DEVELOPMENT OF CHICKEN LYMPHOID SYSTEM

II. Synthesis of Primordial Immunoglobulin M by the Bursa Cells of Chick Embryo*

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In the preceding paper (1), we showed that the bursa cells of the chicken synthesize IgM which is not secreted. In addition to lymphoid cells which synthesize and secrete IgM or IgG, the bursa apparently contain a significant amount of a third population of lymphoid cells that synthesize nonsecretory IgM. Since, in the bursa, IgG-synthesizing cells apparently arise from a clone of IgM-synthesizing cells (2, 3), we suggested that nonsecretory IgM (i.e. IgM-H_0) is synthesized by a clone of lymphoid cells which may be the precursor of 19S IgM (i.e., IgM-H). The present paper provides evidence to support this hypothesis by showing that a quantitative change of IgM-H_0 and IgM-H occurs during development of the chick embryo.

It was also suggested that H_0 chains of the nonsecretory IgM may have a different structure from that of the immunoglobulin (Ig)[1] secreted by the spleen or the bursa cells. We show that the biochemical difference between H and H_0 chains resides in the amount of galactose residues attached to H chains. This finding is discussed in view of the process whereby Ig is secreted by normal lymphoid cells.

Materials and Methods

Animals.—White Leghorn chickens, line 91, were obtained as fertilized eggs from the Basic Research Laboratory, Hy-Line Poultry Farms (Johnston, Iowa), through the courtesy of Dr. G. R. Law. Eggs were incubated in a Jamesway Model 252 Incubator (James Mfg. Div., Fort Atkinson, Wis.).

Incorporation of Radioactive Amino Acids and Carbohydrates.—At various times between 14 and 20 days of incubation, 20–30 bursas were removed by surgical dissection from the chick embryos. The cell suspensions were prepared by mechanical disintegration of the bursa tissues and filtration through stainless steel mesh as previously described (4). The cells were

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[1] *Abbreviations used in this paper:* Ig, immunoglobulin; NP-40, Nonidet P-40 (Shell Chemical Corp.); PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate.

incubated at a concentration of 2×10^7 cells/ml in an Eagle's medium lacking leucine (5), and containing 5.0% fetal calf serum and 20 μCi/ml L-leucine (4,5-^{3}H) (40–54 Ci/mmole, Schwartz Bio Research, Inc., Orangburg, N.Y.), or 5 μCi/ml of L-leucine-^{14}C (262 mCi/mmole, New England Nuclear Corp., Boston, Mass.) and L-threonine-^{14}C (164 mCi/mmole). For incorporation of carbohydrates, a cell suspension (2×10^7 cells/ml) in Eagle's medium containing 0.25 mM glucose was incubated with 50 μCi/ml glucosamine-^{3}H (16 Ci/mmole, Amersham/Searle Corp., Arlington Heights, Ill.) or galactose-^{3}H (18.4 Ci/mmole). Incubations were performed in a humid tissue culture incubator at 37°C in 15% CO_2–85% air.

After the cultures were incubated for 3–4 hr, the incubation mixture was transferred to a centrifuge tube, quickly chilled in an ice water bath, and centrifuged at 3000 *g* for 10 min to separate the cells from the supernatant. The cell pellet was suspended in 0.05 M tris(hydroxymethyl)aminomethane (Tris)-HCl (pH 7.6, 4°C)–0.025 M KCl–0.005 M $MgCl_2$ and lysed by adding Nonidet P-40 (NP-40; Shell Chemical Corp., New York) to a final concentration of 0.5%. The nuclei and ribosomes were removed by centrifugation at 105,000 *g* for 120 min at 0°C (6). Both the cell lysate and supernatant fractions were subjected to serological assay and trichloroacetic acid precipitation to determine total radioactivity (1, 4).

Preparation of Antiserum and Serological Assay.—Antiserum against chicken IgM was prepared in rabbits by injecting purified chicken IgM as described previously (1). Rabbit anti-chicken μ-serum was prepared by solid immune adsorption of anti-IgM with crude chicken IgG. Serological assay for ^{14}C- or ^{3}H-labeled chicken Ig was performed by the indirect precipitation technique. Chicken Ig labeled with radioactive amino acids was complexed with an excess of rabbit anti-IgM and the complexes were precipitated by goat anti-rabbit IgG. The detailed method of preparation of serological precipitates for quantifying radioactive IgM has been described previously (4, 7). Radioactivity was measured by a Beckman Liquid Scintillation Counter LS 233 (Beckman Instruments, Inc., Fullerton, Calif.).

Acrylamide Gel Analysis.—The method of sodium dodecyl sulfate (SDS)–acrylamide gel electrophoresis of serological precipitates has been described elsewhere (4, 8, 9).

Histochemical Staining Method Using Horseradish Peroxidase.—An immunohistochemical method described by Mason et al. (10) was used for staining the bursa cells synthesizing IgM. Briefly, the bursa tissue was fixed for 90 min in 10% formalin plus 0.2% calcium acetate, dehydrated through graded alcohols, embedded in paraffin, and sectioned serially. The tissue sections were hydrated in phosphate-buffered saline (PBS) (i.e. 10^{-2} M phosphate buffer with 0.7% NaCl, pH 7.2) and stained in order with the following agents: (*a*) rabbit anti-chicken IgM; (*b*) goat anti-rabbit IgG; (*c*) rabbit anti-peroxidase; and (*d*) horseradish peroxidase (Sigma Chemical Co., St. Louis, Mo.). The sections were incubated for 10 min with each reagent and the enzyme in a humid chamber on a shaker. Between reactions the sections were washed four times with PBS. The site of bound peroxidase was visualized by incubating for 5 min at room temperature with the substrate solution (0.03% 3.3′-diaminobenzidine tetrahydrochloride in 5 mM Tris-PBS, pH 7.6). Before using, 3 drops of 3% H_2O_2 were added to 5 ml of the substrate solution. After the enzymatic reaction, the tissue was washed in PBS and treated with osmium for 5 min by adding a few drops of a 2% OsO_4 on the stained section. The sections were then dehydrated with graded alcohols, cleared, and mounted in Permount (Fisher Scientific Co., Pittsburgh, Pa.).

The method for preparing antisera for immunohistochemical staining is described elsewhere (4, 10).

RESULTS

Immunohistochemical Staining of the Bursa Tissue.—Serial sections of bursa tissues prepared from the chick embryos of various ages were stained by immunohistochemical method with rabbit anti-chicken IgM to detect Ig-producing cells in the bursa. Nonspecific staining or endogenous peroxidase activity

was revealed by staining with normal rabbit serum in place of specific anti-IgM serum. As shown in Fig. 1, lymphoid cells stained by anti-IgM were detected in the follicles of the bursa removed from 14-day old chick embryos. In the chick embryo younger than 14 days, follicles were not found in the bursa. As the chick

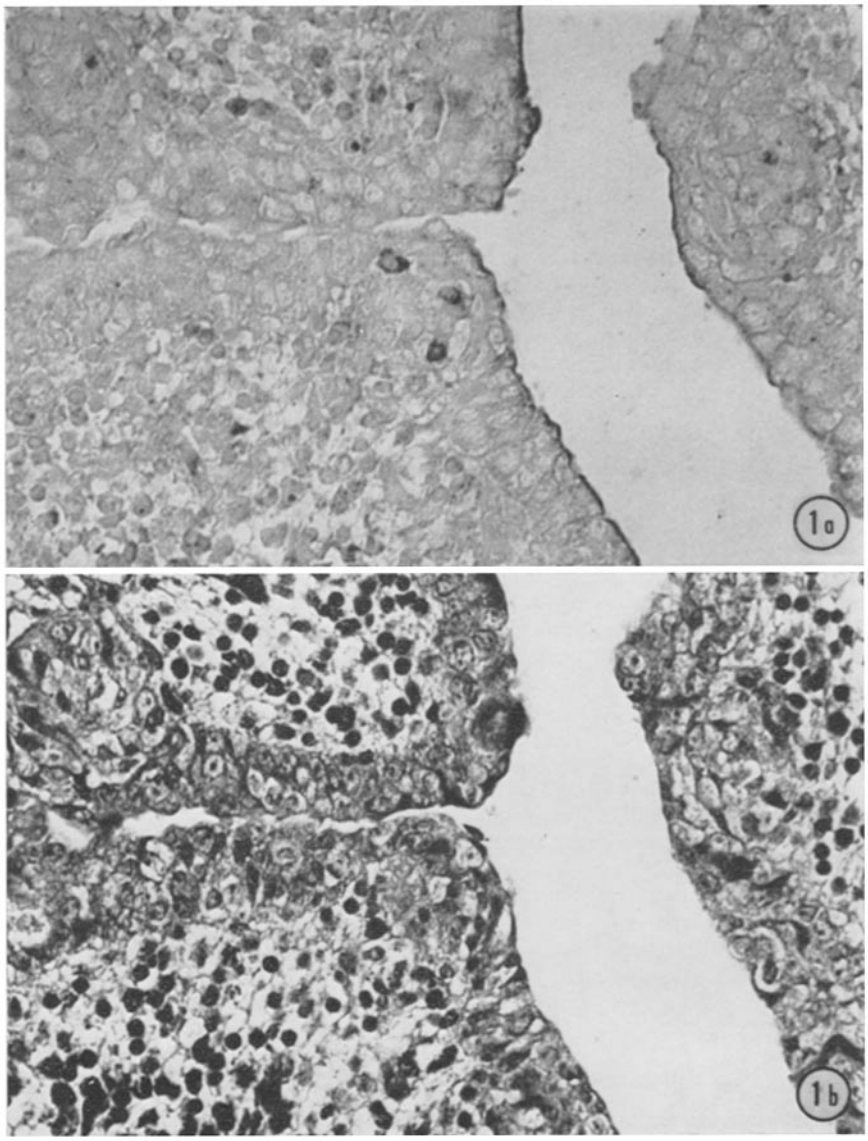

FIG. 1. Immunohistochemical staining of the bursa tissue of a 14 day old chick embryo. (*a*) Stained with anti-IgM as the first component of the immunoglobulin-enzyme bridge. × 480. (*b*) Section serial to that shown in (*a*). Stained with hematoxylin-eosin. × 480.

embryo matured, the number of follicles and the lymphoid composition of the follicles increased along with the number of IgM-producing cells in each follicle, as shown in Fig. 2. Similar observations were previously reported by Kincade and Cooper (11) using anti-μ conjugated with fluorochromes. Before hatching, IgM-containing cells were not found in other lymphoid tissues, but were present in the bursa. The bursa is, therefore, assumed to be an ideal tissue for studying the development of lymphoid cells synthesizing Ig. In contrast to fluorescent antibody staining, the immunohistochemical method using horseradish peroxidase permits easy comparison of cells stained for Ig with serial sections stained for morphologic reference.

Immunoglobulin Synthesis by the Embryonic Bursa Cells.—The results of immunohistochemical staining of the embryonic bursa cells indicated that the capacity of bursa cells to synthesize Ig becomes evident in 14-day old chick embryos. Hence, we chose this embryonic age to begin studying in vitro biosynthesis of Ig by the bursa cells. A cell suspension was prepared from 20 to 30 bursas from chick embryos of various ages, incubated at 37°C for 3 hr with leucine-^{3}H, lysed by NP-40, centrifuged, and subjected to serological precipitation with anti-IgM. Table I shows the amount of Ig synthesized and secreted by the bursa cells of chick embryos. We were able to detect leucine-^{3}H–labeled Ig synthesized by bursa cells of 14-day old chick embryos. It should be noted that the different cultures vary in total cell number. Consequently, a direct comparison of the values for total incorporation of leucine from culture to culture is not meaningful. The essential point to be made is that there is a great increase of Ig synthesized per 10^7 bursa cells as the chick embryos become older. This is consistent with findings by immunohistochemical staining of the bursa tissues obtained from various ages of chick embryos. At 3 hr of incubation, less than 30% of the total Ig synthesized was secreted, suggesting that a large portion of Ig synthesized by the bursa cells is not secreted because, in spleen cells, the amount of secreted Ig becomes greater than that of the intracellular pool in 3 hr of incubation (1).

In the preceding paper (1), we showed that nonsecretory IgM is composed of H_0 as heavy chains, which exhibited a molecular weight of 50,000 in acrylamide gel electrophoresis. If the IgM with H_0 (IgM-H_0) were synthesized by the precursor to the cells synthesizing and secreting IgM with H (IgM-H), we would expect to see the changes of relative amount of H_0 and H synthesized during the development of the chick embryo.

Leucine-^{3}H–labeled Ig which was isolated by serological precipitation from the bursa cells of chick embryos was reduced, alkylated, and analyzed in acrylamide gel electrophoresis as described previously (1). As shown in Fig. 3, the H_0 peak between H and L chain peaks is more remarkable in the chick embryos than in the 8 wk old chicken. In this experiment, leucine-^{14}C-labeled Ig secreted by the spleen cells was used in acrylamide gel electrophoresis as an internal control. We studied only intracellular Ig because in previous experiments the H_0 peak was not detectable in the secreted Ig (1).

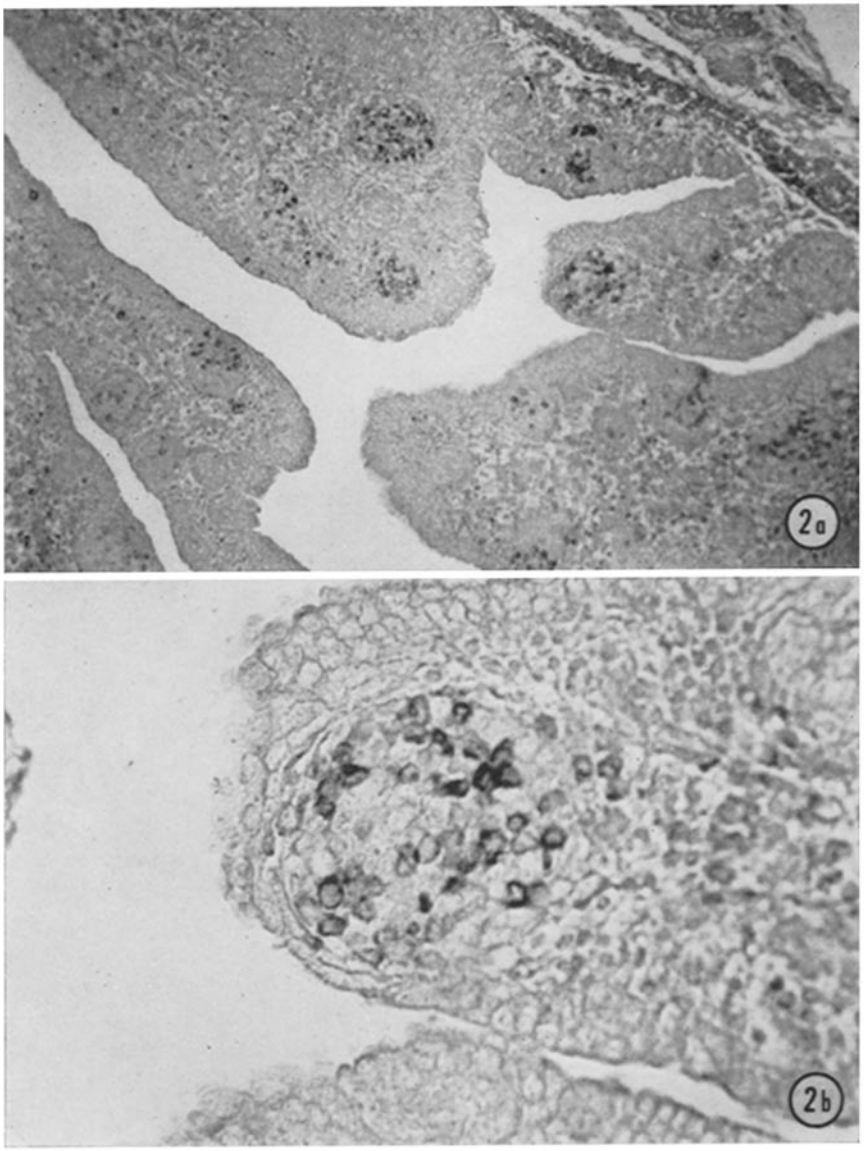

FIG. 2. Immunohistochemical staining of the bursa tissue of an 18 day old chick embryo. The sections were stained with anti-IgM and horseradish peroxidase as described in Materials and Methods. (*a*) × 120; (*b*) × 480.

The actual amounts of the three subunits, H, H_0 and L, were calculated from Fig. 3 and are summarized in Table II. The results are also compared with that obtained from intracellular Ig synthesized by the bursa and spleen cells of an 8 wk old chicken (Fig. 4). During the development of the chick embryo, the

total heavy chain over light chain ratio (H + H_0/L) remains constant (i.e., ~2.0). In the same circumstances, H_0/L decreases from 0.91 to 0.55, while H/L increases from 0.97 to 1.6, reflecting a reciprocal change between H and H_0 chains. As previously discussed (1), we assume that a change of relative amount

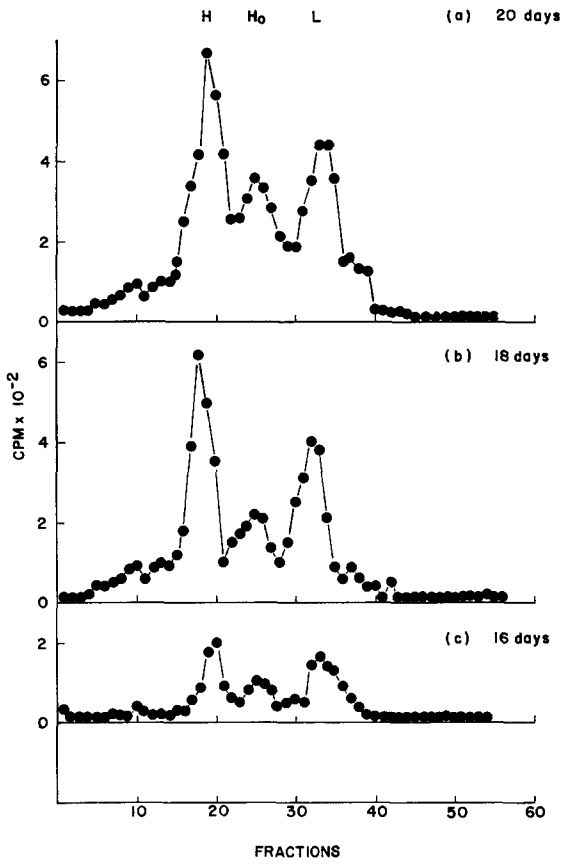

FIG. 3. Acrylamide gel electrophoresis of intracellular Ig synthesized by bursa cells of chick embryos. Intracellular proteins were labeled with leucine-^{3}H by incubating the bursa cells for 3 hr. The detergent-soluble cell fraction was subjected to serological precipitation with anti-IgM, reduced, and analyzed by SDS–acrylamide gel electrophoresis. (a) 20 days; (b) 18 days; (c) 16 day old chick embryo.

of H and H_0 chains reflects a similar change in the populations of the lymphoid cells synthesizing IgM-H and IgM-H_0. It appears that lymphoid cells synthesizing IgM-H_0 develop first in the bursa and that this is followed by development of lymphoid cells synthesizing IgM-H. Thus far we have failed to detect a stage of embryonic development where we can find only IgM-H_0. This is perhaps due to the fact that the amount of serologically precipitable radioactivity in the bursa

cells of younger chick embryo was too small to be analyzed by acrylamide gel electrophoresis.

Carbohydrate Composition of H and H_0 Chains.—In the preceding paper (1), we concluded that H_0 chain is a heavy chain rather than light chain because of calculations based on the heavy over light chain ratios of IgM precipitated by anti-μ serum. It is possible that the difference in mobility in acrylamide gel electrophoresis between two heavy chains may be consequent to a lack of carbohydrate residues and/or to significant differences in protein size (12). H_0 chains may have less protein-bound carbohydrates than H chains of IgM or IgG.

TABLE I

*Immunoglobulin Synthesis by the Bursa Cells of Chick Embryo**

Age	Cell No.	Intracellular Ig	Secreted Ig	Total Ig	Ig/10^7 cells
days		*cpm*	*cpm*		
14	5×10^7	3100	1000	4100	820
16	10×10^7	22,250	12,900	35,150	3500
18	30×10^7	200,040	78,970	279,010	9300

* A cell suspension was prepared from 20 to 30 bursas of chick embryos of various ages, incubated at 37°C for 3 hr with leucine-^{3}H. The incubation media were separated by centrifugation from the cells which were lysed by NP-40 and centrifuged. ^{3}H-labeled Ig in the incubation media and cell lysates were measured by serological assay as described in Materials and Methods.

TABLE II

Subunits of Immunoglobulins Synthesized by the Bursa Cells of Chick Embryo

Age	H	H_0	H + H_0	L	H/L	H_0/L	H + H_0/L
		cpm					
16 days	1100 (51%)	1050 (49%)	2150 (100%)	1140	0.97	0.91	1.9
18 days	2637 (65%)	1367 (35%)	4000 (100%)	1983	1.32	0.68	2.0
20 days	3132 (64%)	1740 (36%)	4872 (100%)	2380	1.31	0.73	2.0
8 wk	3150 (74%)	1100 (25%)	4250	1980	1.60	0.55	2.14
Spleen	2000		2000	750			2.70

The carbohydrate composition of these heavy chains was studied by double-labeling experiments with glucosamine-^{3}H or galactose-^{3}H/leucine-^{14}C and threonine-^{14}C. A batch of 5×10^8 bursa cells was incubated for 3 hr at 37°C with glucosamine-^{3}H (50 μCi/ml) in Eagle's medium minus glucose with 5% fetal calf serum, while a second batch was incubated in galactose-^{3}H (50 μCi/ml)-containing medium. A third batch was incubated with leucine-^{14}C (5 μCi/ml) and threonine-^{14}C (5 μCi/ml) in Eagle's medium minus leucine and threonine with 5% fetal calf serum. The cells were collected by centrifugation after

incubation, lysed by NP-40, and the nuclei and ribosomes were removed by centrifugation and dialyzed exhaustively against PBS. The cell lysates thus prepared were mixed in appropriate ratio of $^{3}H/^{14}C$ and subjected to serological precipitation with anti-IgM. Serological precipitates were analyzed by acrylamide gel electrophoresis after reduction and alkylation.

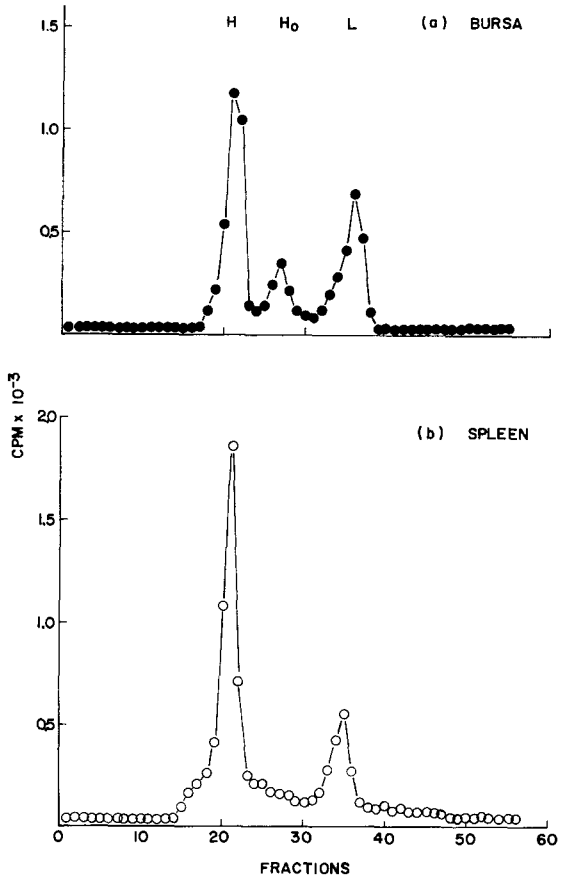

FIG. 4. Acrylamide gel electrophoresis of intracellular Ig synthesized by the bursa cells and spleen cells of an 8 wk old chicken. (a) Ig synthesized by the bursa cells; (b) Ig synthesized by the spleen cells.

Fig. 5 shows acrylamide gel pattern obtained. The $^{3}H/^{14}C$ ratios in the subunits of the bursa Ig are calculated from Fig. 5 and summarized in Table III. Glucosamine-^{3}H was incorporated into all subunits, including L chains. The galactose peak, however, is located mostly in H chains. As exhibited by galactose-^{3}H/leucine-threonine-^{14}C and glucosamine-^{3}H/leucine-threonine-^{14}C, H chains contain more galactose and glucosamine per molecule than H_0 or L

chains. H_0 and L chains contain small amounts of glucosamine, but virtually no galactose. It has been reported that both glucosamine and galactose residues are incorporated into carbohydrate residues of glycoprotein (7, 13). These results indicate that the observed difference in mobilities in SDS–acrylamide gel be-

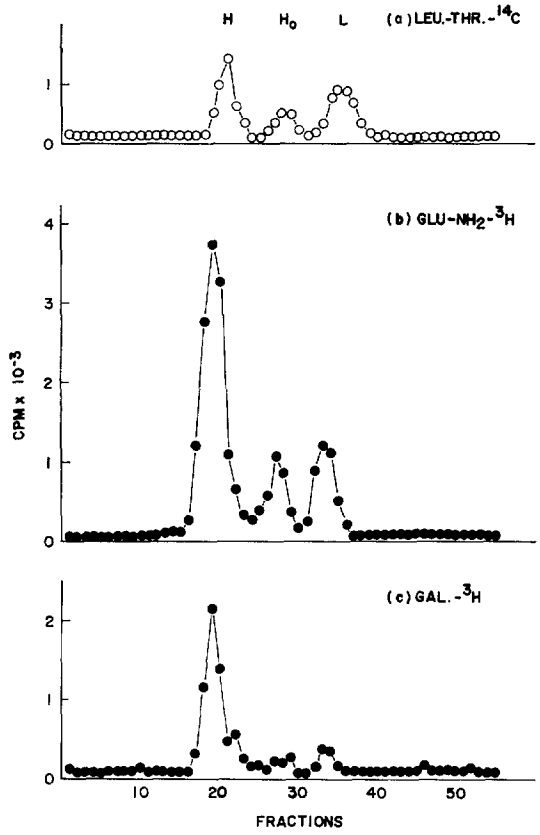

FIG. 5. Incorporation of glucosamine-^{3}H and galactose-^{3}H into intracellular Ig of the bursa cells. Three aliquots of the bursa cells ($\sim 4 \times 10^8$ cells prepared from an 8 wk old chicken) were separately incubated with leucine-threonine-^{14}C, glucosamine-^{3}H, and galactose-^{3}H at 37°C for 3 hr. ^{3}H-labeled intracellular Ig was analyzed by SDS-acrylamide gel electrophoresis after reduction and alkylation. ^{14}C-labeled intracellular Ig was mixed as an internal control before serological precipitation. (a) leu.-thr.-^{14}C-labeled bursa Ig; (b) glucosamine-^{3}H-labeled bursa Ig; (c) galactose-^{3}H-labeled bursa Ig.

tween H and H_0 chains may be due to the difference in carbohydrate content. The slower migration of H chains might be the result of an interaction between carbohydrate and polyacrylamide leading to a disproportionate retardation of H chains relative to H_0 chains that contain little or no galactose. Since proteins were completely denatured in 1% SDS and 9.8 M urea, and reduced and alkyl-

ated, we do not expect a gross conformational difference between H and H_0 chains due to the hydration of the carbohydrate moiety of glycoproteins which might also affect migration.

DISCUSSION

We interpret the changes with age of the amount of H_0 and H chains in the bursa cells of chick embryos as evidence that the lymphoid cells synthesizing IgM-H_0 are the predecessor or probably the precursor to the cells synthesizing IgM-H.

We first showed by immunohistochemical staining, that the lymphoid cells stained with anti-IgM first appear in the lymphoid follicles of the bursa of a 14 day old chick embryo (Figs. 1 and 2). As chick embryo becomes mature, the number of the follicles and lymphoid composition increases markedly. This

TABLE III

Carbohydrate Composition of Bursa Intracellular Ig

Subunits	Leu.-thr.-^{14}C	Gal.-^{3}H	Gal.-^{3}H/^{14}C*	Glu-NH_2-^{3}H	Glu-NH_2-^{3}H/^{14}C*
H	2790	4820	1.7	10,650	3.8
H_0	1180	550	0.5	1880	1.6
L	2870	660	0.2	3080	1.0

* Leucine-^{14}C and threonine-^{14}C.

finding essentially confirms the previous report by Kincade and Cooper (11). At the same time, the amount of leucine-^{3}H–labeled Ig synthesized by $\sim 10^7$ bursa cells also increases (Table I). In the circumstances where total H + H_0/L ratio remains the same, a complementary change in the amounts of H and H_0 synthesized occurs: H_0 decreases while H increases. In the preceding paper (1), we presented evidence that the bursa of an 8 wk old chicken contains three different clones of lymphoid cells synthesizing Ig: one with nonsecretory IgM-H_0, the second with 19S IgM-H, and the third with 7S IgG. In the bursas of chick embryos where IgG synthesizing cells were not detected (11), a reciprocal change in the amounts of H_0 and H synthesized reflects a change in the lymphoid cell populations synthesizing IgM. The cells synthesizing nonsecretory IgM-H_0 apparently precede development of the cells secreting IgM-H.

We studied the structural differences of H and H_0 chains in an attempt to understand the cytodifferentiation of the lymphoid cells in molecular terms. We first studied the carbohydrate structure of IgM because there have been cumulating evidences that the attachment of carbohydrate residues may play a role in the secretion of Ig as well as of other glycoproteins (7, 14–17). As clearly revealed by carbohydrate-^{3}H/amino acids-^{14}C ratios in Table III, H chains of secreted IgM contain more glucosamine and galactose residues per molecule than do H_0 chains.

In the studies with mouse myeloma cells (7, 15, 16, 18, 19), the glucosamine and mannose residues have been shown to be attached to Ig in the rough endoplasmic reticulum fraction where the polypeptides are synthesized on membrane-bound polysomes. When Ig is transported from rough to smooth endoplasmic reticulum, the attachment of galactose takes place before secretion (7). It was also shown that the rate-limiting step in the secretion process is the attachment of galactose residues. If the attachment of galactose residues to H chains has an obligate role in secretion of chicken IgM, the block in secretion of IgM-H_0 could be either due to lack of galactosyl transferase or due to H_0 chain polypeptides which cannot act as an acceptor for galactose transfer by this enzyme. The present work indicates that such a possible block in secretion of IgM persists until a certain stage of cytodifferentiation of lymphoid cells is reached.

After stimulation by antigen, a similar cytodifferentiation of thymus-independent bone marrow or bursa-derived cells based on studies of rosette-forming "B" cells has been observed (20). In the mouse and guinea pig (21–23), antigen-binding cells were detected before any significant development of antibody-secreting cells. The antigen-binding cells were shown to have specificity characteristics resembling precursors of antibody-secreting cells because the receptors possessed by such precursors are Ig molecules essentially equivalent to the antibodies ultimately secreted (24). Thus, antigen-binding cells apparently synthesize Ig which may not be secreted, and in this way resemble chicken IgM-H_0. In the chick embryo, however, it must be presumed that this cytodifferentiation in the bursa occurs apparently in the absence of antigenic stimulation. Crucial to understanding the development of the lymphoid system will be a definition of biological factors controlling this differentiation of the lymphoid cells.

SUMMARY

Development of Ig-synthesizing cells in the bursa of chick embryo was studied by immunohistochemical staining method as well as by in vitro incorporation of leucine-^{3}H into Ig. Ig-synthesizing cells are first detected in the bursa of a 14 day old chick embryo and increase with the maturation of the embryo. Acrylamide gel analysis of leucine-^{3}H–labeled Ig shows that synthesis of nonsecretory IgM-H_0 precedes that of secretory IgM-H, reflecting an ontogenetic sequence of development of lymphoid cells synthesizing IgM.

Since IgM-H_0 is not secreted, we further studied biochemical differences between two heavy chains. The difference is attributable to lack of galactose attachment to H_0 chains. It is proposed that during differentiation of lymphoid cells synthesizing and secreting Ig, attachment of galactose may play an obligatory role in the development of cellular capacity for Ig secretion.

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