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Some Preliminary Studies on the Ontogeny of IgA Producing Cells in Chicken.

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Abstract

Administration of anti-chicken IgG Fc serum in 15-day embryos did not inhibit the IgA production in hatched chicks. The IgA-bearing cell count in the bursa of Fabricius (BF) of chicks from day 18 of incubation to 5 days after hatching consistently exceeded the other immunoglobulin bearing cell counts. When neonatal chicken bone marrow cells were transferred to cyclophosphamide treated chickens, IgA-bearing cells appeared in the BF of the recipients by 2 days after the transfer, and were 3 and 9 times the number of IgM- and IgG-bearing cells respectively.

These results strongly support the hypothesis that IgA-producing cells may be produced independantly of IgM-bearing cells.

Introduction

IgG- and IgA-producing cells in mammals are presumed to be differentiated (class-switch) from immature B cells that have IgM on the cellular membrane.

In experiments using chickens, Leslie & Martin[1], Martin & Leslie[2] and Perey & Bienenstock[3] showed that chicken B cells also undergo a similar class-switch process. However, other studies have reported that lymphocytes having IgA on the cellular membrane exist within the bursa of Fabricius (BF) as early in the embryonal stage as 10 days when neither IgM nor IgA is detected on the B cell surface[4] and that a large number of anti-IgA antibody labelled lymphocytes exist in the BF tissue of 11-day-old embryos[5]. Furthermore, it has also been reported that surgical bursectomy at 60 hours after incubation results in chickens that produce IgA but not IgM or IgG[6]. These studies that in chickens IgA-producing cells may not be differentiated from IgM-producing cells but achieve maturation through an independent route of differentiation.

The purpose of this study is to investigate the origin of IgA-producing cells and the process of ontogenesis including class-switching in chickens.

Materials and Methods

Chickens and eggs.

White leghorn chickens and their eggs of non-inbred strain Hy-Line and an inbred strain Anthony were used. The Anthony strain is homozygous at the major his-

tocompatibility B locus and is of blood type B^AB^A, B^KB^K and B^AB^K.

Preparation of immunoglobulins.

Immunoglobulin M was prepared from pooled chicken serum according to the method of Benedict[7] with some modifications. Basically this consisted of 25 % saturated ammonium sulfate precipitaion, gel filtration on Sephacryl S-300 and Sepharose CL-4 B, DEAE-Sephacel ion-exchange chromatography and purification by adsorption with anti-IgA coupled to a CNBr-activated Sepharose immunoadsorbent column. IgG Fc fragment was also prepared from pooled serum of normal chickens modified from the method of Benedict[7]: through 45 % saturated ammonium sulfate precipitation, Sephacryl S-300 gel filtration, DEAE-Sephacel and CM-cellulose ion-exchange chromatography, treatment with papain, Sephacryl S-200 gel filtration and DEAE-Sephacel ion-exchange chromatography. IgA was prepared from pooled bile of normal chickens modified from the method of Watanabe et al.[8]: through fractionated 10 % acetic acid, 50 % saturated ammonium sulfate precipitation, gel filtration on Sephacryl S-300 and Sepharose CL-4 B and DEAE-Sephacel ion-exchange chromatography. Purity of each of the immunoglobulins was examined by immunodiffusion and immunoelectrophoresis with anti-chicken IgM serum (μ-chain specific), anti-chicken IgG serum and anti-chicken IgA serum (α-chain specific; Miles Laboratories, Inc., USA.) and polyacrylamide gelelectrophoresis.

Preparation of antisera.

Antisera to each of the immunoglobulins were prepared by the sebcutaneous administration of purified immunoglobulin (1 mg) in Freund's complete adjuvant into rabbits. Booster injection of the same antigen (2 mg) were given intravenously a month later. The antisera were obtained one week after the booster injection and inactivated by heating at 56 °C for 30 minutes. The antisera were adsorbed with a CNBr-activated Sepharose immunoadsorbent column; the anti-IgM and anti-IgA serum were adsorbed withh the IgG coupled affinity column, and anti-IgG Fc serum were adsorbed with the IgG Fab'coupled affinity column. These antisera was specific to the antigenic immunoglobulins examined by immunodiffusion or immunoelectrophoresis.

Immunosuppressed chickens.

It is well known that the production of specific antibody is inhibited by either hormonal, chemical or surgical bursectomy. The possibility of obtaining chickens that produce only IgA was examined by means of various methods of immunosuppression from the embryonal stage until the neonatal period. Immunosuppressive chickens (B cell suppression) were prepared in the following 4 ways; (1) treatment at different embryonic stages with testosterone which inhibits the development of BF tissue, (2) teratment at the neonatal stage with colchicine which injures BF lymphoid follicles and BF lymphocytes, (3) treatment at the neonatal stage with cyclophosphamide which destroys the lymphocyte system of BF origin and (4) surgically bursectomy at the time of hatching

Testosterone treatment (hormonal bursectomy: HB) was performed by allantoic inoculation of testosterone propionate (TP; Sigma Chemical Company, USA.). Eggs were injected with 4 mg of TP in 0.1 ml of corn oil on the 8, 10, 12 and 14 days of incubation (TP_8, TP_{10}, TP_{12}, TP_{14}), and surgical bursectomies were performed on the day of hatching.

Chemical bursectomies were performed by a single intrabursal or intraperitoneal injection. For the former the posterior wall of the cloaca was incised to render the bursa

visible, and a colchicine (COL; Wako Pure Chemical Ltd., Osaka, Japan.) injected into the bursal tissue in a volume of 30 μl at a concentration of 1 mg per ml saline solution in newly hatched chickens. The intraperitoneal inoculation consisted of 2.5 mg of cyclophosphamide (cy; Shionogi & Company Ltd, Osaka, Japan.) in saline (25 mg/ml), daily for 4 consecutive days, starting on the day of hatching.

Surgical bursectomy (SB) were performed on the day of hatching. The X-ray irradiation unit was run at 200 KV, 20 mA and with a 0.3 mm copper- and 0.5 mm aluminium filter. The chickens received 650 R at a dose rate of 50 R per minute on the 5 days after SB.

Cytotoxic test.

The cytotoxic test were performen by the following procedure: 0.025 ml of each anti-immunoglobulin serum was incubated with 0.025 ml of a BF cell suspension ($5-10\times10^6$ cell/ml) and 0.025 ml of 1 : 4 saline of diluted, pooled fresh guinea-pig serum (complement) which had been adsorbed with 10^8 chicken spleen cells, in wells in an microtiter tray for 45 minutes at 37 °C. After incubation each mixture received 0.05 ml of a 0.5 % trypan blue solution and was examined for cell viability.

Intravenous injection of embryos with anti-immunoglobulin sera.

In accordance with Kincade et al.[9], the presence or absence of a differentiation process peculiar to IgA-produing cells was investigated. For injection of embryos, a section of shell over a chorionic vessel was removed, and 0.1 ml of each anti-immunoglobulin serum were administrated intravenously into respective groups of 15-day-old embryos. The shells were sealed with vinyl tape and left in an upright position in the incubator and subjected to SB at hatching to inhibit the production of immunoglobulins.

Cell transfer assays.

Six-day-old CY-treated chicks were intravenously transfused with neonatal chick (about 30 birds) femoral bone marrow cells (2×10^7). The ratios of immunoglobulin-bearing cells within recipient BF were determined at intervals of 2 days by the cytotoxic test. After surgical bursectomy within 24 hours after hatching, 18-days embryonic BF cells (2×10^6) were intravenously transferred to 5-day-old chicks that had received whole-body X ray irradiation (650 R) at 3 days of age, BF cells were obtained from about 100 embryos. The serum concentration of each immunoglobulin class was then determined at intervals of 1 week.

Quantitative estimation of immunoglobulins and immunization.

Immunoglobulins were assayed by the single radial immunodiffusion technique (SRID: realisable limit; IgA 5 μg/ml, IgM and IgG 25 μg/ml) on pooled sera of each group. Chickens were injected intravenously with 0.1 ml of a 10 % (v/v) sheep red blood cells (SRBC) at the primary (2-week-old) and secondary (4-week-old) stimulation and serum immunoglobulin concentration were determined one week after each immunization.

Experimental procedures.

a) Experiments in immunosuppression in an attempt to produce a chicken model with marked suppression of IgM and IgG but not IgA.

b) To determine whether IgA may be produced from cells other than those in the

BF. This was undertaken by determining the concentration of immunoglobulins in the serum of chickens which as embryos were inoculated with either anti-IgM or -IgG serum.

c) To determine the percentage of each class of Ig-bearing cells within the BF from the embryonic stage of chickens until after hatching.

d) To determine the percentage of each class of Ig-bearing cells within the BF of cyclophosphamide-treated chickens given a transfer of neonatal bone marrow cells as immunocompetent cells.

e) To determine the relative concentrations of immunoglobulins in surgically bursectomized chickens transfused with BF cells from 18-day-old chick embryos; thus to determine whether the Igs in the serum correlated with (d) above.

Results

Production of each immunoglobulin class in immunosuppressed chickens.

To examine for the presence of maternaly derived antibodies, the serum concentration of IgG antibody from the 14-day-old embryo until 14 days after hatching was determined by the SRID test. The concentration of maternal antibody rose to the maximun on the 4 th day after hatching.

The concentration was reduced to half after 14 days of age. On the basis of this finding, measurement of the serum concentration of immunoglobulin classes was carried out using chickens more than 2-weeks-old.

The results of Immunosuppression: In the TP-treated group, the serum concentration

Table 1. Serum concentration of each immunoglobulin in chickens treated at the embryonic stage with testosterone propionate.

		2 weeks old		4 weeks old	
Group #		No. of chickens	μg/ml	No. of chickens	μg/ml
	IgM	5	169*	2	244
TP_8	IgG	5	1608	2	347
	IgA	5	<5	2	<5
	IgM	6	141	6	130
TP_{10}	IgG	6	1735	6	238
	IgA	6	6	6	<5
	IgM	5	364	5	409
TP_{12}	IgG	6	1554	5	585
	IgA	5	7	5	9
	IgM	5	578	5	614
TP_{14}	IgG	5	1672	5	1385
	IgA	5	18	5	20
	IgM	19	782	16	844
Nor.**	IgG	19	2169	16	2685
	IgA	19	56	16	66

#Age of embryo days at inoculation with TP.

*Pooled serum.

**Normal chicken.

of IgG decreased with age but that of IgM increased. IgA also tended to increase with age in the group treated with TP in 12-day-old embryos (Table 1).

In the COL-treated, CY-treated and SB groups, the serum concentration of IgM tended to increase with age, as it did in normal chickens. The serum concentration of IgG was reduced with age except in the COL-treated group. IgA tended to increase in the COL- and CY-treated group (Table 2). Immunization with SRBC produced a similar results.

As described above, the various forms of immunosuppression failed to produce a chicken group in which the production of IgM and IgG was markedly inhibited while the production of only IgA was stimulated. Thus experimental chicken models for the purpose of determing that IgA producing cells undergo differentiation apart from IgM and IgG producing cells was not obtained.

Changes in serum concentration of immunolgobulin in chickens treated at the embryonic stage with anti-immunoglobulin serum.

The results are shown in Figure 1. In the group treated with anti-IgM serum, IgM, IgG and IgA concentrations were low until 6 weeks of age compared to the normal group. However, in the group treated with anti-IgG Fc serum, although the amount of IgG production stayed constantly lower than normal, the production of IgM and IgA was only a little lower in the normal group. Concentration of each immunoglobulins was determined on pooled sera from 3 to 5 chickens, therefore, statistical analysis were not made.

This demonstrates that the production of both IgG and IgA was inhibited by causing damage to embryonic B lymphocytes, which had IgM on the cell surface, by means of anti-IgM serum.

Table 2. Serum concentration of each immunoglobulin in chickens treated at the neonatal stage with colchicine, cyclophosphamide and surgical bursectomy.

Group #		2 weeks old		4 weeks old	
		No. of chickens	μg/ml	No. of chickens	μg/ml
COL	IgM	7	556*	7	555
	IgG	7	1752	7	1716
	IgA	7	28	7	14
CY	IgM	7	733	6	773
	IgG	7	2256	6	1710
	IgA	7	47	6	48
SB	IgM	8	745	8	818
	IgG	8	1965	8	1612
	IgA	8	48	8	29
Nor.	IgM	19	782	16	844
	IgG	19	2169	16	2685
	IgA	19	56	16	66

COL; Colchicine treated group. CY; Cyclophosphamide treated group.
SB; Surgically bursectomized group. Nor; Normal chicken
* pooled serum.

Changes in each class of immunoglobulin bearing cells within the BF tissue of chicks from the embryonic stage until after hatching.

In the present experiment, the cytotoxic test was used to determine the composition of immunoglobulin bearing cells within the BF of 18-day-old embryos to 5-day-old chicks.

As shown in Figure 2, IgM bearing cells within the BF tissue increased from 18 embryonic days until 3 days after hatching, and quantitatively dominated IgG-bearing cells. Despite an increase with age, the IgG-bearing cell count stayed always lower than the counts of IgM-bearing and IgA-bearing cells. IgA-bearing cells already existed more frequently in 18 day embryos and constantly exceeded the amount of other immunoglobulin bearing cells subsequently except in 1-day-old chickens. In sufficient BF cells were obtained prior to 18 days of embryonic growth to determine their type.

Ratios of immunoglobulin class bearing cells within BF of CY-treated chickens given transfer of neonatal bone marrow cells.

Neonatal bone marrow cells were transferred to CY-treated chickens to study the process by which immature bone marrow lymphocytes, which lack immunoglobulin almost entirely on the cell surface, release each immunoglobulin class within the BF tissue of recipient chickens.

The ratios of IgM-, IgG- and IgA-bearing cells in the transfused femoral bone marrow cells (donor cell) were 0.1 %, 2.8 % and 0.7 % respectively.

As shown in Figure 3, IgM-bearing cells amounted to 16 % at 2 days after the transfer. IgG-bearing cells increased with age from 2 days after the transfer. This increase occurred despite fluctuation in IgM-bearing cells. IgA-bearing cells existed more frequently and in a larger amount from 2 days after the transfer, compared to cells containing the other immunoglobulins. They varied between 35 % and 45 % until 10 days after the transfer. Serum immunoglobulin classes in surgical bursectomized chickens transfused

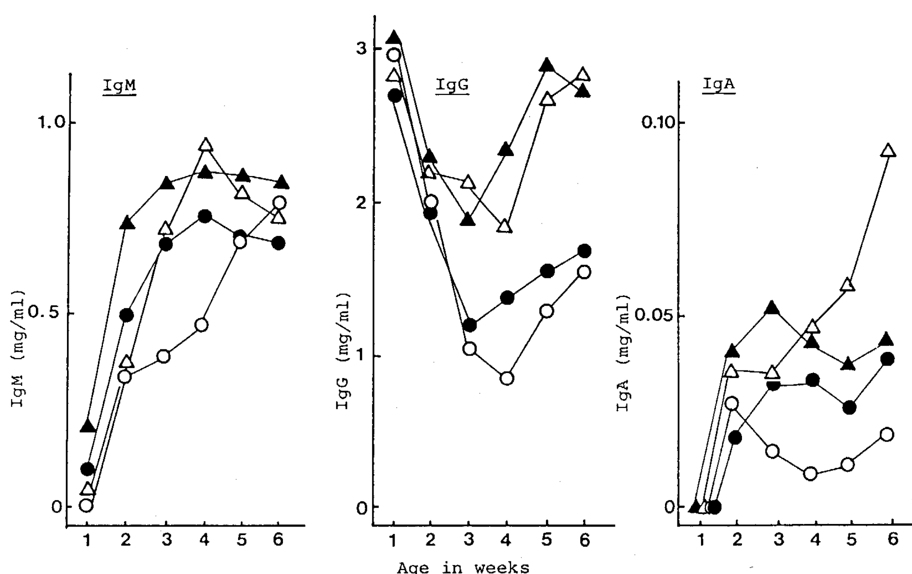

Fig. 1 Changes in serum concentration of immunoglobulin in chickens treated at the embryonic stage with anti-immunoglobulin serum. Fifteen-day embryos were intravenously injected with 0.1 ml of anti-chicken IgM (—○—) or IgG (—●—) serum and subjented to surgical bursectomized at hatching. Surgical bursectomized only (—△—). Normal control (——).

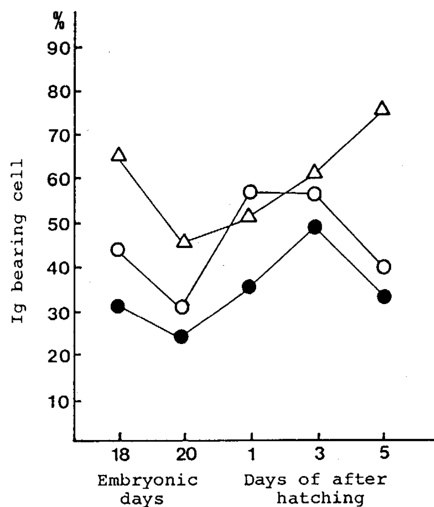

Fig. 2 Percent presence in each immuino-globulin class bearing cells within BF of chicks from the embryonic stage until after hatching. IgM bearing cell (—○—), IgG bearing cell (—●—), IgA bearing cell (—△—).

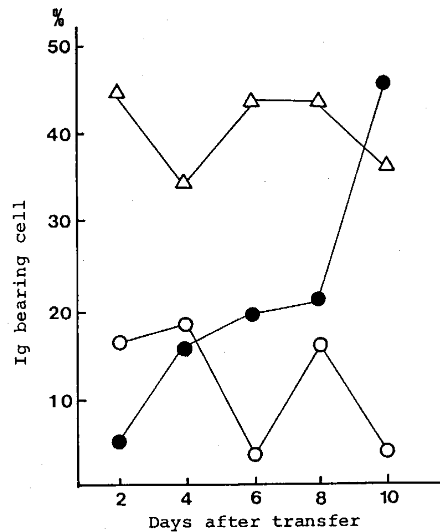

Fig. 3 Ratios of immunoglobulin class bearing cells within BF of CY-treated chickens given transfer of neonatal bone marrow cells. IgM bearing cell (—○—), IgG bearing cell (—●—), IgA bearing cell (—△—).

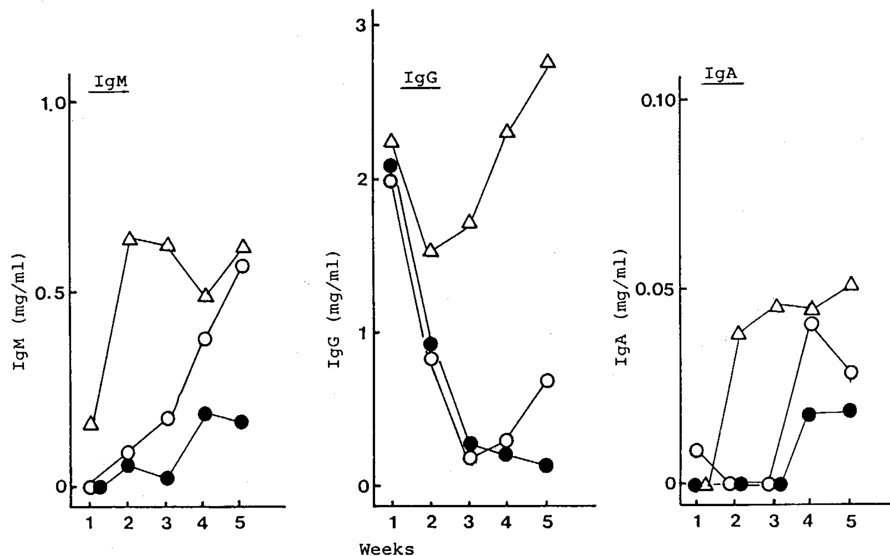

Fig. 4 Serum immunoglobulin classes in surgical bursectomized chickens transfused with 18-day embryonic BF cells to 5-day-old chicks. Surgical bursecto-mized and BF cell transfered (—○—), Surgical bursectomized only (—●—), Normal control (—△—).

with 18-day embryonic BF cells.

The preceding investigations revealed that a larger number of IgA bearing cells always existed within the BF tissue than cells containing other immunoglobulin.

Whether these IgA-bearing cells were IgA-producing cells was then investigated, namely, how the IgA-bearing cell count correlated to the serum concentration of IgA.

The results are shown in Figure 4. The serum concentration of IgA in the recipients

was low until 3 weeks of age. However, almost the same production of IgA as seen in normal chickens was observed after 4 weeks. The serum concentration of IgM showed the same pattern. The serum IgG concentration stayed very low even at 7 weeks of age.

Discussion

In the first immunosuppression experiment, the serum concentration of IgA was found to increase in parallel with that of IgM. This supported differentiation of IgA-producing cells from IgM-producing cells.

In the experiment using embryos treated with anti-immunoglobulin sera, anti-IgM serum caused reduction of serum concentrations of all immunoglobulin classes in hatched chicks, but, only IgM production was recovered by the B lymphocytes from non-bursal site origin[10]. However, treatment with anti-IgG serum caused reduction of IgG but resulted in production of approximately the same amounts of IgM and IgA as in normal chicks.

These results indicate that IgA-producing cells, like IgG-producing cells, are differentiated from IgM-producing cells. This coincides with the results of Leslie's experiment[1,2] with anti-immunoglobulin sera.

If there should exist within the BF tissue a route of differentiation by which IgM-producing cells are transformed into IgA-producing cells, IgM should be released first and be then followed by the appearance of IgA on the surface of BF B lymphocytes. However, on examining the composition of immunoglobulin class bearing cells within the BF tissue with the passage of time after 18 embryonic days, IgA-bearing cells existed always in large quantity. This finding is cosistent with the Albini's report[11] which showed that the number of IgA-beardng cells within the 18-day embryonic BF tissue exceeded that of IgA-bearing cellt and that IgA-bearing cells existed in approximately the same amount as IgM-bearing cells.

Albini & Wick[4] found IgA-bearing cells already within BF of 10-day embryos in which IgM- and IgG-bearing cells were not detected. The existence of IgA-bearing cells in 11-day embryonic BF has also been reported[5].

These descriptions in combination with the present experiment results suggest again the likelihood that IgA-producing cells do not originate from IgM-producing cells within the BF follicles but undergo a peculiar route of differentiation independently of other immunoglobulin producing cells.

On the other hand, Kincade & Cooper[12] found chick BF cells that had two kinds of surface immunoglobulin. These are considered to be the cells just undergoing differentiation from IgM- to IgA-containing cells or from IgM- to IgG-containing cells.

In the experiment in which the composition of surface immunoglobulin bearing lymphocytes in BF was determined, the total value exceeded 100%. This may be explained by the existence of cells having 2 kinds of surface immunoglobulin.

The existence of cells with 2 kinds of surface immunoglobulin may also support the theory that IgA-producing cells are differentiated from IgM-producing cells.

BF lymphocytes from 18-day-old chick embryos were used in the present experiment. Such cells that contained only IgA might have been the cells that had already gone through differentiation completely from IgM-bearing cells. If this presumption is correct, the concept of a peculiar route of differentiation for IgA-producing cells would not be tenable.

Chickens, which had been treated with CY for the destraction of B cells in the BF and peripheral organs were transfused with immature bone marrow cells having no surface

immuno-globulin in order to study the sequence of the appearance of surface immunoglobulin classes in the transferred cells that migrated into the BF. BF lymphocytes in the recipients released IgA on the cell surface as early as 2 days after the cell transfer. The number of IgA-bearing cells amounted to approximately 3 times the number of IgM-bearing cells and to approximately 9 times the number of IgG-bearing cells. This finding again demonstrates that IgA-producing cells are derived from sources other than IgM-producing cells.

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