ONTOGENY OF B CELLS IN THE CHICKEN

I. Sequential Development of Clonal Diversity in the Bursa*

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Early studies on the ontogeny of antibody responsiveness revealed that the capacity to respond to different antigens is developed at different developmental stages in a variety of species (1, 2). The patterns of sequential responsiveness to a given panel of antigens may vary between different species and even between different strains of inbred mice (1–5). The search for an explanation of this "antigenic hierarchy" is complicated by the fact that antibody responses are the products of complex interactions between effector cells, thymus-derived (T) cells and bursa- or bursa-equivalent-derived (B) cells, and accessory cells, e.g., macrophages (6). Each of these cellular participants may develop and achieve functional maturity at different stages during ontogeny (7–10). Central to a solution of the problem is an understanding of the cellular patterns and the responsible genetic mechanisms for generation of a heterogeneous population of B lymphocytes, each of which carries surface antibody receptors with the same specificity as the antibodies to be produced by its plasma cell progeny (11–14).

The generation of B-lymphocyte diversity has been studied mainly in the mouse by examining either the ontogeny of antigen-binding cells (ABC)[1] for several antigens (15, 16) or the ontogeny of antigen-responsive B lymphocytes under conditions in which functionally mature auxiliary cells are present in nonlimiting numbers (17–21). Results obtained by both approaches imply the antigen-independent generation of clonal diversity. Studies employing the latter approach suggest that various clones of antigen-responsive B cells are generated in a genetically predetermined sequence. However, while results of one study suggested that B cells binding one antigen may be generated from stem cells before B cells binding another antigen (22), others have provided evidence that cells binding many different antigens appear simultaneously, increase in parallel with the ontogeny of immunoglobulin-bearing cells, and express the full range of antigen-binding avidities immediately on development (15, 16).

The avian immune system, although less extensively studied in relation to this problem (23–25), provides a unique advantage for studying the development of B-cell diversity due to the presence of the bursa of Fabricius, that normally serves as the single central organ for the generation of B lymphocytes (26–28). We have investigated the ontogeny of B lymphocytes binding sheep erythrocytes via native antibody receptors in relation to development of the overall popula-

tion of IgM-bearing lymphocytes in the chicken. This was examined in the bursa, the circulation and the spleen of birds raised in either germfree or conventional environments. The anatomical distribution of B-lymphocyte clones generated within the bursa was studied using as antigens sheep erythrocytes (SE), key hole limpet hemocyanin (KLH), and poly-L(Tyr,Glu)poly-D,L-Ala-poly-L-Lys (TGAL). Our results imply an antigen-independent and sequential generation of clones in multiple sites within the bursa followed by orderly expansion and seeding into the periphery.

Materials and Methods

Chickens and Eggs. Fertile eggs derived from a cross between Arbre-Acres and Van Tress strains, and chicks were hatched and raised under conditions previously described (27).

In two experiments, germfree chicks were obtained by transferring 19-day old fertile eggs, sterilized by dipping into 3% iodine-iodide solution, to a germfree isolator. After hatching they were fed an antigen-restricted and autoclaved diet (29). The isolator environment and cloacal swabs were tested for sterility by blood agar plates under aerobic and anaerobic conditions and no bacterial growth was observed. Control animals kept in a conventional environment were given the same diet. Surgical bursectomy was performed under general anesthesia on the day of hatching.

Preparation of Cell Suspensions. Bursa, thymus, spleen, embryonic liver, and yolk sacs (freed of yolk by washing in phosphate-buffered saline) were gently dissociated in Hanks' balanced salt solution plus 0.01 M HEPES and 1% fetal calf serum using a loose-fitting glass homogenizer. After filtration through fine steel mesh, all suspensions were washed and allowed to settle to exclude clumps and glass-adherent cells. Separation of bursa into cortical and medullary fractions (30) was performed using bursae from 11-wk-old birds. Bone marrow cells and peripheral blood lymphocytes were obtained as described (31).

The cell viability as determined by trypan blue dye exclusion varied according to the organ sources and ages of donors: bursae up to 18 days of donor incubation age, <50%; bursae after 18 days, >85%; thymus, >90%; embryonic spleens, <50%; posthatching spleens, >70%; embryonic livers, 10%; yolk sac at 14 days, 95%; at 16 days, 60%; bone marrow, >85%; and peripheral blood lymphocytes, >95%. Due to the low yield and viability of embryonic cell suspensions, organs were pooled from 20-50 donors.

Dead cells and erythrocytes were removed by a bovine serum albumin (BSA) gradient-technique (32). Cell suspensions obtained by this method yielded a viability of >95% with the exception of embryonic liver (<50%). Comparisons of separated and unseparated cell suspensions always showed an enrichment of ABC and surface IgM-bearing cells which was directly proportional to the improvement in viability.

Detection of Surface IgM (sIgM). Purified antibodies to chicken IgM were prepared and conjugated with fluorescein isothiocyanate (fluorescein/protein ratio, 2.5) as previously described (27). Cell suspensions were treated with fluoresceinated antibodies at a concentration of 500 μg/ml and examined after washing under incident illumination on a Leitz Orthoplan fluorescence microscope (E. Leitz, Inc., Rockleigh, N. J.).

Enumeration of ABC. Erythrocyte ABC were detected by rosette formation with heterologous erythrocytes using a ratio of 5-10 erythrocytes per lymphocyte,[2] and enumerated by screening 1-4 × 10^{6} lymphoid cells in hemocytometer counting chambers. After preliminary experiments using erythrocytes from mouse, hamster, sheep, horse, goat, cat, dog, rabbit, baboon, and human, all assays were performed with SE from a single donor.

Localization of ABC on Bursal Sections. KLH (Calbiochem, La Jolla, Calif.) and TGAL (a gift from Dr. E. Mozes, Department of Chemical Immunology, The Weizmann Institute of Science, Rehovot, Israel) were labeled with ^{125}I by the chloramine-T method (33). Cryostat sections were

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[2] The conditions for the rosette assay are described in detail elsewhere. Ivanyi, J., E. Fuensalida, and P. M. Lydyard. 1976. Rapid recovery of antigen-binding receptors on chicken B cells following anti-Ig serum treatment. *Eur. J. Immunol.* **6:**25.

prepared from bursae of 14-, 16-, and 18-day embryos and 2-, 5-, and 8-day chicks. They were incubated for 30 min at room temperature with the iodinated antigens (KLH, sp act 18 μCi/mmol; TGAL, sp act 14 μCi/mmol) at 500 μg/ml, washed extensively, treated with 5% TCA, and fixed in acetic-alcohol. Control sections were pretreated with either cold antigens (2 mg/ml) or anti-μ antibodies (1 mg/ml). For autoradiography the slides were dipped in NTB-2 emulsion (Eastman Kodak Co., Rochester, N.Y.), exposed for 4–8 days, developed in D19 (Eastman Kodak Co.), and stained with hematoxylin and eosin.

Inhibition of ABC by Anti-Immunoglobulin Antibodies. Cells (10^6) were treated for 15 min on ice with 25 μl of anti-μ (1 mg/ml) or antilight chain antibodies before addition of erythrocytes for the rosette assay.

Regeneration by Bursal Cells of sIgM and Specific Antibody Receptors. Bursal cells from a 6-wk-old chicken were treated with 1 mg/ml pronase (Sigma Chemical Co., St. Louis, Mo.) at a concentration of 4–6 × 10^7 cells/ml for 30 min at 37°C. After BSA gradient separation the number of cells bearing sIgM by immunofluorescence (sIgM-positive cells) and ABC was determined. The cells were then incubated in Waymouth's medium containing 20% fetal calf serum (Grand Island Biological Co., Grand Island, N. Y.) for 6 h at 37°C, passed again through a BSA gradient, and retested for sIgM and antigen binding. Cycloheximide (Sigma Chemical Co.) inhibition was carried out at a concentration of 20 μg/ml.

Immunization Procedures. Chickens were injected with a crude SE lysate (20% packed erythrocytes in distilled water) either intravenously (2 ml) or intrabursally (5 ml, repeated after 3 days) using the sucking reflex (34), and examined 6 days later. ABC in peripheral blood and the bursa were determined by the rosette assay.

Isolation of SE-ABC. After incubation of peripheral blood lymphocytes from immunized birds with SE, SE-ABC were separated on a Ficoll-Hypaque gradient and the erythrocytes lysed by brief exposure to ammonium chloride solution (35).

Results

Erythrocyte Selection for Detection of ABC by the Rosette Assay. As is the case in mammals (36), various types of mononuclear cells in chickens may bind erythrocytes from other species via nonimmunoglobulin receptors (37, 38). Therefore erythrocytes from 10 species and bursal cells from 4-wk-old chickens were examined for the quality and quantity of rosettes formed and for inhibitability of rosette formation by antilight chain antibodies. Bursal cells formed rosettes with erythrocytes from all the species tested with a frequency ranging between 2.2 per 10^5 (goat) and 32.0 per 10^5 (rabbit). Sheep, goat, cat, and baboon erythrocytes were free of clumps and crenation, and made stable rosettes which could be reliably read. Whereas binding of sheep, goat, and cat erythrocytes was completely inhibited by antilight chain antibodies, with all other species tested the inhibition was incomplete (78–97%). The presence of cross-reactive erythrocyte antigens (15) was examined using peripheral blood lymphocytes from chickens immunized with cat, sheep, and rabbit erythrocytes. The data obtained (Table I) indicate a high degree of cross-reactivity at the ABC level, thereby precluding the use of more than one species of erythrocytes. SE, having a slightly higher frequency of ABC than goat or cat, were chosen for this study.

Nature of the Antigen-Binding Receptor for SE-ABC. More than 20 SE-ABC per 10^5 bursal cells can be detected in chickens older than 2 wk. The binding of SE was inhibitable by pretreatment of bursal lymphocytes with a crude SE lysate. That the surface receptor for SE on bursal lymphocytes is IgM was shown by the complete inhibition of rosette-forming cells with anti-μ as well as antilight chain antibodies. This question was further investigated in peripheral B cells. Whereas SE rosette formation was completely inhibited by antilight

Table I

Evidence for Antigenic Cross-Reactivity of Erythrocytes from Different Species*

Source of erythro-cytes for immuni-zation	Percentage of peripheral blood lymphocytes forming rosettes with erythrocytes from:			
	Cat	Sheep	Rabbit	Horse
None	<0.01	<0.01	<0.01	<0.01
Cat	7.9	2.9	NT‡	3.1
Sheep	3.5	4.6, 4.9	1.1	12.0
Rabbit	NT	0.35	2.1	NT

* In each experiment, two 6-wk-old chickens were immunized with 1 ml of a 4% erythrocyte suspension and bled 4 or 6 days later.
‡ NT, not tested.

chain antibodies in the spleen, thymus, bone marrow, and peripheral blood lymphocytes, it was only partially blocked by anti-μ antibodies; this ranged from 76% inhibition for thymus cells to 92% for peripheral blood lymphocytes. The existence of another immunoglobulin class of receptors on some peripheral B cells is suggested by these results.

To exclude the possibility that bursal cells were binding SE through cytophilic IgM antibodies, we investigated their capacity to regenerate the receptors for antigen after enzymatic stripping of the cell surface. Pronase treatment completely removed surface IgM and ABC receptors detectable by immunofluorescence and binding of SE (Table II). Regeneration, which could be demonstrated after 6 h in culture, was prevented by incubation with cycloheximide.

Ontogeny of sIgM-Positive Cells in the Bursa.   Cells expressing μ and light chain determinants or cells capable of binding SE were not demonstrable in embryonic yolk sac and liver cell suspensions at 14 and 16 days of incubation. Thus under the conditions used, we were unable to confirm the presence of ABC in chicken fetal liver as demonstrated by use of very sensitive assays for antigen on glutaraldehyde-fixed cells (39); the latter treatment was inhibitory for antigen binding by immunoglobulin-bearing cells in our hands.

IgM-bearing cells in very low frequency (<0.1%) were first detectable in the bursa at 12 days of incubation (Table III). By 14 days, 7% of the cells carried sIgM, and the percentage of sIgM-positive cells in the bursa reached adult values by the 18th day of incubation. By contrast, significant numbers of bursal cells (~13 per bursa) capable of binding SE were first detectable on day 18; a background frequency of <1 per 10^6 was considered insignificant.

The population doubling time of the sIgM-positive cells over the period from day 14 to 16 was calculated to be 10.2 h. The data shown in Fig. 1 indicate that around the time of hatching an abrupt change occurred in the rate of increase in sIgM-positive cells. Regression analysis by the least squares method failed to reveal a standard growth curve which closely fitted all the data. The rate of increase up to the 19th day of incubation followed closely an exponential regression curve (using the formula, $\log_{10}$ (sIgM-positive) = $8.85 - 6.3e^{-0.216 \text{ (Time)}}$; $r^2 = 0.997$), whereas the data points after hatching followed a linear logarithmic rate of increase. Since the mean generation time for bursal lympho-

TABLE II

Regeneration of Surface IgM and Antigen-Binding Receptors on
Bursal Lymphocytes from 6-wk-Old Chickens

Time in cul-ture	Treatment with pronase	sIgM-positive cells	SE-ABC/10⁵ *
h		%	
0	−	94	26.9
	+	0	0
6	−	91	17.8
	+	86 (2)‡	39.7

* The frequency of lymphocytes forming rosettes with SE was determined by examination of $>10^6$ lymphoid cells.

‡ Datum in parentheses obtained using lymphocytes incubated in the presence of cycloheximide (20 μg/ml).

TABLE III

Ontogeny of sIgM Bearing* and SE-ABC‡ in the Chicken

Age	Bursa			Spleen		
	sIgM-positive cells	SE-ABC/10⁵	Ratio (× 10⁶) of SE-ABC/ sIgM-positive cells	sIgM-positive cells	SE-ABC/ 10⁵	Ratio (× 10⁶) of SE-ABC/ sIgM-positive cells
	%			%		
12-day embryo	<0.1	NT§	NT	NT	NT	NT
13 ″ ″	1.4	0	0	<0.1	0	0
14 ″ ″	7.0	<0.1	5	0.2	0	0
15 ″ ″	33.4	<0.1	1	NT	NT	NT
16 ″ ″	60.3	<0.1	1	0.3	0	0
17 ″ ″	64.2	<0.1	1	0.4	0	0
18 ″ ″	85.5	0.3 (±0.1)	3	1.0	0	0
20 ″ ″	85.0	0.5 (±0.2)	6	0.9	0	0
2-day chick	82.5	1.1 (±0.5)	13	10.8	0.2	18
5 ″ ″	86.2	2.5 (±1.0)	29	19.6	1.6	82
8 ″ ″	97.1	3.4 (±1.0)	35	32.2	2.2	69
14 ″ ″	84.4	12.0 (±3.1)	141	28.8	12.7	440

* % sIgM-positive cells was determined by immunofluorescence staining.

‡ SE-ABC were determined as the numbers of bursal lymphocytes or spleen mononuclear cells (minus erythrocytes) forming rosettes with SE; for each determination, $>10^6$ cells from pooled organs (5–60 donors) were examined. Data for the bursa are the means for determinations on three to four cell pools with standard errors given in parentheses. Data for the spleen represent the means for two cell pools.

§ NT, not tested.

cytes is approximately the same in 15-day embryos and newly hatched chicks (40), these regression curves imply a persistent loss of bursal lymphocytes which begins slowly over the last few days of incubation and reaches a maximal rate around the time of hatching. In addition to their delayed appearance, SE-ABC also increased in frequency at a linear logarithmic rate which differed signifi-
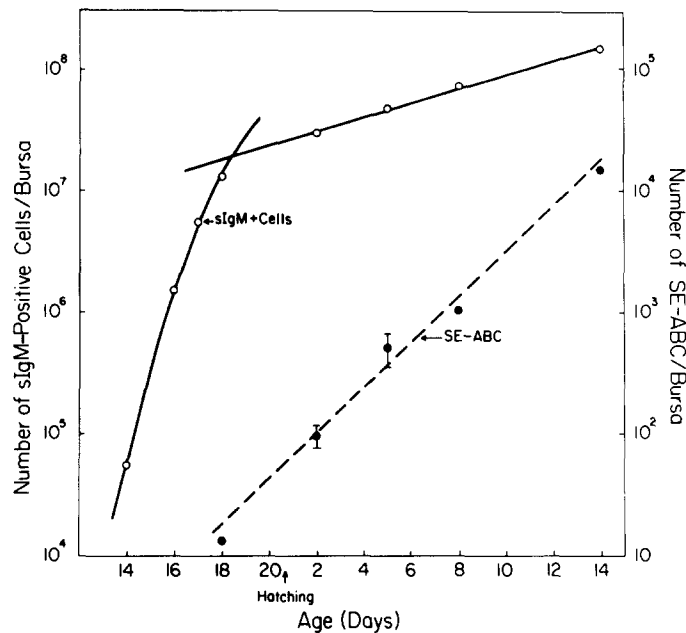
FIG. 1. Ontogeny of sIgM-positive cells and SE-ABC in the bursa. The values indicated by circles (O, sIgM-positive cells; ●, SE-ABC) were calculated from frequency determinations of sIgM-positive cells and SE-ABC in pooled samples obtained from 5 to 50 donors at each data point except at 2 and 5 days of age, when the mean values ± standard errors (⬥) are given for measurements of SE-ABC in individual bursae from 5 donors; these means correlated well with those estimated from pooled samples at the same times 0.85×10^2 and 5.2×10^2 at 2 and 5 days, respectively. The three frequency curves represent regression lines fitted by least squares analysis on log transformed data.

cantly ($P < 0.01$ after hatching) from that of the total bursal population of sIgM-positive lymphocytes.

Anatomical Distribution of ABC Within the Bursa. The lymphoid structure of the bursa is compartmentalized into epithelium-related follicles, which are approximately 10^4 in number. To determine whether or not ABC were generated in one or multiple follicles, two approaches were made using SE, KLH, and TGAL as antigens.

In the first experiments, individual bursae were cut into fragments, each of which was made into a cell suspension and examined separately for SE-binding cells. In bursae from both 2- and 35-day-old animals, SE-ABC were found in every fragment examined, and the frequencies of SE-ABC showed remarkably little variation from one bursal fragment to another (Table IV); the ratio of the highest incidence to the lowest was 2.8/1.

In the next experiments, autoradiography and ^{125}I-labeled KLH and TGAL were used to determine the distribution of ABC more precisely. Cryostat sections of bursae from 16 days of incubation to 8 days after hatching, but not from 14-day bursae, revealed cells binding KLH or TGAL in singlets or in small groups of contiguous cells no more than four in number. These were present exclusively within the follicles (see Fig. 2). Cells binding these antigens were

TABLE IV
Anatomical Distribution of ABC in the Bursa

Donor age	No. of bursal fragments	SE-ABC/10⁵ *
days		
2	4	0.4, 0.8, 0.4, 0.3
35	10 (plicae)	7.7, 9.1, 19.8, 16.7, 8.5, 21.8,
		15.3, 9.8, 19.7, 14.7

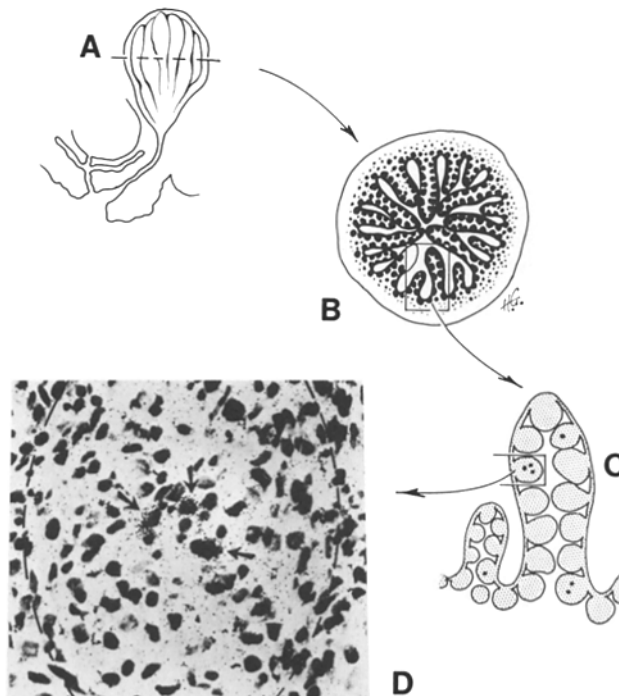* Number of bursal cells in each fragment which formed rosettes with SE.

FIG. 2. Localization of lymphoid cells binding ¹²⁵I-labeled TGAL within bursal follicles. Transection of the bursa of Fabricius (A), a lymphoepithelial organ attached to the dorsum of the cloaca reveals several internal plicae, or folds, each of which contains a large number of lymphoid follicles (B). Cells binding iodinated TGAL were detected within multiple follicles in small foci as illustrated schematically in (C) and in a photomicrograph (D) showing the autoradiographic localization of TGAL-ABC (arrows) within the follicular border (outlined by broken circle). Although at this age (16 days of incubation), 60% of the bursal lymphocytes carry detectable sIgM, the great majority of lymphocytes in the same plane (D) do not bind TGAL, suggesting that they carry IgM antibodies with different specificity.

seen in 0-5 follicles per section containing 30-50 follicles. In follicles containing ABC, the great majority of lymphocytes did not bind the antigen. No labeled cells were detected after pretreatment of sections with cold antigen or anti-μ antibodies.

Development of SE-ABC in Peripheral Lymphoid Organs.   Low numbers of sIgM-bearing cells, but not SE-ABC, were detectable in the spleen before 18

days of incubation (Table III); this was followed by a 10-fold increase in frequency by 2 days after hatching. SE-ABC were first detectable in the spleen on the day of hatching. Thereafter their frequency increased rapidly reaching adult values (12.7 per 10^5) at 2 wk of age; the frequency of SE-ABC at 5 wk was 8.2 per 10^5 and at 11 wk, 9.7 per 10^5 cells. SE-ABC in the spleen showed a homogeneous distribution as indicated by virtually identical frequencies (6.2, 5.1, 4.6, and 5.2 per 10^5 cells) detected in four different spleen fragments from a 23-day old chicken.

Very few sIgM-bearing cells (<0.1%) were detectable in the thymus before hatching and plateau levels (3.5%) were reached by day 8. At this time significant numbers of SE-ABC were detectable (0.6–3.5 per 10^5 cells).

Effect of Bursectomy at Hatching on Development of sIgM-Positive and SE-ABC in the Spleen. The late development of sIgM-positive and SE-ABC in the spleen as compared with the bursa is consistent with the fact that splenic B lymphocytes are bursa-derived. The data in Table III and Fig. 1 suggest a sharp increase in seeding of B lymphocytes around the time of hatching. This possibility was supported by the demonstration of adult frequencies for sIgM-bearing cells in the blood of newly hatched chicks (Table V). This was further explored by comparing the peripheral development of sIgM-positive cells and SE-ABC in normal birds and in birds subjected to bursectomy at hatching. The results of this experiment indicate that bursectomy at hatching results in a drastic reduction of B lymphocytes in the circulation and an early arrest in development of sIgM-positive and SE-ABC in the spleen. This developmental arrest in bursectomized birds was still present at 10 wk (1.4 ± 0.8 SE-ABC per 10^5 spleen cells) and previous observations on sIg-positive cells suggest that this is permanent (26).

Role of Exogenous Antigens on Development and Distribution of sIgM-Positive Cells and SE-ABC. The influence of antigens on development of the total population of sIgM-positive B lymphocytes and those capable of binding SE was examined by (a) reducing the exposure to environmental antigens and (b) immunization with a crude SE lysate.

Chickens hatched into a germfree environment and fed a low antigen diet developed sIgM-positive cells and SE-ABC in the same frequencies within the bursa and spleen as did controls raised on the same diet in a conventional environment (Table VI). Chickens exposed to SE lysate via the bursal lumen and unimmunized controls showed no significant differences in the numbers of SE-ABC in the blood, or among either the cortical or medullary fractions of bursal lymphocytes (Table VII). Likewise, intravenous immunization had no significant effect on bursal lymphocytes, while causing more than a 300-fold increase in the numbers of circulating SE-ABC. As has been shown previously (41), SE-ABC in the medullae were approximately twice as frequent as in the cortices of bursal follicles. The data indicate that the intrabursal generation of SE-ABC and initial population of the spleen by these clones are not governed by antigenic stimulation to an appreciable extent.

Homing Pattern of SE-ABC in Chick Embryos. 15-day embryos were given SE-ABC isolated from the peripheral blood of primed adult birds. Bursae and spleens from recipient and control birds at 20 days of incubation were analyzed

TABLE V
Effect of Bursectomy at Hatching on the Development of sIgM-Positive and SE-ABC in the Spleen and Circulation

Age	Experimental group (no.)	Bursa		Peripheral blood lymphocytes		Spleen	
		sIgM-posi-tive* cells	SE-ABC/10⁵ ‡	sIgM-posi-tive cells	SE-ABC/10⁵	sIgM-posi-tive cells	SE-ABC/10⁵
		%		%		%	
Hatching	Control (5)	85	0.5 (±0.1)	17	1.7 (±0.6)	2	0.3 (±0.06)
15 days	Control (5)	93	7.5 (±1.5)	18	2.9 (±0.9)	29	5.8 (±1.2)
	Bursectomy (5)			0.4	0.05 (±0.04)	6	0.8 (±0.6)

* The % of sIgM-positive cells was determined by immunofluorescence on suspensions of pooled cells from five donors.
‡ The values for SE-ABC represent the means (± standard errors) for determinations on samples from individual donors.

TABLE VI
Influence of Environmental Antigens on Bursal and Spleen Development of sIgM-Positive and SE-ABC

Age	Environment	Bursa		Spleen	
		sIgM-posi-tive cells*	SE-ABC/10⁵‡	sIgM-posi-tive cells	SE-ABC/10⁵
days		%		%	
2	Conventional	88	1.5 (±0.1)	10	0.3 (±0.2)
	Germfree	83	1.4 (±0.5)	9	0.3 (±0.2)
5	Conventional	93	3.0 (±0.1)	20	1.4 (±0.5)
	Germfree	92	3.0 (±0.4)	20	1.8 (±0.1)
8	Conventional	90	5.3 (±0.8)	27	3.0 (±0.8)
	Germfree	92	6.6 (±2.2)	26	2.6 (±0.5)
15	Conventional	87	9.7 (±2.7)	37	7.3 (±2.1)
	Germfree	89	13.3 (±4.9)	35	7.5 (±4.7)

* % sIgM was determined by surface staining with fluorescein labeled anti-μ antibodies.
‡ The numbers of cells forming rosettes with SE were determined by examination of >10⁶ bursal lymphocytes or splenic mononuclear cells minus erythrocytes. Means (± standard errors) were determined from values of three separate pools, each of which contained the cells from three donors.

for frequency of SE-ABC. The data shown in Table VIII demonstrate that SE-ABC homed to the spleen but not to the bursa.

Discussion

Our results indicate that chicken bursal lymphocytes expressing sIgM begin to develop on the 12th embryonic day, and increase in numbers at an exponential rate consistent with a population doubling time of approximately 10 h until significant seeding to the periphery begins. An increased sensitivity of detection achieved by examination of a pooled suspension of viable cells may account for the demonstration of sIgM-positive lymphocytes in the bursa 2 days earlier than in previous studies (27, 42). Still we were unable to confirm the presence of

Table VII

Effect of Immunization on the Frequency of SE-ABC in the Bursa and Circulation

Source of lympho-cytes	Route of immunization		
	Intravenous	Intrabursal*	Unimmunized con-trols
Bursa cortex	27.6 (± 2.7)‡	22.3 (± 2.8)	33.0 (±3.7)
Bursa medulla	74.6 (±21.8)	44.7 (±12.6)	58.4 (±8.8)
Peripheral blood	6,466 (±3,390)	19.1 (±11.1)	14.9 (±1.5)

* This route of immunization involved activation of a sucking reflex which results in an influx of the antigen solution into the bursal lumen and then via pinocytotic epithelium into the medullary areas of the bursal follicles (34, 73).

‡ The mean numbers of SE-ABC/10^5 lymphoid cells (± standard error) were determined using samples obtained from three 11-wk-old donors 6 days after intravenous immunization or 3 days after the second exposure to SE lysate via the bursal lumen.

Table VIII

*Homing of SE-ABC in Embryo Recipients**

Injection	SE-ABC/10^5 cells in recipient tissues	
	Bursa	Spleen
SE-ABC	0.27	5.46
Saline	0.49 (±0.25)‡	0

* 15-day chick embryos were injected with 2 × 10^5 SE-ABC, and organs pooled from five recipients were examined 5 days later. SE-ABC were isolated by density gradient centrifugation from the peripheral blood of adult allogeneic chickens immunized 5 days previously.

‡ Data from saline-injected controls was collected from three groups of five embryos.

native immunoglobulin determinants on the surface or in the cytoplasm of yolk sac cells (43).[3] Thus, the present results are consonant with the prevailing view that the bursa of Fabricius provides the microenvironmental influences responsible for the initial expression of genes coding for immunoglobulin determinants.

Most of the rapid increase in numbers of sIgM-positive lymphocytes within the bursa of chick embryos is attributable to the proliferation of bursal lymphocytes. Between days 14 and 16 of incubation the population doubling time is approximately 10 h (see Fig. 1) and the mean generation time has been calculated to be 8–10 h (40). At this age the influx of stem cells into the bursa apparently has ceased (45). Moreover, the effects of bursectomy at different times during late embryonic life and results obtained in the present studies suggest that lymphocytes begin to migrate from the bursa during the last few days of embryonic life (26, 46). The dramatic rise in the numbers of sIgM-

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positive cells in the spleen between the last day of incubation and the 2nd day after hatching suggests that seeding of bursal cells increases sharply about this time. This may be attributable at least in part to the influence of an increased output of endogenous corticosteroids at hatching (47).

Comparative studies of the kinetics of B-lymphocyte development in the bursae and spleens of birds hatched and housed in conventional or in germfree environments provide additional support for the view that antigens play little if any role in the initial generation and seeding of B lymphocytes. Since an environment entirely free of antigens probably is never achieved and some antigenic materials were probably derived from the hen, the failure to alter the frequency of SE-ABC in the bursa by immunization (reference 41; Table VII) may be the most convincing support for this conclusion.

Erythrocytes provide a convenient source for natural antigens and the rosette assay is a reliable method for detecting lymphoid cells capable of binding erythrocyte surface antigens. That bursal lymphocytes bind SE via IgM antibody receptors was shown by inhibition of rosette formation with antibodies to μ-chain and light chain determinants and with a crude erythrocyte lysate. After pronase stripping of the cell surface, antigen-binding sIgM molecules were regenerated by bursal lymphocytes except when cultured in the presence of cycloheximide. Unfortunately, cross-reactivity between antigens on the surface of erythrocytes from several species precluded the use of erythrocytes from multiple species in studying the ontogeny of ABC for different antigens. However, ^{125}I-labeled KLH and TGAL had the added advantage of being suitable for the study of the anatomical distribution of ABC in sections of the bursa. Binding of these labeled antigens was inhibitable by the appropriate unlabeled antigen preparations and also by anti-μ antibodies.

Although independent markers for chicken T and B cells were not used in the present studies, the fact that the cells which bound SE did so by native IgM receptors indicated that they were B lymphocytes. Others have shown using antisera specific for T and B cells that virtually all of the bursal lymphocytes react only with anti-B antisera (48, 49). Although T cells in peripheral sites can form passive rosettes with SE due to cytophilic IgM antibodies produced by B cells, this has only been demonstrable in antigen-primed animals (50). Since very young unprimed birds were used in our experiments, it seems improbable even in peripheral tissues that significant numbers of T cells were detected as SE-ABC.

Detailed study of the ontogeny of SE-ABC revealed that these clones were generated in the bursa beginning by the 18th embryonic day, i.e., 6 days after the appearance of sIgM-positive cells in this location. The appearance of SE-ABC in the bursa was soon followed by their appearance in the spleen. The rate of increase in numbers of SE-ABC observed in bursae after hatching was doubtlessly influenced by this seeding process. For this reason, a direct comparison of our results with those of Spear et al. (15), who showed parallel increases of surface Ig-positive cells and SE-ABC in the mouse spleen, is probably not valid. Other studies suggest that the mouse spleen is a generation site for B lymphocytes for a short time after birth (51), but most of the splenic B cells in mice probably are derived at first from the liver and later from the bone marrow (52–56).

The relatively late development of SE-ABC in the bursa and the demonstration that SE-ABC are evenly distributed throughout the bursae of 2- and 35-day-old birds, suggested the possibilities that clones of SE-ABC are generated in multiple bursal sites and that other clones of ABC might be generated in a similar fashion earlier in the ontogeny of B lymphocytes. Experimental support for both of these possibilities was obtained in studies of the ontogeny and anatomical distribution of cells capable of binding ^{125}I-labeled KLH and TGAL. Cells binding each of these antigens were detected in small foci within scattered lymphoid follicles of the bursa by the 16th day of embryonic life. We cannot formally exclude the possibility that the assay for cells binding the radiolabeled antigens is more sensitive than the rosette assay for SE-ABC. The available evidence on this point is conflicting (22, 57). However, even if the rosette assay only detects clones of lymphocytes with the highest antibody affinity for SE antigens, these results still suggest that clones of ABC for KLH and TGAL are generated earlier than certain clones of SE-ABC.

The finding of sequential bursal generation of different B-cell clones with subsequent seeding to peripheral tissues predicts that bursectomy at different times during development should result in selective gaps in the overall pattern of antibody responsiveness. This has been difficult to demonstrate experimentally but, by analysis in bursectomized birds of antibody responses to SE, *Bordetella pertussis*, human serum albumin, and influenza virus, Ivanyi has provided suggestive evidence for sequential seeding of B-cell clones (25). Birds subjected to early bursectomy fail to produce antibodies to phosphorylcholine even after repeated immunization with the pneumococcal strain R36a (H. Cosenza, H. Köhler, and M. D. Cooper, unpublished observations).

These results in the chicken complement evidence for sequential development of clonal diversity among responsive B cells in the mouse (17–21). On the other hand, Edelman's group have observed in embryonic mice the simultaneous and parallel development of cells capable of binding many different antigens with the full range of avidities (15, 16), although it may be noteworthy that the assays used detected relatively high frequencies of ABC many of which were not inhibitable by anti-Ig. The simultaneous appearance of full clonal diversity among B cells in mice would suggest that the sequential appearance of antibody responsiveness to different antigens may be primarily related to the functional development of auxiliary cells. Against this hypothesis is the sequential development of responsive B-cell clones in the presence of functionally mature helper cells (17–21). Another possibility is that clonal heterogeneity of mouse B cells is fully developed by the time of sIgM expression but further differentiation is required before all of the clones can be influenced by antigens plus helper activity to develop mature antibody secreting members. This idea is supported by the increased heterogeneity of antibodies produced by mouse fetal liver B cells from the 16th embryonic day onward when stimulated both by antigen and lipopolysaccharide (LPS), whereas B cells from 14-day fetal liver produced antibodies of restricted diversity even in the presence of LPS (58). In view of the facts that cytoplasmic expression of IgM precedes by several days the expression of stable surface IgM receptors in mice (51, 55) and humans (59), and LPS stimulation of B-cell maturation can only be demonstrated after the appearance

of sIgM (60), it is clear that further studies on the ontogeny of ABC in mammals are needed to resolve this important issue. The virtually simultaneous expression of surface and cytoplasmic IgM by bursal lymphocytes[3] could account for the apparent discrepancy between our results on the ontogeny of ABC in the chicken and those reported for mice (15, 16).

The anatomical distribution of ABC for KLH and TGAL being generated in the bursa is especially interesting. Not only were ABC for both antigens found in multiple bursal follicles, but only a few contiguous ABC were present within individual lymphoid follicles; the great majority of lymphocytes in positive bursal follicles did not bind the antigen under study. Since this was the case when nearly all of the bursal lymphocytes had easily detectable sIgM, the most straightforward interpretations of the observations are that multiple clones of ABC are generated within individual bursal follicles and that similar clones of ABC are generated within multiple bursal follicles. We have no evidence indicating that the cells binding the same antigens in different follicles bear identical antibody receptors.

Before speculating on the theoretical implications of these findings, evidence relating to the number of stem cells that initially populate each bursal follicle and the possibility of migration of cells from one bursal follicle to another need to be considered. Data on the precise number of stem cells populating each bursal follicle is presently unavailable, but the experimental results of Le Douarin et al. (45) may allow a reasonable estimate. They studied the influx of stem cells into the bursa by exchanging bursal grafts between chick and quail embryos of different ages. The identification of donor and host cells was made possible by distinctly different chromatin patterns in the nuclei of interphase cells from these two species. Their results show that in the chick, stem cell influx into the bursal mesenchyme begins on the 8th day and continues until around the 12th day. After a slight delay the stem cells subsequently migrate into the epithelium where they give rise to B lymphocytes. Regardless of the age of the donors of bursal grafts, the numbers of individual bursal follicles which developed chimeric lymphoid populations never exceeded 54%; the rest contained only donor quail or solely host chick lymphocytes. While this shows that more than one functional stem cell frequently may enter a single follicle, it strongly suggests the number that do so is very small. Moreover, if lymphoid cells could migrate freely from one bursal follicle to another, essentially all of the individual follicles would eventually be expected to exhibit lymphoid chimerism. The lack of homing of SE-ABC to the embryonic bursa demonstrated in the present experiments (Table VIII) is also against the possibility of interfollicular traffic of ABC. Furthermore, transformed bursal cells in lymphoid leukosis induced by avian leukosis viruses often remain confined to a single follicle for long periods of time (61).

We conclude, therefore, that our observations provide evidence for the generation of multiple clones of B cells from single stem cells. The alternate possibility that the B-cell progeny of each stem cell express only one antibody specificity could only fit our data if different stem cells undergo B-cell differentiation at different but fixed times during ontogeny. That each stem cell should give rise to a number of clones during normal B-cell development is entirely consistent with

previous results indicating that the progeny of a single hemopoietic stem cell may give rise to apparently unlimited diversity of antibody-producing cells in lethally irradiated recipients (22, 62). This has several interesting implications, one of which is that B cells producing identical antibodies may be the progeny of different stem cells. Although having the obvious advantage of reducing the likelihood of gaps in the development of clonal diversity, this would complicate the present concept of a "B-cell clone" as the progeny of a single cell. The term "clonotype" indicating only that the cells under discussion are expressing a particular antibody appears preferable (20).

Previous results from this and other laboratories indicate that, in both chickens and mice, μ-chain expression occurs first in stem cell differentiation along B-cell lines, and that within all B-cell clones expression of constant (C)-region genes for other heavy chain classes occurs subsequently by a sequential switch mechanism that is as yet undefined (20, 51, 55, 63–66). Expression of light chain genes occurs simultaneously with the expression of μ (27, 51). In the chicken this appears not to require a selection process for the stem cell or its progeny because only lambda light chains have been demonstrated (67). Two basically dissimilar patterns could account for the expression of multiple heavy and light chain combinations of variable (V)-region genes by the progeny of a single stem cell (68). First, the switch in expression of V-region sets could occur at the B-cell level of differentiation as is the case for changes in expression of one C-region gene to another. Since cell surface expression of antibodies would precede changes in V-region genes expressed, this model would conform to requirements for somatic mutational theories which have the need for either positive or negative (tolerance) selection pressures to be exerted by antigens of either intrinsic or extrinsic sources (69–71). Since there is convincing evidence that exogeneous antigens do not influence the developmental pattern of cellular expression of V-region diversity (references 15, 16, 18, 20, and 72; Tables VI and VII), it is difficult to envisage that endogenous antigens do so in a positive fashion. This model also has the potential disadvantage of changes in V-region expression occurring after the switch from μ-chain expression to other heavy chain classes thereby precluding the full expression of V-region diversity in all classes of antibodies. The model which we favor (Fig. 3) indicates a sequential preprogrammed selection of different sets of V-region genes occurring at a bursal stem cell level. This model would seem to fit the available data, eliminate some of the disadvantages of the first model and merge best with the germ line theory of predetermined permutation of genetic information expounded by Klinman and co-workers (20). A precise genetic mechanism by which stem cells could shift V-region frames remains to be determined.

Summary

The initial development and distribution of lymphocytes expressing surface IgM (sIgM) and of specific antigen-binding cells (ABC) were studied in the chicken in an attempt to gain information on the process by which B-cell diversity is generated. The antigens used were sheep erythrocytes (SE), keyhole limpet hemocyanin (KLH), and poly-L(Tyr,Glu)poly-D,L-Ala-poly-L-Lys (TGAL). The results indicate that generation of the total sIgM-positive popula-
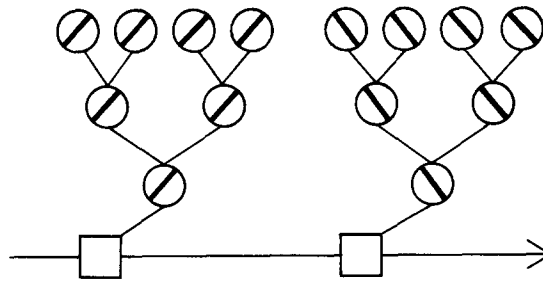
FIG. 3. Hypothetical model for the generation of multiple B-cell clones from individual stem cells. In the bursa or bursa equivalent, immortal stem cells (represented by squares) divide to give rise to (a) a daughter B cell expressing one set of V-region genes determining antibody specificity (indicated schematically by direction of bar across the circles which denote B cells) and (b) another stem cell. By a programmed change in V-gene selection, this stem cell could give rise to another ancestral B cell expressing a new specificity, and so on. Experimental support for this model, some of its biologic implications and possible theoretical advantages are discussed in the text. In devising this schema, we were influenced by Cairns' considerations of the mechanisms by which rapidly dividing epithelial cells avoid accumulation of mutations (74).

tion begins in the bursa and that specific clones of ABC develop in a fixed sequential pattern which is not influenced by either deprivation of or deliberate exposure to exogenous antigens. Cells bearing sIgM by immunofluorescence (IgM-positive cells) were detected first in the bursa on the 12th day of incubation, KLH-ABC and TGAL-ABC by the 16th day, and SE-ABC on the 18th day. The doubling time of the sIgM-positive population of bursal cells was determined to be approximately 10 h before significant antigen-independent seeding to the spleen began a few days before hatching. Clonal expansion of SE-ABC in the bursa also appeared to be antigen independent as was the initial development of SE-ABC in the blood and spleen which ceased abruptly after bursectomy at hatching.

Specific ABC were observed to develop in multiple bursal follicles as small foci of ABC among the much larger total population of sIgM-positive cells within an individual follicle. Intravenously infused SE-ABC homed to the embryonic spleen but not to the bursa. The results are interpreted as favoring a hypothetical model in which individual stem cells give rise to multiple clones of B cells by a predetermined pattern of sequential expression of variable region genes.

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