B-cell precursors in early chicken embryos

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Summary. The ontogeny of B-cell precursors in chicken embryos from day 3 of incubation onwards has been studied. Purified antibodies to chicken Ig L, γ, μ, α chains were used in a sensitive indirect immunofluorescence assay on fixed cell smears and wax-embedded tissue sections; the location and morphology of immunoglobulin positive (Ig+) cells were determined either in phase contrast or after histological staining. Lymphoid cells containing small amounts of cytoplasmic immunoglobulin were found in 3 day and older embryonic yolk sac, 11 and 12 day blood, 11, 12 and 13 day bursal mesenchyme. cIg+ large basophilic cells were first seen in 14 day bursal follicles. It is concluded that cells enter the embryonic bursa at different developmental stages: some appear to be uncommitted stem cells, whilst others have already commenced B-cell maturation in an extra-bursal site.

INTRODUCTION

Lymphoid cells of the B lineage, characterized by their ability to synthesize immunoglobulin, first appear during embryogenesis in mammals (Nossal & Pike, 1972), birds (Thorbecke, Warner, Hochwald & Ohanian, 1968) and amphibia (Du Pasquier, 1970). These early

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B-cell precursors are of crucial interest for investigating the generation of the B-cell repertoire and the establishment of self-tolerance (Raff, Owen, Cooper, Lawton, Megson & Gathings, 1975). Little is known, however, about the genetic and/or micro-environmental factors regulating the onset of immunoglobulin synthesis in embryonic haemopoietic cells. Furthermore, B-cell differentiation appears to be initiated in such structurally and functionally unrelated organs as mammalian foetal liver (Owen, Raff & Cooper, 1974) and the avian bursa of Fabricius (Kincade & Cooper, 1971).

The exact contribution of the avian bursa to B-cell differentiation remains controversial. The embryonic bursa is undoubtedly involved in the development of the humoral immune system, as established by bursectomy experiments (reviewed in Warner, 1967). It is also said to contain the earliest immunoglobulin-positive cells detectable in the chicken embryo. Thus, IgM-containing cells were first located in the follicles of the 14 day embryonic bursa (Kincade & Cooper, 1971) and small numbers of IgM-bearing cells were detected even earlier, at day 12 (Lydyard, Grossi & Cooper, 1976) and at day 10 (Albini & Wick, 1975) of incubation. Furthermore, the *in vivo* injection of anti-μ antibodies at day 13, when combined with surgical bursectomy at hatching, produced a long-lasting and severe reduction of cellular and circulating IgM, IgA and IgG (Lawton, Kincade & Cooper, 1975). These facts were taken to demonstrate that in the chicken embryo the onset of IgM biosynthesis occurs exclusively within the bursal microenvironment, where eventually B-cell precursors 'switch' from IgM to IgG to

IgA synthesis (Lawton *et al.,* 1975) although a 'switch' from IgM to either IgG or IgA cannot be excluded (Martin & Leslie, 1974). This prevailing view has been challenged by several investigators who found residual B-cell activities in bursaless birds (Claflin, Smithies & Meyer, 1966; Rose & Orlans, 1968; Lerner, Glick & McDuffie, 1971; Bryant, Adler, Cordy, Shifrine & Da Massa, 1973; Fitzsimmons, Garrod & Garnett, 1973; Sato & Abe, 1975; Jankovic, Isakovic, Markovic, Rajcevic & Knezevic, 1976) and who detected IgA- and IgM-bearing cells in 5 day and older embryonic yolk sac (Albini & Wick, 1975). Hence, they proposed that at least some B-cell differentiation could occur outside the bursa of Fabricius.

In view of this controversy and of our previous observations of cIg + cells in 12 day bursal mesenchyme (Ritter & Lebacq, 1977) we have investigated 3 day and older chicken embryos for the presence of B-cell precursors. Lymphoid cells containing small amounts of cytoplasmic immunoglobulin were found in 3 day and older embryonic yolk sac, 11 and 12 day blood, and 11, 12 and 13 day bursal mesenchyme. cIg + large basophilic cells made their first appearance in 14 day bursal follicles.

MATERIALS AND METHODS

Biological material
Fertile eggs (Binnings, Ltd, Frilford Heath, Oxfordshire) were incubated at 37·8° in a humid atmosphere with half-hourly automatic turning (Western Incubators, Ltd, London). Five to thirty embryos were sampled daily from day 3 to day 21 of incubation and checked for developmental age by weight. The yolk sac was removed from 3 to 12 day embryos. Blood was taken from the chorio-allantoic vessels at days 11 and 12 and smeared by hand. The bursa and spleen were removed from 10 to 21 day embryos, cleaned from additional tissues and fixed either in cold 5% acetic acid in absolute ethanol or in Bouin's.

Spleen and jejunum were obtained from 4–6 month old Rhode Island chickens that were healthy and clinically free of infections.

Normal serum and bile were taken from 7–9 week old chickens. Sera (1000 ml) were defatted (Burstein & Samaille, 1959) and bile (400 ml) was acidified in order to precipitate some mucinous-like material (Bienenstock, Perey, Gauldie & Underdown, 1972).

Agammaglobulinaemic serum was obtained from a week old Rhode Island chicken that had been injected at day 11 of incubation with 3·5 mg of testosterone propionate and surgically bursectomized at hatching.

Embryonic sera were from 15 day old White Leghorn chicken embryos.

Conventional histology
Embryonic tissues fixed in Bouin's were blocked in polyester wax (British Drug Houses, Poole, England) sectioned at 7 μm and stained in Giemsa R 66 (G. T. Gurr, London, England).

Processing of tissues for immunofluorescence

Tissue sections. Tissues were fixed for 24 h in cold 5% acetic acid in absolute ethanol (renewed twice), dehydrated for 3 h in absolute ethanol (renewed twice) and then transferred into two changes of polyester wax at 37°. Blocks were prepared with freshly melted wax and stored at 4°. Sections, 4–5 μm thick were floated with 1% amylopectin on egg-albumin coated slides (equal parts egg-albumin and 1% NaCl), dried in racks at 37° and stored at 4°. Before use, slides were dewaxed at room temperature in absolute, 90% and 70% ethanol and then transferred to PBS. It was found empirically that pre-staining of tissue sections with a very dilute solution of trypan blue increased the contrast between specific green-labelled cells and a brown autofluorescent background. Slides were left for 1 min in a 0·001% solution of trypan blue (Flow Laboratories, Irving, Scotland) in PBS filtered before use; unfixed dye was washed away in PBS.

Cell smears. Cell suspensions of embryonic yolk sac and adult spleen were prepared at room temperature in Hanks's balanced salt solution (Flow Laboratories) containing 5% foetal calf serum. The area vasculosa of the yolk sac was cut out, carefully washed free of adherent yolk in saline and teased apart with curved forceps on a stainless steel grid. Cells of five to thirty yolk sacs were pooled in each experiment. The cell suspensions were filtered on cheese cloth and washed several times in medium. Living cells were counted in 0·25% trypan blue, using an improved Neubauer counting chamber. Cells were smeared, fixed immediately for 1 h in cold methanol and stained either with Giemsa R 66 or processed for immunofluorescence.

Immunofluorescence
Indirect immunofluorescence. Tissue sections or cell smears were incubated in a moist chamber for 30 min at room temperature with a drop of unlabelled anti-Ig

antibodies. After washing in PBS, the same procedure was repeated with a goat anti-rabbit IgG FITC-conjugate kindly provided by Dr J. P. Vaerman (University of Louvain, Belgium). After further washings, the slides were mounted in glycerol buffered at pH 9 with 1 M Tris-HCl and examined under a Zeiss standard RA microscope equiped with epi-illuminator IV FL, phase contrast and photomicrographic camera. Photographs were taken on Ektachrome High Speed and on Tri-X (Kodak) films and processed for extra-sensitivity. Exposure times varied from 5 to 15 s for adult tissues and from 1 to 2 min for embryonic tissues. In some cases, microscope stage coordinates were recorded for labelled cells so that they could be relocated after Giemsa staining.

Direct and double immunofluorescence. Cell smears were incubated successively and in either order with an anti-L FITC-conjugate (~ 30 μg IgG/ml) and with an anti-γ TMRITC-conjugate (~ 750 μg IgG/ml) (See Antisera and purified antibodies section). Cells were examined both for green (L chain positive) and red (γ chain positive) immunofluorescence.

Specificity of the immunofluorescent labelling. The specificity of indirect immunofluorescence was controlled by testing embryonic cell smears and tissue sections with (1) the goat anti-rabbit IgG FITC-conjugate alone, and (2) the anti-L, anti-γ, anti-μ, anti-α_s, anti-α_b antibodies previously absorbed with an excess of the appropriate purified Ig preparations (see below). Labelled cells were never observed in the absence of first layer antibodies nor when these antibodies had been previously absorbed with antigen.

The specificity of direct immunofluorescence was checked on embryonic cell smears using the anti-L and anti-γ conjugates previously absorbed with purified L chains or IgG, as appropriate.

Determination of the cellular distribution of Ig. Cells containing cytosplamic immunoglobulin (cIg +) were characterized by a uniform fluorescent labelling over the cytoplasm, but excluding the nucleus. Cells with membrane-bound immunoglobulin (sIg +) showed discrete fluorescent labelling over both nucleus and cytoplasm. The classification does not exclude that small amounts of sIg and cIg may also be present on/in cIg + and sIg + cells respectively.

Purification and testing of class specific anti-chicken immunoglobulin antibodies
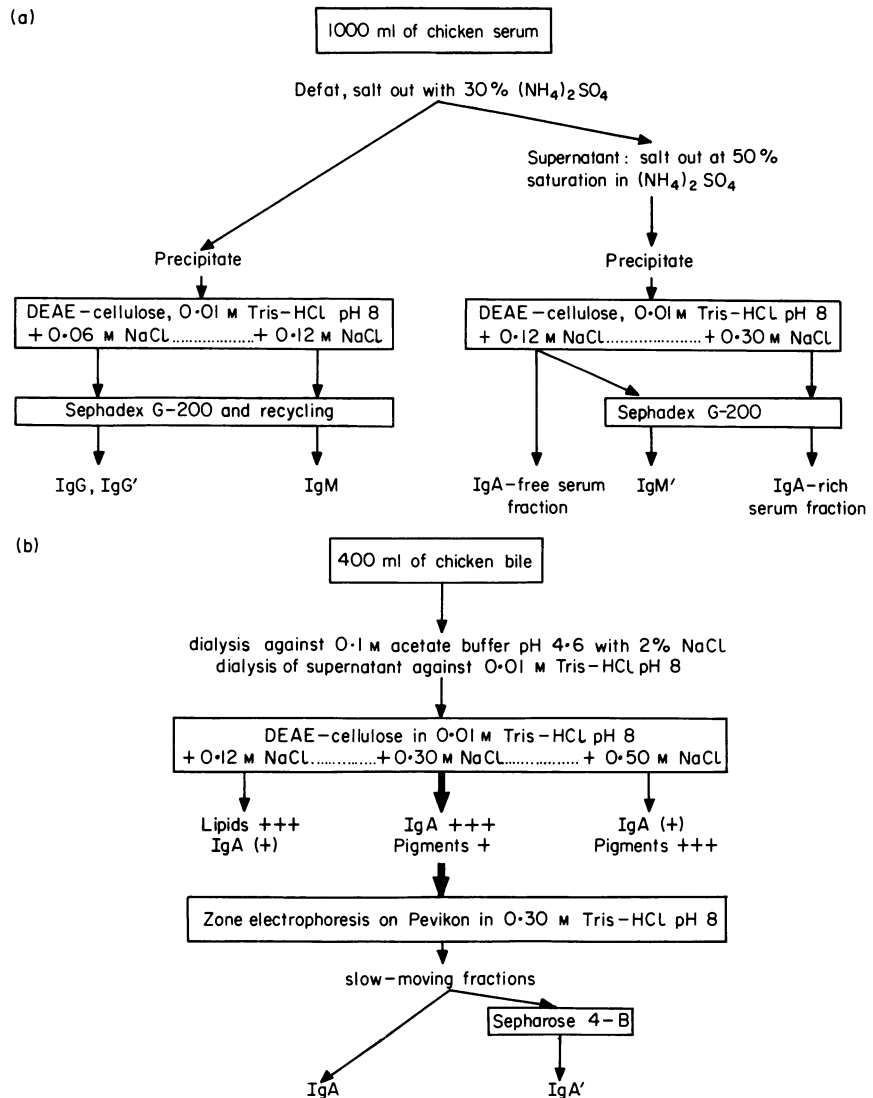   *Chemicals.* Cyanogen bromide, hexamethylenedia-

mine, ammonium thiocyanate and glutaraldehyde were purchased from Koch light, Colnbrook, Bucks, from Schuchardt, München, from Merck, Darmstadt and from Sigma, St Louis, Mo., respectively.

Analytical methods. Crude fractions and purified immunoglobulins (Igs) were assayed by the biuret method and by absorbance at 280 nm respectively. The extinction coefficients used were 13·5 for rabbit IgG (Crumpton & Wilkinson, 1963), 13·2 and 12·7 for chicken IgG and IgM respectively (Leslie & Clem, 1969) and 13·0 was arbitrarily chosen for chicken IgA. Agarose gel electrophoresis, immunodiffusion and immunoelectrophoresis were carried out following well established procedures.

Isolation of serum fractions and of immunoglobulins. These are described in detail elsewhere (Lebacq-Verheyden, Vaerman & Heremans, 1972a; Vaerman, Lebacq-Verheyden & Heremans, 1974; Lebacq-Verheyden, Vaerman & Heremans, 1974; Lebacq 1978) and are summarized in Table 1. IgA-free and IgA-rich serum fractions as well as purified IgG, IgM, IgA and L chains were used in the preparation of immuno-adsorbents. With the possible exception of IgA, these contained only the class of Ig stated as judged by immunoelectrophoresis and Ouchterlony analysis using the previously described rabbit antisera to chicken serum (Lebacq-Verheyden *et al.*, 1972a), bile (Lebacq-Verheyden *et al.*, 1974), immunoglobulin L (Vaerman *et al.*, 1974), γ, μ and α chains (Lebacq-Verheyden *et al.*, 1972a). The IgA gave a double precipitin line against the anti-bile and anti-α antisera, possibly due to the presence of subclasses of IgA (Lebacq, 1978). Ig preparations similar to these, termed IgG', IgM', IgA' and L' were used for the analytical absorption of anti-Ig antibodies. The IgA' preparation displayed a single precipitin line against the anti-chicken bile and anti-α antisera. The IgM' preparation contained substantial amounts of α_2 macroglobulin. The L' and IgG' preparations were undistinguishable from the L and IgG preparations.

Immunoadsorbents. Proteins (25–30 mg/ml) were cross-linked to AH-Sepharose 4-B (Pharmacia, Uppsala) by means of a two-step reaction involving glutaraldehyde as a cross-linking agent (Cambiaso, Goffinet, Vaerman & Heremans, 1975). The amount of protein bound to each immunoadsorbent column was: agammaglobulinaemic serum, 90 mg; embryonic

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Table 1. Fractionation of (a) chicken serum and (b) chicken bile.

(a)

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1000 ml of chicken serum
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Defat, salt out with 30% $(NH_4)_2SO_4$

Supernatant : salt out at 50%
saturation in $(NH_4)_2 SO_4$

Precipitate   Precipitate

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DEAE–cellulose, 0·01 M Tris–HCL  pH 8        DEAE–cellulose, 0·01 M Tris–HCL pH 8
+ 0·06 M NaCL...............+ 0·12 M NaCL      + 0·12 M NaCL...............+ 0·30 M NaCL
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Sephadex  G–200  and recycling               Sephadex  G–200
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IgG, IgG′   IgM   IgA–free serum   IgM′   IgA–rich
                                     fraction   serum fraction

(b)

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400 ml of chicken bile
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dialysis against 0·1 M acetate buffer pH 4·6 with 2% NaCL
dialysis of supernatant against 0·01 M Tris–HCL pH 8

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DEAE–cellulose in 0·01 M Tris–HCL pH 8
+ 0·12 M NaCL...............+0·30 M NaCL.................+ 0·50 M NaCL
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Lipids +++   IgA +++   IgA (+)
IgA (+)   Pigments +   Pigments +++

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Zone electrophoresis on Pevikon in 0·30 M Tris–HCL pH 8
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slow–moving fractions

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Sepharose  4–B
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IgA   IgA′

serum, 90 mg; IgA-free serum fraction, 785 mg; IgA-rich serum fraction, 207 mg; IgG, 383 mg; IgM, 43 mg; biliary IgA, 42 mg; and L chains 60 mg.

Antisera and purified antibodies. The anti-IgM antiserum was obtained by immunizing rabbits (5 mg in Freund's complete adjuvant in the footpads) with serum IgM isolated by Na_2SO_4 precipitations (18, 14, 9 and 5%, w/v) and Sephadex G-200 gel filtrations (Benedict, 1967). Since this also contained some aggre-gated IgG and some polymeric IgA, the native antisera reacted with all three classes of Igs. The preparation of the other antisera has been described in detail elsewhere (Lebacq-Verheyden *et al.*, 1972a; Lebacq, 1978) and is summarized in Table 2.

Anti-Ig antisera were heat-inactivated for 30 min at 56°, defatted (Burstein & Samaille, 1959) and salted out at 40% saturation in ammonium sulphate. Irrelevant antibodies were retained on several immuno-adsorbent columns (Table 2). Eventually, specific

Table 2. Preparation of monospecific antibodies

Antiserum	Immunogen	Absorption with*	Elution from*	Specificity† of purified antibodies
Anti-IgG‡	Washed IgG-anti-IgG precipitates	L chains	IgG	γ chains
Anti-IgM	serum IgM	Embryonic serum IgG Biliary IgA L chains	IgM	μ chains
Anti-serum IgA‡	Washed serum IgA-anti-IgA precipitates	Embryonic serum IgA-free serum fraction L chains	Biliary IgA	α chains (two sub-classes?)
Anti-biliary IgA	Biliary IgA	IgA-free serum fraction L chains	IgA-rich fraction	α chains (two sub-classes?)
Anti-L§	L chains	Agammaglobulinaemic serum	L chains	L chains

* Antigens were cross-linked to AH-Sepharose 4-B.
† As tested by absorption experiments with purified Igs.
‡ Lebacq-Verheyden *et al.*, 1972a.
§ Vaerman *et al.*, 1974.

antibodies were adsorbed on the corresponding insolubilized Igs and eluted with 3 M ammonium thiocyanate at pH 6·7 (Cambiaso *et al.*, 1975).

The IgG of monospecific anti-L and anti-γ antisera were purified by DEAE-cellulose chromatography and labelled for 1 h at room temperature with fluorescein (FITC) or with rhodamine (TMRITC) at ratios of 0·01 and 0·02 mg of dye per mg of protein, respectively. Free dye was removed by fractionation on Sephadex G-50 and the conjugates were stored in small aliquots in the dark at −20°.

Purified antibodies and fluorochrome conjugates were used in immunofluorescence at the following concentrations: anti-L, 15 μg/ml; anti-γ, 100 μg/ml; anti-μ, 15 μg/ml; anti-α_s, 15 μg/ml; anti-α_b, 15 μg/ml; anti-γ TMRITC-conjugate, ~750 μg/ml; anti-L FITC-conjugate, ~30 μg/ml; goat anti-rabbit IgG FITC-conjugate 150 μg/ml.

Specificity of anti-Ig antibodies. First, upon immunoelectrophoresis and Ouchterlony analysis against chicken serum, bile and serum fractions, all monospecific antisera gave a single precipitin line against preparations containing the appropriate antigens (Lebacq, 1978). The anti-L antisera reacted exclusively with immunoglobulin and equally well with IgG, IgM and IgA (Vaerman *et al.*, 1974; Lebacq, 1978).

Second, in indirect immunofluorescence on polyes-polyester wax-embedded tissue sections, anti-L and

anti-H antibodies produced the expected distribution of Igs in adult chicken spleen and jejunum (Lebacq-Vaerman & Heremans, 1972b). In these experimental conditions, plasma cells stained bright green on a brown autofluorescent background, and were clearly distinguishable from yellow autofluorescent red blood cells and granules of heterophils; intra-vascular Igs were also traced. In unimmunized birds anti-γ antibodies stained typically the intra-vascular IgG but few cells in the spleen and in the jejunum. By contrast, anti-μ and anti-α antibodies failed to stain intravascular Igs, which distinguished them from anti-L and anti-γ antibodies. Anti-μ antibodies labelled typically numerous cells in the spleen and few in the jejunum, whereas anti-α antibodies stained many cells in the jejunum and few in the spleen. Anti-L and anti-α antibodies also labelled some IgA that adhered to the brush border of the intestinal epithelium. The IgA distributions produced by anti-α_s and anti-α_b antibodies were indistinguishable.

Third, antibodies were quantitatively absorbed with purified chicken immunoglobulins. Constant amounts of antibodies (3 μg in 100 μl PBS) were mixed with an equal volume of IgG′, IgM′, IgA′ and L′ chains (Table 1), at Ag/Ab ratios of 0, 1, 2, 3, 4, 5 and 10. The mixtures were left for 1 h at room temperature and overnight in the cold; they were carefully decanted and assayed by indirect immunofluorescence on chicken spleen and jejunum sections. Inhibition was considered as complete when no labelled cells were observed
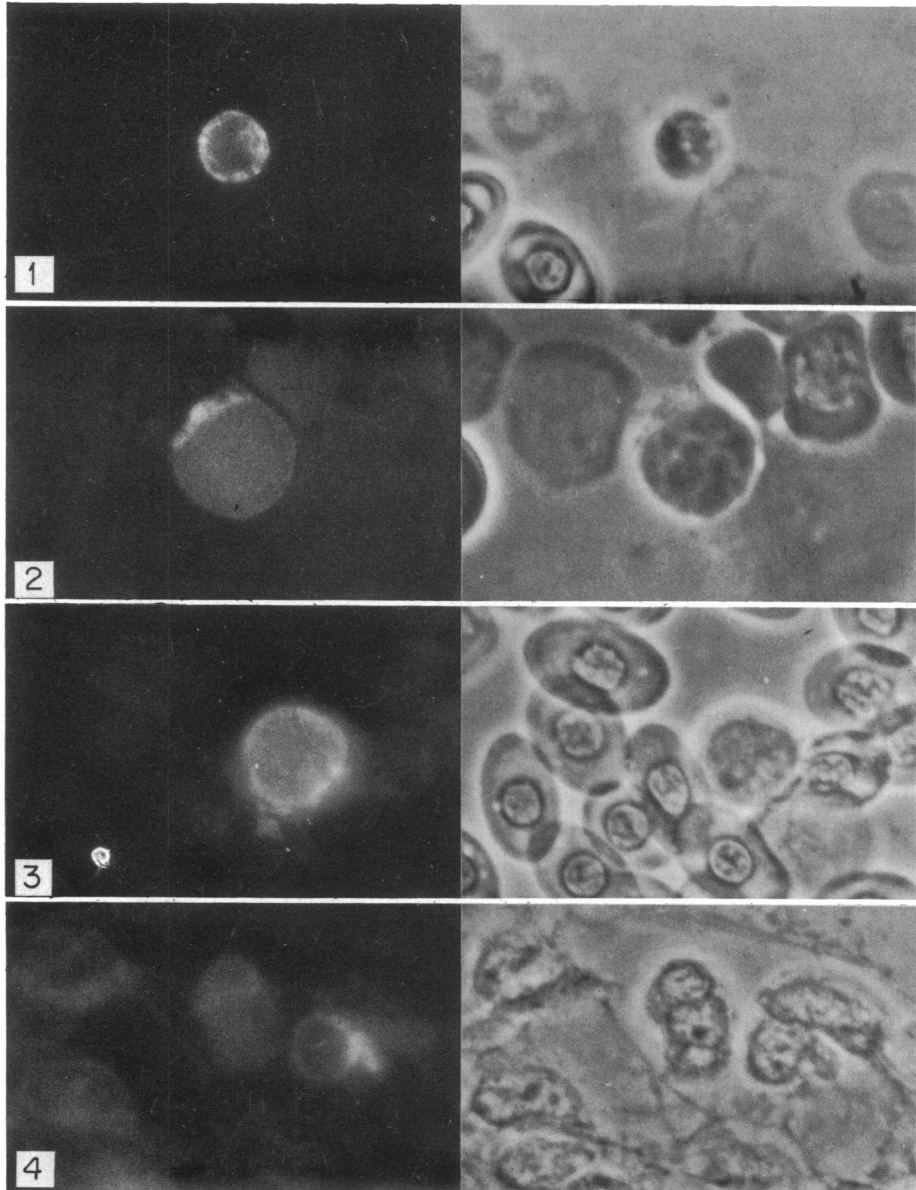
Figure 1. sIg + (1, 3) and cIg + (2, 4) lymphoid cells in adult chicken spleen (1), 3-day embryonic yolk sac (2), 11 day embryonic blood (3) and 12-day embryonic bursal mesenchyme (4). Indirect immunofluorescence with anti-L chain antibodies on fixed cell smears (1–3) and wax-embedded tissue sections (4). Original magnification: × 800; fluorescence left; phase contrast right.

at the highest magnification (× 100 objective) since with the present epi-illumination system, sensitivity increases with magnification (Ploem, 1967). All anti-H chain antibodies stained equivalent numbers of cells before and after absorption with a tenfold excess of L chains or of unrelated Igs. In contrast anti-L, anti-γ

and anti-μ antibodies failed to detect any tissular Igs after absorption with a 1:1, 2:1 and 3:1 excess of purified L chains, IgG and IgM respectively. Neither anti-α_s nor anti-α_b antibodies, however, were completely absorbed with a 2:1 up to 10:1 excess of the IgA' preparation, since some weakly stained cells

Table 3. Frequency of Ig-containing lymphoid cells in 3 to 10 day embryonic yolk sac

Developmental age (days)	Ig-containing cells in yolk sac* (%)		Red blood cells in yolk sac† (%)	Ig-containing cells in non-red blood cells of yolk sac‡ (%)
	DIF	IIF		
3	NT§	0·04	95	0·8
6	0	NT		
	0	NT		
	<0·01	NT		
	0·01	0·02	87·5	0·15
7	0·03	NT		
	0·02	NT		
	0·02	NT		
	0·03	0·03	76	0·12
8	0·02	NT		
	0·01	NT		
	0·02	0·03	86	0·21
10	0·03	NT		
	NT	0·04	NT	NT

* Percentage of Ig-containing lymphoid cells in yolk sac smears (at least 10^4 total cells counted) as tested by direct immunofluorescence (DIF) or indirect immunofluorescence (IIF) with anti-L reagents.

† Percentage of immature and mature red blood cells (at least 500 total cells counted) in accompanying Giemsa-stained yolk sac smears.

‡ Calculated percentage of Ig-containing lymphoid cells in yolk sac cells other than immature and mature red blood cells.

§ NT, not tested.

remained visible in the jejunal submucosa but not in the spleen after absorption. This residual antibody activity may recognize either a subclass of IgA or an as yet undescribed class of chicken Ig (Lebacq, 1978).

RESULTS

Ig + lymphoid cells in 3 day and older embryonic yolk sac

Yolk sac smears from 3–12 day chicken embryos were assayed by both direct and indirect immunofluorescence using anti-L chain reagents (Table 2). Some cIg + cells were found as early as day 3 of development (Fig. 1). In phase contrast they had the appearance of large lymphoid cells with a large nucleus and a thin rim of cytoplasm. Their frequency remained below 0·05% until day 10 (Table 3). Relocation of labelled cells after Giemsa staining showed that they had a basophilic cytoplasm and a large pale nucleus with punctate chromatin. Not all such Giemsa-stained cells were cIg + cells since the former contributed 17% and the latter 0·01% of yolk sac cells.

To exclude cells that might contain maternal IgG,

the presence of L chain positive cells that did not stain for γ chains was investigated by double immunofluorescence in 8 day yolk sac smears. In 0·04% of yolk sac cells the cytoplasm was labelled by the anti-L reagent and did not stain at all for γ chains. Vacuolated cells similar to macrophages, entodermal cells and yolk spheres were, however, brightly labelled by anti-L and anti-γ conjugates.

Finally, 0·03% cIgM + cells were identified by indirect immunofluorescence in 3 day yolk sac smears. The presence of cIgA + cells was not investigated.

Ig + lymphoid cells in 11 and 12 day embryonic blood

Small numbers of lymphoid cells that stained both for cytoplasmic and membrane-bound light chains were identified in 11 and 12 day embryonic blood (Fig 1). Blood was not tested at other developmental stages.

Ig + lymphoid cells in 11 day and older embryonic bursas

Ten to twenty-one day bursas were sampled daily

Table 4. Time of appearance, location and frequency of Ig+ cells during embryonic bursa development

Age of embryo (days)	Location of Ig+ cells*					
	Outer M	Inner M	Touching E	Plain E	Thickened E	Foll
9	−†	−	−	−	−	−
10	−	−	−	−	−	−
11	+	−	−	−	−	−
12	+	+ +	+	−	−	−
13 early	−	+ +	+	+	+	−
13 late		+	+	+	+	+
14		+	+			+ +
16		+ +				+ + +
18		+ +				+ + + +
21		+ +				+ + + + +

* M, mesenchyme; E, epithelium; Foll, Follicle.

† Frequency of Ig+ cells: −, absent in every section scored; +, present in some of the sections scored; + +, present in every section scored; + + +, + + + +, increasing frequencies of Ig+ cells.

Table 5. Time of appearance, location and frequency of IgM +, IgA + and IgG + cells during embryonic bursa development

Age of embryo (days)	Location of Ig+ cells*	Class of cellular Ig†			
		μ	γ	α_b	α_s
11	M	−‡	−	−	+
	E	−	−	−	−
12	M	−	−	−	+ +
	E	−	−	−	−
13	M	+	+	+	+ +
	E-F	+	+	+	+
14	M	+	+	+	+ +
	F	+ +	+	+	+
16	M	+ +	+	NT	+ +
	F	+ + +	+	NT	+ +
21	M	+ +	+ +	NT	+ +
	F	+ + + +	+ + + +	NT	+ + + +

* M, mesenchyme; E, epithelium; F, follicle.

† As tested with the anti-γ, anti-μ, anti-α_s and anti-α_b antibodies.

‡ Frequency of Ig+ cells, as in Table 4. NT, not tested.

(fourteen to nineteen at each stage). At least two sections per bursa were tested for L chain +, IgG+, IgM +, and IgA + cells by indirect immunofluorescence. Labelled cells were identified in phase contrast and some were relocated after Giemsa-staining. The results are summarized in Tables 4 and 5.

No Ig+ cells were observed in the embryonic bursa prior to day 11 of incubation. At day 11, anti-L and anti-α_s antibodies labelled the cytoplasm of occasional lymphoid cells in the outer mesenchyme of a few rudiments.

At day 12, when the embryonic bursa contained epithelial thickenings but as yet no follicles, anti-L and anti-α_s antibodies stained one to five small lymphoid
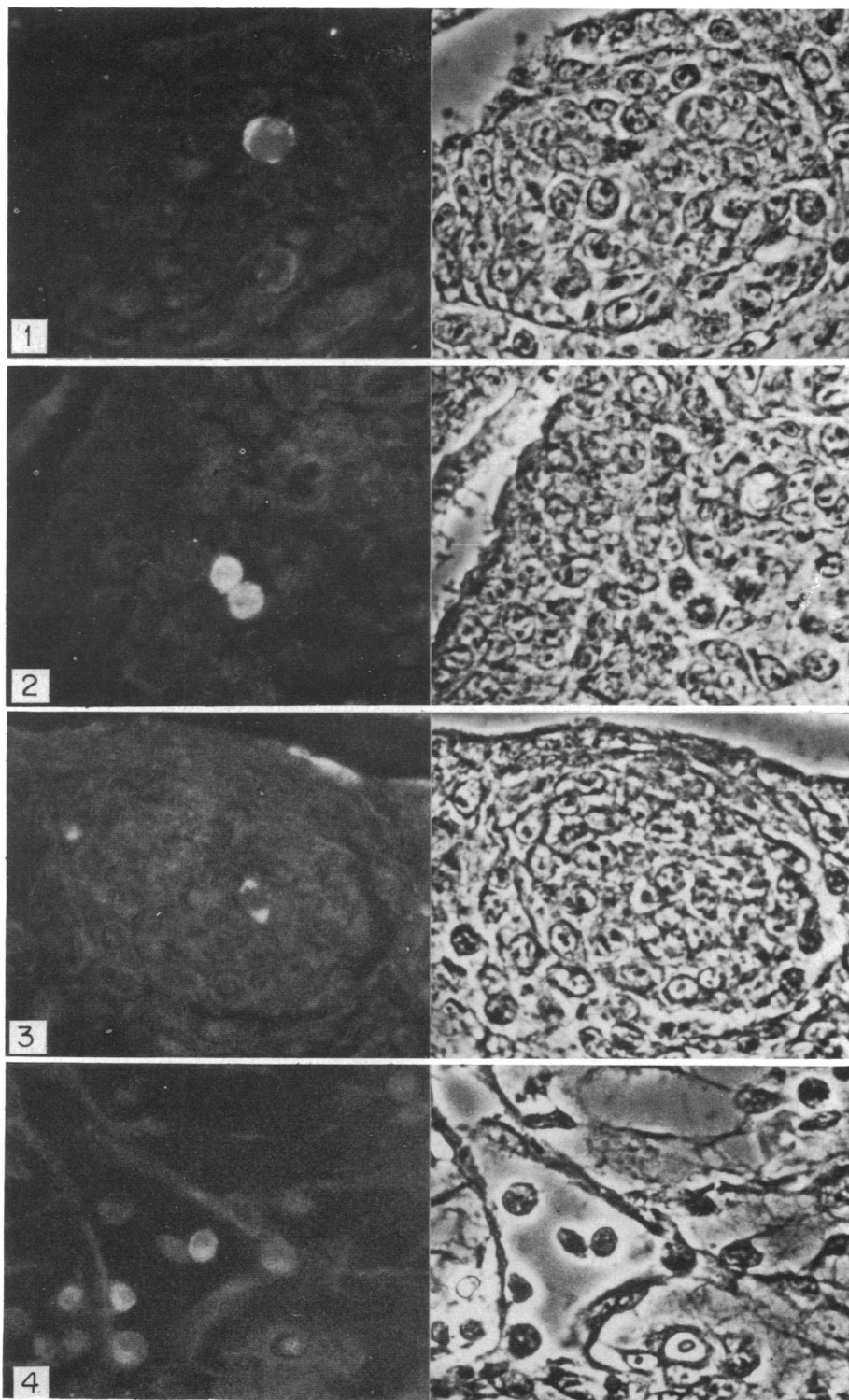
Figure 2. IgM + (1, 2) and IgA + (3, 4) cells in 14 day embryonic bursal follicles (1, 3) and mesenchyme (2, 4). Indirect immunofluorescence on wax-embedded tissue sections. Original magnification: × 320; fluorescence left; phase contrast right.

cells in every section (Fig. 1). These cIg + lymphoid cells were clearly visible in the outer and the inner mesenchyme, but in neither the epithelium nor the epithelial thickenings. At a higher magnification, they showed a typical 'hand mirror' shape (Fig. 1). Complete fading of their fluorescence was achieved within 1 min exposure to ultraviolet light (40 × objective) indicating that they contained only small amounts of labelled Ig.

During day 13, follicles developed increasing quickly in number and in size. Anti-L, anti-γ, anti-μ, anti-α_s and anti-α_b antibodies labelled the cytoplasm of varying numbers of small lymphoid cells in mesenchyme, plain epithelium, epithelial thickenings and follicles at the junction between the follicle and the epithelial lining of the lumen (Tables 4 and 5). It was in this area of follicles that readily detectable cIg + large basophilic cells first made their appearance by the end of day 13.

At day 14, 5–10% of the follicles displayed large cIg + basophilic cells that were brightly labelled by anti-L and anti-μ antibodies (Fig. 2). sIgM + lymphoid cells, however, were also found outside the follicles. The anti-γ, anti-α_s and anti-α_b reagents labelled occasional cells in follicles and mesenchyme (Fig. 2).

By day 16, 40–60% of the follicles contained many cIgM + and occasional cIgA + and cIgG + large basophilic cells as well as some extra-cellular IgA. There were also numerous labelled (sIg +, cIg +) lymphoid cells of all three Ig classes outside follicles.

Before hatching, there were many weakly labelled cIgM +, cIgA and cIgG + lymphoid cells in follicles. Only a few brightly labelled cells were present in follicles and interfollicular spaces.

DISCUSSION

Work presented in this paper was designed to study the possibility of extra-bursal B-cell development in the early avian embryo. Using purified antibodies to chicken Ig L, γ, μ and α chains in a sensitive indirect immunofluorescence assay on fixed cell smears and wax-embedded tissue sections, we were able to identify in 3 day and older embryonic yolk sac, 11 and 12 day blood, and 11, 12 and 13 day bursal mesenchyme a limited population of putative B-cell precursors characterized by the presence of small amounts of cytoplasmic Ig and a lymphoid morphology.

So far, cIg + lymphoid cells have not been reported in early chicken embryos. Thus the earliest cIgM + cells have previously been described in the follicles of

the 14 day embryonic bursa of Fabricius (Kincade & Cooper, 1971), although sIgA + and sIgM + cells have been reported in the yolk sac from day 5, in the bursa and spleen from day 10 (Albini & Wick, 1975) and in the bursa from 12 (Lydyard *et al.,* 1976) of incubation. The following technical points were critical to the identification of weakly labelled cIg + lymphoid cells in the chicken embryo: (1) the specificity of the anti-Ig reagents; (2) the affinity of the anti-Ig reagents, that permitted their use at 15 μg/ml thus making the labelling by otherwise undetected contaminating antibodies very unlikely; (3) the sensitivity of the assay, that allowed the detection of small amounts of labelled Igs; (4) the clear definition of tissues and cells in phase contrast and after Giemsa staining, enabling the cellular distribution of Ig (cytoplasmic/surface) and anatomical location and morphology of labelled cells to be determined.

A crucial question concerns the origin of the intracellular Ig detected in early cIg + yolk sac cells, maternal or embryonic. Since large amounts of maternal IgG are found in the yolk (Rose, Orlans & Buttres, 1974), this is the most likely class of contaminating Ig, after transport from yolk across the entodermal cells and into the foetal circulation. Smaller amounts of IgA and trace amounts of IgM are found in the albumin (Rose *et al.,* 1974). These macromolecules are unlikely to be a source of contamination for early yolk sac cells, however, since the yolk and albumin remain separated by the vitelline membrane and eventually by an extension of the chorion (Romanoff, 1960). Thus in early yolk sac (3 days of incubation onwards) cIg + cells were large lymphoid cells containing IgM, or possibly IgA (not tested with anti-α reagents). The remaining labelled material was excluded from cell counts since it consisted of IgG-containing entodermal cells, vacuolated macrophage-like cells and yolk spheres. In contrast to these, the Ig in the cIg + lymphoid cells was distributed evenly in the cytoplasm and thus unlikely to have been acquired extrinsically by pinocytosis. The formal demonstration of the embryonic origin of IgM and possibly IgA in early yolk sac cells would require the use of allotypic markers.

The large cIgM + cells identified in 3 day yolk sac resemble the large pre-B cells described in murine (Melchers, Von Boehmer & Phillips, 1975; Raff, Megson, Owen & Cooper, 1976) and in human (Gathings, Lawton & Cooper, 1977) foetal liver. The presence of conventional membrane-bound IgM (Raff *et al.,* 1976) cannot be excluded in our system and is cur-

rently being investigated. Their origin remains to be determined. The bursa can formally be excluded since its primordium has not yet started developing at day 3 of incubation (Ackerman & Knouff, 1959).

The small cIg + lymphoid cells identified in 11 to 13 day bursal mesenchyme resemble the small pre-B cells identified in human foetal liver, spleen, lymph nodes and blood (Gathings *et al.*, 1977). As in humans, they were observed at later stages of development and they were stained not only by anti-μ but also by anti-γ and anti-α reagents. Interestingly, studies with anti-H chain reagents revealed that most of these cIg + lymphoid cells were labelled by the anti-α_s but not by the anti-α_b antibodies, suggesting the presence of an additional antibody specificity in the former reagent. This might also have been the case for the two other anti-α reagents that were reported to label the earliest sIg + cells found in the chicken embryonic yolk sac, thymus and bursa (Albini & Wick, 1975). Our anti-α_s and anti-α_b antibody preparations were undistinguishable in immunoelectrophoresis and indirect immunofluorescence on adult chicken spleen and jejunum sections (Lebacq, 1978), differing only when tested on embryonic bursa sections. Clearly, further analysis of the anti-α_s reagent is required in order to study this additional specificity.

What are the interrelationships of the cIg + large lymphoid cells of early yolk sac (from day 3), and blood (days 11 and 12), the cIg + large basophilic cells of bursal follicles (from day 14) and the temporally intermediate cIg + small lymphoid cells of bursal mesenchyme (from day 11)? Bursal lymphocytes are believed to be derived from large basophilic haemopoietic stem cells of yolk sac origin (Moore & Owen, 1967; Le Douarin, Houssaint, Jotereau & Belo, 1975; Ritter & Lebacq, 1977). The presence of cIg + lymphoid cells in blood and bursal mesenchyme prior to the occurrence of cIg + large basophilic cells in follicles suggests that at least some of the blood-borne immigrant precursor cells are already committed to B-cell differentiation, having possibly developed from cIg + large lymphoid cells of the early yolk sac. In the older embryo, cIg + large basophilic cells in bursal follicles may then independently give rise to all subsequent bursal lymphocytes, having themselves derived from the cIg – large basophilic cells that are present from day 9 and 12 in bursal mesenchyme and epithelium respectively (Ritter & Lebacq, 1977).

Later events concerning the fate of Ig + cells in the embryonic bursa are more complex. In addition to cIgM + large basophilic cells, small numbers of cIgA + and cIgG + cells were detected in the bursal follicles from day 14 of incubation. The proportion of cells switching within the bursa from IgM to IgA or IgG biosynthesis (Lawton *et al.*, 1975; Martin & Leslie, 1974) remains unknown since some IgA + and IgG + cells could derive from the early cIgA + and cIgG + lymphoid cells identified in the 13 day bursal mesenchyme.

In summary, the embryonic bursa of Fabricius appears to receive two types of migrant cells, cIg – large basophilic haemopoietic stem cells and cIg + small lymphoid B-cell precursors. The bursa of Fabricius is therefore not the only site of B lymphopoiesis in the avian embryo. The identity of the extra-bursal site is not known although yolk sac (Moore & Owen, 1967) and haemopoietic tissues surrounding the dorsal aorta (Dieterlen-Lièvre, 1975) are good candidates. Thus, B lymphopoiesis in both birds and mammals may initially take place in general haemopoietic tissues. It is only later in avian development that the bursal follicles become available and take over this role.

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