EARLY EMBRYONIC SURGICAL BURSECTOMY: AN INVESTIGATION OF SOME ASPECTS OF THE AVIAN IMMUNE SYSTEM

by

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ABSTRACT

Surgical removal of presumptive bursal tissue at 72 hr *in ovo* results in high mortality before hatching and low survival in the first week thereafter. The viable chicks are underweight and usually remain so. Both the spleen and thymus of the bursectomized (Bx) chicks are reduced in size and lymphoid population. The effect on the thymus may be related to stress and subsequent adrenal cortical activity or may reflect some endocrine function of the bursa necessary for the maturation of thymic lymphoid tissue. The effect on the spleen seems to be more direct. In the absence of the primary level organ which induces maturation of the stem cells, the number of germinal centres in the secondary level organ is reduced. Some of the remaining germinal centres may be of T-cell origin, although these cells are more commonly found in diffuse lymphoid areas. The bulk of the germinal centres found in the Bx spleen must be of B-cell origin.

Repeated stimulation of the humoral immune system with antigens and mitogens elicits a response in some Bx birds. The response is generally not found in the primary stimulation and is rarely of the magnitude of a normal response even after several stimulations. Antibody production may be limited to IgM type immunoglobulin; further studies are necessary to verify this. Autopsy and histological examination of the tissues of the responsive birds failed to show any evidence of residual bursal tissue.

Whether these findings constitute proof of the role of the bursa in the humoral immune response or whether they reflect the broader consequences of bursectomy in the development of the bird requires further study. The inductive capacities of the bursa and the source and autonomous capacity of the stem cells thought to be induced in the bursa should also be investigated.

The effects of early hormonal and late surgical bursectomy

are similar to those found in this study, where an early embryonic surgical technique was used to avoid the possible complications of these other methods.

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INTRODUCTION

The immune system functions to protect an animal against infection, disease, foreign cells and neoplastic cells of self origin. These functions may be fulfilled either by the production of antibodies or by direct cell-to-cell interactions and are carried out by two functionally distinct lines of lymphocytes.

The cellular immune system provides surveillance against neoplastic cells, functions in graft rejection and is involved in delayed hypersensitivity and graft-vs-host reactions. The cells of this system are also necessary for efficient antibody production in some cases. The lymphocytes involved in these reactions appear to be derived largely from the thymus and are termed "T-cells". These cells do not themselves produce immunoglobulin molecules or specific antibodies. They are capable of killing foreign cells on contact and also produce a variety of chemical factors. These include cytotoxin,which lyses cells, chemotaxin and migration inhibition factor for controlling the movements of nearby lymphocytes and macrophages, blastogen, which stimulates the maturation of lymphocytes, and factors for sensitizing bacteria and viruses.

The humoral immune system is the source of specific immunoglobulin molecules termed antibodies (Ab). The Ab-producing cells are thought to be of bone marrow origin in mammals and from the bursa of Fabricius in aves. These "B-cells" produce specific antibodies to a wide variety of substances including chemicals such as dinitrophenol (DNP), cell surface products including proteins and bacterial lipopolysaccharides, viral coat proteins and such substances as bovine serum albumin (BSA) and human chorionic gonadotrophin (HCG). B-cells carry immunoglobulin molecules on their surfaces and do not appear to be direct contact killers (1).

Lymphocytes are found in a variety of adult mammalian tissues such as the spleen, thymus, bone marrow, lymph nodes, tonsils, appendix and peyer's patches of the intestinal wall.

In aves, the caecae and bursa of Fabricius are additional sources of lymphocytes, but the lymph nodes, tonsils and appendix are absent. The organs of primary interest in the developing embryo are the thymus, which is the source of T-cells, and the bursa of Fabricius, which is the source of B-cells. The spleen is also of interest as it is the site of greatest accumulation of mature lymphocytes of both classes.

The existance of the bursa, a single organ postulated to control the development of the humoral immune system, is of great convenience to the immunologist. In most species this function is attributed to widely dispersed tissue. Removal of this primary level organ should enable the investigator to examine the nature and function of this branch of the immune system during its development.

Removal of the bursa can be accomplished by either surgical or hormonal techniques. Hormonal bursectomy, which is performed early in development is, however, somewhat complicated by the side effects of introducing large quantities of steroid hormones into a differentiating system. Surgical removal of the bursa at the time of hatching also has disadvantages. Late removal allows a period of time between the early appearance of stem cells in the bursa and the removal of the organ during which maturing stem cells may escape to seed other lymphoid organs. The sequential seeding of the secondary level organs with cells capable of producing first one then another class of immunoglobulins may explain the selective effects of bursectomy near the time of hatching.

Removal of the bursa or presumptive bursal tissue very early in development should circumvent the problems of the side effects of steroids and of seeding of other organs prior to bursectomy. Such an operation is possible only during a short period between the initiation of the tail fold and the time at which the fragility of the surrounding membranes and blood vessels makes the operation physically impossible. In this investigation, a technique for surgical removal of the

presumptive bursal tissue at 72 hrs *in ovo* was used to produce birds deficient in B-cells and therefore incapable of mounting an humoral immune response (2).

These surgically bursectomized (SBx) birds were raised for periods of up to 10 months and a series of tests to investigate the status of their immune systems was carried out. Their lymphoid tissues were histologically examined for the presence of germinal centres, the sites of lymphocyte proliferation. Their sera were examined by immunoelectrophoresis for the presence of immunoglobulin; they were challenged with specific antigens and tested for antibody production; and their lymphocytes were cultured *in vitro* in the presence of mitogens. The results of these tests were compared with similar tests on normal birds.

In addition to these surgical bursectomies, some hormonal bursectomies were performed using testosterone propionate. These HBx chicks were subjected to many of the same tests as the SBx chicks. They were also tested for their ability to reject skin grafts. Their responses were compared to those of normal and SBx birds.

The data obtained from this study should supplement present knowledge and perhaps aid in clarifying the role of the bursa of Fabricius in the developing avian immune system.

LITERATURE REVIEW

A. The Bursa of Fabricius.

The bursa of Fabricius, discovered in 1621 by Hieronymus Fabricius (3), is a small oval sac of largely lymphoid tissue connected by a short stalk to the dorsal region of the cloacal-colonic junction of the large intestine. The bursa forms early in embryonic life, is fully developed in the immature bird and regresses around the time of sexual maturity (4). The time of maximum mean weight varies among breeds of chickens, ranging from 4½ - 6 weeks in White Leghorns to 10 -12 weeks in Barred crosses. In the White Leghorn, the maximum bursa to body weight ratio at 3 weeks of age is 0.05%. Figure 1 shows bursa development as a percentage of body weight of White Leghorn chickens. The bursa starts to regress at about 7 weeks of age; the rising level of androgens at the onset of sexual maturity hastens the process of involution so the organ nearly disappears by the end of the first year. The size of the bursa tends to correspond with that of the spleen and parallels the growth and involution of the thymus during the first few months of development (5).

The bursa of Fabricius was long thought to be involved either in the digestive process, due to its association with the gut, or in the onset of sexual maturity, as it begins to regress at this time. The nature of its role in the immune system was accidentally discovered by Glick *et al*. (6). Birds which had been surgically bursectomized at 2 weeks of age were found to be incapable of mounting an immune response when used in a demonstration of antibody production.

B. Structure of the Bursa.

The inner surface of the bursa is folded into 11 - 13 primary plicae, each of which has 6 - 7 secondary folds. The plicae are lined with columnar epithelium, below which is located the tunica propria. Each plica contains a vein and an artery. The plicae and the walls of the bursa are thickened

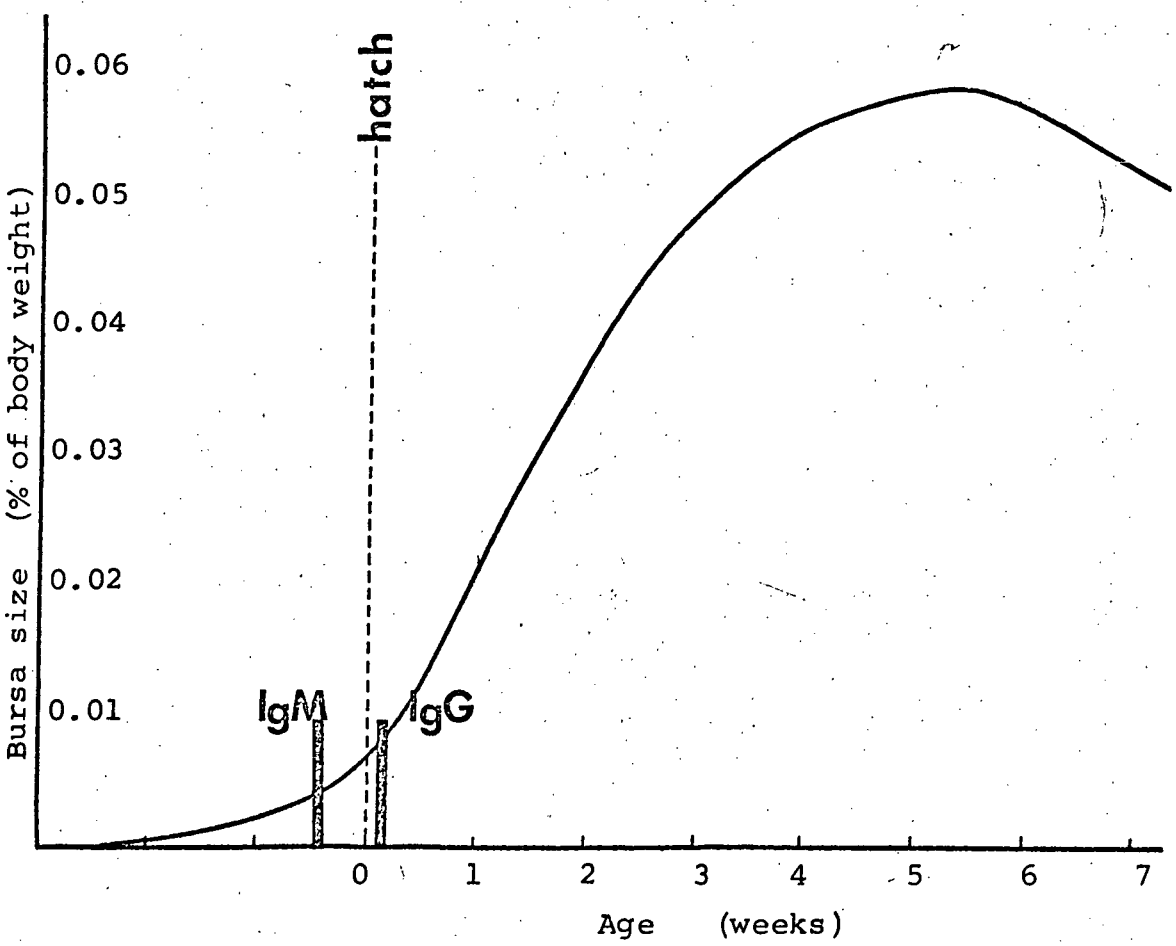

Figure 1. Bursa development shown as a percentage of body weight. The times of initiation of immunoglobulin synthesis are shown.

by numerous large lymphoid follicles which nearly fill the tunica propria (Figure 2). The follicles are found in polyhedral formations, separated from one another by loose connective tissue, small blood vessels and capillaries. Each follicle is divided into a central medulla and a peripheral cortex by a basement membrane which is continuous with the basement membrane of the surface epithelium. The medulla is the main lymphocytopoetic area and contains developing and mature lymphocytes (7).

C. Development of the Bursa.

The bursal primordium first appears at about 4 days of incubation as an epithelial proliferation along the ventral caudal area of contact between the cloaca and the external ectodermal epithelium (8). By day 6 an epithelial anlage projects from the dorso-caudal margin of the urodael membrane. This anlage contains numerous lacunae which, at 7 days, begin to coalesce to form a lumen which becomes continuous with that of the proctodaeum. The bursa then rotates foreward to lie vertically and grows out on a cylindrical stalk. The first plicae appear at 10 or 11 days of incubation and by 15 days the lumen contains about 12 plicae. From the 12th to 16th days the primative epithelium along the basement membrane of the medulla thickens and buds begin to project into the tunica propria. The bud formation is associated with vascularization and an increase in the alkaline phosphatase activity of the subepithelial mesenchyme. At the sites of bud formation the epithelial layer becomes detached from the basement membrane; the buds form solid masses of cells and continue to enlarge.

Lymphoblasts begin to appear in the bursa when the buds reach about 60μ in diameter (about 16 days). Although Ackerman and Knouff (7) consider these cells to originate in the epithelial bud itself, current opinion is that they are of yolk sac origin. Moore and Owen (9), working with parabiotic chick embryos of opposite sexes with a sex chromosome marker,

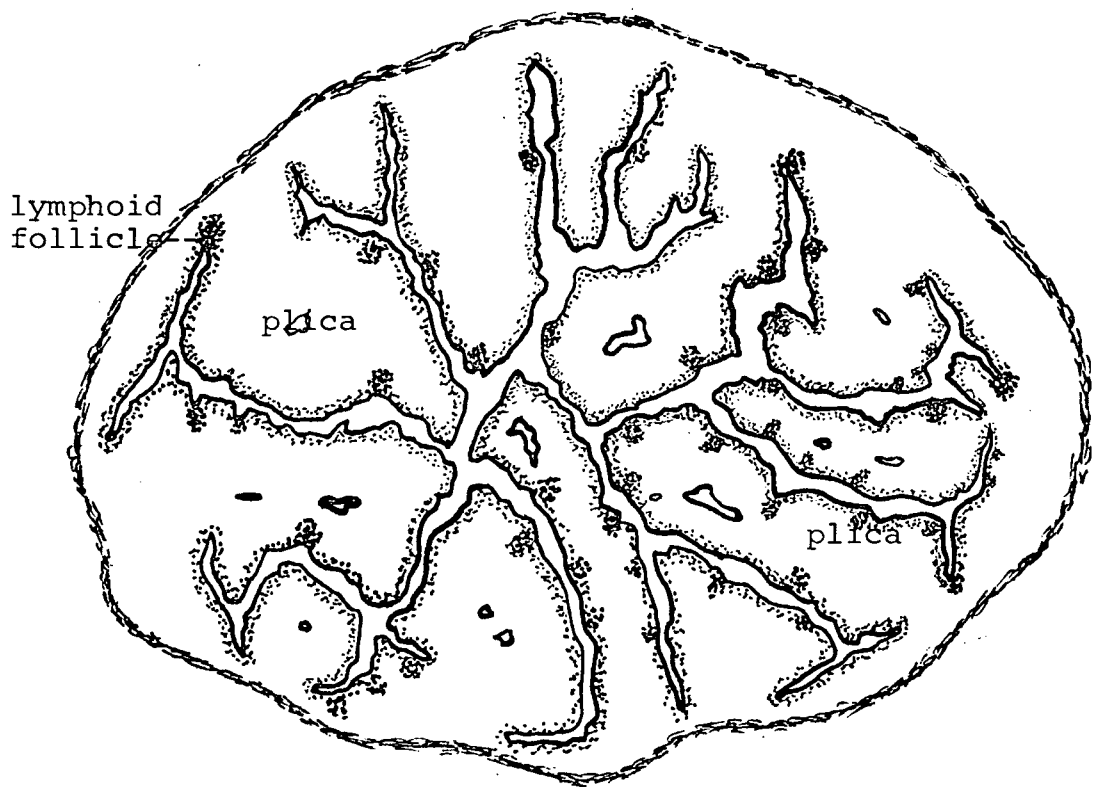

Figure 2. Cross section of the bursa of Fabricius, showing plicae and lymphoid follicles (from Romanoff, 10).

demonstrated that up to 50% of the dividing cells in the bursa are derived from the partner. They take this to indicate an extrinsic source of probably blood-borne cells. The bursal lymphocytes are indistinguishable from those found in other lymphoid tissues. The appearance of lymphoblasts throughout the medullary portion of the follicles continues until the follicles enlarge to 100 - 300 μ .

The lymphoblasts continue characteristic differentiation through decrease in cell size, in nuclear size and basophilia and in a condensation of chromatin. Small lymphocytes appear in the bursa between 16 and 18 days of incubation. The previously undifferentiated cells along the basement membrane become less basophilic and exhibit a decrease in nuclear size at the same time as the small lymphocytes appear.

Two tissues contribute to the formation of the cortex: the mesenchymal cells of the tunica propria and some of the undifferentiated epithelial cells which find their way into the tunica during the early stages of bud formation. As the bud projects into the tunica the subadjacent mesenchymal cells lose their continuity with the basement membrane. Enlargement of the bud crowds the mesenchymal cells into a relatively compact cellular zone about the epithelial bud.

At the start of lymphoblast appearance (16 days) a number of undifferentiated epithelial and blast cells from the periphery of the medulla may pass through the basement membrane into the cortex. The cortex then also transforms into a lymphocytopoetic area with reticular cells and immature lymphocytes.

D. Immunoglobulin Synthesis.

Immunofluorescent staining of bursal cross sections (with fluorescein labelled anti-immunoglobulin sera) shows no immunoglobulin producing cells at 15 days of incubation (11). More sensitive tests for specific immunoglobulins indicate that a small quantity of IgM is produced by 18 days of

incubation (12). Large numbers of competent cells appear in the follicles by 4 - 7 days after hatching. By this time immunofluorescent cells are also found in the spleen and in the germinal centres of the intestine. IgG synthesis in the bursa and spleen follows the appearance of IgM. Similarly, in the response of the mature immunized chicken, IgM type antibody is formed prior to IgG type antibody (13).

Kincade and Cooper (14) used specific antibodies to the IgM (μ) type and IgG (γ) type of heavy chains and to light chains of immunoglobulin molecules to demonstrate the presence of IgM containing cells in the lymphoid follicles of the bursae of 14 day embryos. They also found that IgG containing cells do not appear until 21 days of incubation. Expansion of the IgM and IgG containing cell populations was observed in the bursae of 5 day chicks and the spleens of 8 day chicks. Single cells from the bursa contained both types of heavy chains while single cells from the spleen contained chains of only one or the other class of immunoglobulins. This suggested a developmental switch from IgM to IgG production occuring within the bursa. This theory is further supported by the observation that treatment in ovo with anti-IgM serum followed by neonatal bursectomy resulted in supression of both IgM and IgG synthesis. When the anti-IgM treatment was delayed until after the neonatal bursectomy however, only IgM synthesis was depressed. The authors suggest that the effect of in ovo treatment with anti-IgM serum was the destruction of all IgM producing cells including those which were to switch to IgG synthesis. As all lymphocytes are in the bursa at this time, the secondary lymphoid organs have not been seeded. By the time of hatching, however, the switch in production has already occured and only the IgM producing cells in the peripheral tissues are affected by the treatment. The switch in immunoglobulin synthesis appears to occur exclusively in the bursa; some Bx chicks having supernormal levels of IgM never repair their IgG synthesis mechanism, even over a prolonged period (15).

Immunoglobulin production in the bursa declines by about 3 months. That of the spleen does not decline over time; the spleen is the major source of competent lymphocytes in the mature bird.

The thymus appears to produce no IgM. IgG appears in small quantities at 2 weeks and strengthens by 3 months. Both immunoglobulins appear in the intestinal lymphoid cells after 2 weeks (12).

E. Bursectomy.

The bursa can easily be surgically removed from 18 days of incubation onwards, although there are less complications after hatching (16,17,18,19). Premature regression of the bursa can be induced by the injection of ACTH, adrenal corticoids, sex steroids, and thiouracil or by such stresses as fowl typhoid, muscular exercise or starvation (5).

Injection of 0.65 mg of 19-nortestosterone on the 5th day of incubation causes complete inhibition of bursal development. Smaller doses cause partial inhibition. At 11 - 13 days of incubation, the time of epithelial bud formation, a similar dose of testosterone may cause partial or complete inhibition of lymphoid differentiation in the epithelial bud. This may be due to inhibition of alkaline phosphatase activity in the subepithelial mesenchyme (20). Testosterone propionate, injected into the albumin or absorbed through the shell in an alcohol solution is also an effective agent for hormonal bursectomy.

The bursa can also be caused to regress at the time of hatching by injection of the immunosupressant drug cyclophosphamide (21).

F. Effects of Bursectomy.

Bursectomy, whether by surgical or hormonal means, has been performed by a large number of investigators in the attempt to elucidate its role in the immune system. The effects

of bursectomy at various times during embryonic development and just after hatching provide evidence that the bursa is essential in the development of the stem cells of the humoral immune system. These are the cells which give rise to a population of cells capable of migration and of differentiation into antibody producing cells.

The effects of bursectomy on the immune system appear to depend on the time and method of bursectomy. The direct effects of bursectomy generally include the following:

a) supression of the antibody response. The primary response is usually absent or reduced (22, 23) but the secondary response may be normal or nearly so (24).

b) reduction of immunoglobulin synthesis, usually more restrictive of IgG than of IgM production.

c) reduction of the delayed hypersensitivity reaction to hereditary immune thyroiditis, but not to tuberculin or diptheria.

d) increase of mortality due to avian diseases such as infection by Salmonella typhimurium and to myeloblastosis.

e) changes in the blood and tissue cell populations. Plasma cells and germinal centres are reduced in the spleen and caecal tonsils, although the number of lymphocytes in the intestinal epithelium is increased. The white cell count for lymphocytes and heterophils may be near to normal.

Bursectomy combined with x-irradiation enhances the above effects to the point of aggammaglobulinemia and also results in a deficiency in red blood cells and haemoglobin (25).

The time of bursectomy appears to be critical. Early bursectomy (at 5 days in ovo for example) will prevent or reduce the production of both IgM and IgG type antibodies. Neonatal bursectomy appears to have a major effect on the production of IgG while IgM production is less influenced. Several weeks after hatching, removal of the bursa does not affect immunoglobulin production.

G. The Endocrine Function of the Bursa.

The effects of bursectomy extend beyond the immune system and have led to the proposition of an endocrine function for the bursa. Modification of the adrenal response to stress following bursectomy was first described by Perek and Eilat (26) who noted that adrenal ascorbic acid (AAA) became depleted. Freeman (27) postulated that the bursa produces an hormonal factor which facilitates the development and maturation of the adrenal's ascorbic acid repletion mechanism. Bursectomy, then, would render the animal incapable of maintaining stores of AAA following stimulus depletion.

Pintea and Pethes (28) noted that the rate of radio-iodine uptake by the thyroid and the rate of thyroid hormone synthesis are depressed by bursectomy. The bursa is also postulated as the source of an hormonal factor influencing erythropoesis, as bursectomized and x-irradiated chicks develop severe anaemia when compared to x-irradiated control chicks (29).

The existance of any bursal hormonal product has not been conclusively demonstrated. If such a product does exist, it would have to be classified as a 'developmental hormone' to accomodate the range of its effects.

PART I : Surgical and Hormonal Bursectomy Techniques, Histology and Skin Grafting.

Methods:

i) Early embryonic surgical bursectomy.

Fertile eggs were obtained from White Leghorn stock maintained on the Poultry Farm of the University of British Columbia. All eggs were incubated for 3 days (72 hrs) in a Jamesway Model 252 Incubator under standard conditions. The eggs were placed small end up for 1 hr preceeding the operation. The eggs were surface sterilized with 70% ethanol and the operation was carried out under sterile conditions.

One egg was initially sacrificed to provide albumin and shell membranes for filling and sealing the opened eggs. The empty shells were stored open end down in an egg carton to prevent drying of the membranes.

A small hole was made in the narrow end of the egg, thus preserving the air cell, and enlarged with forceps until the whole embryo was exposed (about 1 cm in diameter). Albumin was added from a syringe to float the embryo to the top of the hole. The embryos were then staged according to Hamburger and Hamilton (30) and only those of stage 17 - 18 were used (Figure 3). Operations on acceptable embryos were carried out under a dissection microscope (mag. 120x). A cataract knife was used to make a small slit in the chorion perpendicular to the embryonic axis just posterior to the leg buds. The tip of the knife was inserted to one side of the midline and the opening was extended to the opposite side. The amnion was cut in the same manner. Care was taken not to puncture the vitelline circulation or the yolk sac. The tip of the tail was then drawn out to lie on top of the chorion (Figure 4). Albumin or chick ringer (Appendix) was added to prevent desiccation of the embryo.

A loop of child's hair, rinsed in 70% ethanol and sterile chick ringer solution was then passed over the free end of the tail and drawn up almost to the leg buds. The knot was tightened sufficiently to ligate the paired dorsal aortae but not

to cut the tail. The tail and the free ends of the hair were trimmed off with iris scissors and removed (Figure 5). The stump was tucked back under the chorion and a little albumin was added to fill the egg. A piece of the double shell membrane was cut to fit and placed, inside down, over the opening in the shell. This was allowed to dry for a few minutes then a layer of high melting point paraffin (mp 65 - 70 C) was painted sparingly over the edges of the patch. The eggs were returned to the incubator small end down and checked after 1 hr for leaks which were patched with additional wax. If both shell membranes were used for the patch few leaks occurred. The eggs were then incubated under standard conditions for the remainder of their term (2).

Initially, a number of sham operations were also performed but as the chicks hatched appeared normal this practice was discontinued. Control birds were normal White Leghorn chicks incubated under standard conditions with the bursectomized chicks.

ii) Early embryonic hormonal bursectomy.

Fertile White Leghorn eggs were incubated for 3 days as above. They were surface sterilized with 70% ethanol and a pair of holes was drilled through the shell and shell membranes. One penetrated into the air cell at the large end of the egg, the other allowed access to the albumin through the side of the egg. Each egg was injected through this latter hole with 0.1 ml of a 20 mg/ml solution of testosterone propionate in peanut oil (this is a final dose of 2 mg/egg). The holes were then sealed with a drop of paraffin and the eggs were returned to the incubator for the remainder of their term.

iii) Preliminary examinations.

All chicks were weighed on the day of hatching and at weekly intervals thereafter. Those which died were examined for the presence of bursal remnants. Chicks were bled and sacrificed at weekly intervals and the spleen, thymus and lower large intestine were excised and prepared for histological

Figure 3

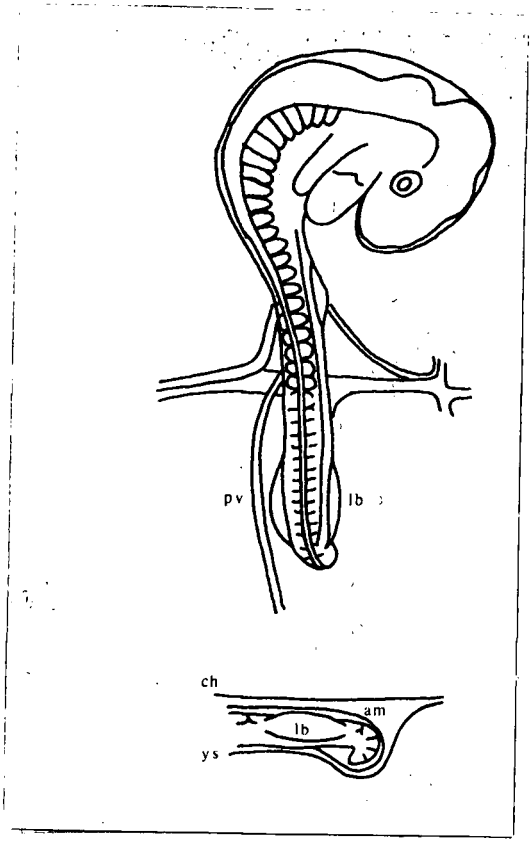

Figure 4

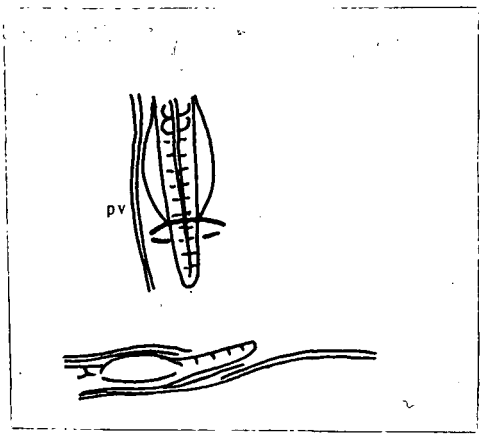

Figure 5

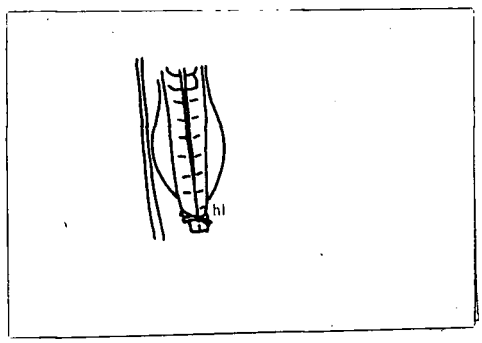

Figure 3. Chick embryo at 3 days (72 hrs) incubation--dorsal view and longitudinal section through tail region.

Figure 4. Tail region (dorsal view and longitudinal section) showing incision through membranes and tip of tail lying free.

Figure 5. Tail region (dorsal view only) following ligation and removal of presumptive bursal tissue.

Abbreviations: am : amnion; ch : chorion; hl : hair loop; lb : limb bud; pv : posterior vitelline vein; ys : yolk sac.

examination. Four normal and 4 bursectomized chicks were sacrificed on the day after hatching and their spleen and body weights were recorded. Records were kept of hatchability and mortality of normal, SBx and HBx chicks. Serum was collected from the blood samples and stored at -20 C until analysed for serum proteins and immunoglobulins.

iv) Histological examination.

Tissues were stored in buffered formalyn (Appendix) until prepared for histological sectioning. Standard wax embedded serial sections were prepared from the spleen, thymus, caecae and trident area of the large intestine for both normal and SBx chicks. Sections were also prepared from the bursa of normal chicks and the cloacal area of SBx chicks. The sections were stained with hematoxylin and eosin and were examined microscopically for the presence of germinal centres and lymphoid cells.

v) Skin grafting.

The capacity for graft rejection by HBx, a few SBx and normal White Leghorn (WL) chicks was tested by reciprocal grafting. Patches of dorsal skin were exchanged between the above mentioned groups and New Hampshire Red (NH) chicks.

Grafting was done as soon as possible after the hatching chicks had dried. The down was plucked from the backs of the chicks and the exposed skin was painted with colloidon to dry and stretch the skin. When this was dry, 0.1 ml of chick ringer solution was injected subcutaneously near the midline over the pelvis, lifting the epidermis free from the underlying tissue and bone (31). A small circular biopsy punch was used to cut out a circle of skin about 5 mm in diameter. Patches of skin were prepared simultaneously from WL and NH chicks and were exchanged between the two. The grafts were placed in their normal anterior-posterior orientation and a drop of colloidon was added to help dry the graft in place. The wound was protected with an Elastoplast spot bandaid.

The grafts were examined at weekly intervals (or at the

time of death) and the prognosis of the graft was noted.

Results and Discussion:

A total of 1466 embryos were surgically bursectomized. Of these, 176 (12%) hatched and 46 (27% of hatch) survived past one week of age. Similarly for the hormonally bursectomized embryos, 51 of 239 (21%) hatched and 16 (31%) survived past one week.

Mortality during the incubation period appeared to be greatest just after the operation, probably due to wounding during the operation. There was another peak of mortality at 18 days of incubation. These embryos usually had malformed limbs and the yolk sac was remaining outside the body cavity. Some of the chicks were able to pip the shell but could not break out of the shell without help. The mortality peaks of the Bx embryos correspond to mortality peaks in normal developing embryos, indicating critical requirements which must be met at this time.

During the first week after hatching the high mortality of the Bx chicks was largely due to compaction of the large intestine owing to poor development of the anal sphincter. Those chicks which survived the first week continued to develop relatively normally until the time they were sacrificed. Four birds died after 6 - 8 weeks, all suffering from acute nephritis.

The SBx chicks were about 6 gm lighter at hatching than normal chicks and by 8 weeks were about 130 gm lighter (Table I). Autopsy indicated that the Bx birds lacked the bursa, about 1/3 of the large intestine and the last few vertebrae. The caecae were not affected and the cloaca appeared normal in birds several weeks of age. Development of the oviduct in female chicks appeared normal. Several hens were kept for over 10 months and produced normal eggs.

The spleens of newly hatched SBx chicks weighed an average of 0.012 g (0.040 % of body weight). Those of normal chicks

weighed an average of 0.22 g (0.054% of body weight). The difference in spleen to body weight ratios indicates that the spleens of the Bx chicks were really smaller than those of normal chicks (Table II). The spleens of the Bx chicks appeared darker than those of normal chicks, perhaps because of the relatively higher proportion of red cells. Histologic sections showed the Bx spleens to have fewer germinal centres than normal chicks (Figure 6).

The thymus glands of the Bx chicks also appeared smaller than those of the normal WL chicks, but the diffuse nature of the tissue and the abundance of associated fat deposits made reliable weight data difficult to obtain. Histological sections, however, reveal that the small size of the thymic lobes is due to incomplete development of the cortex (Figure 7).

The prognosis of the skin grafts on SBx, HBx, normal White Leghorn and normal New Hampshire chicks was recorded at 7, 14 and 28 days after grafting (Table III). The grafts were observed to pass through a series of stages in the rejection process. Reddening and slight swelling of the area was followed by swelling of the graft site to about 5 mm in height. The edges of the grafted patch then separated from the host tissue and finally the graft blackened and fell off. Successful grafts did not pass beyond the slight swelling stage; this soon subsided and the graft patch appeared to be incorporated into the skin of the host. Grafts were recorded as rejected when the edges of the patch separated from the host tissue as rejection seemed to be irreversible from this point.

As a large number of the Bx chicks died within the first week, the complete history of the graft was obtained in only about 20% of the cases. These results do, however, show some trends. The NH birds rejected the grafts within the first week in 80% of the transfers. Normal WL chicks were somewhat slower, but all rejected the grafts within 28 days. Both the SBx and HBx groups, however, contained a few birds which showed no signs of graft rejection at the end of 28 days. At 8 weeks

of age, as feathering neared completion, the grafts still appeared intact. Observation of the thymus gland through the skin of the neck of these birds showed them to be smaller than the thymus glands of normal birds observed in the same way. If this smaller size was due to the absence or decrease of the thymic cortex as was found in other Bx birds, the poor graft rejection may be explained as a lack of competent lymphocytes. It is also possible that some B-cells are necessary in the process of graft rejection and that some Bx birds were unable to build up a sufficient population of these cells to carry out rejection.

Table I Average weights of normal and surgically bursectomized chicks from hatch to 8 weeks of age.

Age	Normal			SBx		
	Weight (g)	Error	Sample Size	Weight (g)	Error	Sample Size
Hatch	41.2	4.1	29	36.3	4.4	29
1 wk.	63.8	7.3	8	47.9	2.2	6
2 wk.	197.6	25.3	8	93.3	14.4	6
3 wk.	288.1	30.8	8	141.3	22.9	3
4 wk.	435.0	17.7	2	210.0	40.6	2
5 wk.	433.8	85.5	6	247.8	60.5	2
6 wk.	-	-	-	310.7	94.1	3
8 wk.	808.3	265.7	3	675.3	124.8	3

Table II Spleen and body weights for day old normal and surgically bursectomized chicks.

	Spleen Wt.	Body Wt.	Ratio
Normal	0.0183 g	41.9 g	0.00043
Normal	0.0217	43.1	0.00050
Normal	0.0213	36.2	0.00058
Normal	0.0246	38.0	0.00064
Average	0.0227	36.8	0.00054
SBx	0.0123 g	29.8 g	0.00041
SBx	0.0160	36.3	0.00044
SBx	0.0144	37.0	0.00038
SBx	0.0079	19.8	0.00039
Average	0.0126	30.7	0.00040

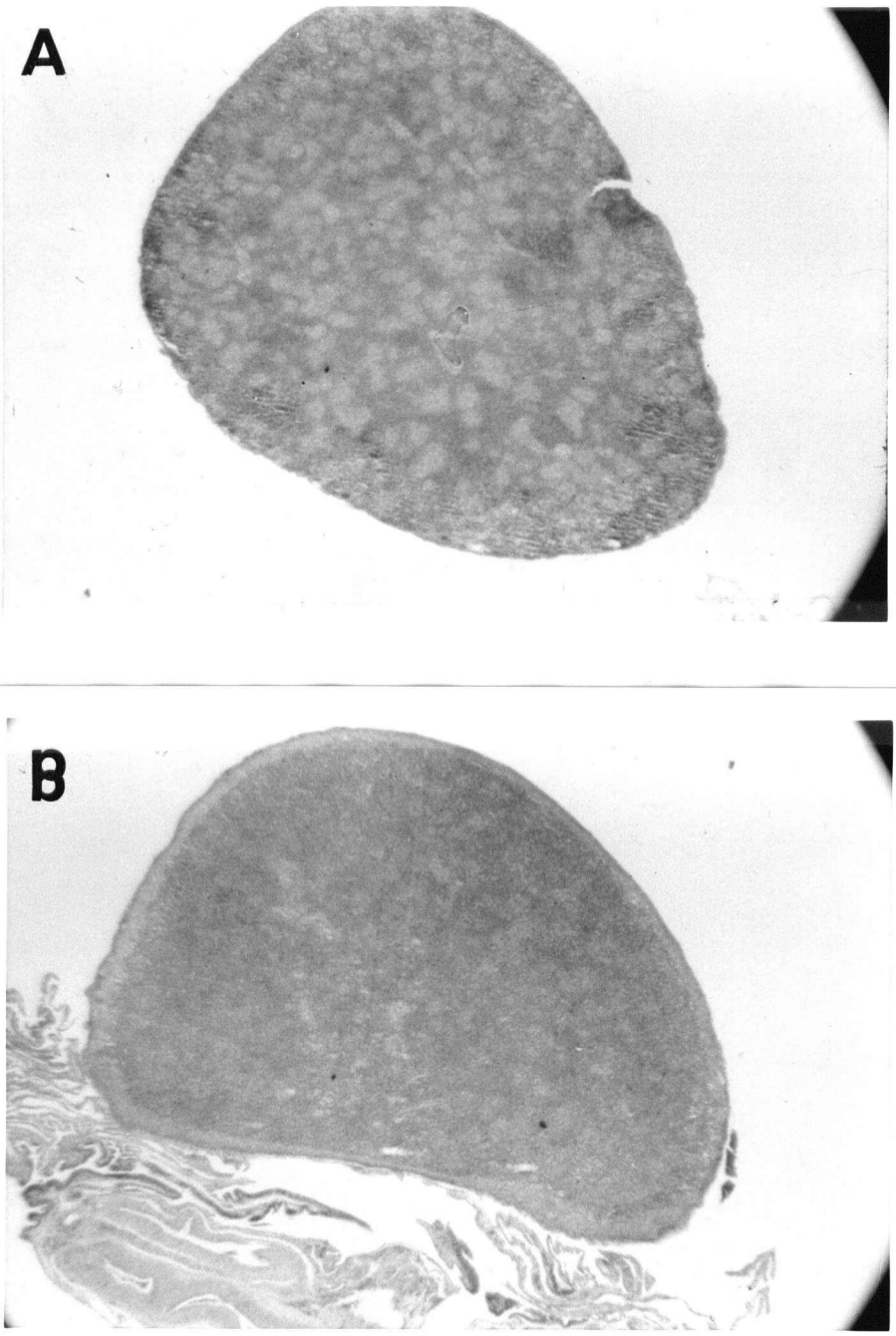

Figure 6. Histological sections of normal (A) and bursectomized (B) spleens; note the reduced number of germinal centres in Bx spleen. H & E stain. Magnification 120 x .

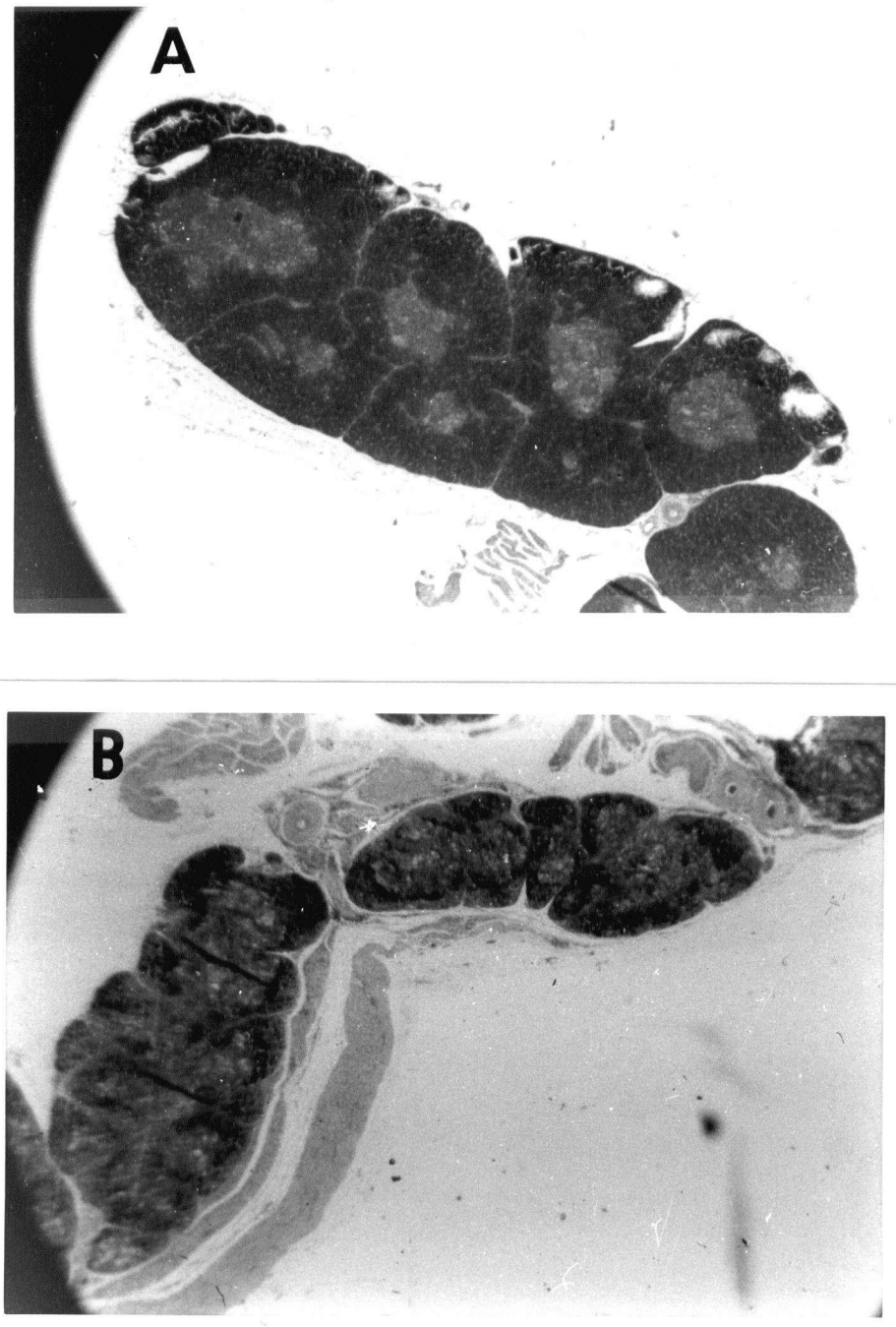

Figure 7. Histological sections of normal (A) and bursectomized (B) thymic lobes; note the reduced thymic cortex in Bx thymus. H & E stain. Magnification 120 x.

Table III Prognosis of skin grafts at 7, 14, and 28 days after grafting.

Host	(No)	Donor	7 days (-)	7 days (+)	died before 14 days	14 days (-)	14 days (+)	28 days (-)	28 days (+)
WL	(16)	NH	4	12	5	2	5	5	0
SBx	(5)	NH	0	5	3	0	2	0	2
HBx	(26)	NH	6	20	16	1	3	1	2
NH	(16)	WL	9	7	2	4	1	1	0
NH	(26)	HBx	23	3	1	1	1	1	0

Those grafts scored as a (-) in one observation are not included in the next observation.

PART II : Tests of the Humoral Immune System.

Methods:

i) Serum samples.

Blood was collected at weekly intervals by cardiac puncture or from the jugular vein of young birds and from the wing vein of birds over 5 weeks of age. At least 0.5 ml of blood was collected from chicks and up to 5.0 ml was taken from the older birds. The blood was allowed to clot at room temperature for 3 - 4 hrs, then refrigerated overnight. The serum was then removed with a pasteur pipette and stored in covered test tubes at -20 C until used.

ii) Preparation of antisera.

Adult chicken serum was used in the preparation of rabbit anti-chicken sera. A modification of the method of Hudson et al. (32) for the separation of ovine serum immunoglobulin was used to obtain purified chicken IgM. Whole adult chicken serum was applied to a G-200 Sephadex column and eluted with a 0.1 M Tris-HCl buffer of pH 8.2 (Appendix). The first protein peak detected by UV spectrophotometry (Pharmacia UV Monitor Model 100) was collected and concentrated by nitrogen pressure dialysis.

Five mg of protein (either whole serum or the IgM purified above) in a 2% solution in chick ringer was mixed 1:1 with Freunds complete adjuvant and injected subcutaneously into rabbits at 2 one week intervals. Rabbit blood was collected from an ear vein one week after the second injection. The blood was allowed to clot as above and the serum containing anti-chicken antibodies was stored at -20 C.

iii) Immunoelectrophoresis.

A standard immunoelectrophoresis procedure (chicken serum in wells, rabbit antiserum in trough) was used to obtain serum protein patterns. Serum samples from day-old chicks through to those several weeks of age were tested in this way for all serum proteins and specifically for IgM. The precipitin curves were identified according to Tureen et al. (33).

iv) Test for specific antibody production.

Sheep red blood cells (SRBC) were used to stimulate specific antibody formation in normal, SBx and HBx birds. Five week old birds were injected intraperitoneally with 0.5 ml of a 2% solution of SRBC in chick ringer solution. Five days later the birds were bled from the wing vein and given a second injection of SRBC. These serial bleedings and injections were repeated 3 - 4 times. Serum was collected from the clotted blood as above and stored at -20 C.

The serum from these stimulated birds was tested for the presence of specific anti-SRBC antibody by microtitre haem-agglutination of SRBC. Twenty-five μl of chicken serum was placed in the first well of a microtitre plate and doubling dilutions were made through 11 more wells each containing 25 μl of chick ringer. The serum dilutions were incubated with 25 μl of 5% SRBC for 30 min at 37 C. The end point titre was recorded as that dilution of serum which last caused agglutination of the SRBC.

Results and Discussion:

According to Tureen et al. (33) immunoelectrophoresis of sera from growing chicks and older birds shows a changing pattern of serum proteins as the birds mature. Figure 8 shows their serum pattern for a 5 day old chick. The prealbumin and beta-lipoprotein bands show the greatest changes with age. Both are present from the time of hatching until 8 days when the beta-lipoprotein band disappears. On the 10th day the prealbumin band starts to become fainter and disappears by the 20th day. The gamma globulins appear as three curves corresponding to IgA, IgM and IgG. All three are present by 5 days in normal chicks. A fourth globulin fraction, perhaps IgD, is also known to occur but is not constantly found. Transferrin appears as a prominant band near the origin in the younger birds and sometimes disappears in older birds. The beta globulins appear more prominantly in older birds.

The serum samples from the normal White Leghorn chicks showed the expected immunoelectrophoretic patterns for transferrin, albumin, lipoprotein and the serum globulins. IgM was present from the time of hatching and IgG appeared in the first few days. Figures 9A and 10A show the immunoelectrophoretic patterns for normal 14 day chick sera against anti-whole serum and anti-IgM respectively. The anti-IgM plates show several bands because the heavy chains of immunoglobin molecules are common to all immunoglobulins resulting in cross reactivity. The transferrin band is present because of incomplete purification. These extra bands could have been removed by incubation of the antiserum with embryonic chick serum prior to electrophoresis.

The immunoelectrophoretic patterns for 14 day SBx chick serum against rabbit anti-whole chicken serum and anti-IgM (Figures 9B and 10B respectively) show the normal patterns for transferrin, lipoprotein and albumin. The serum globulins, however, are incomplete; IgM and IgG immunoglobulin bands are absent. In some cases (not shown here) there were traces of IgM appearing several days after hatching, but IgG was not found.

The haemagglutination tests for specific antibody to SRBC (Figure 11) indicate that the SBx birds do not produce detectable Ab following a primary challenge. However, as the production of antibody to SRBC requires co-operation between B- and T-cells, the absence of the response may not be entirely due to an absence or reduction of bursa-derived cells. Table IV gives the results of a series of challenges for normal, SBx and HBx birds. Some of the HBx birds respond to the primary challenge, but the degree of the response is far below that of the normal birds. Hormonal bursectomy does not always impair the development of the thymic cortex. The antibody titres of both Bx groups slowly rise following successive challenges and would probably reach normal levels if the series were continued. The reduced lymphocyte populations of the Bx birds require a

longer time in which to build up sufficient cells producing a specific antibody than do those of normal birds. The HBx birds appear to approach normal levels more rapidly than the SBx birds, perhaps suggesting that the effects of hormonal bursectomy are less severe than those of early surgical bursectomy.

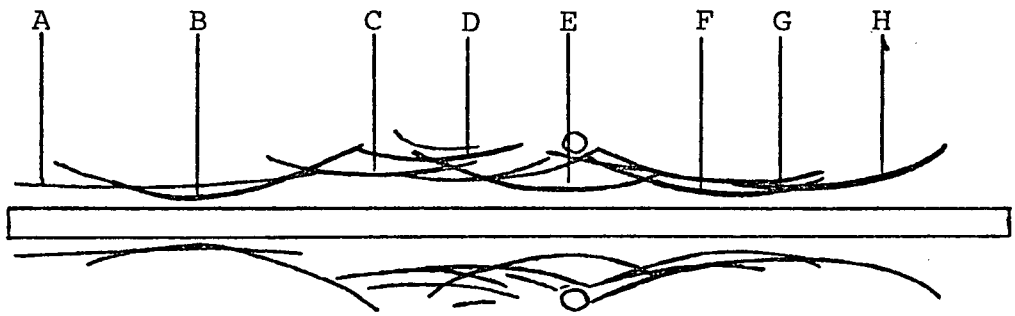

Figure 8. Precipitin bands from immunoelectrophoresis of normal 5 day chick serum (from Tureen et al., 33).

Key:

A. prealbumin
B. albumin
C. alpha lipoprotein
D. beta lipoprotein
E. transferrin
F. IgA gamma globulin
G. IgM gamma globulin
H. IgG gamma globulin

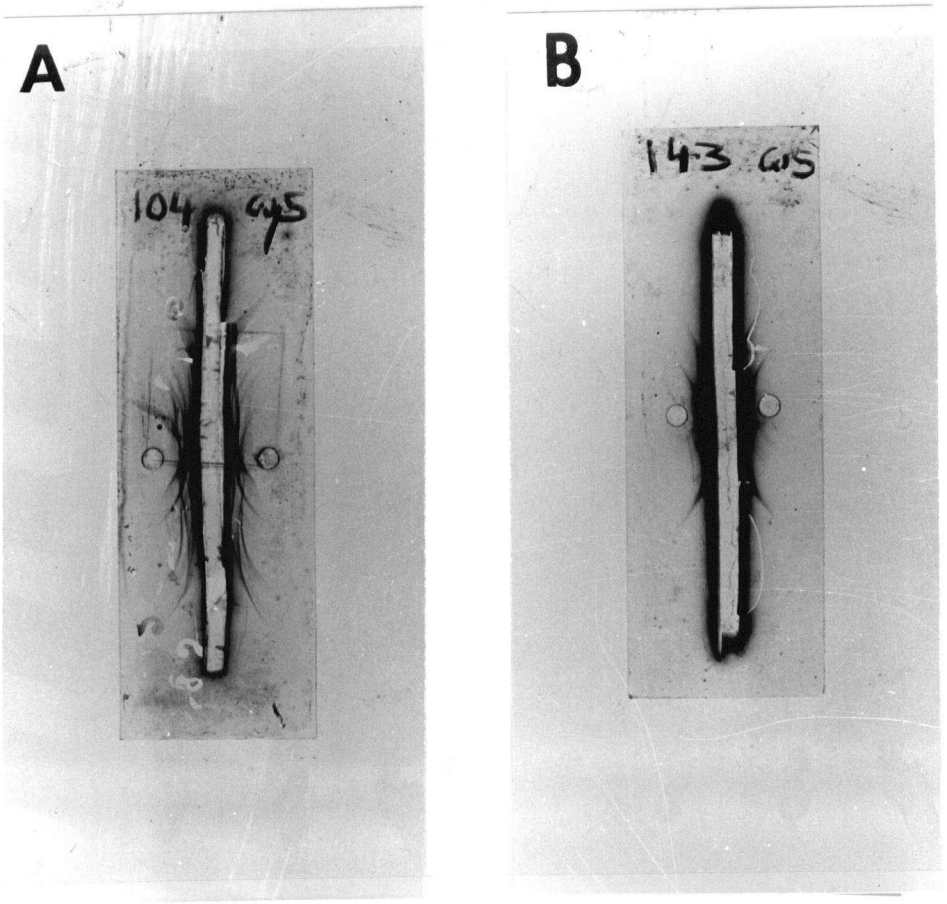

Figure 9. Precipitin bands from immunoelectrophoresis of 14 day normal (A) and bursectomized (B) chick sera against rabbit anti-whole chicken serum.

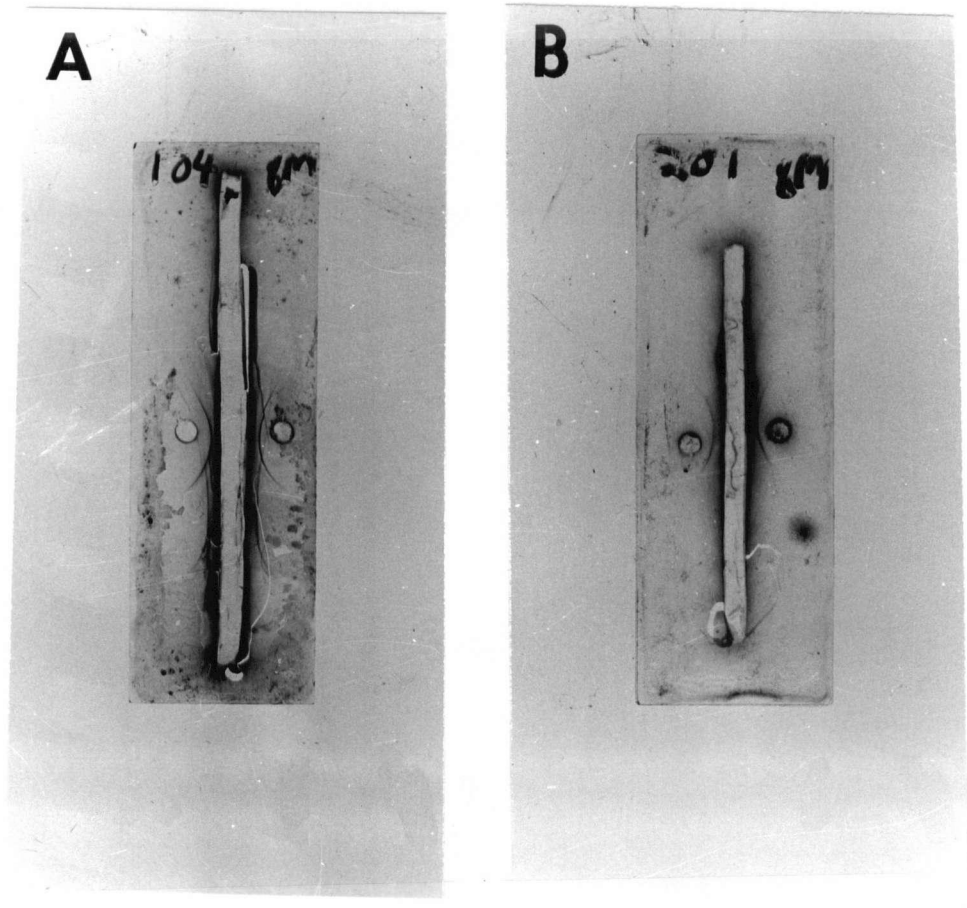

Figure 10. Precipitin bands from immunoelectrophoresis of 14 day normal (A) and bursectomized (B) chick sera against rabbit anti-chicken IgM.

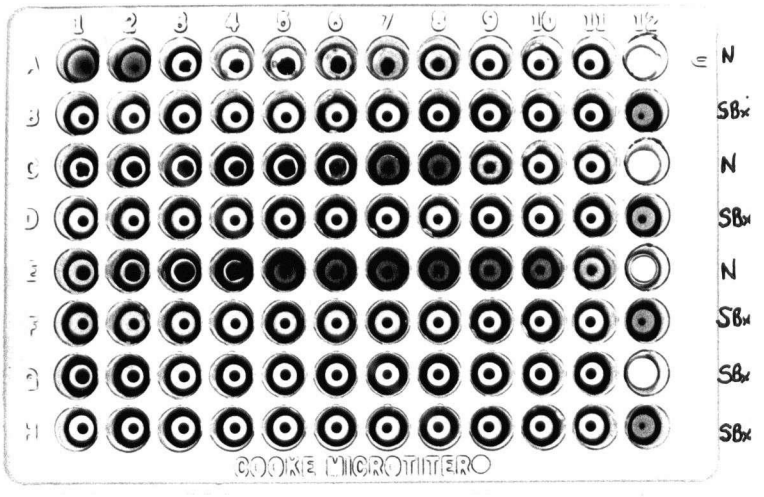

Figure 11. Microtitre haemagglutination of SRBC by normal (N), surgically bursectomized (SBx) and hormonally bursectomized (HBx) chicken sera following primary stimulation with SRBC.

Table IV Haemagglutination titres for normal, SBx and HBx chicken sera following successive stimulations with SRBC.

Birds (No)	Primary	Secondary	Tertary
Normal (10)	128*	256 - 512	1024 - 2048
SBx (6)	-	8 - 32	256 - 512
HBx (8)	4	8 - 64	512 - 1024

* a titre of 128 indicates that a dilution of serum 1/128 in chick ringer is the greatest dilution which still contains sufficient anti-SRBC antibody to agglutinate 25 µl of 5% SRBC.

PART III : Stimulation of spleen, thymus and bursa cells with mitogens.

Introduction to mitogens:

Several plant proteins known to be red blood cell agglutinins and the bacterial product endotoxin have been shown to stimulate the transformation of leukocytes *in vitro*. Some of these mitogens are thought to specifically stimulate T-cells as they have no effect on mouse lymphocytes treated with anti-theta to kill all thymus-derived cells (34). Others, which have little effect on T-cells are thought to be specific stimulants of B-cells, although the evidence for this is less concrete than that for the T-cell specific mitogens.

Phytohaemagglutinin (PHA) is derived from the red kidney bean, *Phaseolus vulgaris*, by saline extraction. Its mitogenic properties were discovered in 1959 by Hungerford *et al.* (35). Concanavalin A (ConA) is a similar extract from the jack bean. These two mitogens stimulate division of mixed leukocyte cultures but fail to stimulate anti-theta treated cells and are thus considered to be T-cell specific mitogens. They stimulate rapid cell division and the transformation of undifferentiated lymphocytes into blast cells. PHA-induced transformation does not appear to require the presence of macrophages necessary for antigen-induced transformation (34).

Endotoxin is a lipopolysaccharide product of bacteria, usually obtained from cultures of gram negative bacteria such as *E. coli*. It has been shown to induce DNA synthesis in B lymphocytes and has no effect on thymus-derived lymphocytes (36,37). Endotoxin is capable of stimulating B-cells in the absence of T-cells--it is a T-cell-independant antigen (38).

The response of an *in vitro* cell population to a variety of cell-specific mitogens allows for speculation as to the relative proportions of B- and T-cells in that population. For this purpose, spleen and thymus cells from bursectomized birds and spleen, thymus and bursa cells from normal birds were cultured in the presence of mitogens.

Methods:

Spleen and thymus cells from 5 to 8 week old SBx chickens and spleen, thymus and bursa cells from normal chickens were cultured in the wells of microtitre plates and were stimulated with 2 T-cell specific (PHA and ConA) mitogens and one B-cell specific (endotoxin) mitogen in an effort to determine the effect of bursectomy on the B- and T-cell populations.

Cells for culture were prepared in the following manner. The birds were bled to provide autologous serum for the culture medium then were killed by intracardiac injection of 10 cc of air. The appropriate organs were removed aseptically, trimmed of fat and washed in chick ringer.

Each organ was then placed in 5 ml of chick ringer and 1 ml of autologous serum and the cells were gently teased from the organ with two pairs of forceps. A single cell suspension was obtained by passing the tissue fragments through a 1 ml syringe equipped with a 26 guage needle.

The cells were washed 2 times in chick ringer and finally resuspended in RPMI 1640 culture medium (Appendix) supplemented with 1% penicillin/streptomycin. A sample of the suspension was then diluted 1/100 in culture medium and the viable lymphocytes counted with a hemocytometer in a 1/1 mixture with trypan blue.

The cell suspension was then diluted to obtain 5 ml samples of lymphocyte concentrations of 2.5×10^7 and 2.5×10^6 cells per ml in both serum free and 5% autologous serum supplemented culture medium. The cells were dispensed with a Hamilton syringe into the wells of microtitre plates to final concentrations of 5×10^6 and 5×10^5 cells per well for both media. Triplicate wells were made for controls and for each type of mitogen; all cell types were stimulated with all mitogens. The mitogens were added in the following concentrations: PHA - 10 g/ml, ConA - 20 g/ml, endotoxin - 20 g/ml each in 0.05 ml of saline. The total volume of each well was made up to 0.25 ml with the appropriate medium and the cultures were

incubated at 37 C for 48 hr. At this time 0.05 ml of ^{3}HTdr (tritiated thymidine) or 20 μci/ml of isotope was added to each well and the cultures were incubated for a further 12 hrs.

The cells were harvested by a vacuum collection machine, constructed for this purpose, onto glass fibre filters. The cells were washed 5 times with tap water to remove excess radioactive material, to lyse the cells and to precipitate the DNA all in one step. The filters were then dried under a heat lamp, placed in scintillation vials with 3 ml of scintillation fluid (Liquifluor and toluene, no methanol) and counted in a scintillation counter. Dpms were not determined as the results were expressed as a ratio of two counts.

$$\text{Stimulation by a mitogen} = \frac{\text{CPM of mitogen stimulated culture}}{\text{CPM of a non-stimulated culture}}.$$

Results and Discussion:

A series of preliminary tests with various combinations of cell density, serum types and serum concentrations determined that homologous, preferably autologous, serum at a concentration of 5 - 10% and a lymphocyte density of 5×10^{5} to 5×10^{6} produced the most consistant results. Among these, cultures of 5×10^{5} cells per well cultured without serum appeared to be the healthiest and were least often infected with bacteria. The serum was most often the carrier of contamination.

The unstimulated or control cultures gave cpms in the range of several hundred, those of the mitogen stimulated cultures were 3 to 300 times greater.

Normal spleen cells at 5×10^{5} cells per well without serum were stimulated 25 times with PHA, 17 times with ConA and 40 times with endotoxin. Spleen cells from an SBx chicken were stimulated 50 times with PHA, 15 times with ConA and only 15 times with endotoxin. The spleen of the SBx bird, however, was expected to be deficient in B-cells. If this were the case, a sample of 5×10^{5} lymphocytes from such a spleen would

contain a relatively smaller proportion of B-cells and a relatively larger proportion of T-cells. This appears to be further supported by the large stimulation by PHA, a T-cell mitogen. The results of the ConA stimulation do not give support to this idea, but are not sufficiently consistent to disprove it either (Table V).

At 5×10^5 cells per well in the absence of serum the stimulation of normal and SBx chicken thymus cells was very similar. Although the thymus glands of the SBx birds were much smaller than those of the normal birds, a standard size sample of cells showed the same ability as cells from a normal bird to respond to mitogens. The response to endotoxin was remarkably high for a predominantly T-cell population (Table VI).

The bursa cells responded only to endotoxin, supporting the view that the bursa contains only B-cells (Table VII).

Table V Average stimulation* of normal (N) and SBx chicken spleen cells with mitogens.

No. of cells	Serum	Endotoxin		ConA		PHA	
		N	Bx	N	Bx	N	Bx
5×10^5	-	40	15	17	15	25	50
5×10^5	+	11	3	14	15	18	26
5×10^6	-	5	7	65	15	44	10
5×10^6	+	5	20	26	30	41	58

* Stimulation = $\frac{\text{CPM of mitogen stimulated cultures}}{\text{CPM of non-stimulated (control) cultures}}$

Table VI Average stimulation of normal (N) and SBx chicken thymus cells with mitogens.

No. of cells	Serum	Endotoxin		ConA		PHA	
		N	Bx	N	Bx	N	Bx
5×10^5	-	120	125	20	24	17	20
5×10^5	+	5	-	25	-	10	13
5×10^6	-	25	16	133	26	30	30
5×10^6	+	14	8	-	-	10	13

Table VII Average stimulation of normal chicken bursa cells with mitogens.

No. of cells	Serum	Endotoxin	ConA	PHA
5×10^5	-	5	-	-
5×10^5	+	-	-	-
5×10^6	-	8	-	-
5×10^6	+	11	-	-

GENERAL DISCUSSION

Present evidence favours the view that the bursa of Fabricius is an anatomical and physiological site where undifferentiated stem cells of yolk sac origin serve as precursors of a developing lymphocyte population that differentiates into immunoglobulin producing cells late in embryonic life. In the ontogeny of the immune system, cells producing IgG appear to develop exclusively from cells which previously synthesized IgM. Once seeded from the bursa, cells seem to be irrevocably committed to the production of a single class and specificity of antibody. This implys a role for the bursa in directing the genetic events leading to antibody heterogenecity (39).

Removal of the bursa at the time of hatching or supressing its growth by injection of hormones during its development rarely produces totally agammaglobulinemic birds. Neonatal bursectomy followed by total body irradiation results in agammaglobulinemia and an inability to produce Ab after repeated challenges in only about 40% of the birds treated (25). Hormonal bursectomy *in ovo* can result in agammaglobulinemia in 40 - 50% of treated birds (40) but this can be raised to more than 90% effective if coupled with repeated cyclophosphamide (an immunosupressant) treatments during the first few days after hatching (41). This treatment, although highly effective, results in high mortality.

The technique developed here for early embryonic surgical bursectomy produced viable bursa-less birds in only about 2% of all embryos treated. This low survival rate has been noted by other workers using both surgical and hormonal treatments (42). Early treatment with hormones results in abnormal development of the cloaca. Meyer *et al.* (40) noted stunted growth of the ventral anal lip, increased musculature of the dorsal lip and attenuation of the proctodaeum. These abnormalities, similar to those noted following early surgical bursectomy, resulted in intracloacal accumulation of faeces, usually terminating in death. It would appear, then, that

early mortality is the result of physical abnormalities resulting from the treatment rather than the result of any developmental requirement for the bursa.

Although the body weight data in Table I are insufficient for growth rate analysis, the weight differences between the normal and Bx chicks appear to be greater than can be attributed to loss of the tail structure and lower large intestine. It is possible that the stress of the operation retarded the growth of the embryo, but one would not expect these effects to persist 8 weeks after hatching. Freeman (43) noted that bursectomized birds showed a reduced response to stress from handling. The effect of bursectomy on the general physiological state of the bird is not clear and may extend beyond the immune system.

Spleen to body weight ratios for day old chicks (Table II) indicate that the spleens of Bx chicks represent a somewhat smaller proportion of the body weight than do those of normal chicks. This, again, may reflect a general physiologic effect of bursectomy as the lymphoid tissues appear to be the most sensitive to such stresses. Histological sections of the spleen, however, indicate that the reduced size of the Bx spleen may be the result of a reduced lymphoid population (Figure 6). The reduced number of germinal centres is an indication of a deficiency of humoral lymphocytes. The origin of the B-cells forming germinal centres in the Bx birds is not known. No bursal remnants were found in these birds, nor were any germinal centres found in the histological sections of the caecae and large intestine. Auxilliary bursae are occasionally found at the junction of the bursal stalk and the cloaca, but were not found in this study. It is possible that the B-cells in the spleen are of direct yolk sac origin and have migrated here in the absence of the bursa. The germinal centres of the gut-associated tissues do not appear until about 10 days after hatching and so are unlikely to be involved in the seeding of other lymphoid tissues. Jankovic *et al.* (24) noted that

neonatal bursectomy reduces the number of plasma cells and germinal centres in the caecae.

Thymus weights were not obtained as, in aves, this gland is formed as a series of lobes embedded in fat along the length of the neck extending almost to the level of the thyroids. The thymic lobes of the Bx chicks appeared smaller than those of normal birds; cross-sections at the same magnification (Figure 7) support this observation. These sections also show that the reduced size of the lobes is due to a deficiency in the thymic cortex, the main area of lymphocytopoesis. The same stresses, such as ACTH and adrenal corticoids, which cause premature regression of the bursa have a similar effect on the thymus. The pattern of early growth and maturity followed by regression in the adult animal found for the bursa is also true for the thymus. The removal of the bursa could affect the development of the thymus in several ways. The stress of the operation itself is unlikely to be a major factor as sham operated birds showed apparently normal thymic development. The effect of bursectomy on the general physiological state of the bird may result in incomplete cortical development. It is also possible that the bursa may play a direct or hormone mediated role in the development of the thymic branch of the immune system.

Atrophy of the thymic cortex following hormonal bursectomy at 13 days *in ovo* was noted by Warner *et al.* (44). They observed a variety of effects ranging from very little change in 60% of the chicks to severe reduction of the cortex and an inability to reject skin grafts in 10% of the chicks treated. These data are difficult to interpret as bursectomy was accomplished by the injection of steroid hormone rather late in development, when the effects may have been wide and varied. The observation of similar effects following surgical bursectomy suggest that the phenomenon may be the result of a bursal effect rather than a side effect of hormonal treatment.

The capacity for skin graft rejection was tested in a

small number of SBx chicks and a group of chicks hormonally bursectomized at 72 hr *in ovo*. The results of this study (Table III) indicate that the SBx birds were least capable of skin graft rejection. The time required for the edges of the graft to separate from the host tissue was at least twice that required for a normal chick. Hormonally bursectomized chicks also showed a retarded time course of graft rejection but most of this group showed signs of rejection within the test period. The birds used in this test were not killed and autopsied as they were required for other tests. Superficial examination of the lobes of the thymus through the skin of the neck indicated that the glands were considerably smaller in those birds which required an extended time course for graft rejection. It is probable that these birds, similarly to those of Warner *et al.* (44), were lacking in thymic cortex.

The data to this point, then, do not clearly indicate the role of the bursa in the immune system. Removal of the organ obviously has effects extending beyond the immune system to the general physiological state of the bird. The direct effects on the immune system appear to be a reduction of the germinal centres of the peripheral lymphoid tissues and perhaps an effect on the lymphocyte population of the thymus.

Immunoelectrophoresis of serum samples through the first few weeks after hatching showed the normal (33) patterns for prealbumin, albumin, lipoprotein and beta globulin fractions. The transferrin band remained strong throughout the four week sampling period. The sera of the bursectomized birds, however, showed reduction or absence of the gamma globulin bands. The presence of these bands immediately after hatching can be attributed to the presence of maternal immunoglobulin, but by the latter part of the test period could only have been produced by the chick. The sera from most of the Bx chicks were entirely devoid of IgG. In some cases an IgM band was found but tended to be rather weak. It is possible that the globulins were being produced in such small quantities as to be

undetectable by this method. The immunoglobulin bands of the normal chicks were easily detectable.

Stimulation of the humoral immune system by the introduction of antigenic substances increases the amount of circulating immunoglobulin. This stimulation did elicit the production of specific antibody in some of the bursectomized birds.

Surgically and hormonally bursectomized chickens, stimulated for the first time at 5 weeks of age with SRBC produced little or no antibody. Microtitre haemagglutination analysis (Table IV) of the sera indicated a titre of 128 for normal chickens and titres of 0 to 4 for the bursectomized birds. Neonatally bursectomized birds are also known to have reduced antibody titres following the primary challenge (22,23,24).

A second challenge with the same antigen may result in nearly normal antibody production in some Bx birds (24). The results in Table IV indicated that some of the Bx birds did produce antibody in the 2^{o} response although the level of Ab did not approach normal levels. Further stimulation with the same antigen did cause the Ab titres to rise toward normal levels; the HBx birds appeared to respond to the serial stimulations more rapidly than did the SBx birds. The low number of germinal centres found in the spleens of Bx birds suggests that the gradual response is the result of a gradual increase in humoral cell numbers. As the increase in numbers is logarithmic following stimulation, and as only a small number of the lymphocytes present are capable of responding to any one antigen, the process of building up the cell population takes some time. This is probably not the only factor involved in the slow humoral response; the production of antibody to a number of antigens requires the cooperation of T- and B- cells. A deficiency in the thymic cortex and thus in T-cells would also affect the production of antibody following stimulation with SRBC.

Jankovic and Isakovic (24), working with neonatally Bx

birds, found that no antibody was produced following the primary challenge. After a secondary challenge these birds produced a small amount of 19S (IgM) type antibody. In a normal response, IgM is produced in the primary reaction and is of much less importance in the subsequent reactions. IgG, which is normally undetectable in the primary response, rises to much higher levels in the secondary response. In the Bx birds mentioned above, no IgG was found. These results may in part be due to the time of bursectomy more than the fact of bursectomy as neonatal removal seems to mainly impair IgG production.

Early surgical bursectomy seems to have a similar effect on the class of immunoglobulin produced. Immunoelectrophoresis of serum from Bx birds twice challenged with bovine serum albumin (BSA) shows the presence of small amounts of IgM (2).

Other workers also report low Ab titres in Bx birds following stimulation with a variety of antigens, some of which require B-T cell cooperation, others which do not. Warner et al. (44) found primary antibody titres of less than 10 following challenge with Brucella suis, influenza type A virus, human chorionic gonadotrophin or BSA. Primary titres for normal birds stimulated with these antigens ranged from 320 to 20480.

The residual capacity to synthesize Ab following neonatal bursectomy can be removed by total body x-irradiation. This treatment indiscriminantly kills all rapidly dividing cells including those lymphocytes which are seeding the spleen and other peripheral lymphoid tissues (22).

Warner et al. (44) noted that even though cytotoxic antibody was absent, surgically and hormonally bursectomized birds were capable of rejecting skin grafts in the normal time. In a later publication however, they found that a small percentage of their hormonally bursectomized birds exhibited atrophy of the thymic cortex and were incapable of rejecting grafts (45). Others, with intermediate damage to the thymic cortex, showed

a graded ability proportional to the extent of damage.

The effects of bursectomy on the humoral immune system cannot be entirely divorced from the effects of bursectomy on the rest of the immune system and on the bird generally. Surgical and hormonal bursectomy appear to have similar effects on the production of immunoglobulins and the synthesis of specific antibody, although the effect of early surgical bursectomy appears to be more severe. Bursectomy at any time seems to be more damaging to the IgG producing cells than to the IgM producing cells. It may be that the most critical part of the role of the bursa in the humoral immune system is in the switch from IgM to IgG production in some cells before they are seeded to the peripheral tissue.

The B- and T-cell subpopulations of a mixed lymphocyte population can be individually estimated by stimulation with specific mitogens. These substances appear to be capable of stimulating all of one on these subpopulations rather than just a few select cells as in the case of a normal antigen. A comparison of the stimulation of normal and Bx chicken spleen cells with B- and T-cell mitogens leads to the speculation that the spleen of the Bx bird contains relatively fewer B-cells and relatively more T-cells per standard sample than does the spleen of a normal bird. The cells of the thymus, although reduced in number, do not appear to be impared in their ability to respond to mitogens.

SUMMARY AND CONCLUSION

The general effects of early embryonic surgical bursectomy are similar to those found by other workers employing both surgical and hormonal techniques for bursectomy. Many of the effects of bursectomy cannot be considered only in terms of a reduced humoral lymphoid population but must also be examined in terms of the effects on the thymus, on B-T cell cooperation and on the physiological state of the bird. The depletion of lymphoid populations, especially those of the humoral system, and the reduction of immunoglobulin synthesis support the postulated role of the bursa in the ontogeny of the immune response. There appear to be some lymphoid cells which are capable of differentiation into cells at least capable of producing IgM type antibody even in the absence of the bursa. The source and degree of autonomy in the differentiation of these cells is unclear. It is possible that they are of yolk sac origin or of bone marrow origin and they appear to be capable of self determination to the point of IgM synthesis but unable to switch to IgG synthesis. It may be that the major role of the bursa is in the division of the humoral lymphocytes into IgM and IgG producing populations. Further studies on the nature of the antibody producing cells in the bursectomized bird are necessary to answer this question. A more extensive investigation of the source of the stem cells entering the bursa and the capacity of these cells to develop into competent lymphocytes in the absence of the bursal environment would be of value.

BIBLIOGRAPHY

1. Gordon, B.L. and D.K. Ford, 1971, "Essentials of Immunology", F.A. Davis Co., Philadelphia, Pa.

2. Fitzsimmons, R.C., E.M.F. Garrod, D. Mason and I. Garnett, 1973, The bursa of Fabricius and the ontogeny of the avian immune response, Cellular Immunology, in press.

3. Fabricius, H., 1621, De formatione ovi et pulli tractatus accuratissimus, Patavii, Ex officina aloysii Bencij bibliopolae.

4. Jolly, J., 1914-15, Archs. Anat. microsc. 16:363 - 547.

5. Payne, L.N., 1971, in "Physiology and Biochemistry of the Domestic Fowl" Vol. 3 (D.J. Bell and B.M. Freeman, eds., 3 volumes) pp. 984 - 1037, Academic Press, London.

6. Glick, B., T.S. Chang and R.G. Jaap, 1956, The bursa of Fabricius and antibody production, Poultry Science 35:224 - 225.

7. Ackerman, G.A. and R.A. Knouff, 1959, Lymphocytopoesis in the bursa of Fabricius, Am. J. Anat. 104:163 - 205.

8. Ruth, R.F., C.P. Allen and H.R. Wolfe, 1964, in "The Thymus in Immunobiology" (R.A. Good and A.E. Gabrielson, eds.) pp. 183 - 205, Harper & Row, N.Y.

9. Moore, M.A.S. and J.J.T. Owen, 1966, Experimental studies on the development of the bursa of Fabricius, Dev. Biol. 14:40 - 51.

10. Romanoff, A.L., 1960, "The Avian Embryo, Structural and Functional Development", p. 506, The Macmillan Co., N.Y.

11. Bäck, O., 1970, Studies on the lymphocytes in the intestinal epithelium of the chicken III The effect of thymectomy, Int. arch. Appl. Immunol. 39:192 - 200.

12. Thorbeck, G.J., N.L. Warner, G.M. Hochwald and S.H. Ohanian, 1968, Immune globulin production by the bursa of Fabricius in young chickens, Immunology 15: 123 - 154.

13. Aspinall, R.L., R.K. Meyer, M.A. Graetzer and H.R. Wolfe, 1963 Effect of thymectomy and bursectomy on the survival of skin homografts in chickens, J. Immunology 90:

of skin homografts in chickens, J. Immunology 90: 872 - 877.

14. Kincade, P.W. and M.D. Cooper, 1970, Development and distribution of immunoglobulin-containing cells in the chicken, J. Immunology 106:371 -382.

15. Kincade, P.W., A.R. Lawton, E.D. Bockman and M.D. Cooper, 1971, Supression of immunoglobulin G synthesis as a result of antibody-mediated supression of immunoglobulin M synthesis in chickens, Proc. Nat. Acad. Sci. USA 67:1918 - 1925.

16. Takahashi, K., 1967, Immunobiologic role of the bursa of Fabricius in the development of the competence to produce serum antibodies in chickens, Japanese J. Microbiology 11(1):1 - 12.

17. Van Alten, P.J., W.A. Cain, R.A. Good and M.D. Cooper, 1968, Gamma globulin production and antibody production in chickens bursectomized as embryos, Nature Lond. 217:358 - 360.

18. Ivanyi, J., H. Marvanova and E. Skamene, 1969, Anti-immunoglobulin sera in bursectomized chicks, Immunology 17:325 - 331.

19. Cooper, M.D., W.A. Cain, P.J. Van Alten and R.A. Good, 1969, Development and function of the immunoglobulin producing system I. Effect of bursectomy at different stages of development on germinal centres, plasma cells, immunoglobulin and antibody production, Int. arch. Allergy 35:242 - 252.

20. Ackerman, G.A. and R.A. Knouff, 1963, Testosterone supression of mesenchymal alkaline phosphatase activity and lympho-epithelial module formation in the bursa of Fabricius of the embryonic chicken, Anat. Rec. 146: 23 - 27.

21. Weber, W.T., 1972, abstract, Proceedings of the 7th Leukocyte Culture Conference.

22. Mueller, A.P., H.R. Wolfe, R.K. Meyer and R.L. Aspinall, 1962, Further studies on the role of the bursa of Fabricius in antibody production, J. Immunology 88: 354 - 360.

23. Cooper, M.D., R.A. Peterson, M.A. South and R.A. Good, 1966, The function of the thymus system and the bursa system in the chicken, J. Exptl. Med. 123:75 - 102.

24. Jankovic, B.D. and K. Isakovic, 1964, The role of the thymus and the bursa of Fabricius in immune reactions in chickens II. Cellular changes in lymphoid tissue of thymectomized, bursectomized and normal chickens in the course of the first antibody response, Int. arch. Allergy 24:296 - 310.

25. Bäck, O., 1970, Studies on the lymphocytes of the intestinal epithelium of the chicken IV. Effect of bursectomy, Int. arch. Appl. Immunol. 39:342 - 351.

26. Perek, M. and A. Eilat, 1960, The bursa of Fabricius and adrenal ascorbic acid depletion following ACTH injections in chicks, J. Endocrinology 20:251 - 255.

27. Freeman, B.M., 1970, The effects of ACTH on adrenal weight and adrenal ascorbic acid in the normal and bursectomized fowl, Comp. Bioch. Physiol. 32:755 - 761.

28. Pintea, V. and G. Pethes, 1967, Sb. vys. Sk. zemed, les Fac. Brne B 36:449 - 452.

29. Alm, G.V. and R.D.A. Peterson, 1971, Anaemia in bursectomized irradiated chickens, Nature, Lond. 229:201 - 202.

30. Hamburger, V. and H.L. Hamilton, 1951, A series of normal stages in the development of the chick embryo, J. Morphology 88:49 - 67.

31. Polley, C.R., A.E. Grosse and J.V. Craig, 1960, A skin grafting technique for use in genetic studies in chickens, Transplantation Bull. 7:425 - 428.

32. Hudson, R.J., P.J. Bandy and W.D. Kitts, 1970, Immunochemical quantitation of ovine immunoglobulins, Am. J. Vet. Res. 31:1231 - 1236.

33. Tureen, L.L., K.Warecka and P.A. Young, 1966, Immunophoretic evaluation of serum proteins, Soc. exptl. Biol. Med. 122:729 - 732.

34. Kallos, P. and B.H. Waksman, 1968, The action of PHA *in vivo* and *in vitro*, a review, Prog. Allergy 12:1 - 14.

35. Hungerford, D.A., A.J. Donelly, P.C. Nowell and S. Beck, 1959, The chromosome constituent of a human phenotypic intersex, Am. J. hum. Genet. 11:215 - 235.

36. Peavy, D.L., W.H.Adler and R.Y. Smith, 1970, Effects of endotoxin and staphlococcal enterotoxin B on mouse spleen cells and human peripheral lymphocytes,

spleen cells and human peripheral lymphocytes, J. Immunology 105:1453 - 1458.

37. Andersson, J., O. Sjoberg and G. Moller, 1972, Induction of immunoglobulin and antibody synthesis in vitro by lipopolysaccharides, Eur. J. Immunol. 2:349 - 353.

38. Gery I., T. Kruger and S.Z. Spiesel, 1972, Stimulation of B-lymphocytes by endotoxin, J. Immunology 108:1088 - 1091.

39. Weber, W.T. 1972, in "Immunity to Animal Parasites" (E.J. L. Soulsby, ed.) pp 34 - 55, Academic Press, N.Y.

40. Meyer, R.K., M.A. Rao, and N.L. Aspinall, 1969, Inhibition of development of the bursa of Fabricius in the embryos of the common fowl by 19-nortestosterone, Endocrinology 64:890 - 897.

41. Lerman, S.P. and W.P. Weidanz, 1969, Antibody deficiency resulting from cyclophosphamide treatment of bursectomized chicks, Bacteriol. Proc. abstract.

42. Glick, B., 1973, personnal communication.

43. Freeman, B.M., 1969, The bursa of Fabricius and the Adrenal cortex, J. Endocrinology 29:248 - 253.

44. Warner, N.L., A. Szenberg and F.M. Burnett, 1969, The immunological role of different lymphoid organs of the chicken, Aust. J. exptl Biol. med Sci. 40:373 - 388.

45. Warner, N.L. and A. Szenberg, 1964, The immunological function of the bursa of Fabricius in the chicken, Ann. Rev. Microbiol. 18:253 - 268.

APPENDIX : Formulae for solutions and culture media.

A. Chick Ringer

NaCl	7.00	g
KCl	0.42	g
$CaCl_2$	0.24	g
dist. H_2O	to 1000	ml

B. Buffered Formalyn

commercial formaldehyde	100	ml
Na_2HPO_4 (anhydrous)	6.5	g
NaH_2PO_4 (anhydrous)	3.5	g
dist. H_2O	to 1000	ml

C. Tris-HCl Buffer

Tris (Tham)	15.75	g
NaCl	117.90	g
HCl	7.0	ml
dist. H_2O	to 1000	ml

D. RPMI 1640 Tissue Culture Medium (Gibco)

$Ca(NO_3)_2 \cdot 4H_2O$	100.0	mg/l
Glucose	2000.0	"
$MgSO_4 \cdot 7H_2O$	100.0	"
KCl	400.0	"
$Na_2HPO_4 \cdot 7H_2O$	1512.0	"
NaCl	6000.0	"
L-Arginine (free base)	200.0	"
L-Asparagine	50.0	"
L-Aspartic acid	20.0	"
L-Cystine	50.0	"
L-Glutamic acid	20.0	"
L-Glutamine	300.0	"
Glutathione (reduced)	1.0	"
Glycine	10.0	"
L-Histidine (free base)	15.0	"

cont. RPMI 1640

L-Hydroxyproline	20.0	mg/l
L-Isoleucine (allo free)	50.0	"
L-Leucine (methionine free)	50.0	"
L-Lysine HCl	40.0	"
L-Metionine	15.0	"
L-Phenylalanine	15.0	"
L-Proline (Hydroxy free)	20.0	"
L-Serine	30.0	"
L-Threonine (Allo free)	20.0	"
L-Tryptophane	5.0	"
L-Tyrosine	20.0	"
L-Valine	20.0	"
Biotin	0.2	"
Vitamin B_{12}	0.005	"
D-Ca pantothenate	0.25	"
Choline Cl	3.0	"
Folic acid	1.0	"
i-Inositol	35.0	"
Nicotinamide	1.0	"
Para-Aminobenzoic acid	1.0	"
Pyridoxine HCl	1.0	"
Riboflavin	0.2	"
Thiamine HCl	1.0	"
Phenol Red	5.0	"
$NaHCO_3$	2000.0	"