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Suppression of Immunoglobulin G Synthesis as a Result of Antibody-Mediated Suppression of Immunoglobulin M Synthesis in Chickens*

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Abstract. Development of heterogeneity of immunoglobulin classes has been investigated in the chicken by studying the effects of antibody-mediated suppression of IgM synthesis. Treatment of 13-day embryos with purified goat antibodies to IgM resulted in the elimination of IgM-containing cells from the bursa of Fabricius of 16- and 19-day embryos. When combined with bursectomy at hatching, administration of anti-IgM *in ovo* suppressed the synthesis not only of IgM but also of IgG. A number of experimental birds lacked detectable circulating immunoglobulins, plasma cells, and germinal centers when killed at 10 weeks of age. Contrasting results were obtained when IgM synthesis was suppressed *after* bursectomy at hatching. Birds so treated produced little or no IgM but synthesized normal amounts of IgG. The results suggest that, within the bursal environment, IgG-producing cells arise exclusively from cells that previously synthesized IgM. A model for generation of antibody variability is presented.

The chicken has been extremely useful for studying differentiation of immunoglobulin-producing cells, primarily because development of this line of cells depends on the functional integrity of the bursa of Fabricius.[1] Effective bursectomy prior to or at the time of hatching, combined with procedures to destroy cells already seeded from the bursa, results in agammaglobulinemia.[2–5] The ability to form germinal centers, plasma cells, and immunoglobulins[6] and to respond to antigen[7] may be conferred upon bursectomized recipients by bursal lymphocytes. Immunoglobulin synthesis first occurs in the bursa[8,9].

Impetus for the experiments described in this report stemmed from recent observations made in this laboratory on the ontogeny of immunoglobulin synthesis in chickens.[9] With the aid of sensitive fluorescent-antibody techniques, it was found that IgM- and IgG-containing cells appear first in the bursa of Fabricius of 14- and 21-day embryos, respectively. The time of appearance of each class of immunoglobulin is unrelated to exogenous antigenic stimulation. In the mature bursa, contiguous localization of IgM and IgG was observed in the medullary areas of most follicles. IgG-containing cells were found only in the medullas of follicles that contained IgM, whereas some follicles

were found to contain *only* IgM. Deposition of IgM and IgG within the same cells was not observed in any other tissue.

Two models were proposed[10] to explain these phenomena. The first assumed that stem cells of yolk sac origin arrive in the epithelial anlage of the bursa of Fabricius precommitted to the eventual synthesis of one or the other class of antibody, and that the ontogenetic sequence, IgM followed by IgG, depends upon a difference in time of arrival of stem cells or a longer period of induction for IgG synthesis. The second hypothesis proposed that IgG-synthesizing cells arise from cells that have previously synthesized IgM.

The success of antibody-mediated suppression of development of immunoglobulin allotypes in rabbits[11] and of adult hemoglobin synthesis in frogs[12] suggested the approach used in these experiments. Were the first model correct, suppression of IgM synthesis prior to the onset of IgG synthesis should not affect the latter. The opposite result—suppression of both IgM and IgG synthesis by administration of anti-IgM—would support the second hypothesis.

Materials and Methods. Fertile eggs of an Arbor Acre–Van Tress cross, obtained locally, were used in all experiments. Goat antisera to chicken μ chains was prepared as described elsewhere.[9] Immunoadsorbent columns of semipurified IgM, semipurified IgG, and agammaglobulinemic chicken serum were used to isolate antibodies and render them specific. The preparations of anti-μ chain antibodies formed a single precipitin arc against normal adult chicken serum and failed to react with newborn chicken serum (containing maternal IgG but no IgM) when tested by Ouchterlony analysis and immunoelectrophoresis at concentrations of 20 mg/ml. In preliminary experiments, bovine serum albumin (Pentex, Kankakee, Ill.) was injected as a control protein. In later experiments we used normal goat globulin, prepared by precipitation of goat serum with 18% (w/v) Na_2SO_4, dialysis against 0.15 M NaCl, and passage over the IgM immunoadsorbent column to ensure that it contained no antibodies to chicken IgM. All solutions were dialyzed against 0.15 M NaCl, concentrated to 10–20 mg/ml and sterilized by filtration through a 0.45-μm Millipore filter prior to intravenous injection. Protein concentrations of goat globulin and anti-IgM were estimated by absorbance at 280 nm, using the extinction coefficient $\epsilon_{1\ cm}^{1\%} = 14.0$.

For injection of embryos, a section of shell over a chorionic vessel was removed. A drop of mineral oil was placed on the exposed membrane and the vein was cannulated with a 30-gauge needle. After infusion of anti-IgM or the control protein in a volume of 0.1–0.2 ml, the defect in the shell was sealed with warm paraffin and the egg was returned to the incubator.

Circulating immunoglobulins were measured by single radial diffusion as previously described.[13] Dilutions of a serum pool previously analyzed for IgG content were used to construct a standard curve for each determination. IgM was prepared from chicken serum by the method of Benedict[14] and further purified by zone electrophoresis in agar. The final preparation gave a single arc in immunoelectrophoresis against anti-chicken serum at a concentration of 6 mg/ml. The protein content of this preparation was measured by absorbance at 280 nm, using the extinction coefficient $\epsilon_{1\ cm}^{1\%} = 12.7$ (ref. 15). This material was used to determine the amount of IgM in the serum pool. IgM could be detected at a concentration of approximately 1 mg/100 ml and IgG at approximately 5 mg/100 ml.

Tissues obtained at necropsy were stained for routine microscopy with hematoxylin and eosin and with methyl green pyronin. Tissues and reagents were prepared for fluorescence microscopy as previously described.[9] Tissues were prepared for electron microscopy using modifications of a previously described[16] sequence of standard techniques.

Results. A pilot study was performed to determine the dose of antibody to IgM necessary to suppress IgM synthesis. 13-day embryos were given a single

injection of 1.9, 0.95, or 0.48 mg of antibody. Control animals received an equivalent amount of bovine serum albumin. Part of each group was bursectomized at hatching. Plasma IgM and IgG levels were determined at weekly intervals. The largest dose of anti-IgM (1.9 mg), when followed by bursectomy at hatching, completely prevented development of IgM- and IgG-synthesizing cells; the bird had no detectable immunoglobulins when killed at 9 weeks of age. No cells containing IgM or IgG could be demonstrated by fluorescence microscopy in the lymphoid tissues obtained at autopsy. The intermediate dose (0.95 mg) of anti-IgM also resulted in significant suppression of IgM and IgG in bursectomized birds. Three birds in this group lacked IgM and had very low levels of IgG at 4 weeks, but modest repair of these deficits was noted thereafter. The smallest dose of antibody (0.48 mg) had a very transient suppressive effect, which was followed by development of supernormal levels of both immunoglobulin classes. All dosages of anti-IgM produced only transient suppression of IgM and IgG in chickens not also bursectomized at hatching.

In the next experiment, 15 embryos were each injected with 2 mg of anti-IgM at 13 days of incubation. The control group (17 embryos) received 2 mg of normal goat globulin. Surviving birds (10 experimental and 14 controls) were bursectomized at hatching. Fig. 1 shows mean plasma levels of IgM and IgG at various ages in the experimental and control groups. Both IgM and IgG levels were markedly suppressed in the animals receiving anti-IgM; on termination of the experiment at 10 weeks of age, five of eight surviving birds lacked detectable IgM and four of these 5 lacked detectable IgG as well.

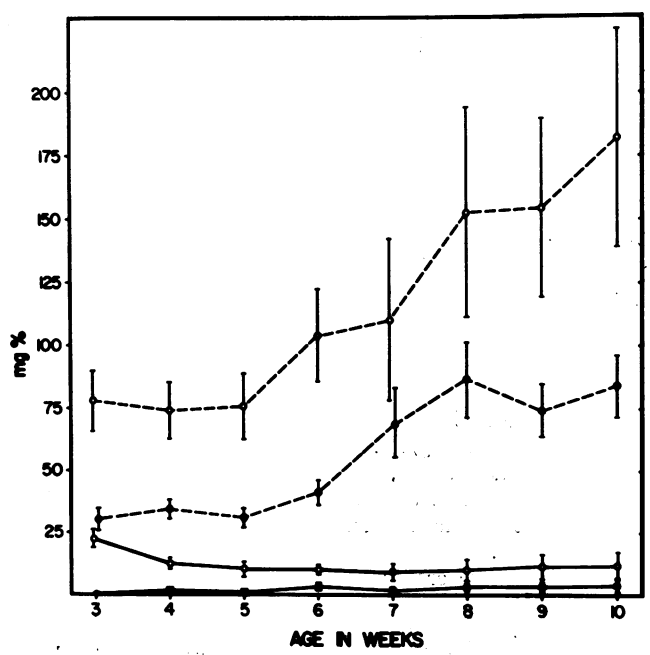

FIG. 1. Mean plasma immunoglobulin levels ± SE in 10 chickens injected *in ovo* (13 days of incubation) with 2 mg of anti-IgM (*solid lines*) and in 14 controls injected with normal goat globulin (*broken lines*) and bursectomized at hatching. Mean IgM levels are shown by closed circles and IgG levels by open circles.

Integrity of cellular immune mechanisms was assayed in all birds at 8 weeks of age by application of wattle skin grafts.[17] All grafts were rejected by the 9th day; no differences in the two groups were observed.

Lymphoid tissues from all agammaglobulinemic animals were examined at 10 weeks of age by light and fluorescence microscopy. No germinal centers, plasma cells, or cells staining with fluorescein-labeled antibody to IgM or IgG were found in their spleens, cecal tonsils, or thymuses. Tissues from control birds were normal with respect to these features. Thymus-dependent areas were histologically normal in both groups.

Suppression of IgG as well as IgM synthesis by embryonic administration of anti-IgM antibodies suggested that cells synthesizing IgG might be derived from IgM-producing precursors. To determine whether IgM and IgG cells would function independently once outside of the bursa, and also to provide evidence for the selective toxicity of the antibodies for IgM-containing cells, the following experiment was conducted.

21 chicks were bursectomized on the day of hatching. Members of one group (5 birds) were given a single 4-mg dose of anti-IgM; those of a second group (5 birds), 2 mg of anti-IgM immediately and 2 mg at 3 days of age. Members of control groups (5 and 6 birds, respectively) were given equivalent doses of normal goat globulin according to the same schedule. Serial determinations of circulating IgG and IgM levels are shown in Fig. 2. Since no differences resulted from the two dosage regimens, the data from control and experimental groups respectively were pooled. All anti-IgM treated birds synthesized small

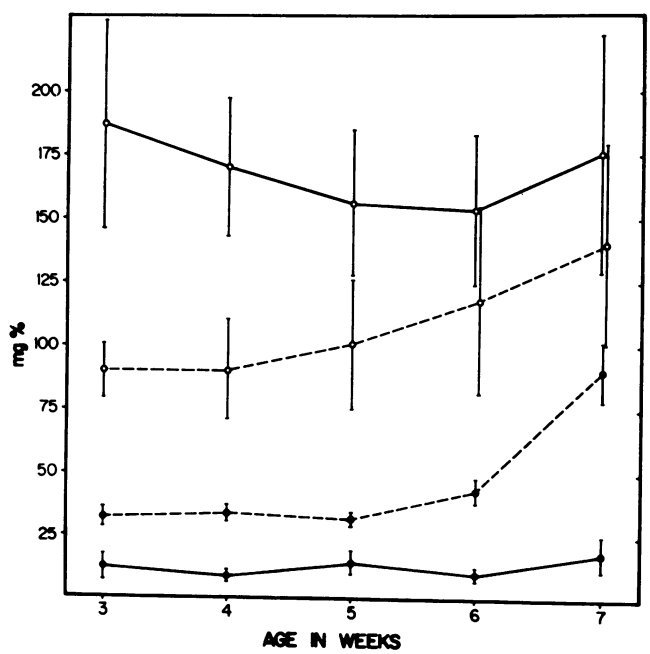

FIG. 2. Mean plasma immunoglobulin levels ±SE in 10 chickens injected with 4 mg of anti-IgM (*solid* lines) and in 11 chickens injected with normal goat globulin (*broken lines*) after bursectomy at hatching. Mean IgM levels are shown by closed circles and IgG levels by open circles.

amounts of IgM after 4 weeks of age, but far less than control birds. In contrast, the levels of IgG in experimental birds exceeded or equalled those in control groups. Similar results were obtained by giving three injections of 1 ml of whole anti-IgM serum on alternate days after bursectomy at hatching.

Attempts were made to determine the mechanism of antibody-mediated suppression by histologic methods. Several embryos that had received anti-IgM were killed at 16 and 19 days of incubation and their bursas were removed; a larger number of bursas obtained at hatching were also examined. By light microscopy, bursas from chicks treated with anti-IgM were easily distinguished from those of controls. Most lymphoid follicles of the former were smaller than normal, were sparsely populated with lymphoid cells, and showed some degenerative changes. Some follicles appeared normal. Electron microscopy revealed normal and even dividing cells adjacent to necrotic cells (Fig. 3). Bursas stained with fluorescein-labeled anti-IgM revealed only rare fluorescent cells. Attempts to demonstrate cell-bound anti-IgM antibody, using fluorescein-tagged rabbit antibodies to goat immunoglobulin, were unsuccessful.

Discussion. The feasibility of antibody-mediated suppression of synthesis of a particular immunoglobulin class has been demonstrated by these experiments. Administration of a sufficient dose of anti-IgM on the day prior to the appearance of IgM-containing cells in the bursa, coupled with bursectomy at hatching, resulted in complete elimination of the plasma cell line, and of its end-products,

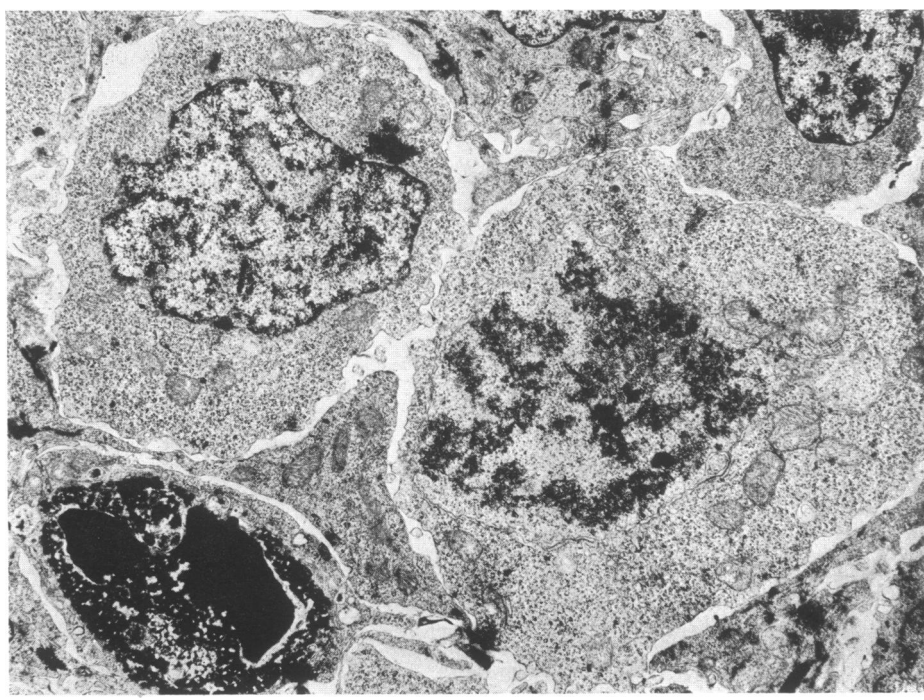

FIG. 3. Bursa from newly hatched chick given 0.95 mg of anti-IgM antibodies on the 13th day of incubation. A degenerating cell (*lower left*), with closely associated processes of epithelial cells, lies adjacent to apparently normal interphase (*upper left*) and dividing lymphoid cells. ×9360.

IgM and IgG. Agammaglobulinemic birds had intact cellular immune functions, and differed from bursectomized-irradiated[17] or hormonally bursectomized[5] birds only in that they were healthier and survived longer.

Several explanations for the ablation of IgG synthesis by embryonic administration of antibodies specific for IgM are possible. Lack of specificity of the antibodies used is highly unlikely. The antibodies were monospecific by reasonably sensitive criteria.[9] Further, serum of chick embryos at 13 days of incubation contains approximately 10 mg of maternally derived IgG per 100 ml (unpublished observations). Cross-reactive antibody combining with circulating IgG would not be expected to arrive at the appropriate target cell, presumably in the bursa. A second possiblity is that potential IgG-producing cells were destroyed as "innocent bystanders." Selectivity of cell destruction in treated birds, as demonstrated by electron microscopy, makes this explanation unlikely. Moreover, administration of the same antibody after bursectomy at hatching produced suppression of IgM synthesis without affecting IgG synthesis. The most reasonable explanation for the effect of embryonic adminstration of anti-IgM on IgG synthesis is that the cells that synthesize IgG are exclusively derived from cells that previously synthesized IgM.

Results of the second experiment, in which anti-IgM was administered shortly after hatching and bursectomy, indicate that once seeded from the bursa, IgM- and IgG-producing cells function autonomously. Plasma IgG levels of birds treated with anti-IgM at this stage of development were even higher than those of controls, while IgM levels were markedly diminished. One treated bird had normal IgG levels despite the fact that it synthesized no detectable IgM until 5 weeks of age.

Chickens subjected to bursectomy at 19 days of incubation often develop supernormal levels of IgM but have little or no detectable IgG.[4] IgM and IgG levels have been determined serially in such birds over a period of 8 months. IgM levels remained high, but the deficit in IgG synthesis was never repaired (unpublished data). These observations suggest that IgM-producing cells in peripheral lymphoid tissue do not switch to synthesis of IgG. The pathogenesis of the dysgammaglobulinemia produced by bursectomy of 19-day embryos is probably similar to that of the dysgammaglobulinemia produced by bursectomy of 21-day embryos followed by treatment with anti-IgM, although each procedure affects a different cell line. In the first case, the defect probably results from removal of the bursa prior to the appearance of more than a few IgG cells in peripheral tissues. In the second, it seems likely that most IgM-producing cells already in peripheral tissues are destroyed, leaving IgG cells in the majority.

The concept that cells synthesizing one class of immunoglobulin may switch to synthesis of another class is not new. Using double fluorescent labeling, several investigators[18-20] have searched for cells producing more than one class of immunoglobulin in peripheral lymphoid tissues. Only a very small percentage of double producers were found. While an early report[21] suggested that significant numbers of cells were engaged in synthesis of both IgM and IgG during a stage of the immune response, more recent studies have shown that no more than 1–4% of cells simultaneously synthesize two classes of antibody.[22-24] In certain pathological situations, however, individual cells making two classes of

immunoglobulins have been observed[25-27]; dual synthesis by individual cells appears stable over many generations of cells in tissue culture.[28]

Our results suggest that IgG producers arise from IgM-synthesizing cells within the bursa; accordingly, cells producing both classes might be expected to occur frequently in this site. In a previous study, we found that approximately 50% of individual bursal lymphocytes that stained with fluorescein-labeled anti-IgG could also be stained with rhodamine-labeled anti-IgM. Less than 5% of spleen cells similarly examined contained both classes of heavy chains.[9] These observations provide further support for the concept that a switch from IgM to IgG synthesis occurs within the bursa, but does not occur with any frequency in peripheral tissues.

Dreyer and Bennet have proposed[29] that separate genes encode for the variable and common regions of immunoglobulin heavy chains. They theorized that during differentiation a gene for a given variable (V) region aligns with a constant (C) region gene to direct heavy chain synthesis. This hypothesis has recently received strong experimental verification. Wang *et al.*[30] demonstrated that heavy chains isolated from IgM and IgG myeloma proteins occurring in the same patient have identical N-terminal amino acid sequences for the first 27 residues. Light chains from these two proteins likewise appear to be identical even though separate cells are responsible for synthesis of the two classes.[31] Penn *et al.*[32] described common idiotypic determinants on the light and heavy chains of IgM and IgG myeloma proteins from a similar patient. Oudin and Michel have reported[33] that IgM and IgG antibodies raised to the same antigen in rabbits share idiotypic specificities. This suggests that the gene for a single V region may be expressed in association with more than one immunoglobulin class in the normal process of antibody formation. All these observations are consistent with the hypothesis that during differentiation a single clone of cells may switch from the expression of one C_H gene to another without alteration in the expression of V_H and V_L genes.

We have shown that cells producing IgG develop exclusively from cells that previously synthesized IgM in the bursa of Fabricius, as a normal event of differentiation of the plasma cell line in chickens. In contrast, a switch from IgM to IgG synthesis in peripheral tissues is a rare occurrence. Individual antibody-producing cells seem committed to synthesis of a single specificity, and presumably a single set of V region sequences.[34,35] However, a single sequence in the V region of the heavy chain may be associated with more than one class of heavy chain.[30] On the basis of this information it is reasonable to postulate that antibody variability, as well as class heterogeneity, is generated within the bursa of Fabricius in chickens. Selection of a particular set of V_L and V_H genes, present in the germ line or generated by somatic mutation, prior to the switch from IgM to IgG synthesis would constitute an efficient mechanism for assuring that the same specificity is associated with immunoglobulin molecules having varied biologic advantages. Once seeded from the bursa, cells may be irrevocably committed to synthesis of a single class and specificity of antibody. This model reemphasizes the importance of the bursa in the induction of the immunoglobulin-producing line of cells and suggests that the bursal environment may have a critical role in directing the genetic events by which antibody

heterogeneity is generated. The relevance of this model to development of the plasma cell line in mammals and to human immunologic deficiencies has been more fully discussed elsewhere.[10]

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