*a*), but the influence of vitamin E on total or antigen-specific IgA antibody levels has not been evaluated in chickens. However, the potential for vitamin E supplementation to increase IgA antibody production has been demonstrated in rodents fed diets containing between 0·1 % (Kaku *et al.* 1999) and 0·2 % (Gu *et al.* 1999) tocopherol. More specifically increased IgA productivity of lymphocytes in the mesenteric lymph nodes (Gu *et al.* 1999; Kaku *et al.* 1999), and elevated IgA antibody levels in serum (Kaku *et al.* 1999) and at the intestinal surface (Watson & Messiha, 1987) have been observed.

The induction of an antigen-specific antibody-based immune response requires the antigen to be expressed by antigen-presenting cells in association with class II major histocompatibility molecules (MHC-II). The Ia antigen is an MHC-II product, and its expression provides an indication of the level of antigen presentation via MHC-II. Downstream from antigen-presentation IgA antibody production is closely regulated by T-cells, in particular T-helper (Th) cells (Kiyono & McGhee, 1994), and, an increase in the Th cell population favours increased antibody production. A number of studies investigating the impact of vitamin E on immune responses have observed alterations in Ia+ and T cell subset populations (Chang *et al.* 1994; Erf *et al.* 1998). In the present study the percentage of Th cells (CD4+), T suppressor cells (CD8+) and Ia+ cells in peripheral blood (PB) were determined before and after vaccination of the chickens.

The present study was designed to determine if dietary supplementation with vitamin E at levels above those required to meet nutritional requirements (National Research Council, 1994), and, including levels shown to increase IgA production in rodents (Gu *et al.* 1999), would upregulate IgA antibody production in chickens. Chicks received vitamin E-supplemented diet (VESD) for the duration of the study, and IgA antibody titres were determined before and after vaccination with an immunisation protocol which stimulates significant IgA antibody at the intestinal surface (Muir *et al.* 1995). In particular the titre of total and anti-antigen IgA antibody at the intestinal mucosa and the percentage of CD4+, CD8+ and Ia+ cells was determined.

Materials and methods

Chickens and diets

One-d-old male broiler chickens (Cobb) were obtained from Inghams hatchery (Casula, Australia). Before dispatch the chicks were vaccinated with Marek's disease virus and infectious bronchitis virus. Chicks were housed in wire-floor brooders until 18 d old, and then transferred to carry-on cages in a continuously illuminated room maintained at 22°C. Feed and water were available *ad libitum*.

At 1 d old all chicks were randomly allocated to twenty-five pens, each containing six chicks. Five pens were randomly allocated to each dietary treatment, which the chicks received for the duration of the study. All treatment groups received a maize-based basal diet (Table 1) containing 120 mg tocopherol acetate/kg determined by HPLC analysis (Lumley, 1993). Of this, 50 mg vitamin E/kg was derived from the commercial broiler premix (BASF, Homebush, NSW, Australia). To prepare the VESD, the basal diet was supplemented with 0, 25, 250, 2500 and 5000 mg dl-α-tocopherol acetate/kg in Lutavit E 50 S (BASF), which consisted of 50 % dl-α-tocopherol acetate in a milk powder–gelatin matrix. Between days 21 and 42 feed intake was determined for each treatment group, and individual bird weights were recorded on days 21 and 42. Feed conversion ratio was calculated for this period.

Table 1. Composition of the basal experimental diet

Ingredients	g/kg
Maize	566·8
Soyabean meal	360·0
Vegetable oil	30·0
Limestone	16·6
Dicalcium phosphate	14·3
Vitamin–mineral premix (broiler)*	5·0
Sodium chloride	3·0
DL-methionine	2·3
Choline chloride	2·0
Calculated analysis	
Metabolisable energy (MJ/kg)	12·9
Crude protein	210·0
Lysine	11·8
Methionine–cystine	9·0
Ca	10·1
Total P	6·9

* Vitamin–mineral premix (BASF, Homebush, NSW, Australia) provided (/kg diet) vitamin A, 3·6 mg, vitamin D_3, 60 μg, thiamine, 0·8 g; vitamin B_{12}, 6 mg; folic acid, 0·6 g; Co, 0·04 g; I, 0·2 g; Mo, 0·04 g; riboflavin, 5 g; pyridoxine, 2·4 g; vitamin E, 10 g; menadione, 0·8 g; pantothenic acid, 6 g; niacin, 24 g; biotin, 0·06 g; Mn, 40 g; Zn, 32 g; Mg, 40 g; Fe, 32 g; Cu, 4 g; Se, 0·04 g; endox, 50 g; liquid paraffin white, 10 g.

Ethical considerations

All experimental procedures were approved by the University of Sydney Animal Ethics Committee, and complied with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Antigen and vaccinations

A crude preparation of tetanus toxoid (T. toxoid), prepared by formalin treatment of *Clostridium tetani* culture supernatant fractions was obtained from Commonwealth Serum Laboratories, Melbourne, Australia. This was adjusted to 1500 limit flocculation units/ml by the addition of sterile PBS (pH 7·3).

At 21 d of age all chickens received a primary, intraperitoneal immunisation with T. toxoid emulsified in a vegetable oil-based adjuvant (Husband, 1993), with Quil A (Superfos, Denmark; 1 mg/dose). At 35 d of age all chickens received an oral booster immunisation.

Sample collections

Blood samples were collected from the jugular vein of each

chicken on days 21 and 42. Serum was prepared and stored at −20°C until assayed. On days 19, 26, 33 and 40 samples of PB were collected from six chickens in each treatment group for determination of CD4+ and CD8+ T cell subsets and Ia+ cells, using flow cytometry.

At the end of the experiment all chickens were euthanased by intravenous administration of sodium pentobarbitone. Samples of intestinal scrapings were obtained from the length of jejunum after the serosal and mucosal surfaces were washed in ice cold PBS. The samples were immediately frozen on dry ice and stored at −80°C. The intestinal scrapings supernatant fraction (ISS) was collected for antibody determination following ultracentrifugation at 24000 ***g*** for 60 min (Duncan *et al.* 1978).

Antibody detection by ELISA

An indirect ELISA was used to determine T. toxoid-specific IgA and total IgA in serum and ISS. To determine IgA antibodies to T. toxoid polysorb ELISA plates (Nunc Immuno, Medos Company, Sydney, Australia) were coated with 100 μl purified T. toxoid (diluted 1:100 in carbonate buffer), and incubated overnight at 4°C. All subsequent incubations were for 1 h at 37°C. Between each incubation, plates were washed twice in washing buffer (0·05 % Tween 20 and 0·5 M NaCl in PBS) and PBS, then tapped dry. Plates were blocked with 0·25 % gelatin in washing buffer. Serum was diluted 1:25, ISS 1:20 and the horseradish peroxidase-conjugated goat anti-chicken IgA (Bethyl Laboratories, Montgomery, TX, USA) was diluted 1:100 in washing buffer containing 1 % bovine serum albumin. Neat 2·2′-azino-d:[3-ethyl-benzthiazoline sulfonate] (ABTS) substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA) was incubated for 30 min at room temperature and the reaction stopped with 1 % sodium dodecyl sulfate (Kirkegaard and Perry Laboratories).

For total IgA analysis, maxisorb ELISA plates (Nunc Immuno; Medos Company, Sydney, Australia) were coated with mouse anti-chicken IgA (Southern Biotechnology Associates, Birmingham, AL, USA) diluted 1:1000 in carbonate buffer. Serum collected on day 21 was diluted 1:200, day 42 serum was diluted 1:400 and ISS diluted 1:500 in washing buffer. All other steps were identical to the indirect ELISA for measuring anti-T. toxoid IgA.

Absorbance values were read at 405 nm in a Spectro Max 250 (Molecular Devices, Springvale, CA, USA) plate reader. A negative buffer blank and a hyperimmune positive reference standard were included in each plate, and all samples were analysed in triplicate. Sample dilutions were adjusted to provide optical density readings on the linear portion of the standard curve, approximately 20 % below saturation point. Optical densities are expressed as a percentage of the hyperimmune positive control.

Hyperimmune antiserum

Hyperimmune antisera were obtained from chickens immunised intraperitoneally with 1500 limit flocculation units T. toxoid/ml in a vegetable oil-in-water adjuvant and Quil A (1 mg/dose) at days 19 and 33 of age. A 0·5 ml oral booster of 1500 limit flocculation units T. toxoid/ml was delivered by gavage at 47 d of age. Blood was collected 7 d later, and serum prepared and stored at −20°C.

Preparation of lymphocytes for flow cytometric analysis

Blood taken from the jugular vein was collected into heparinised tubes. A total leucocyte count was determined manually by preparing a 1:200 dilution of whole blood in Natt and Herrick's solution (Campbell, 1995).

Heparinised blood was centrifuged at 1300 ***g*** for 20 min. The buffy coat was diluted in an equal volume of PBS and then layered onto 5 ml of Ficoll Paque (Pharmacia Biotech, Sweden). Following centrifugation at 900 ***g*** for 15 min the layer at the interface was removed and washed twice in PBS–5 % fetal calf serum by centrifugation at 300 ***g*** for 5 min, before enumeration and viability assessment by trypan blue exclusion.

Flow cytometric analysis

Approximately 10^7 lymphocytes in 100 μl PBS–fetal calf serum were incubated with 50 μl optimal dilution of either monoclonal mouse anti-chicken CD4, CD8 or Ia (Southern Biotechnology Associates) at 4°C for 20 min. Each sample was washed twice in 2 ml PBS, then incubated with 100 μl fluorescein isothiocyanate-conjugated affinity purified goat anti-mouse immunoglobulin (Southern Biotechnology Associates) for 20 min at 4°C in the dark, and washed as before. The cells were fixed in 0·1 % paraformaldehyde in MilliQ water (Millipore, North Ryde, NSW, Australia), stored at 4°C overnight and analysed within 24 h of fixing. For each sample, data were collected from 10000 events using an Ar ion laser providing light at 488 nm. Analysis was performed on the gated lymphoid population visually selected from the forward angle and 90° light scatter properties of the dot plot cloud. Both acquisition and analysis were undertaken on a FACScan flow cytometer (Becton Dickinson, Sydney, Australia) using Cell Quest software (Becton Dickinson). For each chicken a positive control tube was analysed to ensure minimal non-specific binding of the anti-mouse fluorescein isothiocyanate secondary antibody.

Statistical analysis

To account for heterogeneity of variance all data were log-transformed before analysis. A one-way ANOVA was performed to determine between treatment group means. Between group comparisons were determined using a Tukey's test (Sokal & Rohlf, 1995). Statistical significance was set at $P<0·05$.

Results

Bird performance

Birds were clinically normal throughout the study and between days 21 to 42 there were no significant differences

between treatment groups in average body weight gain or feed conversion ratio (data not shown).

Total immunoglobulin A antibody

Total IgA present at the intestinal surface, ISS, on day 42 (Fig. 1) was significantly enhanced (P=0·05) in birds receiving the 5000 mg/kg VESD and notably elevated (P=0·06) in birds receiving the 250 mg/kg VESD. Interestingly, the group mean total IgA antibody titres in the ISS in birds receiving either the 25 or the 2500 mg/kg VESD did not differ from that of the control birds.

Mean group serum total IgA antibody titres for days 21 and 42 are presented in Fig. 1. On day 21, birds receiving the 250 mg/kg VESD had significantly higher (P=0·03) total IgA titres than the control group. On day 42, significantly higher total IgA antibody titres were measured in the serum of birds receiving the 250 mg/kg VESD (P=0·05) and the 5000 mg/kg VESD (P=0·003) compared with the control group. However, birds fed the 2500 mg/kg VESD did not show any upregulation in total IgA antibody titres.

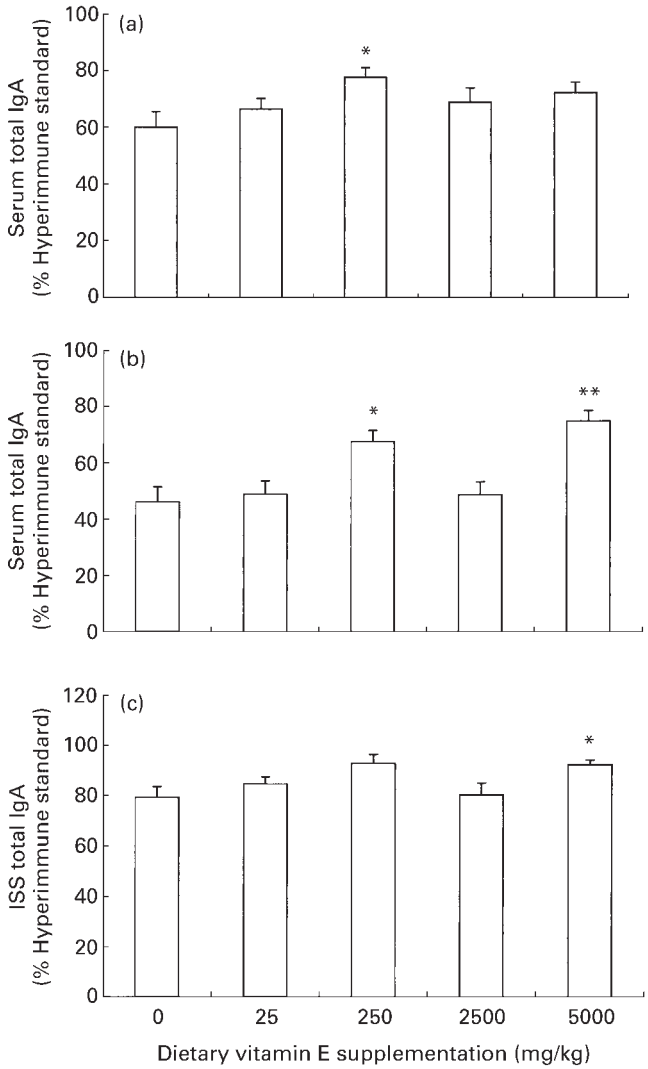

Fig. 1. Total immunoglobulin (Ig)A antibody, determined by ELISA, in serum at day 21 (a), day 42 (b), and in intestinal scrapings supernatant fraction (ISS) on day 42 (c) of birds receiving basal diet only or basal diet with 25, 250, 2500 or 5000 mg supplementary vitamin E/kg diet from day 1–42. Data are expressed as a percentage of hyperimmune positive serum. Histograms are the mean of data from thirty birds per group and vertical bars are standard errors. Mean values are significantly different from that of the control group: *P<0·05, **P<0·01.

Anti-tetanus toxoid immunoglobulin A antibody

Similar trends to those observed in total IgA antibody titres are evident in serum and ISS anti-T. toxoid IgA antibody titres on day 42 (Fig. 2). Group average serum anti-T. toxoid IgA titres were highest in birds receiving the 250 mg/kg VESD, followed by birds receiving the 5000 mg/kg VESD; however, statistically there were no significant differences between VESD treatment groups. ISS anti-T. toxoid IgA antibody titres were significantly elevated only in birds receiving the 5000 mg/kg VESD (P=0·02).

Peripheral blood T cell subsets and Ia+ cells

The percentage of CD4+ and CD8+ T cell subsets and Ia+ cells in PB were determined on days 19, 26, 33 and

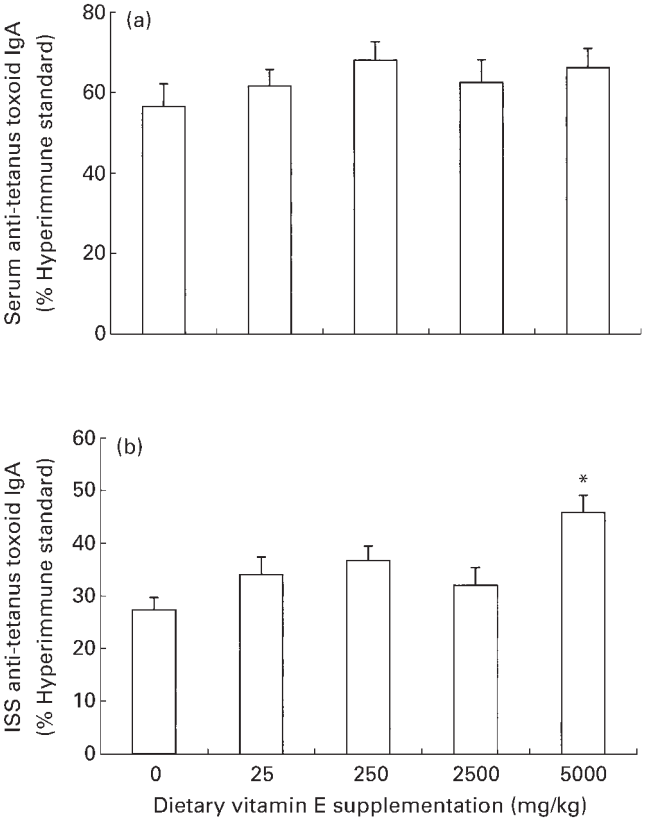

Fig. 2. Anti-tetanus toxoid immunoglobulin (Ig)A antibody, determined by ELISA, in serum (a), and intestinal scrapings supernatant fraction (ISS) (b) on day 42 of birds receiving basal diet only or basal diet with 25, 250, 2500 or 5000 mg supplementary vitamin E/kg diet from day 1–42. Data are expressed as a percentage of hyperimmune positive serum. Histograms are the mean of data from thirty birds per group and vertical bars are standard errors. Mean value is significantly different from that of the control group: *P<0·05.

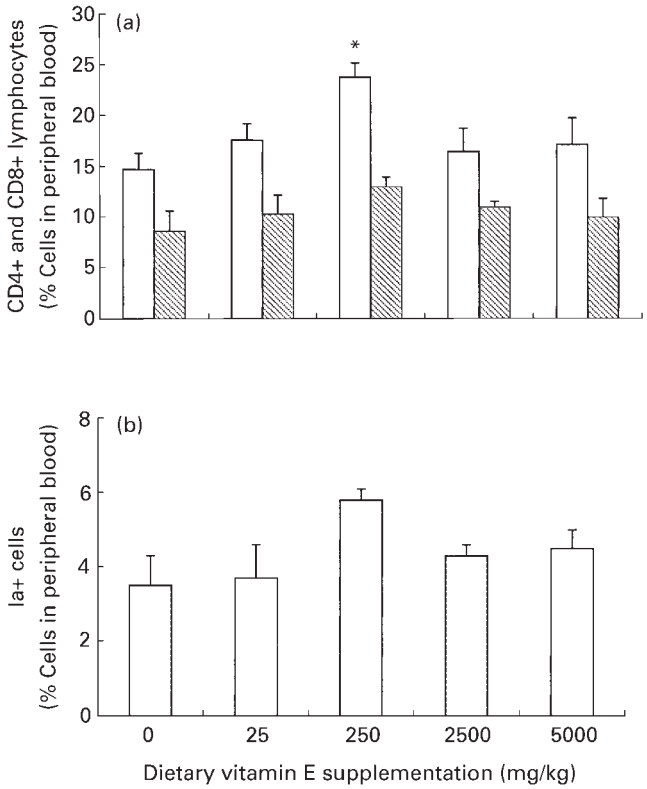

Fig. 3. CD4+ (□) and CD8+ (▨) T cell subset (a) and Ia+ cells (b) distribution determined by flow cytometry on day 26 of birds receiving basal diet only or basal diet with 25, 250, 2500 or 5000 mg supplementary vitamin E/kg diet from day 1–42. Data are expressed as a percentage of leucocytes in peripheral blood. Histograms are the mean of data from six birds per group and vertical bars are standard errors. Mean value is significantly different from that of the control group: *$P<0{\cdot}05$.

40. It was only on day 26, 5 d post primary immunisation, that any statistically significant alterations were observed. On day 26 all groups receiving VESD had an increase in the group mean percentage of CD4+ cells compared with the control group. However, birds receiving the 250 mg/kg VESD had a statistically significant increase ($P=0{\cdot}05$) in the percentage of CD4+ cells (Fig. 3), and an increase in the total number of CD4+ cells (data not shown). These birds also demonstrated a notable increase in the percentage of Ia+ cells (Fig. 3) ($P=0{\cdot}06$) in PB. Despite the alterations in antibody titres seen in birds receiving the 5000 mg/kg VESD (Figs. 1 and 2), no significant alterations in PB cellular subsets were observed in this group.

Discussion

IgA-producing plasma cells located in the intestinal lamina propria, which originate from local sites such as the bursa of Fabricius and caecal tonsils (Muir *et al.* 2000*b*), are the main source of IgA found at the avian intestinal surface. The local intestinal production of IgA antibody is the first line of defence against pathogens which gain entry to the host through the intestinal mucosa (Muir *et al.* 2000*a*). From our previous studies it was expected that upregulation of intestinal IgA antibody production would improve host defence against intestinal pathogens (Muir *et al.* 1998). The present study is the first report demonstrating the ability of vitamin E to enhance mucosal IgA antibody production in chickens. Previous avian studies have not examined IgA production but have shown the potential of VESD to increase total circulating antibody (Heinzerling *et al.* 1974; Tengerdy & Brown, 1977; Jackson *et al.* 1978; Franchini *et al.* 1986; Friedman *et al.* 1998; Boa-Amponsem *et al.* 2000).

The immunisation protocol utilised is specifically designed to stimulate a local intestinal immune response; however, the trend in IgA antibody titre at the intestinal site is mirrored in serum IgA antibody titres. Consistently, the most significant increases in total IgA in the ISS and serum were observed in birds receiving diets supplemented with 250 and 5000 mg vitamin E/kg. Similar trends in total IgA antibody levels were observed in anti-T. toxoid IgA antibody titres in ISS and serum at day 42. Despite this, no significant immunomodulatory effect was observed in birds fed the 2500 mg VESD/kg in either total or antigen-specific IgA antibody production. A non-linear dose effect of vitamin E supplementation on avian immunoglobulin production has been previously reported (Jackson *et al.* 1978; Friedman *et al.* 1998). However, it is difficult to compare these studies as a number of variables, including the age and strain of the birds, the level of vitamin E supplementation and the immunisation and/or challenge conditions differ between experiments.

The differentiation and maturation of B cells into IgA-producing plasma cells is closely orchestrated by Th cells, through their cytokine profile and cell–cell contact (Husband & Dunkley, 1990; Hodgkin *et al.* 1991). Alterations in IgA antibody production may therefore be accompanied by changes in the percentage of CD4+ Th cells. A number of reports have identified the potential for dietary vitamin E supplementation to invoke CD4+ Th cell activity (Tanaka *et al.* 1979), enhancing antibody responses and improved disease resistance. Erf *et al.* (1998) observed increased percentages of CD4+CD8− thymocytes and CD4+ T cells in the spleen, in 7-week-old birds which had been maintained on a diet containing 87 mg dl-α-tocopherol acetate/kg feed. No significant impact was observed on splenic B cells. Further, lymphocytes of athymic nude mice (Cook, 1991) failed to demonstrate a 'typical' improved response following vitamin E supplementation. Chickens receiving diets deficient in vitamin E experienced a reduced proportion of T cells, particularly CD4+ T cells, in PB (Chang *et al.* 1994).

In the present study, a significant ($P = 0{\cdot}05$) increase in the percentage of CD4+ cells of birds receiving the 250 mg/kg VESD, compared with the control birds, was observed on day 26, 5 d after primary intraperitoneal immunisation with T. toxoid. Further, on days 33 and 40 these birds demonstrated an elevation (not statistically significantly) in the percentage of CD4+ cells and, on day 42, higher mean serum and ISS total and anti-T. toxoid IgA compared with control birds (Figs. 1 and 2). The alterations in PB derived CD4+ cells observed during the present experiment reflect the relationship between PB and the focal point of the immune response

at that time, as dictated by the immunisation protocol. Alterations in PB-derived CD4+ T cells will be most evident following stimulation of the systemic immune system, as with the day 21 primary intraperitoneal vaccination used in the present study. A subsequent oral booster immunisation will invoke a local immune response at the intestinal site. The small increase in PB-derived CD4+ cells observed on day 40 reflects an increase in intestinal lamina propria CD4+ cells involved in the induction of the local intestinal immune response. These outcomes contrast with reports from rodent studies where VESD increased lymphocyte IgA production concurrently with a reduction in the percentage of CD4+ cells derived from the spleen (Gu *et al.* 1994, 1999) and mesenteric lymph nodes (Gu *et al.* 1999). However, in contrast to the present study, the rats were not immunised.

On day 26, birds receiving the 250 mg/kg VESD also had a notable increase ($P=0{\cdot}06$) in the mean percentage of Ia+ cells in PB (Fig. 3). The Ia antigen is expressed on B cells, mitogen-activated T cells and a subpopulation of monocyte–macrophages (Ewert *et al.* 1984), which are closely involved in the induction of a humoral immune response. Day 26 was the only occasion when a significant alteration in Ia+ cells was observed. It is interesting to note that the birds receiving the 250 mg/kg VESD had significantly enhanced total IgA antibody titres and elevated anti-T. toxoid IgA antibody titres following immunisation and also demonstrated increases in the percentage of CD4+ and Ia+ cells in PB at day 26. If viewed in isolation, it could be concluded that these cells are the main contributors to enhanced antibody production by vitamin E. However, birds receiving the 5000 mg/kg VESD had statistically significant increases in IgA antibody titres in the absence of any significant alterations in the percentage of these cellular subsets. As alterations in cellular subsets can be transitory, changes in subset percentages outside of the sampling time may have occurred.

The mechanism/s of vitamin E immunomodulation require further elucidation. Vitamin E has been shown to influence cell function and homeostasis, and prostaglandin synthesis, which, through a number of interrelated processes mediate immune function (Meydani, 1995; Meydani & Beharka, 1996; Moriguchi & Muraga, 2000). Avian studies also support the complex interrelationships between vitamin E, prostaglandin production, phagocytosis and antibody production (Tengerdy & Brown, 1977; Likoff *et al.* 1981; Dietert *et al.* 1983; Gore & Qureshi, 1997; Qureshi & Gore, 1997). The present study has demonstrated the potential for some levels of dietary vitamin E supplementation to modulate IgA antibody titres. Upregulation of IgA antibody titres was observed in unimmunised birds, as total IgA antibody, and following immunisation, as anti-antigen IgA antibody. Increased IgA antibody was present in the circulation and at the local intestinal site. It would appear that a number of mechanisms are involved in the induction of elevated antibody production, including IgA, during dietary supplementation with vitamin E.

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