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Development of Surface Immunoglobulins in the Chicken

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Summary. B-cell maturation in the chicken has been evaluated by the appearance of membrane immunoglobulins on cells in the spleen, the thymus, the bursa and the bone marrow during embryonic development and shortly after hatching. The majority of the bursa cells acquire demonstrable membrane immunoglobulin between days 16 and 18 of incubational age and show significantly increased amounts of membrane immunoglobulin between days 18 and 20, even though immunoglobulin-bearing cells can be found in the bursa as early as day 14 of embryonic age. The spleen shows cells possessing immunoglobulin receptors on their membranes (Ig^+) only after the bursa cells have reached full membrane immunoglobulin maturation as reflected in the number of Ig^+ cells and the amounts of membrane immunoglobulin. The thymus is practically devoid of Ig^+ cells in the embryo and it is not clear whether there are any Ig^+ cells in the bone marrow.

There are two phenomena which stand out in the observations. One is that there appears to be a gradual increase in the quantity or quality of the surface immunoglobulins on individual cells with advance in the embryonic development as reflected in the gradual increase in the staining intensity. The other is that there appears to be a polar distribution of membrane immunoglobulin in some cells especially in younger embryos. This polar distribution is seen under conditions where immunoglobulin capping is prevented by inhibitors and where immunoglobulin capping is impossible, such as with monomeric Fab. Immunoglobulin capping has been found to occur readily in embryonic cells and under conditions which would normally inhibit capping in adult cells.

INTRODUCTION

Chicks bursectomized chemically early in the embryonic development, for example between days 3 and 12 of incubation, show greatly reduced or abolished primary antibody response to bacteriophage ΦX 174, but slightly reduced or near normal secondary response.

The degree of depression depended on the time of chemical bursectomy. Testosterone given before day 5 of embryonic development when the bursa starts to appear resulted in less depression of the antibody response later in life than that given later such as on day 12 after incubation. Repeated injections often resulted in normal levels of serum antibodies. It is generally understood that the bursa is the only site where antibody-forming cells

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normally develop in the chicken (reviewed by Kincade, Self & Cooper, 1973). Therefore, the question arose where and how the antibody-forming cells developed in those animals, which apparently showed no bursa throughout the embryonic development and adult life. It was hoped that some clue might be found if we studied the development of cells carrying membrane immunoglobulin markers in the embryo of normal and bursectomized chicks. This report will present the temporal appearance of immunoglobulin-bearing cells in the chick embryo in various lymphoid organs studied and the maturation phenomena of such cells particularly in the bursa. No clue could be found for the origin of the B cells in bursectomized chicks.

MATERIALS AND METHODS

Animals

Outbred White Leghorn Chickens bred and maintained in the Basel Institute Chicken Farm were used. Fertilized eggs were incubated for the first 18 days in an Ehret incubator with automatic turning (Emmendingen-Kollmarsrente, Baden, Germany) at 37° and 80 per cent humidity, and for the following 3 days in an Ehret hatcher. Testosterone was used for chemical bursectomy (Glick and Sadler, 1961). With embryos younger than 5 days, eggs were dipped in 2 per cent testosterone (in alcohol) for 5 to 10 seconds. With embryos older than 11–12 days, eggs were either dipped in the testosterone solution or were injected with 0·25 ml of 25 mg of testosterone/100 ml of corn oil as described by Warner, Uhr, Thorbecke and Ovary (1969).

Cell suspensions

Thymus, spleen, bursa and bone marrow from six to seven animals were pooled respectively. From embryos of 14–18 days of age, cell suspensions were prepared by teasing the tissues with needles and forceps in BGJb medium (Grand Island Biological Company, Grand Island, New York), supplemented with 1 per cent bovine serum albumin (BSA) (30 per cent stock solution, Sigma, St Louis, Missouri) and 12·5 mM NaN_3. With older embryos and young chicks, cell suspensions were prepared by gently pressing the tissues through a stainless steel gauze. The cell suspensions were filtered through double layered gauze to remove cell clumps. The cells were washed three to five times by centrifugation at 150 ***g*** for 10 minutes and the pellet was finally resuspended in fresh medium usually in a concentration of 25×10^6 cells/ml. All the procedures were performed in the cold.

Reagents

Rabbit anti-chicken γ-globulin antiserum, a generous gift from Dr J. R. L. Pink, was raised against chicken γ-globulin (CγG), prepared by agarose block electrophoresis followed by 18 per cent Na_2SO_4 precipitation and Sephadex G-200 gel chromatography. The isolation of rabbit IgG (RIgG-α-CγG), the preparation of monovalent antibody fragments (RFab-α-CγG) and the conjugation of both reagents with tetramethylrhodamine isothiocyanate (TRITC) (Baltimore Biological Laboratories, Becton Division, Cockeysville, Maryland), were made as described by Loor and Roelants (1974). The following conjugates were used for these studies: TRITC–RIgG-α-CγG with a ratio OD_{280nm}/OD_{515nm} of 2·0–3·0, and TRITC–RFab-α-CγG with a ratio OD_{280nm}/OD_{515nm} of 2·7, and TRITC–SIgG-α-RIg (sheep IgG against rabbit IgG) with a ratio OD_{280nm}/OD_{515nm} of 2·4. All the fluorescent reagents were routinely absorbed with chicken red

blood cells, and sometimes thymus cells from embryos or newly hatched chicks to remove any possible, though undetected, natural rabbit antibody against chicken membranes.

Concanavalin A (Con A) (Grade IV, Sigma, St Louis, Missouri) was used without further purification. Cytochalasin B (ICI Research Laboratory, Alderley Park, Cheshire), which was used in some experiments, was dissolved in the medium at 50 μg/ml in 0·5 per cent DMSO (dimethylsulphoxide) and controls had 0·5 per cent DMSO only.

Immunofluorescence (IF)

This was performed as previously described (Loor, Forni and Pernis, 1972; Loor and Roelants, 1974) for observation of fresh cells in suspension. Briefly, before staining, the cells were preincubated with NaN_3 (12·5 mM NaN_3 in medium supplemented with 1 per cent BSA) at 37° for 15 minutes to block the capping machinery. For indirect IF, the cells were incubated with TRITC–RIgG-α-CγG at 0° for 15 minutes and at 37° for 10 minutes, washed three times, further incubated with TRITC–SIgG-α-RIg at 0° for 15 minutes and at 37° for 10 minutes, washed again and observed in suspension with the fluorescence microscope (Leitz Orthoplan microscope equipped with a vertical illuminator). In some experiments, cell smears were fixed in ethanol to enable prolonged and more detailed observation and to facilitate the detection of faint fluorescence (Loor and Roelants, 1974). The enumeration of fluorescence positive cells and total cells were determined with the aid of a phase contrast objective (Leitz PhaCONP oil 100/1·30 objective) and eye piece (Periplan GW, ×10).* Cells considered to be specifically showing fluorescence are considered to possess immunoglobulin receptors on their membranes (Ig^+cells).

RESULTS

SITES OF APPEARANCE OF Ig^+ CELLS. EFFECTS OF EMBRYONIC BURSECTOMY

To obtain the percentage of cells bearing membrane immunoglobulin, a total of at least 200–4000 cells were counted for each preparation depending on the proportion of Ig^+ cells. Indirect IF was used in this series of experiments.

The cells from the bursa are mostly large, blast-like cells. The frequency of brightly positive Ig^+ cells in the normal bursa was found to increase markedly between day 16 and day 20 developmental age and reach approximately 95 per cent a few days after hatching (hatching was usually at 21–22 days after fertilization), as shown in Table 1.

The data also show that significant number of Ig^+ cells appear in the spleen only after hatching when most of the cells in the bursa were Ig^+.

On gross examination, chick embryos whose bursectomy treatment was started on day 13 (B-less II and B-less III) showed bursas of progressively decreasing size. Microscopically, the cells showed a progressive degeneration and death following such chemical bursectomy. Very few cells could be obtained from such a bursa a week later and no viable cells could be found following hatching. The bursa can no longer be found macroscopically a few weeks after hatching. Together with the degenerative process seen morphologically, many cells which gave positive immunofluorescence showed only a few spots in contrast to what was seen with normal bursal cells of comparable age. During the progressive degeneration of the bursa, the cells in the spleen also showed some damage to a certain extent as reflected by the diffuse staining of some cells. (The non-specific diffuse staining of damaged or dead cells can be distinguished easily from the specific membrane staining.

* It has been found after the conclusion of the experiments that this combination of lenses would allow the detection of 'brightly positive' cells only (Loor and Roelants, 1974).

TABLE 1

PERCENTAGE OF CELLS WITH EASILY DETECTABLE MEMBRANE IMMUNOGLOBULINS IN NORMAL AND BURSECTOMIZED CHICK EMBRYOS OR YOUNG CHICKS

Chicken age (days)		Normal*				B-less I*†				B-less II*‡				B-less III*§			
Embryo	Chick	T	BM	S	B	T	BM	S	B	T	BM	S	B	T	BM	S	B
14		0·03	n.d.	0·76	2·32	0·17	n.d.	0·39	n.e.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
16		< 0·05	< 0·05	< 0·07	6·92	0·12	0·14	0·11	n.e.	0·06	< 0·07	0·24	4·13	< 0·07	0·37	0·52	8·30
20		< 0·04	< 0·1	< 0·05	65·27	0·07	< 0·1	0·13	n.e.	< 0·02	< 0·1	< 0·05	33·05	< 0·02	< 0·1	< 0·07	54·73
	1	< 0·04	< 0·1	< 0·1	75·07	< 0·05	< 0·1	< 0·2	n.e.	< 0·1	< 0·2	< 0·2	n.v.	< 0·05	< 0·2	< 0·2	n.v.
	3	n.c.	0·21	3·47	94·50	n.c.	0·20	0·41	n.e.	n.c.	n.c.	1·01	n.v.	n.c.	n.c.	< 0·25	n.v.
	6	< 0·10	0·55	10·98	94·12	< 0·05	0·11	1·80	n.e.	< 0·05	< 0·08	3·64	n.v.	< 0·05	0·03	< 0·58	n.v.

The results showing a percentage of < 0·5 are uncertain.
n.c. = not counted, no positive cells by scanning. n.d. = not determined. n.e. = non-existent. n.v. = no viable cells seen.

* T = thymus; BM = bone marrow; S = spleen; B = bursa of Fabricius.
† B-less I = chemical bursectomy at day 5 of developmental age.
‡ B-less II = chemical bursectomy at day 12 of developmental age by injection of testosterone.
§ B-less III = chemical bursectomy at day 12 of developmental age by dipping in testosterone.

The former appears homogeneous and the latter appears as a ring or as spots).

Cells from the thymus are mostly small cells resembling small lymphocytes in contrast to the large cells from the bursa. These cells were never found to show membrane immunoglobulin. When occasional Ig^+ cells were found from the thymus, they were always 2–3 times larger in diameter than the mean thymocyte size.

The cells from the bone marrow did not morphologically resemble those of the lymphoid series. The staining if and when it occurred was not clear. It was always weak and it is difficult to ascertain whether it is on the membrane or intracytoplasmic.

MATURATION OF Ig^+ CELLS IN THE BURSA

With the maturation of the embryo, the Ig^+ cells showed a progressive 'maturation' with time, as reflected in the progressive increase in the staining intensity of their membranes. The Ig^+ cells showed only faint staining when they first appeared at about day 14 of developmental age. Gradually, with increasing age, more Ig^+ cells developed, and the intensity increased until it reached maximum at a time when the maximum percentage of Ig^+ cells was reached. A series of normal embryos of various developmental age were used to demonstrate this phenomenon. The immunofluorescent staining was carried out at the same time and with the same reagents to avoid any possible variables. Direct IF technique was used. The cells in suspension were incubated with TRITC–RIgG-α-CγG, washed fixed on microscope slides and examined for fluorescence with a combination of Leitz oil objective lens ×63/1·30 and Leitz H ×6·3 M eye pieces (this combination of lenses for observation is found to increase the sensitivity of detection due to increased brightness of the field). The degree of fluorescent brightness of individual Ig^+ cells is scored as follows: 'very faint' (+); 'faint' (++); 'bright' (+++); 'very bright' (++++) cells. The results are presented in Fig. 1. The results show clearly that

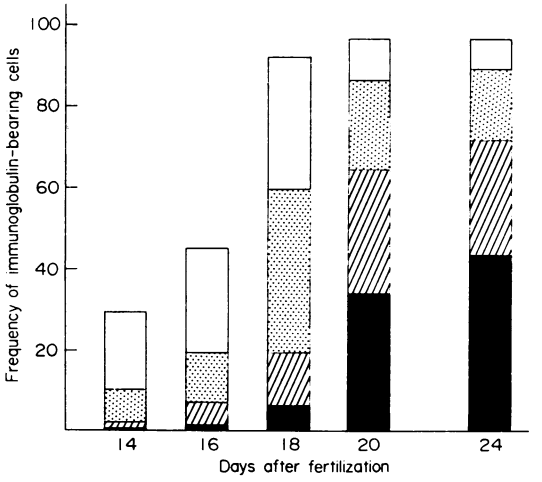

FIG. 1. Diagram showing the frequency of immunoglobulin-bearing cells in the chick bursa at various developmental days post-egg fertilization. Cells were labelled with TRITC–RIgG-α-CγG and scored for degree of fluorescence brightness. There is a net increase in proportion of cells with detectable membrane immunoglobulin, followed by an increase in the mean density of membrane immunoglobulin on the immunoglobulin-bearing cells. Blank columns (+); stippled columns (++); hatched columns (+++); solid columns (++++).

with progressive maturation of the embryo, there is a progressive increase not only in the percentage of bursa cells bearing membrane immunoglobulin, but also in the density of such immunoglobulin per cell as reflected in the increase in staining intensity.

DISTRIBUTION OF MEMBRANE IMMUNOGLOBULINS ON BURSA CELLS

Despite the preincubation with 12·5 mM NaN_3 and its presence throughout the staining procedure, many of the Ig^+ cells in the bursa showed a polar distribution of the membrane immunoglobulin in a pattern similar to the cap of immunoglobulin (Taylor, Duffus, Raff and Petris, 1971; Loor *et al.*, 1972). In young embryos when some bursa cells started to show membrane immunoglobulin, the amount appeared to be small, as reflected in the faintness of the staining, and the distribution tended to be at one region. As the cells acquired more membrane immunoglobulin, as reflected in the increased staining intensity, a faint ring of membrane immunoglobulin all around the cells was seen in addition to the 'cap'. With cells which had reached maximum brightness, only the homogeneously distributed pattern as a ring was seen. The classification of the cells as cells with an homogeneous ring of fluorescence or cells with a cap or a gradient of fluorescence is extremely

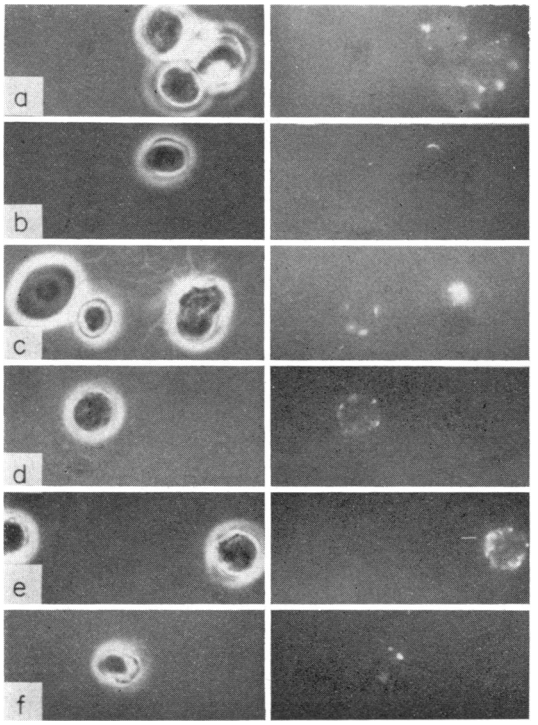

FIG. 2. Membrane immunoglobulins on bursa cells from chick embryo, 14 days after fertilization. Detection in IF, by TRITC–RIgG-α-CγG, in conventional 'non-capping' conditions (0°, 12·5 mM NaN_3). Left hand side: phase contrast image of the cells in suspension. Right hand side: IF. (a) Clump of three cells with only a few IF spots per cell. (b) Moving cell with membrane immunoglobulin detectable only as a small cap. (c) A red cell, a lymphocyte with only a few spots and another with a polar cap. (d) Cell with a few spots. (e) One cell without detectable IF, another with many IF spots. (f) Moving cell with a few IF spots.

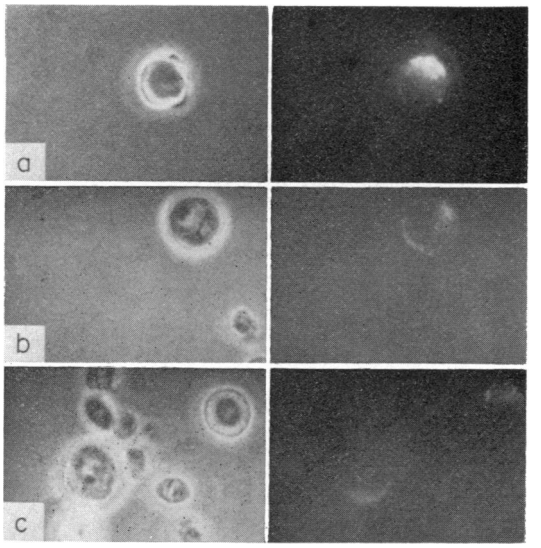

FIG. 3. A few typical cells having the occasional irregular distribution of the detectable membrane immunoglobulin. Detection in IF, by TRITC-RIgG-α-CγG, in conventional 'non-capping' conditions (0°, 12·5 mM NaN_3). Left hand side: phase contrast image of the cells in suspension. Right hand side: IF. (a) Cell with a gradient of fluorescence (from a chick embryo, 16 days after fertilization). (b) Cell from a chick embryo, 17 days after fertilization; in addition to the ring of fluorescence, there is a brighter area of IF. (c) Cell from a chick embryo, 17 days after fertilization; both these cells show a polar distribution of most of their membrane immunoglobulin.

subjective; thus we did not attempt to establish quantitatively the frequency of the various cells with the various membrane immunoglobulin distribution. However, the majority of the cells with membrane immunoglobulin in the bursa of embryos 14 days after egg fertilization were found with only a few spots, often distributed at one cell pole; later the proportion of cells with regularly distributed membrane immunoglobulins appeared to rise gradually to reach 90–95 per cent of the Ig^+ bursa cells found at days 22–24 after egg fertilization. Samples of fluorescent cells from days 14 and 16–17 embryos are given in Figs 2 and 3. It should be stressed that cells with this irregular localization of membrane-bound immunoglobulins were restricted only to the bursa; when Ig^+ cells were found in the spleen, immunoglobulin was always homogeneously and uniformly distributed.

To obtain more evidence for the native polar distribution of the membrane immunoglobulin in some young bursa cells, two more approaches were used to ensure further the absence of capping. One was to use agents other than NaN_3 to block capping, such as 5 per cent BSA, whose inhibitory effect on mouse membrane immunoglobulin spotting and capping is known (Loor *et al.*, 1972; Loor, 1974), 100 μg/ml of concanavalin A (con A), which is known strongly to reduce immunoglobulin receptor mobility (Loor *et al.*, 1972; Yahara & Edelman, 1972) or 10^{-4} M cytochalasin B (Loor, 1974). Even though the polar distribution was seen under all of these conditions, it was still not certain that this was not due to some type of capping phenomenon, because it was observed that capping could take place slowly with time during observation and when the temperature was intentionally raised in spite of the high BSA content and the inhibitors in the medium. These inhibitory conditions, however, were able to block capping of membrane immunoglobulin

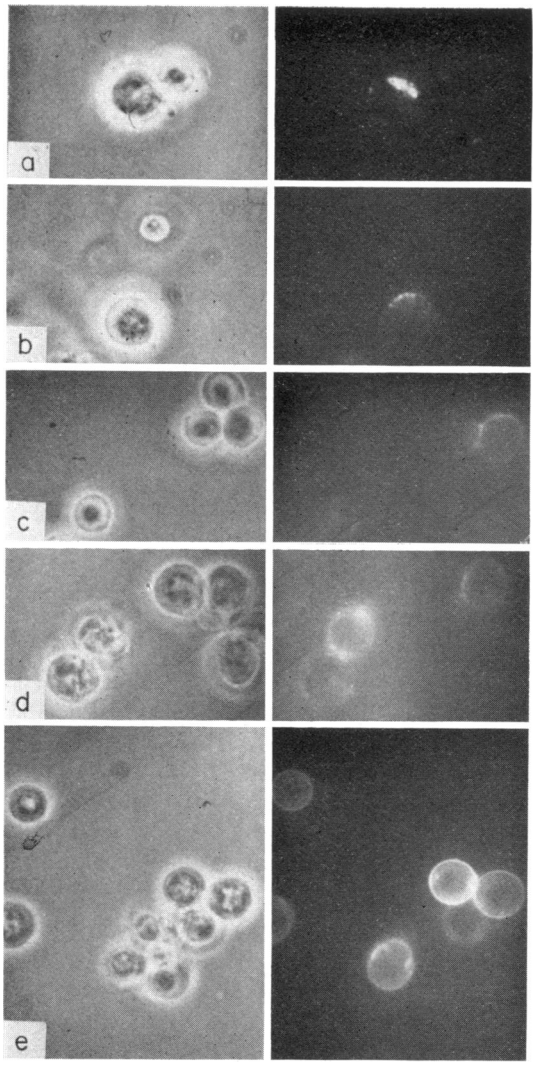

FIG. 4. Irregular distribution of membrane immunoglobulin on some cells, despite their IF staining under extreme 'non-capping' conditions (see text, other conditions as for Fig. 2). Left hand side: phase contrast image of the cells in suspension. Right hand side: IF. (a)–(d) Bursa cells from chick embryo 17 days after fertilization: (a) and (b) stained by fluorescent divalent antibody, in the presence of a blocking dose of con A (100 μg/ml); these cells show a local higher concentration of membrane Ig; (c) staining by monovalent antibody; two cells are stained as homogenous rings, one has fluorescent label on only a third of the surface; (d) labelling in presence of high (5 per cent) BSA concentration: label is faint, and two cells show unequal distribution of membrane Ig. (e) Young chick, 4 days after hatching, same labelling procedure as (d). The IF rings of immunoglobulin are brighter and perfectly homogenous.

effectively on Ig^+ cells from chicks of more than 2 weeks of age. The other approach was to use rabbit monovalent Fab against CγG (TRITC–RFab-α-CγG) instead of the divalent antibody (TRITC–RIgG-α-CγG) used normally. Monovalent Fab is known to be unable to redistribute as spots and caps (Taylor *et al.*, 1971; Loor *et al.*, 1972). Direct IF was carried out with fluorescent TRITC–RFab-α-CγG. The results confirm the observations described above in that there was the presence of 'caps' or 'gradient' of fluorescence in some early bursa cells and that there was never any change from ring to spots or caps under any conditions with Fab. Fig. 4 shows some examples.

DISCUSSION

Among the chick embryo organs tested the bursa is definitely the first site of appearance of cells with detectable membrane immunoglobulin: the number of Ig^+ bursa cells increases with increase in developmental age, as does their degree of fluorescence brightness, probably reflecting an increase in the quantity of immunoglobulin receptors, to reach a maximum at about the time of hatching. As we could not get viable bursa cell suspension from day-13 embryos, we do not know if cells with membrane immunoglobulin are present in the bursa before day 14 of developmental age.

Our observations that the great increase in the frequency of Ig^+ bursa cells occurs between day 16 and day 18 after fertilization agree with other reports (Albini and Wick, 1973; Hudson and Roitt, 1973) even though we find more Ig^+ bursa cells at earlier times, and higher maximum values at time of hatching. We further show that there is a sharp increase in membrane Ig density on the Ig^+ bursa cells which occurs between day 18 and day 20 after egg fertilization.

Significant numbers of Ig^+ cells started to appear in the spleen only after Ig^+ bursa cells had reached their maximum number and shown the maximum density of immunoglobulin receptors. Few Ig^+ cells were found in the normal embryo spleen, as found by Albini and Wick (1973), and appreciable numbers were first found a few days after hatching. During embryogenesis and soon after hatching, the thymus remains without Ig^+ cells, as found by Albini and Wick (1973), but in contradiction to the findings of Hudson and Roitt (1973). It is quite possible that significant numbers of Ig^+ cells are present in the bone marrow (Albini and Wick, 1973; Hudson and Roitt, 1973), but we were unable to distinguish cells of the lymphoid lines from cells of other lines by phase contrast microscopy. When mononucleated cells were stained, the staining was faint and its specificity was dubious.

As one follows the Ig^+ cells in the normal developing bursa as it differentiates and matures with increase in developmental age, three phenomena stand out: (1) there is a progressive increase in the proportion of Ig^+ cells with maturation; (2) there is a progressive increase in the quantity or quality of the immunoglobulin receptors on the membrane as reflected in the intensity of staining; (3) many cells in the early developing bursa show a gradient of receptors on the membrane. When Ig^+ cells first start to appear, all the membrane immunoglobulin seems to locate at one pole only and the staining is faint. With maturation of the embryo, Ig^+ cells can be seen which showed immunoglobulin at one pole only, immunoglobulin at one pole and in addition around the surface and immunoglobulin distributed evenly around the cell surface. This apparent polar distribution is interesting in that it may represent an important differentiation process occurring naturally *in vivo*. These could be a budding area for vesicles with immunoglobulin

to move to the cell surface as they are gradually produced inside the cell in a way similar to that suggested by Hirano, Parkhouse, Nicolson, Lennox and Singer (1972). This polar distribution could also be due to the point association with the bursa epithelial cell.

Another phenomenon which the developing Ig^+ bursa cells exhibit is a tendency to form 'caps' in the presence of various inhibitors which would inhibit 'capping' in adult cells. This is in variance with the experiences of Albini and Wick (1973) who could obtain only very poor capping.

Since the antibody-forming capacity in those embryonically bursectomized chicks which survive is only slightly impaired, there should logically be an alternative pathway for the development of these antibody-forming cells in the absence of the bursa which is the only site of differentiation for such cells. It was hoped that by following the development of cells bearing membrane immunoglobulin, a clue could be found as to what this alternative pathway might be. Unfortunately, the distribution of Ig^+ cells in bursectomized chicks is similar to that in normal chicks with the exception of the absence of the bursa. Therefore the question remains open.

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