only a negligible amount of D-alanine was detected in the GF mice.

However, we have found that the free D-amino acid content in the serum, kidney, liver, brain and small intestine[9], of GF mice was not different from that of SPF mice. This suggests that the free D-amino acids in those tissues are not microbial origin.

The time of appearance and the rate of increase of D-amino acids were examined using suckling mice (fig.). Mutant mice lacking DAAO were used in the experiment, since the amounts of free D-amino acids were not large enough for the accurate detection of a slight quantitative alteration in normal animals. The mutant mice look normal, and have a life span similar to other mouse strains. It seems probable, therefore, that the free D-amino acid content in the mutant mice may show what the D-amino acid level in normal mice would be if it were not for the presence of DAAO. DAAO activity also increased with age in the normal suckling mice. Thus a possible interpretation for the DAAO activity observed in the present experiment may be that the enzyme activity had increased to eliminate the free D-amino acids which had increased with age in the young normal mice, and the D-alanine administration altered the enzyme activity no further in the adult animals.

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Distribution of the 1,25 dihydroxy-vitamin D_3 receptor in the bursa of Fabricius of chicken

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Abstract. The vitamin D_3 metabolite $1,25(OH)_2D_3$ is probably involved in B lymphocyte ontogeny. We therefore determined the distribution of the $1,25(OH)_2D_3$ receptor in the bursa of Fabricius and spleen cells of 7-day-old chicks, by immunohistochemistry using a monoclonal antibody against the chick intestinal cell $1,25(OH)_2D_3$ receptor. The bursa cells of young (7-day-old) chicks contained large amounts of receptor while the spleen cells did not. The bursa cells of older (35-day-old) chicks contained fewer receptors, but the number of receptors in the spleen increased.
Key words. Vitamin D_3 receptor; dihydroxy-vitamin D_3; bursa of Fabricius; ontogeny of immune system; B lymphocytes.

Since the first reports suggesting a role for 1,25 dihydroxy-vitamin D_3 ($1,25(OH)_2D_3$)[1,2] and its receptor[3] in lymphocyte functions, the vitamin D_3 metabolite $1,25(OH)_2D_3$ has been considered to be an immunomodulating hormone. A specific receptor for $1,25(OH)_2D_3$ has been shown by radiobinding[1] to be present in activated human T and B lymphocytes. Vitamin D_3 affects activated lymphocytes in various ways: it inhibits lymphocyte proliferation, the production of gamma interferon[1], and the production of antibodies[4,5]. Inhibition of IL-2 production by activated lymphocytes has also been reported[6]. However, the antiproliferative activity of $1,25(OH)_2D_3$ seems to be related to the mitogens used and to the presence of monocytes in lymphocyte culture[7]. The production of colony-stimulating factors (CSF) by lymphocytes is also modulated by $1,25(OH)_2D_3$: CSF production is reduced when lymphocytes are activated by PHA[8] but it seems to be increased when lymphocytes are activated by Con A[9]. These data suggest that $1,25(OH)_2D_3$ plays a role in the physiology of the immune system and that it may be considered to be an immunomodulating hormone[2].

The chicken bursa of Fabricius is a spherical organ connected to the cloaca by a duct. 12 to 14 longitudinal plicae of lymphoid tissue surrounded by epithelial cells protrude into the bursal lumen. The organ contains

lymphoid follicles, with medullary and cortical segments. The cortex and medulla are separated by a layer of lattened epithelial cells directly connected to the surface epithelium. The bursa medulla contains macrophages and reticuloepithelial cells (REp) in addition to lymphocytes. Desmosomes connect the REp cell processes to one another and to the cortico-medullar border epithelium, forming a three-dimensional network [10]. The bursal follicle is crowned by a tuft of cells, the follicle-associated epithelial (FAE) cells. The cells lie directly upon the medulla with no intervening basal membrane between, and bulge into the bursal lumen among the surrounding surface epithelial cells [11]. B lymphocytes differentiate in the bursa of Fabricius, which is the primary organ controlling the ontogeny of B lymphocytes in avian species. We evaluated the distribution of the $1,25(OH)_2D_3$ receptors in the bursa and spleen of young chickens in order to clarify vitamin D_3 involvement in B lymphocyte ontogeny.

Materials and methods

Chickens. Ten 3-day-old Red and Grey chickens were housed under standard conditions and sacrificed for experiments when they were 7 or 35 days old.

Immunoperoxidase assays. The 9A7gamma monoclonal antibody (rat IgG 2b) developed against the chicken $1,25(OH)_2D_3$ receptor was kindly provided by Dr J. W. Pike. This monoclonal antibody was developed through immunization of Lewis rats with a partially purified chick intestinal receptor for $1,25(OH)_2D_3$ followed by spleen cell fusion with murine myeloma SP2/0 Ag14 cell. The antibody interacts directly with the receptor and binds the occupied and unoccupied receptors. 9A7gamma has no effect on $1,25(OH)_2D_3$ binding whereas it alters the receptor's association with nuclei. The development and characterization of the 9A7gamma antibody has been described by Dr Pike [12].

Immunoperoxidase histochemistry was performed on cryosections of chicken tissues fixed in acetone/methyl alcohol (1:1 v/v) for 10 min at room temperature. Endogenous peroxidases were inhibited by incubating the fixed sections in 0.3% H_2O_2 in methyl alcohol for 15 min. Sections were then incubated with 9A7gamma monoclonal antibody diluted in PBS (1:500) for 2 h at 37 °C. Sections were washed 3 times and incubated for 30 min with a peroxidase-labeled goat anti-rat IgG antibody (1:100). The sections were finally incubated for 10 min at room temperature in a solution of diaminobenzidine tetrachloride and 0.001% hydrogen peroxide in 0.1 M phosphate buffer, pH 7.2. Sections of chicken intestine were used as positive controls; negative controls were sections of bursa incubated without the 9A7gamma monoclonal antibody.

Bursal cell separation. The bursae were removed under sterile conditions and gently squeezed; the cells thus obtained were allowed to sediment in RPMI 1640 culture medium for 10 min. The supernatant was thus enriched with cortical lymphocytes whereas the intact medullas, which were released from the bursal plicae [13], remained at the bottom of the vessel. The medullas were washed in RPMI 1640, shaken and again allowed to sediment. The medullary lymphocytes were recovered from the supernatant; the REp cells were collected from the sediment by further gentle washes. Contaminating lymphocytes were removed by culturing the REp cells for 24 h [14].

Dot blot. Bursal purified lymphocytes (35 days), REp cells and peripheral blood lymphocytes were suspended in 500 µl of 10 mM Tris/HCl, 50 mM KCl, pH 7.4 containing 1% Triton X-100 and 1 mM phenyl-methyl-sulfonyl fluoride for 30 min at 4 °C with gentle shaking as previously described [15]. Solubilized material was recovered in the supernatant after centrifugation at 100,000 g for 30 min. Volumes (containing 100 µg of protein) were spotted on nitrocellulose filters by means of a Bio-Dot apparatus (Bio Rad). The unreacted binding sites on the nitrocellulose sheets were saturated with 5% skim milk for 30 min. The sheets were then incubated with the primary 9A7gamma monoclonal antibody (1:1000 v/v in PBS) for 30 min at room temperature, washed three times in PBS, for 10 min each time, and incubated with a peroxidase-labeled goat anti-rat IgG (1:1000 v/v in PBS) (KPl inc.). The sheets were washed and the reactions developed with 3,3'-diaminobenzidine.

Counting of positive cells. Ten 7-µm sections were observed for each age group in order to count the positive cells, using a ×10 eyepiece with a square grid and a ×100 objective (immersion). Fifty microscopic fields were observed for each section at the level of the bursal follicle cortex, medulla and spleen. The counting was carried out in randomly-chosen fields. The reaction of a cell was considered to be positive when at least one clear granule of reaction products was found inside it. The data obtained are expressed as the mean of the percentage of cells that were positive to the immunoperoxidase test. The ranges of variability are also reported.

Results

Immunocytochemical reactivity was shown by stained dots located over the nuclei (fig. 1).

7-Day-old chicks. Cells in both the cortex and medulla of the lymphoid follicles reacted with the 9A7gamma monoclonal antibody (fig. 2). The spleen contained a few immunoreactive cells in the white pulp region. The count of reacting cells resulted in mean percentages of 76 (range 62.5–87.5) in the cortex of the bursal follicles and 61 (range 42.8–76.9) in the medulla (table).

35-Day-old chicks. Many of the cells in the cortex were positive but they were only dispersed as single positive cells in the medulla. Thus the immunocytochemical pattern of the bursa of Fabricius of the 35-day-old chicks was very similar to that of 7-day-old ones. The percentages of positive cells were 72 (range 50–90) in the cortex and 54 (range 40–70) in the medulla (table). However, there were many positive cells in the spleen, mainly locat-

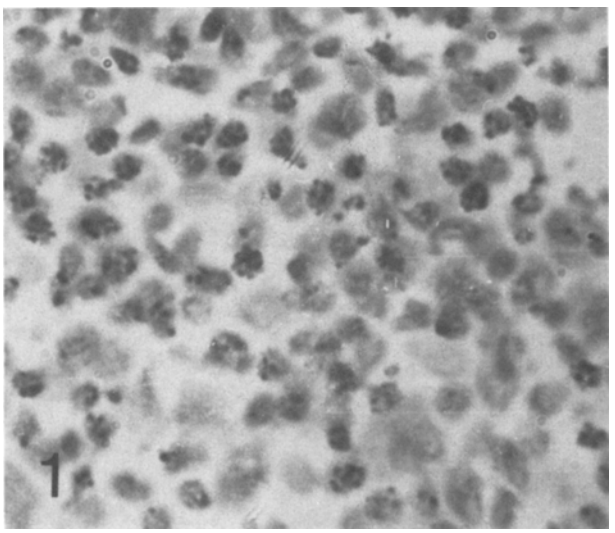

Figure 1. Section of the bursal follicle in 7-day-old chick. Cryostat; acetone-methanol, immunoperoxidase, methylene blue counterstain. The reactivity for anti-1,25$(OH)_2D_3$ receptor monoclonal antibody is dot-shaped; it is localized in the perinuclear area. ×1000

Count of positive cells in the bursal follicles and in the spleen

Days of life	Bursal cortex	Bursal medulla	Spleen white pulp	Spleen red pulp
7	76 (62.5–87.5)	61 (42.8–76.9)	–	–
35	72 (50–90)	54 (40–70)	72 (57.1–85.7)	10 (0–25)

The numbers in brackets indicate the ranges of variability.

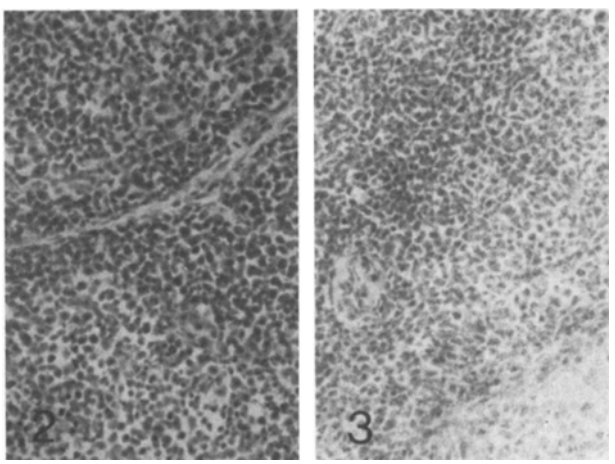

Figure 2. Section of the bursal follicle in 7-day-old chick. Cryostat; acetone-methanol, immunoperoxidase, methylene blue counterstain. Both cortex and medulla are positive. The number of reacting cells is greater in the cortex than in the medulla. ×450

Figure 3. Section of the spleen in 7-day-old chick. Cryostat; acetone-methanol, immunoperoxidase, methylene blue counterstain. Positive cells are mainly located in the perivascular area of the white pulp. ×450

ed in the perivascular area of the white pulp (fig. 3). Positive cells were not found elsewhere in the spleen. The percentages of positive cells were 72 in the white pulp (range 57.1–85.7) and 10 (range 0–25) in the red pulp.

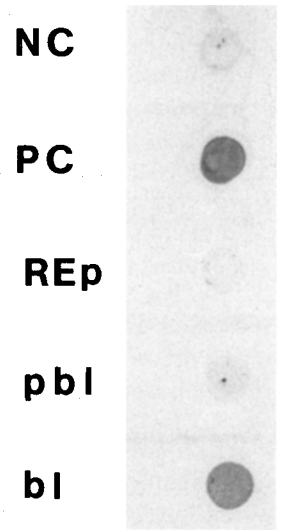

Figure 4. Dot blot of soluble extracts. NC, negative control; PC, positive control; REp, Reticulo-epithelial cells; pbl, Peripheral blood lymphocytes; bl, Bursal lymphocytes; Explanation of the results is given in the text.

Dot blot. The dot blot test confirmed the immunocytochemical data. Soluble extracts of REp cells did not react with the 9A7gamma antibody. Conversely, soluble extracts of bursal lymphocytes recovered at 35 days reacted strongly with the monoclonal antibody; the reaction was comparable to that obtained with an extract from a human monoblastic cell line (U-937). Soluble extracts of chicken resting peripheral blood lymphocytes showed no reaction with the anti-1,25$(OH)_2D_3$ receptor antibody (fig. 4).

Discussion

The effect of 1,25$(OH)_2D_3$ on the ontogeny of the immune system has not been extensively evaluated. It has been suggested that the large mitotically-active lymphocytes from the thymus of rats carry 1,25-$(OH)_2D_3$-specific receptors [16]. These results have been confirmed by showing that a high affinity receptor is present on thymic medullary lymphocytes (PNA negative) but not on cortical immature thymic cells [17]. The addition of 1,25$(OH)_2D_3$ to thymocyte cultures inhibits their mitogenic response to PHA and IL-2 and decreases the rate of spontaneous lysis of the PNA-negative subpopulation [18].

In the present paper we describe the detection of 1,25$(OH)_2D_3$ receptors in the bursa of Fabricius and the spleen of 7- and 35-day-old chicks. These studies were performed using a monoclonal antibody to the 1,25-$(OH)_2D_3$ receptor [12], which identifies a 17-residue antigenic determinant located close to the DNA-binding domain [19]. Our data suggest that the 1,25$(OH)_2D_3$ receptor is present in bursal lymphocytes at hatching and persists in 5-week-old chicks. Both cortical and medullary lymphocytes strongly reacted with the monoclonal antibody to the 1,25$(OH)_2D_3$ receptor, but non-activated

peripheral blood lymphocytes do not. In contrast, the white pulp of the spleen proved to be negative at seven days and positive at 35 days.

The function of the receptors for $1,25(OH)_2D_3$ in primary lymphoid organs has still to be clarified, but it is tempting to hypothesize that $1,25(OH)_2D_3$ plays a role in cell differentiation, possibly by inhibiting cell proliferation. The presence of the receptor in human activated peripheral blood lymphocytes[1] and in chicken spleen at 35 days might also be explained in this way. We are aware that, as far as lymphoid cells are concerned, the term 'differentiation' is confusing: resting lymphocytes may undergo proliferation and further differentiation depending upon the antigenic (or mitogenic) challenge. However, the reported inhibition of mitogenesis and interleukin production might be considered to be an anti-proliferating rather than, as previously suggested[20], an anti-differentiating activity, at least at the earlier ontogenetic stages.

The presence of $1,25(OH)_2D_3$ receptor in the bursa of chicks as well as in the thymus of rodents[16] suggests that the steroids play an important part in the ontogeny of both T and B lymphocytes. The bursa of Fabricius is considered to be the primary organ for B lymphocyte differentiation during embryonic life and the first week after hatching[21–23]. At that time the role played by the bursa becomes that of a secondary lymphoid organ[24]. Starting from the first week of life, the B lymphocytes which differentiate in the bursa of Fabricius migrate towards the spleen and other secondary lymphoid organs. These cells show surface IgG from the 8th day onwards[25]. The presence of the $1,25(OH)_2D_3$ receptors seems to follow the appearance of surface IgG antigens. The fact that receptors are already present in the bursal cell at the 7th day of life, and appear in the spleen cells later, allows us to hypothesize that $1,25(OH)_2D_3$ is involved in the complete maturation of B lymphocytes into antibody-producing cells. $1,25(OH)_2D_3$ has been described as inhibiting the expression of IL-2 receptors; consequently, $1,25(OH)_2D_3$ interaction with its receptors might modulate the balance between the factors involved in the full development of immune responsiveness. It will be interesting to evaluate the activity of the $1,25(OH)_2D_3$ receptor during embryonic development in order to correlate the presence of the receptor with B lymphocyte ontogenesis.

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