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Ontogeny of humoral immune function in normal chickens: a comparison of immunoglobulin-secreting cells in bone marrow, spleen, lungs and intestine

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SUMMARY

A reverse haemolytic plaque assay was employed to study the ontogeny of immunoglobulin (Ig) secreting cells of either IgG, IgA, or IgM class in normal chickens. After hatching, IgM-secreting cells were detectable in the spleen by 3 days of age whereas IgG- and IgA-secreting cells were first noted at 6 days. Adult levels of Ig-secreting cells of all three classes were attained by 31 days of age in bone marrow and two separate lymphoid populations (lamina propria and intraepithelial lymphocytes). By contrast, adult levels of Ig-secreting cells were not obtained in either the spleen or the lungs until after 50 days of age. In the case of the spleen, the delay in attainment of adult levels of total Ig-secreting cells reflected the smaller spleen size in immature birds, whereas the percentages of cells secreting Ig of each class were in the adult range by 31 days. By contrast, the numbers of cells recovered from the lungs of 50-day-old chickens were near the adult range, while the percentages of cells secreting either IgG, IgA, or IgM were much fewer than those seen in the lungs of adult chickens. These data indicate that the lungs of normal chickens are populated more slowly with Ig-secreting cells than either the bone marrow, spleen, or intestine. At all ages studied, greater numbers of Ig-secreting cells, particularly of the IgG and IgM classes, were recovered from the bone marrow and spleen as compared to the lungs and intestine. Since only a portion of the total bone marrow population was studied, these data indicate that the bone marrow may be a major site of Ig-secreting cells in chickens beginning shortly after hatching.

INTRODUCTION

Our current understanding of the development of humoral immune function stems from earlier observations on the role of the bursa of Fabricius in antibody production in chickens (Glick, Chang & Japp, 1956). Ablation of this lymphoepithelial organ of hind-gut origin, whether by surgical, chemical, or hormonal means, may radically alter serum Ig levels and the magnitude of specific antibody responses to antigen (Cooper *et al.*, 1966, 1969; Warner *et al.*, 1969). Suppressor cells for

Abbreviations: Ig = immunoglobulin; RHPA = reverse haemolytic plaque assay; LP = lamina propria; IEL = intraepithelial lymphocyte; PBS = phosphate-buffered salt solution; SRBC = sheep red blood cell; BALT = bronchus-associated lymphoid tissue; BM = bone marrow.

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one or more Ig class may as well be induced by bursectomy (Blaese *et al.*, 1974, 1977; Palladino, Lerman & Thorbecke, 1976) and can perpetuate Ig deficiencies when transferred to recipient birds. The unique ability to manipulate the bursa-derived immune system in chickens has been an invaluable tool in defining humoral immune function.

While the bursa of Fabricius in chickens (or the bursa equivalent in other species) may influence humoral immune development, antibody formation occurs primarily in the peripheral lymphoid organs such as the spleen (Askonas & Humphrey, 1958; Humphrey & Sulitzeanu, 1958). However, both the lungs (Bienenstock, Johnston & Perey, 1973) and the intestine (Crabbe & Heremans, 1966a, 1966b; Craig & Cebra, 1971) may also contribute substantially to antibody formation. Indeed, in a recent study, we determined that the lungs and intestine had appreciable numbers of Ig-secreting cells, particularly of the IgA class, as measured by a reverse haemolytic plaque assay (RHPA) (Lawrence *et al.*, 1979). The present study was designed to assess the rate at which the major Ig-secreting organs of normal chickens, including the lungs and intestine, attain adult numbers of cells secreting either IgG, IgA, or IgM and the relative contribution of each organ to total Ig production.

MATERIALS AND METHODS

Chickens. Newly hatched line 6, subline 1, chicks, homozygous at the major histocompatibility locus (B2/B2), were obtained from the USDA Regional Poultry Research Laboratory, East Lansing, Michigan. These birds were maintained in the animal facilities of the NIH without special pathogen-free precautions.

Cell preparations. After hatching, groups of birds were anticoagulated with sodium heparin and killed by exsanguination. The lungs, spleen, small intestine and femora were removed as previously described (Arnaud-Battandier *et al.*, 1980; Lawrence *et al.*, 1979). The respective organs from several birds were pooled and single-cell suspensions prepared and then purified via Ficoll–Hypaque centrifugation (Arnaud-Battandier *et al.*, 1980; Lawrence *et al.*, 1979). The lymphoid populations from the bone marrow, spleen, lung and intestinal lamina propria (LP) and intestinal intraepithelium (IEL) were counted and adjusted to concentrations ranging from 1×10^6 to 5×10^7 per ml for assay of Ig-secreting cells. Data were expressed as Ig-secreting cells per million mononuclear cells, as well as total Ig-secreting cells per organ, calculated from the number of mononuclear cells obtained from individual birds. Ten adult birds of greater than 180 days of age were studied for comparison.

Medium. Phosphate-buffered salt solution (PBS) was prepared in the NIH media unit and was used throughout these experiments.

Reagents. Chicken IgG and IgM were purified from chicken serum, and IgA from chicken bile. Antisera against each chicken Ig were raised in rabbits and then purified by solid-phase adsorption against insolubilized chicken serum deficient in one or more Ig class. The purified antisera were then checked for monospecificity by Ouchterlony analysis and immunoelectrophoresis. Protein A (*S. aureus*) was obtained from Pharmacia Fine Chemicals, Piscataway, New Jersey, and coupled to SRBC with chromic chloride ($CrCl_3$) as previously described (Lawrence *et al.*, 1978).

Assay of immunoglobulin-secreting cells by RHPA. The details of the RHPA have been previously described for both humans (Lawrence *et al.*, 1978) and chickens (Lawrence *et al.*, 1979). Briefly, mononuclear cells from the respective chicken organs were mixed with protein A–SRBC and agar in glass tubes preheated to 43°C. This mixture was transferred to petri dishes precoated with 4 ml of agarose and swirled into a monolayer. The dishes were incubated for 1 hr at 37°C in a humidified, 5% CO_2 incubator with 1 ml of diluted developing antisera. The dishes were then incubated for an additional hr with 1 ml of SRBC-absorbed guinea-pig complement. Excess fluid was aspirated and the dishes stored at 4°C overnight. On the following day, the dishes were examined under × 7 magnification and the numbers of plaques in duplicate dishes were determined. Control plates containing no developing antisera were performed in parallel experiments, but no significant numbers of plaques were demonstrated.

RESULTS

Early population of chicken spleen by Ig-secreting cells

Previous studies have documented the sequential appearance of lymphocytes containing cytoplasmic IgM, followed by cells with cytoplasmic IgG, in the spleens of chickens shortly after hatching (Kincade & Cooper, 1971). We studied the sequence of appearance of cells actively secreting IgG, IgA, or IgM in young chicken spleens using the RHPA (Table 1). IgM-secreting cells were first apparent by 3 days of age while IgG- and IgA-secreting cells were not seen until 6 days after hatching. These results are thus in accord with the observations of Kincade & Cooper (1971) using cytoplasmic immunofluorescence.

Development of Ig-secreting cells in normal chickens between 9 and 50 days of age

A major purpose of the current study was to determine the rate and sequence by which the major lymphoid organs of normal chickens attain the adult complement of Ig secretory function. Beginning at 9 days after hatching, groups of birds from the same inbred line were killed and Ig-secreting cells determined.

The recovery of mononuclear cells from each organ was determined following purification by Ficoll–Hypaque density centrifugation. The cell recovery for each organ at each age studied is shown in Table 2, as is the adult range. By 31 days of age, recovered cells from bone marrow and intestine were within the adult range. However, the number of recovered cells from the spleen had not reached the adult range even after 50 days of age. In the lungs, the numbers of recovered cells were at the lower limits of the adult range by 50 days of age.

The percentages of cells secreting IgG, IgA, or IgM in young birds were compared with adult levels (Table 2). In the bone marrow, the percentages of Ig-secreting cells of all three classes were within the adult range by 31 days of age. For the spleen, the percentages of IgM-secreting cells were within the normal range on the first day studied (day 9), whereas the percentages of IgA-secreting cells fell within normal range by day 23, followed by the IgG-secreting cells which attained adult proportions by day 31. In both the intestinal LP and IEL populations, the percentages of IgM-secreting cells reached the adult range by day 16, followed by the IgA- and IgG-secreting cells which reached adult proportions by day 31. In marked contrast to the maturation of IgG-, IgA- and IgM-secreting cells in the bone marrow, spleen and intestine by at least 31 days of age, the lungs failed to attain adult percentages of Ig-secreting cells of any class until after 50 days of age.

Another purpose of this study was to determine the contribution of each of the major lymphoid organs of chickens to the total Ig production. Accordingly, the total number of Ig-secreting cells recovered was determined by multiplying the percentages of IgG-, IgA- or IgM-secreting cells by the number of mononuclear cells recovered from each organ.

The data for total Ig-secreting cells have been plotted on a log scale for each lymphoid population as a function of age in Fig. 1. Also shown are the adult ranges for total Ig-secreting cells for each population. At all ages studied, bone marrow and spleen had greater numbers of total

Table 1. Early population of chicken spleen by Ig-secreting cells

	Day after hatching			
	2	3	6	8
IgG-SC*	0	0	8	30
IgA-SC	0	0	38	125
IgM-SC	0	63	145	1,960

* Data expressed as IgG-, IgA-, or IgM-secreting cells (SC) per 10^6 mononuclear cells.

Table 2. Development of Ig-secreting cells in normal chickens between 9 and 50 days of age

	Day after hatching						
	9	16	23	31	38	50	Adult range*
Bone marrow							
Cell yield†	92	133·3	430	540	625	n.d.	468–1,003
IgG-SC‡	226	92	251	1,556	682	n.d.	1,079–4,468
IgA-SC‡	<1	5	20	203	31	n.d.	47–226
IgM-SC‡	116	106	122	770	306	n.d.	520–891
Spleen							
Cell yield	4·66	28	90	143·5	355	470	1,313–2,122
IgG-SC	674	468	964	1,616	1,356	1,612	1,073–2,404
IgA-SC	47	9	44	66	29	38	22–46
IgM-SC	502	440	564	1,308	391	484	296–562
Intestine LP							
Cell yield	0·92	4·6	1·9	7·5	n.d.	10	2·3–9·9
IgG-SC	25	198	1,053	3,160	n.d.	3,310	1,396–5,767
IgA-SC	13	95	596	1,256	n.d.	2,340	1,116–5,182
IgM-SC	5	63	266	128	n.d.	13	48–268
Intestine IEL							
Cell yield	1·6	4·2	3·2	18	n.d.	26	10·5–33·3
IgG-SC	10	36	235	565	n.d.	845	406–1,290
IgA-SC	3	7	85	427	n.d.	500	108–339
IgM-SC	3	12	63	73	n.d.	13	2–23
Lung							
Cell yield	20	15	53	55	130	110	112–500
IgG-SC	9	23	86	151	100	185	836–1,600
IgA-SC	1	2	9	66	30	20	130–318
IgM-SC	1	21	22	32	39	15	190–560

* Ninety-five per cent confidence limits for mean of birds ⩾180 days of age.

† Mononuclear cells recovered per organ, per bird, $\times 10^6$.

‡ Data expressed as IgG-, IgA- or IgM-secreting cells (SC) per 10^6 mononuclear cells.

n.d. = Not determined.

recovered IgG- and IgM-secreting cells than either intestinal LP, IEL, or the lung populations. The differences between the various organs is less striking for IgA-secreting cells, but bone marrow also had the greatest number of recovered IgA-secreting cells from 16 days of age onward. Considering that only a fraction of total chicken bone marrow, perhaps 1/10 or less, was processed for these studies, the contribution of bone marrow to total Ig secretion may be considerable.

Adult numbers of total recovered IgG-, IgA- and IgM-secreting cells were reached by 31 days of age in bone marrow and both intestinal populations, while neither the spleen nor the lungs had reached adult levels by 50 days of age. In the case of the spleen, the percentages of Ig-secreting cells were within the adult range, but the number of recovered mononuclear cells was less than in adult birds (Table 2). By contrast, the number of recovered mononuclear cells from chicken lungs was at the lower limit of the adult range by 50 days of age, whereas the percentages of Ig-secreting cells had not reached adult levels by this date (Table 2). Thus, the failure of the spleen to reach adult levels of total Ig-secreting cells by 50 days of age was due to its smaller size; the paucity of total Ig-secreting cells in chicken lungs of this age reflected a delay in populating the lungs with Ig-secreting cells.

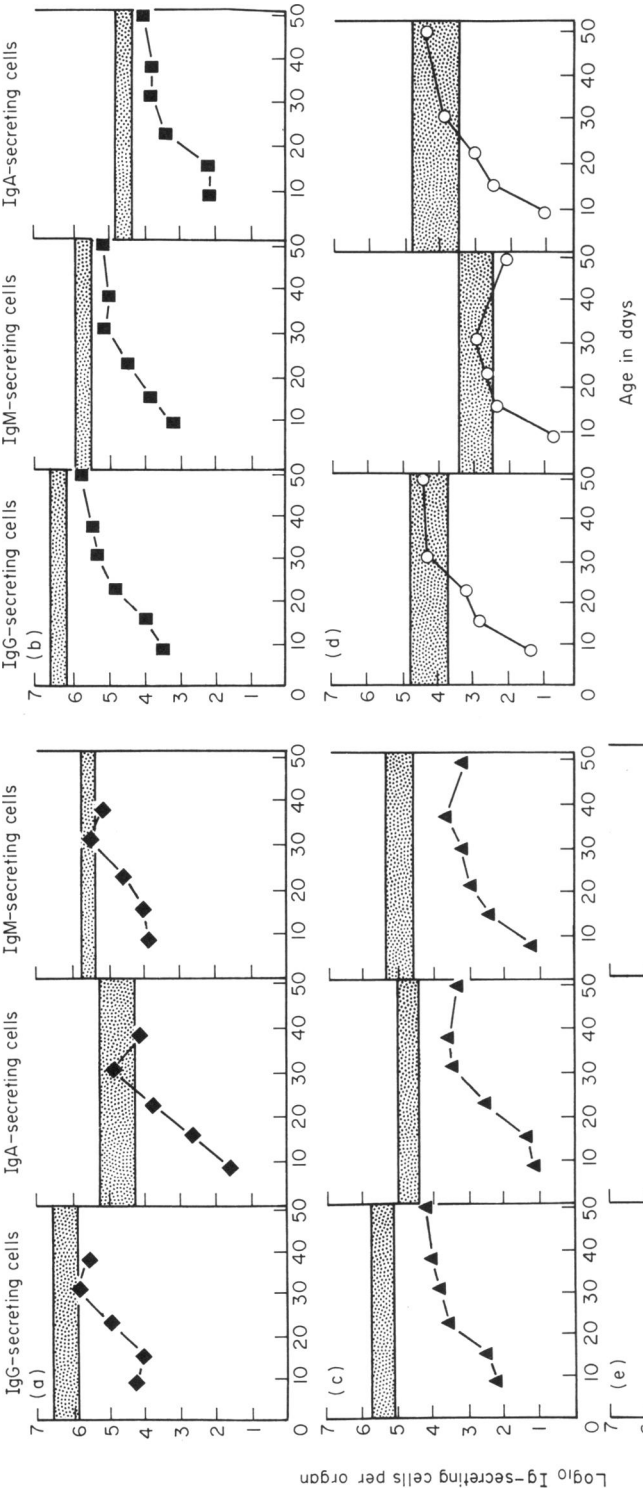

Fig. 1. Ontogeny of Ig-secreting cells in chickens between 9 and 50 days of age. (a) Bone marrow (◆), (b) spleen (■), (c) lung (▲), (d) intestinal LP (○), (e) intestinal IEL (●). Data for total Ig-secreting cells of each class are plotted on a log scale for each organ. The shaded areas indicate corresponding range (geometric mean ± 2 s.e.m.) for adult birds of greater than 180 days of age.

DISCUSSION

We have studied B cell function in chickens using a recently developed RHPA, which is a sensitive assay for individual cells actively secreting Ig (Arnaud-Battandier *et al.*, 1980; Gronowicz, Coutinho & Melcher, 1976; Lawrence *et al.*, 1978, 1979). Specific antibody is not measured, but rather polyclonal Ig of a given class, depending on the specificity of the developing antisera employed. Using this assay, we have previously defined the relative frequencies and tissue distribution of cells secreting either IgG, IgA, or IgM in normal adult chickens (Lawrence *et al.*, 1979). A noteworthy finding of that study was the relatively high percentage of Ig-secreting cells in the lungs and intestine of adult chickens, especially cells secreting IgA.

Kincade & Cooper (1971) have used cytoplasmic immunofluorescence to study the ontogeny of B cell function in chickens. Prior to hatching, these workers found cells within the bursa which stained for both IgG and IgM whereas peripheral lymphoid organs possessed cells staining for only one Ig class. Moreover, in both the bursa prior to hatching and the spleen after hatching, IgM-bearing cells always preceded IgG-containing cells. From these observations, they have postulated a developmental switch from IgM to IgG synthesis within the bursa and the subsequent population of peripheral lymphoid organs by first IgM- then IgG-containing cells. In the current study, we have studied the population of chicken spleen by Ig-secreting cells, as measured by the RHPA. IgM-secreting cells were noted 3 days after hatching while IgG- and IgA-secreting cells were first evident at 6 days of age. Our results thus confirm the earlier observations of Kincade & Cooper (1971) using another technique.

One purpose of the current study was to determine the rates at which the major lymphoid organs of normal chickens attain adult capabilities for Ig secretion. The bone marrow and intestinal lymphoid populations (LP and IEL) reached adult levels for total cells secreting IgG, IgA, or IgM by 31 days of age. This corresponded to a similar maturation in both the numbers of recovered mononuclear cells and the percentages of Ig-secreting cells. By contrast, the spleen had not yet attained adult levels of total Ig-secreting cells by 50 days of age. The percentages of spleen Ig-secreting cells were into the adult range by 19 days of age, whereas total recovered cells were still less than that of adult birds. Thus, the spleens of 50-day-old chickens are simply not as cellular as those of adult birds. The lungs failed to attain adult numbers of total IgG-, IgA-, or IgM-secreting cells by 50 days of age. This lag in maturation was due partly to fewer numbers of recovered mononuclear cells, but was mainly due to smaller percentages of cells secreting Ig. Thus, compared to bone marrow and intestinal lymphoid populations, the lungs were relatively deficient in humoral immune response capabilities until at least 50 days of age.

The lungs of adult animals may contribute substantially to both local and systemic humoral immune functions (Askonas & Humphrey, 1958; Emery & Dinsdale, 1973; Hand & Cantley, 1974; Kaltreider & Turner, 1976; Lawrence *et al.*, 1978, 1979; Milne, Bienenstock & Perey, 1975; Palladino *et al.*, 1976). However, humoral immune capabilities in the lungs of embryos or neonates have not been carefully studied. Bronchus-associated lymphoid tissue (BALT) is not found until after birth in either rabbits (Bienenstock *et al.*, 1973) or humans (Emery & Dinsdale, 1973). Milne *et al.* (1975) studied the development of Ig-containing cells in mouse fetal intestine and lungs transplanted subcutaneously into syngeneic recipients. While transplanted fetal intestine developed IgA-secreting cells in about the same numbers as intestine from germ-free mice, very few Ig-containing cells could be identified in either normal or transplanted lungs. The data in mouse lung (Milne *et al.*, 1975) and our own data from young chickens in the current study suggest that the lungs lag behind the remainder of the immune system in the development of humoral immune competence.

Another purpose of the present study was to compare the humoral immune capacities of various lymphoid tissues at different ages. Total recovered IgG-, IgA-, or IgM-secreting cells were calculated for each organ by multiplying the mononuclear cell yield by the percentages of Ig-secreting cells per mononuclear cell for each class in that organ. The greatest numbers of Ig-secreting cells were recovered from the bone marrow and spleen, while the recovery from the intestine and lungs were at least 10- to 100-fold less. These differences were similar at all ages studied.

These data would indicate that the bone marrow and spleen have the greatest humoral immune capabilities at all ages studied, based on the total numbers of Ig-secreting cells recovered. However,

the actual humoral immune potential of the bone marrow may be greatly underestimated since only a portion, perhaps 1/10 or less, of the total bone marrow was sampled. The entire spleen was studied and the numbers of cells recovered probably reflect the potential of the spleen for Ig secretion. The numbers of Ig-secreting cells recovered from the lungs were also reasonably accurate since both lungs were processed in their entirety. The same holds true for the intestine since most lymphoid cells should be in the region of the small intestine which was studied. However, the lamina propria of the intestine was difficult to disrupt and the number of recovered cells was probably significantly underestimated. Despite these provisos, one could reasonably conclude that the bone marrow has by far the largest reservoir of total Ig-secreting cells, followed by the spleen. This would be consistent with the known major role of the bone marrow and lymphoid organs such as the spleen in specific antibody production (Askonas & Humphrey, 1958).

The bone marrow is known to possess many B cell precursor populations including stem cells, pre-B cells and immature B cells (Gathings, Lawton & Cooper, 1977). Our data would emphasize that the bone marrow as well as the other major lymphoid organs of chickens have large numbers of mature B cells which are actively secreting immunoglobulin. However, the prominence of Ig-secreting cells in the bone marrow relative to the other organs is evident as early as 9 days after hatching and persists to adulthood.

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