University Of Glasgow

The Bursa Of Fabricius : Its Relationships To The Thymus And Cellular Development And Function Of The Peripheral Lymphoid Tissues.

by

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GENERAL SUMMARY

The experimental works reported in this thesis were undertaken as a contribution to the understanding of the physiology of the immune response with respect to the two central lymphoid organs of the chicken - the bursa of Fabricius and the thymus gland.

Production of antibody by a system independent of the bursa of Fabricius was first investigated in bursectomized-irradiated chickens immunized to 'phage ϕX174 and human serum albumin (HSA). Two highly sensitive tests - the 'phage neutralization test and the Farr test - were employed to detect the possibility of production of antibody by these birds. The bursectomized-irradiated chickens were however found to be completely deficient in production of antibody 8 days after intraperitoneal injection of 'phage ϕX174 and up to 28 days after intramuscular (i.m.) inoculation of HSA in Freund's complete adjuvant (FCA).

The intact-irradiated and thymectomized-irradiated chickens which were inoculated with HSA in FCA showed a biphasic antibody response with two major cycles of antibody in their circulations. Neonatal thymectomy coupled with whole body irradiation was however found to reduce the circulating antibody level to intramuscular injection of HSA in FCA. Further study of the antibody response of the thymectomized-irradiated and intact-irradiated chickens immunized to HSA in FCA or to HSA in water-in-oil emulsion suggested that potentiation of antibody response by the Freund's complete adjuvant was dependent on the cooperation of the thymus.

Surgical ablation of the bursa in ovo on day 18th of incubation suppressed almost totally production of antibody to sheep red blood cells and reduced significantly circulating IgG levels.

Histological examination of the spleens and caecal tonsils of the bursectomized-irradiated chickens showed total absence of plasma cells and germinal centres. Furthermore histological evidence was presented which showed that development of plasma cells and formation of germinal centres in the umbilical cord residue (Meckel's diverticulum) are also bursa dependent.

The bursa of Fabricius was shown to contribute substantially to the cellular make up of the thymus. Thus surgical bursectomy in ovo on day 18th of embryonic development brought about a significant reduction in the weight of the thymus. This finding was discussed in relation to a transport of cells from the bursa to the thymus during the development of the chick embryo.

Neonatal thymectomy followed by whole body irradiation did not bring about any apparent depletion of cells in the bursa of Fabricius. Contrariwise, the spleens of thymectomized-irradiated chickens were found depleted of lymphocytes. This ~~which~~ was very marked in the peri-arteriolar sheath of the white pulp.

Both the thymus and the bursa of Fabricius were shown to contribute to the cellular make up of the epitheloid-granuloma which formed in the pectoral muscles at the site of inoculation of HSA in Freund's complete adjuvant. Neonatal thymectomy coupled with whole body irradiation impaired infiltration of lymphocytes, epitheloid and giant cells in the granuloma. Neonatal bursectomy followed by whole body irradiation completely suppressed development of plasma cells and formation of germinal

centres in the granuloma.

The experiments which are presented in part two of this thesis were designed to study migration of bursa and thymus cells, using the technique of autoradiography.

Bursa cells were shown to migrate to the spleen, thymus, caecal tonsils and Meckel's diverticulum. Thymus cells were found to migrate to the spleen and caecal tonsils.

Distribution patterns of radio-isotope labelled bursa and thymus cells in the spleen showed that the bursa and thymus cells populate the areas in the spleen which correspond respectively to the depleted areas in the spleens of bursectomized and thymectomized chickens. Thus bursa cells were found to home preferentially to the peri-ellipsoidal zone, germinal centres and the red pulp, whereas the thymus cells were found to home preferentially to the peri-arteriole sheath of the white pulp and to the red pulp.

Bursectomized chickens offer an ideal model whereby one can study the fate of antigen in the body in the absence of specific antibody. In part three of this thesis, the experiments are presented in which the effect of bursectomy combined with whole body irradiation on distribution and localization of intravenous injection of native and aggregated HSA were investigated.

Neonatal bursectomy combined with whole body irradiation was found to deprive the bird from the immunological mechanism responsible for localization of these antigens to the dendritic cells of the white pulp and germinal centres of the spleen. Provision of specific antibody as HSA- anti HSA complexes was found to restore the ability of the bursectomized-irradiated

chickens to localize HSA to the dendritic cells but did not restore formation of germinal centres with antigen-bearing dendritic cells. It was concluded that the failure of the bursectomized-irradiated chickens to localize these antigens was due chiefly to the absence of specific antibody not absence of dendritic cells. Furthermore these studies indicated that B-lymphocytes as well as antigen, specific antibody and dendritic cells are necessary for formation of germinal centres.

Aggregated HSA and HSA-anti HSA complex did not localize in the bursa and thymus after intravenous injection. Lack of localization of these antigens in the two central lymphoid organs of the chickens is probably due to the fine vascular system in these organs which reduce considerably the inflow of circulating antigens in these organs.

GENERAL INTRODUCTION

One of the main methods for elucidating the role of an organ in the body is to remove it from the living animal and then determine what changes have occurred. The functions of the two lymphoid organs - the bursa of Fabricius and the thymus gland first came to light employing this method. The discovery of the immunological functions of the bursa of Fabricius preceded that of the thymus gland.

The bursa of Fabricius is a lymphoid organ which is found in birds only. It is an unpaired sac-like organ arising as a posterior diverticulum from the cloaca and it is connected to the posterior portion of the cloaca by a short duct or stalk.

HIERONYMUS FABRICIUS reported the existence of the so-called bursa of Fabricius in 1621. He believed that the bursa was a semen reservoir (Aldelman 1942, quoted by Warner *et al* 1964). The point that this organ was present in both sexes was overlooked by Fabricius. The following names illustrate other functions atributed to the bursa: egg reservoir, genital apparatus, seminal vesicle, prostate, third caecum, anal gland, bladder and Cowper's gland (Retterer *et al* 1913 quoted by Glick 1964). The first indication that the bursa of Fabricius had some connection with production of antibody came in 1956 when Glick and his co-workers found that chicken bursectomized at an early age failed to produce specific antibody to heat inactivated *Salmonella typhimurium*.

The discovery of the role of the bursa of Fabricius with production of antibody reawakened interest in the thymus gland and its connection with immunity. Lack of formation of plasma cells in the thymus of immunized rabbits (Fagreus 1948) and production of antibody by the thymectomized rabbits (Harris *et al* 1948, MacLean *et al* 1956) had suggested that thymus is not the

lymphoid organ responsible for humoral immunity. However, the main indication that the thymus plays a part in immune response came in 1961 by J.F.A.P. Miller. As part of an experiment which he was following on the role of thymus on leukaemia, he performed neonatal thymectomy in mice and found that thymectomy reduced the numbers of small lymphocytes in the blood and prolonged survival of skin grafts in the thymectomized mice. The thymectomized mice became ill and wasted. The wasting disease of the thymectomized mice could be prevented by grafting a syngeneic thymus back into the animal (Miller 1961, 1962). Miller's findings threw light into the mysterious function of the thymus and opened many lines of investigations into the physiology of the immune responses.

As the result of employing chicken in experimental immunology, a major progress was made toward the immunological functions of the main lymphoid organs. Investigations which were carried out in bursectomized and thymectomized chickens revealed that there is dissociation of immunological responses in chicken and there exist two separate immune systems in this bird each system under the control of one lymphoid gland. Bursectomy impairs certain immune responses only and does not abolish the ability of the bursaless birds to reject a skin homograft or to show a normal delayed hypersensitivity skin reaction to tuberculin. Thus the bursa of Fabricius is involved mainly in production of antibodies whereas the thymus gland is responsible for cell-mediated immune reactions. (Warner et al 1962, Cooper et al 1966).

Chicken as an experimental animal for the research worker in the field of immunology gained much reputation as it soon became apparent that the thymus gland in mammals does not cover the functions attributed to the bursa of Fabricius. Yet there are some indications that in certain mammalian species there exists

a lymphoid organ which is a homologue of the bursa of Fabricius.

The experimental works presented in this thesis are submitted as a contribution to the understanding of the physiology of the immune response with respect to the two central lymphoid organs of the chicken - the bursa of Fabricius and the thymus gland.

The experiments which are presented in the first part of this thesis were designed to add further evidence and extend the existing knowledge on the parts which bursa of Fabricius and the thymus play in production of antibody and the role of these organs in cellular development of the peripheral lymphoid tissues such as the spleen and caecal tonsils. The antigens employed in these studies were suspended in saline or [illegible] incorporated into the Freund's complete adjuvant. In the latter case, the object of the study was to elucidate the pattern of circulating antibody levels in bursectomized and thymectomized chickens over a long period of time after immunization. Furthermore, the contributions of the bursa and the thymus to the cellular make up of the granuloma which forms at the site of inoculation of HSA in FCA were investigated.

The part which bursa and thymus play in the development and cellular composition of the spleen and gut associated lymphoid tissues were further substantiated by a series of experiments in which cell migration from the two central lymphoid organs of the chickens to other parts in the body was investigated. These studies involved labelling of the bursa and thymic cells with 3H - thymidine or 3H - adenosine and tracing the labelled cells in the lymphoid tissues by the technique of autoradiography.

The part which specific antibody plays in localization of an antigen to the dendritic cells was studied in bursectomized-irradiated (BX-IR) chickens. BX-IR chickens are excellent

experimental models for investigation on this line since specific antibody, which is involved in localization of an antigen to the dendritic cells, is absent in BX-IR chickens. Furthermore, germinal centres which subsequently embody the antigen - bearing dendritic cells, do not form in the lymphoid tissues of these birds. Fluorescent antibody technique was employed to detect localization of native HSA, aggregated HSA and HSA-antiHSA complexes to the dendritic cells of the spleens of BX-IR and IN-IR chickens.

ABBREVIATIONS

ABC	=	Antigen binding capacity
Ab	=	Antibody
Ag	=	Antigen
BSA	=	Bovine serum albumin
BX-IR	=	Bursectomized-irradiated
°C	=	degrees Centrigrade
Ci	=	Curie
Cm	=	Centimetre
DEAE	=	Diethylaminoethyl
FCA	=	Freund's complete adjuvant (water-in-oil emulsion containing heat killed Mycobacteium tuberculosis)
g	=	Gramme
G	=	Relative centrifugal force of gravity
HGG	=	Human gamma globulin
HSA	=	Human serum albumin
Ig	=	Immunoglobulin
i.m.	=	Intramuscular
IN-IR	=	Intact-irradiated
i.p.	=	Intraperitoneal
i.v.	=	Intravenous
Kg	=	Kilogramme
M	=	Molar
mg	=	Milligramme
ml	=	Millilitre
ug	=	Microgramme
mu	=	Millimicron
mm^2	=	Square millimetre
p	=	Probability
PBS	=	Phosphate buffered saline
PFU	=	Plaque forming unit
RBC	=	Red blood cells
TX-IR	=	Thymectomized-irradiated
UP	=	Unna-Pappenheim

1 - Materials:

Chickens - Day old male chicks were kindly supplied by Thornber Brothers Hatchery Ltd., Ayrshire. The chicks were Thornber strain 808, a cross between White Leghorn and Light Sussex. Thornber 808 embryonated eggs were also obtained and were put in Westernette egg incubator for the whole incubation period of the chick embryo (21 days). The chicks were all kept in the animal house of the Department of Bacteriology and Immunology, Western Infirmary. They were given "Baby Chick Crumbs" made by British Oil and Cake Mills', up to the age of five weeks. Thereafter they were given "Intensive Grower Pellets" supplied by the same manufacturer.

Rabbits - Newly adult New Zealand White (NZW) rabbits were used for production of antiserum. They were obtained from Kingford Conies, Great Tey, Colchester, England.

HSA - Crystalline human serum albumin was obtained from Behringwerke Marburg, Germany. This was a purified preparation and was supplied in vials containing 1g. It was stored in a refrigerator at 4°C until use.

Bacteriophage ϕX174 - The original suspension of 'phage ϕX174 and its host organism Escherichia coli strain C were kindly supplied by Dr. W.A. Fleming, now at the Queen University of Belfast, Northern Ireland.

Mycobacterium tuberculosis - The tubercle bacilli, avian type (strain 04 ER) and human type (strain C) were used as one of the components of Freund's complete adjuvant. Both types were heat killed, freeze dried preparations which were kindly supplied by the Ministry of Agriculture, Central Veterinary Laboratory, Weybridge, England.

Arlacel A - This was obtained from Evans Medical Ltd., Arlacel A

(mannide mono-oleate) is a product of the Atlas Powder Company, Welmington, Delaware, U.S.A.

Bayol 55 - This mineral oil was obtained from Esso Petroleum Company Ltd., Scotland Branch, Blythswood Street, Glasgow.

Drakeol 6 VR - This mineral oil was obtained from Pennsylvania Refining Company, Kansas City, Butlev , Pa., 16001, USA.

Fluorescein Isothiocyanate - This dye was obtained from the British Drug Houses (BDH) Laboratory, Chemical Division, Poole, England.

Iodine - 131 (^{131}I) - This isotope was obtained from the Radiochemical Centre, Amersham, England. It was supplied as thiosulphate-free iodide, preparation 1853 to the Department of Biophysics, Western Infirmary Samples of 1 uci ^{131}I per ml was prepared in this department which was collected on the same day and was used for labelling HSA.

^{3}H - adenosine - This radioisotope was obtained from Radio-Chemical Centre, Amersham, England. The specific radioactivity of this preparation was 4·65 ci/milli-mole. It was used for *in vitro* labelling of bursa and thymus cells.

^{3}H - thymidine - This radioisotope was also obtained from Radio-Chemical Centre, Amersham. The specific radioactivity of this preparation was 18·1 ci/milli-mole. It was used for *in vivo* labelling of bursa cells.

Sheep Red Blood Cells - Fresh sheep blood was collected from the Glasgow slaughter house and the red cells in the blood were separated by centrifugation within two hours after it had been collected from the sheep.

Indian Ink (Colloidal carbon suspension). The Indian ink was obtained from Pelikan Ink Manufacturers, Gunther Wagner, Hanover, Germany. It was diluted 1 in 5 with 0·85% sterile saline for intravenous injection into the chickens.

Materials Used for Bursectomy:

A pair of small dissecting forceps.

A pair of 5 inch artery forceps.

A pair of 5 inch Mayo scissors.

A scalpel handle loaded with blade No.15.

Retort stand and clamp.

A small bulldog type paperclip.

A large glass jar with cotton wool pad at the base soaked with "ether".

Universal container with ether-soaked swab.

Sterile gauze swabs, cotton wool and cotton drape.

Cork board and mapping pins.

Surgical silk No. 4/0 and suture needles No.20.

Materials Used for Thymectomy:

A pair of small blunt dissecting forceps.

A pair of fine dissecting forceps.

Two pairs of small artery forceps.

A pair of small Mayo scissors.

Cork board and mapping pins.

Sterile gauze, cotton drape and cotton-wool swabs.

A narrow cotton cloth strip.

Suture needle No.20 and surgical silk No. 4/0.

A large glass jar with cotton wool pad at the base soaked with Trilene (Trichloroethylene).

Universal container with Trilene-soaked swabs.

Neonatal Bursectomy - This operation was carried out on chicks under 10 hours old.

The surgical instruments listed on page 11 were initially sterilized by autoclaving at 121°C and 15 pounds pressure for 20 minutes. The instruments were boiled for 20 minutes between successive operations.

The chick was initially anaesthetized with ether in a closed jar. During the operation, anaesthesia was continued, as required by the application of a small mask containing an ether-soaked swab to the nose of the bird.

The feathers between the cloacal vent and the base of the tail were plucked out. The bird was then placed ventral side down on the cork board with its tail towards the operator. The body, legs and wings were covered by the cotton drape which was pinned down at all four corners. The tail was drawn through the slit in the drape and held upright by a bulldog type paper clip hanging on a string from the retort stand.

A horizontal incision, about 7 mm long, was made mid-way between the cloacal vent and the base of the tail. The bursa was exposed by blunt dissection. Care was taken not to lacerate the ureters and the genital tubes which run over the organ on both sides and terminate in the cloaca (Fig. 1) The bursa was grasped with dissecting forceps and pulled out gently. By blunt dissection it was freed from its attachments to mesenteric membranes and the cloaca. The organ was then excised as closely as possible to its cloacal attachment. The incision was then closed with three stitches and the bird was freed.

It was found that the most important point to consider in a complete bursectomy was to make the incision in the skin without lacerating the underlying bursa. It was then very easy to excise

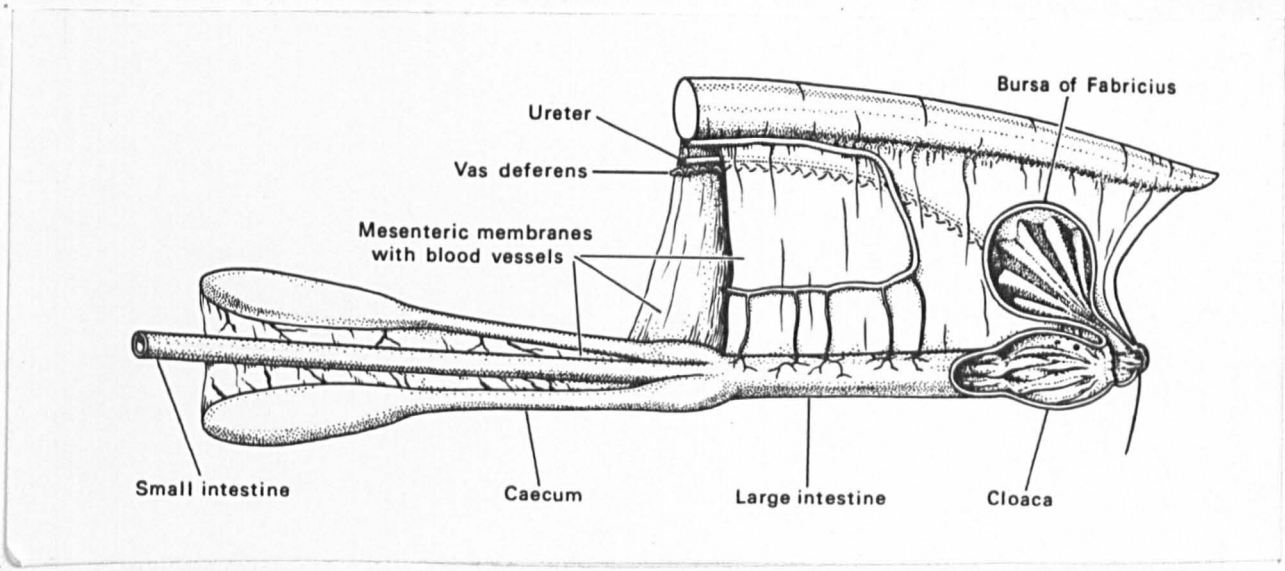


Fig. 1 - This drawing shows the bursa of Fabricius in Chicken and its relationship to cloaca and large intestine. A longitudinal section of the bursa and cloaca were drawn in order to show the plicae in the lumen of bursa and the duct which connects this organ to the cloaca. The bulging in the beginning of each caecum is the caecal tonsil.

the whole organ completely rather than removing it by bits and pieces. The bursectomized chickens were examined after an autopsy at 6 - 10 weeks of age and were all found free from any bursa remnant. Each operation took about 5 minutes. There was no post operative complication or mortality.

Bursectomy "in ovo" - This operation was carried out on chick embryos on day 18th of incubation (adapted from Aitken and Penhale 1973).

Fertile eggs were put in the Westernette egg incubator vertically with their broad ends up. The temperature of the incubator was set at 40·5°C and humidity was provided by putting a container with water in the lower shelf of the incubator. A constant and steady rotation of an electric fan maintained an even distribution of heat and humidity inside the incubator.

In this operation it is essential to know the area of the

shell which immediately lies over the tail of the embryo since an opening has to be made in that area of the shell. The tail of the embryo lies close to the narrow end of the egg (Fig. II). However, the exact location of the tail was determined by candling the embryonated eggs - the broad end of the egg was held against an electric lamp in a dark room. The air sac appeared as a light area, the embryo together with the yolk and albumen filled the rest of the space in the egg and appeared very much darker than the air sac area. The margin of the air sac with the embryo is well demarcated. This margin is convex in one surface of the embryonated egg (Fig.IIIa) and it is concave when the egg is rotated 180° (Fig.IIIb). The tail of the embryo lies under the lower left of the latter surface.

The shell over the tail region (Fig.IIIc) was cleaned with surgical spirit. A rectangular opening 10 mm x 10 mm was made in the shell over the tail region, using an electric dental drill. The shell was removed and the thin membranes covering the area were gently torn. The tail was held upright by an alligator clip, hanging on a string from a retort stand.

The feathers under the tail were plucked out by the aid of a pair of forceps. A horizontal incision about 4 - 5 mm long was made mid-way between the cloacal vent and the base of the tail with a pair of fine scissors. The bursa was exposed and grasped with the fine forceps and it was pulled out gently and was cut as closely as possible to its cloacal attachments. The tail, then was freed and pushed inside the egg. The two edges of the incision were brought close together. This incision was not stitched. The opening in the shell was sealed with Sellotape. The egg was put back into the incubator. To prevent any leakage of fluid from the opening in the shell, the egg was placed firmly in a stationary position with the opening uppermost. The chicks

were hatched on day 21st of incubation.

The mortality of the bursectomized chick embryos was very high (up to 70 per cent). However, the operated chicks which hatched were found to be quite healthy and in good condition.

Neonatal Thymectomy - This operation was carried out on chicks under 20 hours old.

The thymus of chicken is situated along the neck. There are fourteen thymic lobes, seven on each side. The first pair of lobes are located almost within the upper part of the thoracic cavity. The next pair of lobes are well hidden between the neck and the scapulae. The remainders, five lobes on each side of the neck, are easy to expose for ablation. All the thymic lobes lie closely to jugular vein and have short vascular connections to this vein.

The surgical instruments listed on page 11 were initially sterilized by autoclaving. Between successive operations, the instruments were cleaned and boiled for 20 minutes.

The chick was initially anaesthetized with Trilene in a closed jar. During the operation, anaesthesia was continued, as required, by application of a universal container containing a Trilene-soaked swab to the nose of the bird.

The feathers at the back of the neck were plucked out and the chick then was placed ventral side down on the cork board with its tail towards the operator. The body, legs and wings were covered with a cotton drape which was pinned down to the cork board at all four corners. The neck was left uncovered. A pillow made of gauze was placed under the neck to raise it up. A narrow ribbon of cloth was passed over the head and pinned down at both ends.

The skin was picked up with forceps near the scapula and

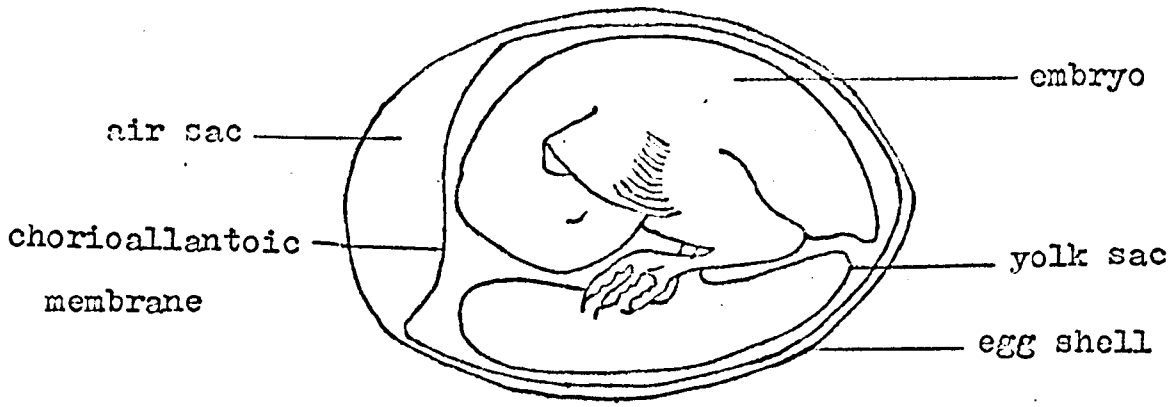


Fig.II- Diagrammatic representation of chick embryo on days 17th-19th of incubation. The tail of the embryo lies near the narrow end of the egg. The air sac is separated from the embryo,s compartment by chorioallantoic membrane.

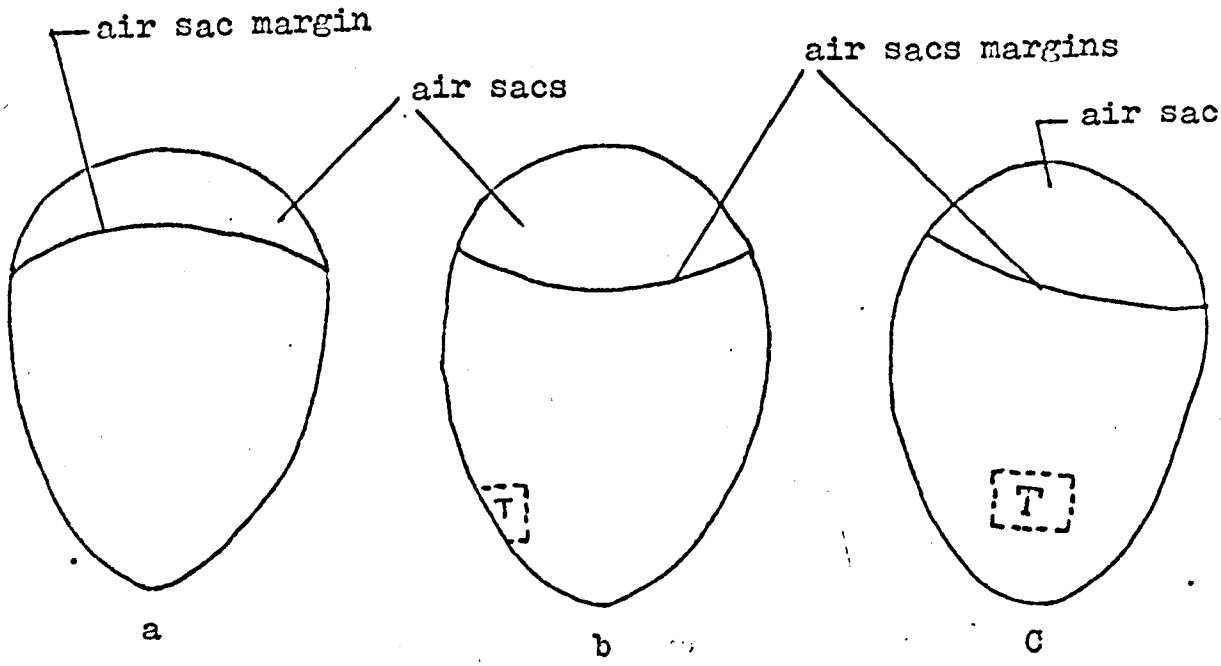


Fig.III-These diagrams show the margin of the air sac with the embryo,s compartment after candling the embryonated hen,s egg. On one surface of the egg, the margin of the air sac with the embryo,s compartment appears convex (Fig.IIIa), whereas if the egg is rotated 180° on its long axis, this margin appears concave(Fig.IIIb). The tail of the embryo lies under the shell in the lower left of the latter surface of the egg. The egg shell in the tail region (T,Fig.IIIc) is removed in the cource of bursectomy in ovo.

using scissors, a dorsal mid-line incision was made. This began at the level of the scapula and was carried up to the back of the skull. The skin on one side of the neck, then laid open as a large flap with two pairs of artery forceps. The fascia covering the thymic lobes was gently torn apart and starting with the thymus lobe nearest to the head, each lobe was gently plucked out with fine dissecting forceps. To avoid lacerating of the jugular vein, scalpel or scissors were not used at this stage. The vascular connections to the jugular vein were not ligated as in general no or very little bleeding occurred. Care was taken to remove each lobe completely.

The last two lobes were visualized by gently pulling the jugular vein from the thoracic cavity with a pair of blunt dissecting forceps. The deepest lobe was always difficult to find. Several attemps had to be made, with the risk of lacerating the jugular vein or pleura and the lobe often had to be removed bit by bit. Doubt always existed, therefore, as to whether or not the last lobe had been completely removed. Thus a post mortem examination of the area at the end of each experiment was carried out. The lobes on the other side of the neck were removed in a similar manner. Finally the two flaps of skin were brought together with a continuous suture. This was removed 10 days later. Because of the pain along the neck the chicks did not show interest in food and water. To prevent them from dehydration 2 - 3 ml. of physiological saline was given to each bird by intraperitoneal injection for a few days until recovery. Thymectomy took about 30 minutes per bird and there was always a high post operative mortality.

Whole Body Irradiation - The neonatally bursectomized and thymectomized chicks, along with their intact controls were whole body irradiated on the second day after hatching. The chicks

were taken to the Department of Biophysics at the Western Infirmary. There, the chicks were made immovable by tying their legs with strings to a wire-mesh and were put under the source.

The source of γ-radiation was AEI-Urbiton radio-isotope ^{60}Cobalt. The chicks were always put under the source with a source skin distance of 70 cm and they were exposed to 800 or 840 rads of γ-rays. It took 8 minutes to expose the birds to 840 rads of γ-rays. However, due to the gradual decay of the radioisotope over a period of four years, the exposure time increased to 12 minutes.

On completion of irradiation, the chicks were released and were given leg bands or wing numbers. The irradiated chicks were given 0·5 per cent sulphadimi-dine sodium (I.C.I.) in their drinking water for 10 days in order to protect them from infection.

Preparation of Antigen Mixtures - For i.v. or i.p. inoculation, the antigen (HSA, 'phage ϕX174 or the immunoglobulin) was suspended in 0·85 per cent sterile saline. They were always made up freshly before inoculation into the animals.

Water-in-oil emulsion was made up as described by Herbert (1973). The mineral oil Bayol 55 (or Drakeol 6VR) and the emulsifier Arlacel A were mixed together in the ratio of nine parts by volume of oil to one part of the emulsifier. The mixture was shaken well and then sterilized by filtration with Seitz filter.

The antigen was dissolved in saline. A known volume of oil-Arlacel mixture was placed in a universal container. A small portion of the antigen solution was then taken up in a hypodermic syringe and squirted vigorously into the oil through a 0·5 mm needle the point of which was held below the surface of the oil. The container was then capped and shaken vigorously before another

portion of the antigen solution was added in the same way. After the second or the third addition of antigen solution, the mixed emulsion was taken up into the syringe through a wide bore (1 mm) needle and squirted out again through a fine bore (0·5 mm) needle. More portions of the antigen solution were added and the process was repeated until the mixture appeared as a white viscous fluid. However, the dispersion of the antigen solution into the oil-Arlacel was only considered complete if a drop of the mixture on the surface of tap water in a beaker did not spread rapidly but remained as a discrete drop.

The Freund's complete adjuvant (FCA) was prepared exactly as above with the addition of heat killed Mycobacterium tuberculosis (avian or human type). Heat killed, freeze-dried M. tuberculosis was weighed in a universal container. 0·5 or 1 ml of the oil-Arlacel was added to the Mycobacterium in the universal container. A glass bead was then placed in the universal container. The container was capped and was shaken vigorously for two hours by means of Griffin Flask Shaker (Griffin and George Ltd., England). The tubercle bacilli disintegrated and a homogenous suspension was then obtained. The suspension was then added to the antigen in water-in-oil emulsion, which had been previously made up. The mixture was mixed thoroughly by cycling repeatedly through a syringe and a hypodermic needle. It was then tested over the surface of water in a beaker as described previously.

Inoculation of Antigen And Collection of Blood Samples In Chicken - The legs and wings of the bird were tied up by two pieces of string to restrain its movement (Adapted from Lepine et al 1964).

One piece of string was used to tie up the legs. Another piece of string passed through the left wing feathers. It was

next passed over the front side of the neck and then passed through the right wing feathers. The head and the neck of the bird were then turned back and were held under the wings. Finally, the two wings were tied up firmly over the back of the bird.

This technique was found extremely useful. The chicken remained completely immobile hence no assistant was needed to hold the bird during inoculation of antigen or bleeding the bird through the wing vein.

Intravenous Injection - The bird was restrained with two pieces of string as described above. The feathers over one of the basilic veins (a tributary of the brachial vein in undersides of the wings) were plucked out. Holding the syringe at approximately 5° angle relative to the vein, the hypodermic needle was placed over the vein. The needle was then inserted into the vein by a quick thrust. The antigen solution was injected into the vein very slowly. After injection of the antigen had been completed, the needle was withdrawn quickly from the vein and immediately the point of needle insertion was pressed with the finger to stop the flow of blood and thus prevent seepage of blood into the surrounding tissues.

Intraperitoneal Injection - In chicken, the broad sternum extends downward and covers a large part of the abdomen. The area between the sternum and the cloacal vent is suitable for intraperitoneal injection. The chicken was first laid down on its back and was held by an assistant. The skin together with the abdominal muscles were grasped with left hand fingers and were raised up slightly to produce a gap between the abdominal muscles and the viscerals. Holding the syringe in the right hand with 10° angle relative to the body, the antigen solution

was injected into the abdominal cavity.

Collection of Blood Samples - The bird was tied up and access was gained into the vein as described previously. Blood, one ml in volume, was withdrawn into the syringe and was immediately transferred into bijou bottles. The chicken was then released by untying the string.

Inoculation of Antigens And Collection of Blood in Rabbit - The whole body of this animal, except the head, was wrapped in a blanket to secure its movement. The antigen solution was injected into the marginal vein of the ear which runs along the posterior margin of both ears.

To bleed the immunized rabbit , the animal was restrained by wrapping it up in a blanket as described above. The hair over the marginal vein of the ear was shaved. A diagonal incision was made across the marginal vein by a sharp scalpel blade. Blood was collected in a universal container which was held under the ear. At a single bleeding 15 - 20 ml blood was collected from each rabbit. The animal was freed and returned to the cage after the flow of the blood from the vein had completely stopped.

Separation And Preservation of Sera - The freshly drawn blood was left on bench at room temperature for 30 minutes or so for clot formation. The clot was carefully separated from the wall of the bottles with a steel spatula. The bottles, then, were either put in an incubator or in a water-bath at 37 °C. for 1 hour. The clear sera released from the clots were transferred into test tubes by means of Pasteur pipettes and were spun in an MSE refrigerated centrifuge for 15 minutes at 800G. The sera either were used for estimation of antibody on the same day or they were transferred into the bijou bottles or universal containers

and were kept in deep freeze at -20°C for a week or two until use.

Preparation of Antiserum In Rabbits - Antisera to HSA, 'phage 174 and chicken immunoglobulin were prepared in rabbits using similar immunization schedules for all these three antigens.

Newly adult rabbits were given an initial injection of 0·5 ml water-in-oil emulsion containing 5 mg HSA and 1 mg human type Mycobacterium tuberculosis. The mixture was divided and injected in equal amounts into the subcutaneous tissues of the inner thigh of both legs. The rabbits were boosted, 40 days later, with an intravenous injection of 2 mg HSA in 0·5 ml saline. They were then bled on day 10 and day 14 after the secondary immunization.

Antiserum to 'phage ϕX 174 was prepared in two rabbits in the same way using 10^4PFU 'phage ϕX174 in Freund's complete adjuvant for the primary immunization. The rabbitswere then boosted, 40 days later by intravenous injection of 10^2PFU 'phage 174 suspended in 0·5 ml saline. They were bled on day 10 after the secondary immunization.

Chicken immunoglobulin was obtained by addition of ice-cold saturated ammonium sulphate to non-immunized adult chicken serum. The precipitated immunoglobulin was then dialysed against 0·1M saline to remove ammonium sulphate ions from the final immunoglobulin solution (detailed accounts of these procedures are given on page 31).

Anti chicken immunoglobulin antibody was prepared in two rabbits in the same way as described above for production of anti HSA antiserum. The rabbits were initially immunized subcutaneously with 5 mg chicken immunoglobulin in Freund's complete adjuvant. They were boosted, 40 days later, with

intravenous injection of 2 mg chicken immunoglobulin suspended in 0·5 ml saline. The rabbits were then bled on day 10 and day 14 after the secondary immunization. Anti-chicken immunoglobulin antisera were checked for specificity and potency by immunoelectro-phoresis.

Monovalent rabbit anti chicken IgG was kindly supplied by Dr. Valentine I. French.

Heat Aggregation of HSA - A 20 mg/ml solution of HSA was heated in a water bath at 70°C with mechanical shaking until the rise in optimal density at 550 nm reached 1. This took between 10 and 20 minutes. The antigen solution was left in the cold room at +4°C until use.

Preparation of HSA - Anti HSA Complex - Anti HSA antibody was prepared in adult chickens by intravenous injection of 10 mg HSA. The chickens were bled 10 days later. After an interval of 3 - 4 weeks the birds were boosted with an intravenous injection of 10 mg HSA and were bled on day 10 after the secondary immunization.

A tube precipitation test was carried out on each serum in 8·5% saline to determine the optimal reacting proportions of the anti HSA antibody with its corresponding antigen. The optimal amount of the antiserum was then mixed with 10 mg HSA made up in 8·5% saline. The mixture was left at room temperature for two hours, by then all visible HSA - anti HSA complexes had precipitated. The supernatant was decanted and the precipitate was washed by addition of 0·85% saline, and after 15 minutes, the HSA - anti HSA complexes were recovered by centrifugation at 250G for 5 minutes. The precipitate was then mixed with a solution of HSA in 0·85% saline, containing 100 mg/10 ml. The mixture was left in the cold room at +4°C. The visible HSA -

anti HSA complexes usually dissolved completely after standing overnight at +4 C.

Isotope Labelling of Human Serum Albumin - Radioisotope labelled HSA was used in the Farr Test for measurement of anti HSA antibody in the serum.

HSA was labelled with radioisotope iodine 131 (^{131}I) by direct oxidation technique of Hunter and Greenwood (1962) using chloramine T as an oxidizing agent.

2 mg HSA was disolved in 0·2 ml PBS and it was added to 0·5 mc iodine - 131. Chloramine T, 250 ug in 0·25 ml PBS, was added to the vial containing the isotope - HSA mixture and the contents allowed to interact for 3 minutes at room temperature. 240 ug sodium metabisulphate in 0·1 ml PBS was added to the isotope - HSA mixture to neutralize any remaining chloramine T by reducing this oxidizing agent and also any unreacted iodine to iodide. Finally 2 mg potassium iodide in 0·2 ml PBS was added to the mixture to act as carrier iodide.

Separation of the iodinated HSA from unreacted iodide and other low molecular weight reactants was achieved by passing the whole mixture through a G25 coarse grain Sephadex column (30 cm x 1 cm), equilibrated with 0·15 M saline. The fractions were collected in 2 ml amounts in test tubes. The radioactivity of each fraction was then estimated with a Geiger counter. The level of radioactivity which is achieved by this labelling technique is about 0·5 uc/ug and experience has shown that 35 - 40% of the ^{131}I could be attached to the HSA (Stark, 1969)

The protein content of the fraction with highest radioactivity was determined by reading its optical density at 280 mu in a spectrophotometer and from a standard graph. The labelled HSA was diluted in 0·1M saline to give a final concentration of 1 ug protein per ml.

Antigen Binding Capacity (ABC) Test, "The Farr Test" - This test was introduced by Farr in 1958. It is based on the principle that ^{131}I labelled albumin is soluble in 40 per cent saturated ammonium sulphate, whereas ^{131}I - albumin/antibody complexes are insoluble under the same conditions. A constant amount of labelled antigen is added to the serial dilutions of antiserum, a point is reached when antigen excess is achieved. Upon addition of an equal volume of saturated ammonium sulphate, the soluble ^{131}I - albumin not bound to antibody remains in the supernatant. A measurement of precipitated radioactivity, thus, indicates antigen binding cacacity of the antiserum. The test is extremely sensitive and measures antibody of all immunoglobulin classes and sub-classes.

This test was carried out for quantitative estimation of anti HSA antibody by a modification of the method of Farr (1958) as described by White, French and Stark (1970).

Tubes containing undiluted test serum and test serum diluted 1 in 5, 1 in 25 and 1 in 125 in normal chicken serum, in 0·1 ml volumes, were set up in duplicate. If a high titre of antibody was expected the antiserum was diluted 1 in 10, 1 in 100 and 1 in 1000 as above. 0·2 ml of the ^{131}I labelled HSA solution (containing 0·2 ug HSA) was added to each tube. The tubes were shaken in an electric shaker for few seconds and were left in the cold room at 4 °C overnight. The following day 0·2 ml of cold saturated ammonium sulphate was added to each tube. The materials in the tubes well mixed by vigorous shaking for few seconds using the electric shaker and were left in cold room at 4 °C for 3 hours. The tubes were then spun in an MSE refrigerated centrifuge at 800G for 15 minutes. The supernatant was discarded and the precipitate was washed once with 40% cold saturated ammonium

sulphate. The final precipitate was disolved in 0·5 ml physiological saline.

The "controls" in this test consisted of four tubes, each containing 0·1 ml normal chicken serum. One pair of the tubes in the controls was treated as were the test sera to determine the minimal precipitations of ^{131}I labelled HSA in the absence of anti HSA antibody. To the other pair of tubes 0·3 ml of 10% trichloracetic acid (TCA) was added to determine the maximum precipitable radioactivity of ^{131}I labelled HSA. The resulting precipitate was centrifuged and washed in 0·6 ml of 10% TCA and resuspended in 0·5 ml physiological saline.

The washed precipitates from all tubes and the supernatant from normal chicken serum and TCA tubes were counted in a well type sodium Iodide Crystal Scintillation Counter.

The radioactive counts of the precipitates were plotted against the serum dilution on semi-log paper and the dilution (d) of 0·1 ml serum equivalent to 30% precipitation determined (where 100% = total TCA - precipitable proteins minus the normal serum precipitate). The amount of antigen bound per millilitre of serum was determined at the 30% end point by the following calculations :

$$0.1 \times d = \frac{30}{100} \times 0.2 \text{ ug HSA}$$

$$ABC_{30} \text{ in 1 ml neat serum} = \frac{30 \times 0.2 \times 10}{100 \times d} \text{ ug / ml.}$$

Preparation of stock suspension of 'phage ΦX174 - The bacteriophage ΦX174 was prepared by the agar layer technique (Adams, 1959). The 'phage suspension, in sufficient concentration to give barely confluent lysis, was added to a 4 hour culture of Escherichia coli c strain c in nutrient broth. The mixture was then added to molten agar at 46 °C and mixed thoroughly. It was then poured over the surface of several agar plates and allowed to set at room temperature. The E. coli cultures were incubated for 14 hours at 37 °C. After incubation, the culture plates were quickly frozen in a -20°C deep freeze, then allowed to thaw at room temperature. Any fluid

extruded from the thawed culture plates were collected, and were centrifuged at 2400G for 20 minutes to remove bacterial debries. The crude suspension of bacteriophage was then brought to 40% saturation with ice-cold saturated ammonium sulphate, and allowed to stand at 4 °C overnight. On standing, a precipitate formed which was suspended in Tris - EDTA buffer and centrifuged at 5400G for 45 minutes. The supernatant was discarded and the precipitate re-suspended in Tris - EDTA buffer and further centrifuged at 31500G for 5 hours. The precipitate was suspended in 4 ml Tris - EDTA buffer. The titre of the 'phage suspension was then determined (see below) and the suspension was kept at 4 °C until use.

Tris - EDTA Buffer - The 'phage ϕX174 was suspended in this buffer at pH 8·2.

90 ml of 0·1M solution of Tris (hydroxymethy amino methane) was added to 10 ml 0·01M solution of EDTA (ethylene - diaminetetra acetic acid) and the pH was checked with a pH meter.

Titration of 'phage ϕX174 - Tenfold dilution of the 'phage suspension, starting from 10^{-1} up to 10^{-12} were made in 0·1M saline. 0·5 ml of each 'phage dilution was added to 2 ml of a 4 hour culture of E. coli strain c in nutrient broth and mixed thoroughly. 0·5 ml of this 'phage - E. coli mixture were added to each of four test tubes containing 4 ml melted 0·6% nutrient agar maintained at a temperature of 46 °C in a water bath, mixed well and the entire mixture poured over the surface of a nutrient agar in a petri dish. After the agar had solidified, the plates were incubated at 37 °C overnight and any plaques then visible were counted. The titre of the 'phage stock suspension was then calculated from the equation below:

$$\text{Titre} = N \times 10^{n+1}$$

where:

N = average numbers of plaques for four

plates at any one dilution.

n = log 10 of the dilution of the 'phage.

Each new stock of 'phage suspension was titrated against a standard rabbit anti-'phage serum to ensure that the 'phage neutralization occurred logarithmically.

The Bacteriophage Neutralization Test - When a suspension of a 'phage is mixed with its specific antibody and the mixture is added to the 'phage host - the bacterial culture, the infectivity of the 'phage is inhibited. This effect of antibody can be measured quantitatively by observing the reduction in the number of plaques formed in the mixture after incubation when subsequently added to the bacterial culture. The test is very sensitive and very low level of antibody against the virus can be detected.

Antibody activity in the sera of the bursectomized and intact birds immunized to 'phage ϕX 174 was measured by the method of Wilkinson, Fleming and White (1967). The controls consisted of two normal sera from two young adult chickens. The normal sera were tested along with the sera of the immunized birds.

A mixture of the bacteriophage and chicken serum was allowed to react at 37 °C and samples of this mixture, taken at different times, titrated for infective 'phage.

The appropriate dilution of the 'phage ϕX174 was prepared and incubated at 37 °C for 20 minutes. Then 0·1 ml of the 'phage suspension was added to 0·9 ml of the test serum or serum dilution which had also been pre-incubated at 37 °C. 0·1 ml samples of this 'phage - antiserum mixture were withdrawn at five minute intervals up to 20 minutes, and the samples immediately diluted in 9·9 ml ice-cold nutrient broth to stop the antigen-antibody reaction. 0·5 ml of this dilution were then added to the 2 ml of a 4 hour culture of E. coli strain c and after incubation at 37 °C overnight,

the remaining infective bacteriophages, which developed plaques on the E. coli culture plates, were counted and the titre of the 'phage was calculated as described previously. The remaining infective bacteriophage in each test serum was plotted against time on a semi-log scale which gave a straight line, indicating that the test progressed logarithmically with time. The antibody activity against the 'phage which is expressed as inactivation constant (K) was calculated from the following equation:

$$K = \frac{2 \cdot 3D}{t} \log \frac{No}{N}$$

where: D = dilution of the serum

t = time in minute

No = 'phage titre at time o

N = 'phage titre at time t

Haemagglutination Test - This test was employed to detect antibodies directed to sheep red blood cells (SRBC).

Fresh sheep blood was collected from Glasgow slaughter house in a sterile glass jar containing large numbers of paper clips. The jar was continuously shaken for one hour. This procedure prevented blood from clotting and the fibrin clung to the paper clips. The defibrinated blood was then centrifuged and the precipitated red cells were washed five times with sterile 0·85% saline. Centrifugation in all cases was carried out at 500G for 15 minutes.

The test sera which had been collected from the bursectomized and intact chickens immunized to SRBC were first incubated in a water bath at 56°C for half of an hour to inactivate the complement, thus destroy the haemolytic activity of the sera.

A normal serum obtained from an adult non-immunized chicken was

used to serve as a control in this test. The control serum was incubated along with the immune sera in water bath at 56°C for half an hour. In addition, to remove natural antibodies to SRBC which are present in chicken serum, the control serum was then absorbed with SRBC three times: to 5 ml of the control serum 1 ml packed SRBC was added and thoroughly mixed and it was left in room temperature for 2 hours. It was then spun at 500 G for 15 minutes. This process was repeated three times. The control serum was then kept in the deep freeze at -20°C until use.

A set of 12 Widal test tubes was set up in a rack for each serum. To all test tubes 0·2 ml of 0·85% saline was added. Doubling dilution of each serum was achieved by adding 0·2 ml of the serum to the first test tube. After mixing the serum with the saline, 0·2 ml of the diluted serum from the first tube was transferred to the second tube. Thus the serum in the first tube was diluted with saline in ratio of $\frac{1}{2}$ and in the second tube in ratio of $\frac{1}{4}$. The transfer of diluted serum in 0·2 ml volumes from one test tube to the next tube containing saline only was carried out up to tube No.10. Finally 0·2 ml of the diluted serum from test tube No.10 was discarded. Thus all test tubes contained diluted serum in 0·2 ml volume.

A suspension of the SRBC in 0·85% saline was made up. 0·1 ml of this suspension containing 6×10^6 SRBC was added to the diluted sera in the test tubes. The tubes were gently shaken and were put in the water bath at 37°C for $1\frac{1}{2}$ hours. The controls in the test consisted of one tube containing $\frac{1}{2}$ dilution of the absorbed non-immune chicken serum plus SRBC. The other control consisted of SRBC suspended in 0·2 ml saline.

Agglutination of the red cells was read 3 hours after incubation. Doubtful agglutination was checked under the microscope. The titre

of each serum was recorded in terms of the reciprocal of the highest dilution of the serum which caused agglutination of the red cells.

Optimal Proportion Precipitation Test - The potency of rabbit anti HSA antisera was determined by this test (Kabat and Mayer,1961).

A 100 ug/ml solution of HSA in 0·85 per cent saline was made up. Dilutions of the HSA solution in 0·85% saline were made up in a row of 5 test tubes, in which the first test tube contained 20 ug HSA/ml and the last test tube contained 100 ug HSA/ml

A constant amount of undiluted antiserum (0·1 or 0·2 ml) was then added to the antigen solution in each test tube. Two controls were also set up which consisted of two test tubes in which either antigen or antiserum had been omitted. The tubes were shaken gently and were left on the bench at room temperature and they were observed very closely. The test tube in which the cloudy HSA - anti HSA floculates appeared prior to that of the other test tubes in the row and the amount of its subsequent precipitate was more than the amount of the precipitate formed in the other tubes, was considered to contain antigen - antibody in optimal proportion. Any serum which, in 0·1 ml volume showed such optimum reaction with 80 or 100 ug HSA was considered to contain a high level of anti HSA antibody. Such serum was used for fluorescein labelling of anti HSA antibody.

In the case of antisera prepared in chicken, this test was carried out in 8·5% saline as the amount of visible precipitate which form in 8·5% saline in chicken serum is considerably higher than that which form in lower concentrations of sodium chloride.

Fluorescein Labellings of Anti -HSA and Anti Chicken Immunoglobulin Antibodies - Rabbit anti - HSA and anti chicken immunoglobulin

antisera were used. The sera showing high antibody levels were selected for labelling with fluorescein iso thiocyanate. Titres of anti HSA antisera were estimated by the optimal proportion precipitation test. Anti chicken immunoglobulin antisera were checked for specificity and potency by immunoelectrophoresis. The same procedure was followed for fluorescein labellings of anti HSA antibody and anti chicken immunoglobulin antibody (White, French and Stark 1970).

Stage 1: Separation of immunoglobulins from Albumin - Separation of immunoglobulins in the antiserum from the albumin was achieved by "salting out" technique, using ammonium sulphate. Immunoglobulins are relatively insoluble in 30 - 40% saturated ammonium sulphate whereas albumin is soluble in these concentrations of ammonium sulphate. The immunoglobulins in the antiserum thus can be separated by precipitation with this salt.

To 10 ml of antiserum 5 ml cold saturated ammonium sulphate was added dropwise. The solution was left in the cold room at 4°C on a magnetic stirrer for one hour. It was then centrifuged at 2400G for 10 minutes at 4 °C. The precipitated immunoglobulin was collected and re-suspended in 20 ml of 40% cold saturated ammonium sulphate in 0·15 M saline and was stirred and centrifuged as before. The precipitated immunoglobulin was then resuspended in 5 ml of 0·15 M saline.

Stage 2: Elimination of the Ammonium Sulphate Ions from Immunoglobulin solution - The remaining ammonium sulphate ions in the final immunoglobulin solution were eliminated by "dialysis". The solution was placed in a cellulose dialysis sac and the sac was suspended in a large graduated cylinder containing 0·1M saline. This was left in cold room at 4°C for two days. The dialysate was changed twice daily. At the end of the second day of dialysis,

the dialysate was tested for presence of sulphate ions. To 2ml of the dialysate 4 drops of N/10 HCl and few drops of 2% barium chloride were added. Presence of sulphate ions was indicated by a white precipitate of barium sulphate. When the test showed absence of sulphate ions in the dialysate, the dialysis of the immunoglobulin solution was discontinued.

The immunoglobulin solution was then centrifuged at 600G for 10 minutes. The supernatant was collected and its protein content was estimated by reading its optical density in U.V. spectrophotometer at 280 mu. The optical density of 1 was considered to be equivalent to 1 mg immunoglobulins per one ml.

Stage 3: Conjugation of Fluorescein Isothiocyanate (FITC) To Immunoglobulin - 3 mg fluorescein isothiacyanate was dissolved in 3 ml bicarbonate-carbonate buffer at pH9. This was then added to 7 ml of immunoglobulin solution containing 100 mg immunoglobulins. The mixture, in a universal container, was stirred continiously on a magnetic stirrer in the cold room at 4 °C for 18 hours.

Stage 4: Elimination of Unreacted Fluorescein from the Conjugate - The unreacted fluorescein from the fluorescein conjugated immunoglobulin was removed by "gel filtration" using coarse grain Sephadex G25. Sephadex G25 acts as molecular sieve and retains molecules up to 5000 molecular weight which includes fluorescein whereas the immunoglobulins with higher molecular weights (150,000 - 180,000 molecular weights) pass readily through a glass tube filled with this grade of Sephadex.

5g of Sephadex G25 powder was suspended in PBS in a beaker. The suspension was stirred gently with a glass rod for few minutes. The gel granules were allowed to sediment. The supernatant fluid which contained very fine granules was discarded. This process was repeated and the Sephadex granules were then re-suspended

in PBS and was left at room temperature for 3 - 4 hours to allow adequate swelling of the gel granules.

A glass tube (about 60 cm long with internal diameter of 1·5 cm) was then set up vertically. This glass tube was held by a retort stand. A rubber tube was connected to the lower end of the glass tube and the rubber tube was clipped by a spring compressor clamp. A small amount of cotton wool was sent down into the lower end of the glass tube to prevent the outflow of the gel granules.

The suspension of Sephadex granules was then gently poured into the glass tubes in small volumes and with great care to allow an even column of gel to set in the glass tube. The sephadex in the glass tube was then equilibrated with PBS.

The fluorescein conjugated immunoglobulin solution was then carefully added to the top of the sephadex column in small volumes using a pasteur pipette. The outflow of the glass tube was simultaneously opened. The conjugate thus passed down through the gel granules slowly. PBS was then added in small volumes to the top of the Sephadex column.

The effluent was collected into a series of test tubes. The fractions which contained a high concentration of fluorescein-linked to immunoglobulin, as ascertained by their yellow colours were pooled together.

Stage 5: Elimination from the Conjugate the molecules which cause non-specific staining - The conjugate which was obtained by the above-mentioned procedures consists largely of immunoglobulin-linked to FITC. Yet if used for staining of tissues it may also give some non-specific staining. This is due to some protein molecules which are excessively labelled during conjugation and cause non-specific staining through their enhanced electrostatic

binding capacity. Two methods were employed to remove from the conjugate these highly charged molecules. The conjugate was divided into two equal parts. One half was absorbed by DEAE cellulose (Whatman DE 11) and the other half was absorbed by acetone-dried mouse liver powder:

a - Absorption by DEAE Cellulose - A glass column was set up filled with DEAE cellulose (1 g DEAE cellulose per each 1 ml of conjugate). PBS was run through the column overnight until it was equilibrated. The conjugate was then layered gently on the top of the column and it was washed through with PBS. All green eluates were collected and pooled together. During this process, the conjugate became diluted. To concentrate the conjugate to the original volume, it was transferred to a dialysis sac by means of a pasteur pipette and the sac was placed in a perspex box and was covered with carbowax. Depending on the volume of the conjugate and the diameter of the dialysis sac, it usually took between 2 and 4 hours to get the conjugate concentrated to the original volume. The conjugate was then transferred to a test tube. Finally, one drop of 12% sodium azide was added to the conjugate to preserve it from bacterial contamination. The conjugate was stored in the refrigerator at 4°C until use.

b - Absorption by Mouse Liver Powder - 100 mg acetone dried mouse liver powder was added to each 1 ml of conjugate, mixed thoroughly by means of a glass rod. It was then centrifuged at 31500 G for 40 minutes. The supernatant was collected and the process of mixing and centrifugation was repeated using 50 mg mouse liver powder per each 1 ml of the conjugate. After centrifugation, the supernatant was transferred to a test tube. One drop of 12% sodium azide was added to the conjugate and it was kept at 4°C until use

Preparation of Frozen Sections - The immunized chickens were killed by an overdose of i.v. injection of Sodium Pentobarbitone.

Spleen, bursa and one thymic lobe were removed from the bird immediately after the bird had died. These lymphoid organs were placed separately in glass tubes. The glass tubes were sealed with a rubber bung and were placed in a flask containing acetone and solid CO_2 (known as dry ice or Carbide) for 30 minutes. Sections, 4u thick were cut in a cryostat (refrigerated microtome) at -20 °C. The sections were placed on a glass microscope slide, melted and dried in a stream of warm air and were fixed in absolute methanol at 20 - 24 °C for 15 minutes. The sections then were left at room temperature for 30 minutes to get dry for staining.

Immunofluorescence staining of the Frozen Sections - Single layer and sandwich techniques of stainings were used to demonstrate the presence of antigen or antibody respectively in the sections of the lymphoid tissues (White et al 1970).

a - Single Layer Technique - This technique was employed to demonstrate the presence of HSA or chick immunoglobulin in the tissues. One drop of fluorescein labelled anti - HSA or anti chick immunoglobulin antibody was applied directly over the sections by means of a pasteur pipette. It was mixed well over the sections by drawing up and down in the pipette and any excess of conjugate was transferred to the next section to be stained similarly. The slides were then left in a closed wet perspex box for 30 minutes at room temperature. They were then dipped in PBS in a Coplin jar for 10 minutes with intermittent shakings. The slides were removed from the Coplin jar, dried except for the areas of the sections. Finally, the sections were mounted with a cover slip in either 0·9% saline or 20% glycerine in saline. The edges of the cover slip were sealed with nail varnish to prevent sections from drying up.

b - Sandwich Technique - This technique was employed to detect

antibody containing cells in the frozen sections of the spleen, bursa and the thymus of the intact and bursectomized chickens which had been immunized to HSA.

A solution of antigen in physiological saline containing 2 mg HSA per ml was made up freshly. The frozen sections of the lymphoid organs were dipped in PBS and the slides were dried as described previously. One drop of the antigen solution was placed on the section by means of pasteur pipette. The slides were then left in a closed wet perspex box for 30 minutes at room temperature. They were then left in PBS in the Coplin jar for 5 minutes and were dried except for the areas of the sections. At this stage one drop of the conjugate (FITC labelled anti HSA antibody) was applied over each section. The conjugate was mixed well over the sections by drawing up and down in the pipette and any excess of the conjugate was transferred to the next section to be stained similarly. The slides were then left in the moist perspex box for another 30 minutes at room temperature. Finally the slides were gently washed under a stream of PBS and mounted with a cover slip as described above for the single layer technique.

Fluorescence Microscopy - The frozen sections of the tissues which had been stained with FITC labelled antibody were examined under a Leitz Ortholux Fluorescence or Wild Fluorescence microscope, fitted with an Osram HBO 200 high pressure mercury arc, oil-immersion objectives and a Cardioid bi-reflecting dark ground condenser. The exciter filters were BG12 (2 mm) and BG38 (3 mm). The barrier filter was Wratter gelatin No.12 or Schott OG1.

Autoradiography - This technique was employed in the study of migration of bursa and thymus cells to other sites in the body.

In this technique, a tissue section containing a radioisotope material is coated with photographic emulsion. The photographic

emulsion serves as a thin layer of film and is in close contact with the tissue. After a given period of exposure, the film is developed and the precise localization of the radioisotope in the tissue section is determined from the pattern of darkening on the film.

The procedures which were followed at various stages of autoradiography were those of de Sousa (1971) Dr. de Sousa has used this technique extensively in her research works and collaborated also with the author in this technique and in counting the labelled bursa and thymus cells in the autoradiographs.

1 - "In-vitro" Labelling of The Bursa and Thymus Cells - Bursa and thymus cells were labelled in-vitro with ^{3}H-adenosine.

The bursa of Fabricius was exposed through an incision mid way between the cloacal vent and the base of the tail. The whole organ was then excised and the incision in the skin was closed with three stitches.

In another group of birds, the thymic lobes were exposed through a dorsal mid line incision in the neck. Five to six thymic lobes were removed from each bird. The incision in the skin was then closed with a continuous suture.

The bursa and the thymic lobes from individual birds were immersed in Eagle's Medium (M.E.M.) containing 10% normal adult chicken serum. The whole bursa or thymic lobes were transected into 5 - 10 slices with a pair of fine scissors and the individual sections teased apart with fine forceps to liberate individual cells. The big lumps of tissues which did not come apart were discarded. The cell suspensions were washed once in warm (37°C) PBS and re-suspended in 10 ml warm (37°C) PBS.

Each cell suspension was then incubated with 50 uci ^{3}H - adensodine at a concentration of 10 uci/ml in a volume of 5 ml for 1½

hours in a shaking water bath at 37°C.

After incubation, the cells were washed twice in Eagle's medium to remove any amount of ^{3}H - adensosine which had not been utilized by the cells. The cells then were suspended in 0·5 - 1 ml PBS. The viability of the cells was determined in a 1% solution of trypan blue. The cells which did not take up this dye were considered as live cells. About 90% of the cells in individual cell suspensions were found to be alive. The live cells were counted in a Neubauer counting chamber.

The labelled cells were then injected intravenously into the birds in volumesof 0·1 - 0·5 ml. Each bird was injected, into the wing vein, with its own bursa or thymus cells.

In-vivo Labelling of the Bursa Cells - With bird under ether anaesthesia, the bursa was exposed through an incision in the skin mid way between the cloacal vent and the base of the tail. A volume of 0·1 - 0·2 ml of PBS containing 40 uci ^{3}H - thymidine was injected, in small volumes, into the substance of the bursa from a syringe fitted with a 30 gauge needle. The needle was re-introduced 2 or 3 times in different sites to distribute the radio-isotope throughout the substance of the bursa. Immediately after 0·5 ml unlabelled thymidine solution containing 0·5 mg thymidine was flushed over the surface of the bursa and 2·5 mg of the unlabelled thymidine was also injected int raperitoneally into the bird. Finally, the incision in the skin was closed by three stitches.

Preparing the Histological Sections - The birds were killed at different intervals after in situ labelling of the bursa or after i.v. injection of either labelled bursa cells or labelled thymus cells. Spleen, two thymic lobes, caecal tonsil and the bursa (where applicable) were removed from the bird. In some birds, umbilical cord residue, a lobe of lung and a lobe of liver were also removed.

All organs were then embedded in paraffin wax and sections, 5u thick, were cut with microtome. The sections were mounted on the glass slides which had previously been treated with "subbing solution" (see below).

The mounted sections were then de-waxed through a series of xylene and alcohol bath lasting 5 minutes each (xylene, industrial methylated spirit, 90% alcohol, 70% alcohol, 50% alcohol, 30% alcohol, distilled water).

Coating - The histological sections were coated with photographic emulsion using "Dipping technique". The coating was carried out in the dark room, working under a safe red light.

Depending on the numbers of slides to be coated, sufficient amount of Ilford emulsion (Ilford Limited, Essex, England) was placed in a Coplin jar and was heated in a water bath at 45°C for 10 minutes. After melting, the emulsion was diluted 1 in 4 with warm (45°C) distilled water. The slides were dipped one by one into the emulsion for five seconds. They were then placed horizontally in a standing metallic rack and were dried at the room temperture. Finally, the slides were placed in a light-tight box. This was left in the cold room at +4°C for four weeks.

Developing and Staining - The slides were placed in developer solution (Kodak developer D-19B, Kodak, London) at 18°C for 5 minutes in the dark room. They were next dipped in tap water for one minute and then transferred to fixing solution (Amfix, May & Baker Ltd., England) for 4 minutes. They were then washed in slow running tap water and stained with UP.

Cell Countings - The histological sections were examined under a Vickers Patholux microscope. Cell counts in sections of all lymphoid organs were carried out under X100 oil-immersion lens and X10 eye piece. Cells with 10 or more developed silver grains were considered labelled. The per centage of the total labelled

cells found in each micro-compartments of the spleen (Red pulp and white pulp including the ellipsoids, peri-ellipsoidal zones and germinal centres) was also calculated.

Subbing solution (Gelatin solution) - 3g gelatin was dissolved in 200 ml distilled water and the solution was left on bench overnight at room temperature. A solution of chrome alum (1·5g chrome alum in 100 ml distilled water) was then made up and it was mixed with the gelatin solution. The gelatin - chrome alum mixture was then diluted by addition of 100 ml distilled water.

The slides which were used for mounting the histological sections in autoradiography were first washed in boiling water containing a trace amount of HCI. They were dried and dipped in the subbing solution for 5 - 10 seconds. They were then drained and dried in a dust free cabinet.

Phosphate Buffered saline pH 7·2 - The stock solution of this buffer was made up by dissolving the following salts in 1000 ml distilled water:

Na Cl	36·0 g
Na^2 H Po^4	7·4 g
KH^2 po^4	2·15 g

The stock solution was kept in cold room at 4 °C. When required, it was diluted 1:4 with distilled water and the pH was checked with a pH meter.

Single Radial Immunodiffusion Test (Mancini Test) - This test was employed to determine the serum IgG levels in bursectomized chickens with reference to those of their corresponding intact controls.

In this test the antigen is allowed to diffuse radially from a circular well in a thin layer of agar containing antiserum. The antigen reacts with the antibody in the agar, consequently a ring

of precipitate will form in the agar after a given period of time. The quantitative aspect of the test is based on the principle that the higher the concentration of the antigen, the greater the diameter of the precipitate ring (Mancini et al. 1965). It has also been shown that there is a direct proportionality between the area enclosed by the precipitate ring and the antigen concentration (Vaerman et al. 1969).

The test was carried out in Oxoid Ionagar No.2 containing 9% sodium chloride. Rabbit anti chicken IgG was warmed in a water bath at 48°C for 15 minutes. 1 ml of the warm antisarum was then added to 10 ml of molten agar at 50°C. The antiserum and the agar were thoroughly mixed and 2·5 ml of the agar-antiserum mixture was poured over the surface of 10 cm x 4 cm plastic plates (Hyland product) to give a uniform layer of 2 mm thickness. The agar plates, when set, were stored in cold room at 4°C until use.

Circular wells were cut with equal diameters in the agar layer on the plates and in each well 0·04 ml of the neat serum from either bursectomized or intact chicken was added. The plates were placed in moist perspex boxes and they were left on bench at room temperature for 48 hours. The radii of the precipitate rings in the agar were measured by a ruler and the surface area enclosed by each precipitate ring was calculated by multiplying 3·14 by the radius squared. No standard serum was included in this test as a standard reference serum for chicken immunoglobulin is not available. The conclusion which is drawn from this test is therefore based on the comparison between the Ig levels in the sera of a group of bursectomized chickens with those of their corresponding intact controls.

Preparation of the electrophoresis plate – A clean sheet of glass (20 cm x 10 cm) was used. Few drops of silicone fluid were added

on the upper surface of the glass and it was evenly spread all over the glass sheet, and the excess silicone washed off with tap water. The glass sheet then was dried. The Ionagar was prepared as follows:

Ionagar No.2 (oxoid)		8·0g
merthiolate		0·1 ml
barbitone buffer	to	1 litre

The Ionagar prepared as above was melted and poured over the glass plate to give an agar layer of thickness 3 - 4 mm. The agar was allowed to set in situ. The agar plate was kept in a wet chamber at 4° C until use.

Immuno-electrophoresis - Troughs and wells were cut on the agar plate using a No.1 cork borer, a ruler, a scalpel and a template drawn on graph paper. The ager plate was then placed over the central compartment of a Shandon electrophoresis tank, and filter paper wicks (Whatman filter paper No.3) immersed in 0·05 M barbitone buffer in the electrophoresis compartment on either side. The test sera were then placed in the wells and a small amount of bromophenol blue dye was added to one of the test sera. This dye attaches itself to the serum albumin and forms a marker for the progress of protein separation. A constant voltage of 160 volts of electric current was then applied to the plate. When the blue spot reached the edge of the plate, the current was cut off. At this stage, the troughs were filled with the appropriate antisera. The plate was left in a wet chamber for 48 hours and examined for precipitin lines.

Histological Techniques - Histological changes in the lymphoid tissues of the bursectomized, thymectomized and intact chickens were investigated after they had been immunized. The organs which were studied histologically included: Spleen, bursa of

Fabricius, thymus, caecal tonsils, umbilical cord residue and the granuloma which formed in the pectoral muscles at the site of injection of HSA in Freund's complete adjuvant. The animals were sacrificed with an overdose of intravenous injection of sodium pentabarbitone. The organs were removed immediately after the bird had died and were placed separately in small bottles containing 10% formalin (formol-saline). The organs were left in this fixative for 48 hours. They were then trimmed and embedded in paraffin wax. Sections, 5 u thick, were cut with a microtome and stained with methyl-green pyronin (Unna-Pappenheim) and/or haematoxylin, counter stained with eosin.

To count the germinal centres in the spleen sections, a grid was drawn with ballpen on the coverslips, overlaying the spleen section on the glass slides. The germinal centres were counted under the microscope at low power magnification.

Statistical Analysis - Arithmetic means and probabilities of differences between groups of values were calculated using a Hewlett Packard calculator.

PART I

THE EFFECT OF BURSECTOMY AND THYMECTOMY ON PRODUCTION OF ANTIBODY AND DEVELOPMENT OF PLASMA CELLS, GERMINAL CENTRES AND GRANULOMA.

Review of the Literature

Investigation on the immunological function of the bursa of Fabricius began with the findings of Glick *et al.* (1956) and Chang *et al.* (1957) who showed that surgically bursectomized chickens were deficient in production of antibody to *Salmonella typhimurium*.

In 1959 Meyer and his co-workers reported that administration of 19-nortestosterone in chick embryos at an early stage of development completely inhibited development of the bursa of Febricius. The chicks were hatched without a bursa. Inhibition of the bursa of Fabricius *in ovo* by steroid hormones - the so-called hormonal bursectomy, was employed by several groups of workers to study the humoral immune response in bursaless chickens. Mueller *et al.* (1960) employing both surgically and hormonally bursectomized chickens found that prenatal inhibition of the bursa by injections of 0·63 mg of 19-nortestosterone into the albumen portion of the fertilized eggs on the 5th day of incubation brought about greater interference with production of antibody to BSA in comparison with surgical bursectomy at one or two weeks of age. Surgical bursectomy early in life caused a deficiency in antibody response to BSA whereas ablation of this organ later in life had little or no effect on humoral immune response of the bursectomized chickens (Mueller *et al.* 1960, 1962). Mueller and his co-workers put forward a hypothesis for time dependency of the bursa extirpation and its effect on the chicken immune response. They suggested that the bursa may not be a site for antibody production per se, but a place where the cells are sensitized prior to migration to other sites. The data which later were provided by other workers on this line added weight to this hypothesis.

Study of the immunological function of the lymphoid tissues

in chicken was extended to that of the thymus gland. Warner et al.(1962b) found that while bursectomized chickens failed to produce antibody to a variety of antigens, the thymectomized chickens apparently had no deficiency in production of circulating antibody. The antigens tested included: BSA, HGG, formol killed Brucella suis, "O" antigen of Salmonella adelaide and influenza type A virus. However, survival of skin homograft was prolonged in the thymectomized chickens and their blood cells were found to be incompetent in producing lesions on the chorio-allantoic membrane. These results indicated that there was a functional division or dissociation of the immunological responsiveness in chicken.

The concept of dissociation of immunological responsiveness in chicken was substantiated by the findings of Graetzer et al. (1963) and Jankovic et al. (1963a, 1963b) who found production of haemagglutinin was affected by bursectomy but not by thymectomy. Bursectomy did not interfere with normal development of delayed wattle reaction to tuberculin. The development of tuberculin reaction, however was found to be inhibited in the thymectomized birds.

Although bursectomy by a hormone had been found to be more effective in suppression of antibody production than surgical bursectomy (Mueller et al. 1960, Pierce et al. 1966), hormonally bursectomized chickens were found to have varying degrees of atrophy of the thymus (Warner et al,1962, Mueller et al. 1962 and Pierce et al. 1966).

Cooper et al.(1965) developed a technique by which the bursa and bursa-dependent lymphoid tissues were eliminated from the birds without causing any permanent damage to the thymus gland - the bursa was surgically ablated on the hatching day and the

bursectomized chickens were whole body x-irradiated the following day. Such bursectomized-irradiated chickens were found to be incompetent to produce any detectable amount of circulating antibodies to BSA and Brucella abortus at 7 weeks of age. Furthermore, both IgG and IgM were found to be absent in the sera of the bursectomized-irradiated chickens.

Surgical thymectomy on the hatching day followed by whole body x-irradiation on the second day of life was also employed to test the immunological functions of the thymus gland in chicken. Neonatal thymectomy combined with whole body x-irradiation suppressed development of delayed hypersensitivity reaction to tuberculin and prolonged survivals of skin homografts but did not bring any deficiency in antibody production to BSA and B. abortus (Cooper et al. 1965, Cooper et al. 1966). These results clearly established the concept that in chicken humoral immunity and cell-mediated immunity are anatomically represented by two different lymphoid organs. Furthermore, these findings suggested that the bursa and the thymus gland can develop and function independently in the absence of one another.

Several groups of workers produced evidence which suggested that bursaless chickens were able to mount a humoral immune response to a variety of antigens. Pierce et al.(1966) found that chickens bursectomized surgically on the day of hatching produced a low level of antibody to BSA at 3 weeks of age. They accordingly suggested that the continued presence of bursa in chicken was not necessary for antibody production. Claflin et al. 1966) also found that both hormonally bursectomized chickens and chickens bursectomized surgically on the day of hatching produced antibody to B. abortus at 6 weeks of age. A Similar finding was reported by Lerner et al.(1971) who found hormonal

bursectomy and surgical bursectomy on the day of hatching inhibited but did not totally eliminate production of antibody to sheep R.B.C. Furthermore they found that inhibition of antibody synthesis in the bursaless birds was not accompanied by a similar marked reduction in the serum levels of IgG and IgM. According to these findings Lerner and his co-workers (Lerner, Glick and McDuffie) suggested that the bursa was not essential for antibody and immunoglobulin synthesis.

Development of a technique by which the bursa of Fabricius could be ablated surgically *in ovo* several days before hatching paved the way for the study of ontogeny of humoral immunity particularly immunoglobulin synthesis in chicken. Van Meter *et al.* (1968) and Cooper *et al.* (1969) found that bursectomy *in ovo* on day 19th of embryonic development reduced serum levels of IgG in chicken at 5 - 6 weeks of age but did not suppress IgM synthesis. A gross deficiency of both IgG and IgM were found in the chickens bursectomized *in ovo* on day 17th of incubation. These findings were substantiated by Aitken *et al.* (1972) who found 29% of the chickens bursectomized on day 17th of incubation had no serum IgG or IgM.

There is, thus, a general agreement that bursa of Fabricius plays a major part in the development of humoral immunity particularly production of antibodies to different antigens. It is also evident that contribution of the bursa of Fabricius to the development of humoral immunity in chicken begins well before hatching. However, it appears that there is not a general concensus among different groups of workers with regard to bursa of Fabricius as the only lymphoid organ in chicken responsible for production of antibody and immunoglobulin.

Histological Chances in the Lymphoid Tissues of Chickens After

bursectomy or thymectomy - Histological examination of the lymphoid tissues of the bursectomized and thymectomized chickens revealed that the bursa of Fabricius and the thymus play a major part in the cellular development of the peripheral lymphoid tissues. Carey et al. (1964) showed that inhibition of the bursa of Fabricius by administration of testosterone propionate in ovo suppressed normal development of plasma cells and germinal centres in the spleen. Surgical ablation of the bursa of Fabricius on the day of hatching was also found to impair the mechanism responsible for the proliferation of plasma cells (Isakovic et al. 1964). In addition, these workers showed that implantation of the bursa from newly hatched or 8 week old chickens into neonatally bursectomized chickens significantly increased their ability to produce antibody to heterologous erythrocytes and caused proliferation of plasma cells in the spleen. They accordingly suggested that the way in which the bursa of Fabricius exerts its immunological function is probably by means of a humoral factor.

The role of the thymus in the cellular development of the spleen was also revealed by surgical ablation of this organ. Neonatal thymectomy did not suppress proliferation of plasma cells in the spleen. In the spleen of thymectomized chickens, however, masses of small lymphocytes surrounding arteries, schweiger-seidel sheaths and lymphatic nodules were found markedly depleted (Jankovic et al. 1964). These findings clearly indicated that the bursa of Fabricius and the thymus contribute to the development of two different populations of lymphoid cells in the spleen.

Another finding of considerable importance was that of Pierce et al. (1966) who found that inhibition of bursa of

Fabricius *in ovo* by *testosterone propionate* brought greater reduction in the numbers of germinal centres and plasma cells in the spleen than surgical bursectomy on the day of hatching. Surgical bursectomy *in ovo* on day 17th of incubation was similarly found to be more effective in reducing the numbers of plasma cells and germinal centres in the spleen and caecal tonsils than surgical bursectomy on the day of hatching (Van Meter *et al*. 1969, Cooper *et al*. 1969). It became, thus, clear that the part which bursa of Fabricius plays in development of plasma cells and germinal centres begins well before hatching.

Complete absence of plasma cells and germinal centres were found in the spleens and caecal tonsils of chickens which had been surgically bursectomized on the day of hatching and had been whole body x-irradiated on their second day of life (Cooper *et al*. 1965). Similar study in the thymectomized-irradiated chickens added weight to the previous finding (Jankovic *et al*. 1964) that neonatal ablation of the thymus brings about a depletion of lymphocytes in the white pulp of the spleen.

Studies on migration of the lymphoid cells in chickens have shown that there is a migration of cells from the bursa and the thymus to the spleen and caecal tonsils (Warner 1965, Linna *et al*. 1969, Hemmingsson 1972). It appears therefore that neonatal bursectomy and neonatal thymectomy cut off the supply of lymphoid cells from the bursa and thymus to the spleen and caecal tonsils, consequently these peripheral lymphoid organs show marked cell depletion.

The Role of Granuloma in Development of Immunological Reactions - Epitheloid granulomas are chronic inflammatory reactions which are characterized microscopically by focal or diffuse collections of epitheloid cells with variable numbers of macrophages, lymphocytes

and giant cells. Formation of epitheloid-granuloma has been associated with the development of both humoral and cell-mediated delayed hympersensitivity immune responses. Using fluorescent antibody technique, White, Coons and Connolly (1955) demonstrated large numbers of antibody-containing plasma cells in the granuloma which formed in rabbits at the site of inoculation of ovalbumin or diphtheria toxoid precipitated by aluminum phosphate. They accordingly suggested that production of specific antibody in the granuloma contributed to the increased antibody production.

In chicken, intramuscular injection of HSA in FCA results in formation of a large epitheloid-granuloma at the site of inoculation. Antibody production in such immunized chickens occurs in two separate phases as antibody levels in the circulation show two major cycles (French, Stark and White 1970). French and her co-workers found that the granuloma at the second phase of antibody response showed an intense infiltration of plasma cells and the tissue extract of the granuloma showed very high anti HSA antibody.

The epitheloid-granuloma has also been shown to contribute to the development of cell-mediated immune reactions. Thus Wilkinson and White showed that there is a relationship between the granuloma which forms in guinea pigs at the site of inoculation of ovalbumin in FCA and delayed hypersensitivity reaction to the test antigen. Delayed hypersensitivity reaction was tested by injection of ovalbumin under the surface of cornea 21 days after the primary immunization and the degree of opacity of the cornea was measured at 24 and 48 hours after injection. They found that there was a correlation between the size of the granuloma with the severity of the cornea response to the test antigen (Wilkinson & White 1966, Wilkinson 1966).

Reid and McKay (1967) similarly found that in human patients undergoing treatment for pulmonary tuberculosis, the size of granuloma which formed at the site of inoculation of heat killed M. tuberculosis correlated with the severity of the delayed hypersensitivity skin reaction. In chickens, Aiyedun (1972) showed that formation of a granuloma at the site of inoculation of HSA in FCA was essential for the development of the delayed hypersensitivity response to the test antigen as indicated by the increased rate of clearance of colloidal carbon from the circulation.

It is likely that the contribution of the granuloma to the enhancement of the delayed hypersensitivity reaction and increased antibody production is due to the fact that the granulation tissue consists of the lymphoid cells which mediate these immunological responses. In chicken, it is conceivable that both bursa of Fabricius as well as the thymus gland may contribute to the cellular development of the epitheloid-granuloma.

Experiment 1 - The Effect of Bursectomy on Production of Antibody to 'phage ΦX174 and to Formation of plasma cells and Germinal centres in the Lymphoid Tissues.

Introduction - The bursa of Fabricius in chicken is considered to be the only lymphoid organ which contributes to the development of humoral immunity on the grounds that its inhibition *in ovo* by hormone or its surgical ablation soon after hatching deprives the bird of the entire immune system responsible for production of antibody to *Brucella suis* and *HGG* (Warner *et al.* 1962), *Haemocyanin* and *Brucella abortus* (Cooper *et al.* 1966) and *sheep RBC* (Alm *et al.* 1969).

Several groups of workers, however, found that both surgically and hormonally bursectomized chickens can produce a low level of specific antibody to *Brucella abortus* (Claflin *et al.* 1966) and *sheep RBC* (Lerner *et al.* 1971). Production of antibody to *BSA* in chickens bursectomized on the day of hatching was also reported by Pierce *et al.*(1966). Lerner and his co-workers together with Bryant *et al.* (1973), who found specific antibody to *sheep RBC* as well as to *Mycoplasma gallisepticum* in hormonally bursectomized chickens came to the conclusion that bursa of Fabricius is not the only organ essential for development of humoral immunity in chicken and a possible bursa-independent humoral immune system in chicken was suggested by these workers.

The general agreement between all workers, however, is the fact that bursectomy profoundly impairs the full production of specific antibody in chicken. It is conceivable, thus, that these variable results on the production of antibody by the bursa-less birds are merely due to the different techniques used for ablation of the bursa of Fabricius or/and the methods used for detection of antibody. The workers who did not find any amount

of antibody in the sera of bursectomized chickens may in fact have used too insensitive method for detection of antibody. It is also probable that the workers who reported presence of specific antibody in the sera of bursectomized chickens may have failed to deprive the birds completely of the entire bursa dependent lymphoid cells. Any bursa cell which escapes elimination may, upon antigenic stimulation,produce specific antibody.

An experiment was therefore designed to verify whether the bursa of Fabricius is the only lymphoid organ in chickan which contributes to formation of circulating antibody.

Brief outlines of the Materials and Methods (detailed accounts of the materials and methods were given in the "General Materials and Methods")

Birds - Thornber 808 chicks were used in this experiment.

Surgical Operation and Irradiation - The chicks were bursectomized surgically on the day of hatching.

In order to eliminate the cells which had migrated, during the embryonic period, from the bursa to other sites in the body the bursectomized chicks were whole body irradiated the day after the surgical operation. A group of non-bursectomized chicks were similarly irradiated on their second day of life to serve as intact irradiated controls.

^{60}Cobalt was used as the source of radiation. The chicks were kept under the exposure of 840 rads of γ-rays with skin source distance of 70 centimetres.

A group of bursectomized and a group of non-bursectomized birds were also included in this study to serve as non-irradiated controls (table 1).

Antigen and Immunization Schedule - Bacteriophage ØX174 was chosen for this investigation since the 'phage neutralization test which

is used to detect antibody against this antigen is one of the most sensitive serological tests (Humphrey and White 1970).

At five weeks of age, each chick was injected intraperitoneally with 10^9 plaque forming units (PFU) of 'phage ØX174 suspended in 1 ml saline. On day 8 after immunization, blood was collected from each chick's wing vein for estimation of antibody to the 'phage and for immunoelectrophoretic analysis. The chicks then were killed by an overdose of sodium pentabarbitone. The spleen, thymus, caecal tonsils and bursa of Fabricius (where applicable) were removed from each bird for histological examination. Histological examination of these lymphoid tissues were undertaken to determine the effect of neonatal bursectomy and whole body irradiation on the cellular make-up of the lymphoid tissues.

RESULTS

Antibody Production - The result of the neutralization test on the sera of the bursectomized and non-bursectomized chickens 8 days after an i.p. inoculation of 'phage ØX174 is presented in table 1. High antibody activity was detected in the sera of both intact non-irradiated and intact-irradiated chickens. The sera of intact non-irradiated chickens showed higher antibody activity than those of the intact irradiated chickens and the difference was found to be statistically significant ($P=0{\cdot}08$). No antibody activity was detected in the sera of the bursectomized-irradiated birds. The bursectomized non-irradiated birds, however, were found to have produced a low level of antibody against the 'phage.

Immunoglobulin synthesis - The immunoelectrophoretic analysis of the sera of the bursectomized non-irradiated, intact non-irradiated and intact-irradiated chickens showed no difference from one another.

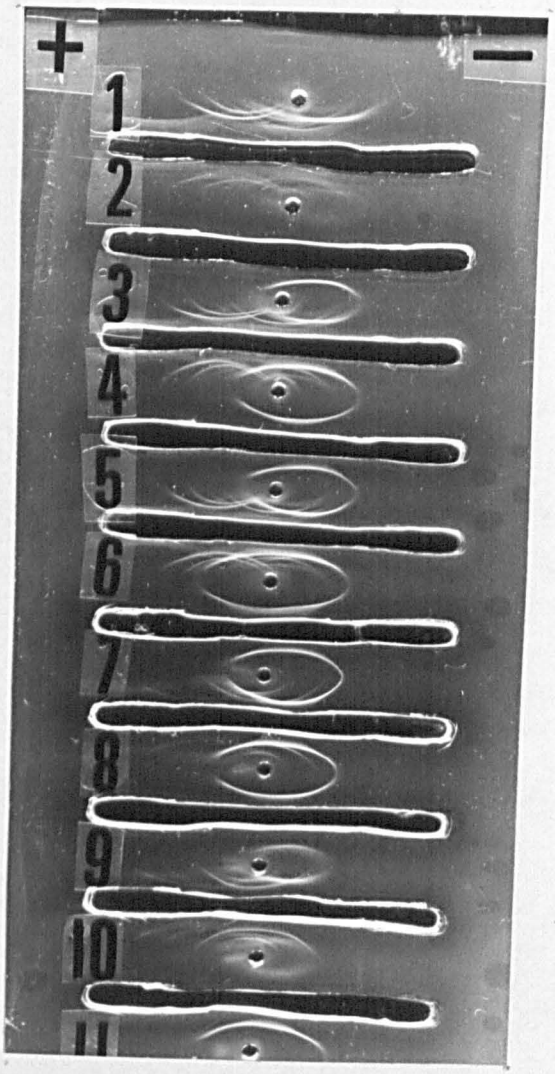


Plate 1 - Agar gel immunoelectrophoresis of sera from bursectomized-irradiated and intact-irradiated chickens eight days after i.p. inoculation of 10^9 PFU 'phage ØX174. Troughs contained either polyvalent rabbit anti chicken immunoglobulins or monovalent rabbit anti chicken IgG. Wells 2, 3, 5, 7, 9 and 10 contained sera from bursectomized-irradiated chickens. Wells 1, 4, 6, 8 and 11 contained sera from intact-irradiated control chickens. Note absence of IgG in the serum 2. The precipitin arcs corresponding to IgG in the sera 9 and 10 also are very faint indicating marked reduction of IgG levels in these sera. Sera 2, 9 and 10 similarly show reduction in the serum levels of other classes of immunoglobulins.

Among bursectomized-irradiated chickens there was one serum (14•3%) which lacked IgG and two sera (28•5%) had barely detectable IgG (plate 1).

HISTOLOGICAL FINDINGS

Spleen - Histological sections of the spleensof individual birds wereexamined under the microscope. At low magnification, the two pulps of the organ - the red and white pulps with the germinal centres are recognized distinctly (plate 2). The very apparent difference in the spleens of the bursectomized-irradiated chickens with that of non-bursectomized birds, was the total absence of germinal centres in the entire white pulp areas of the majority of the sections examined under the microscope. These centres were counted in the four sections of the spleen cut at four different levels, the results are presented in table 2. Intact-irradiated chickens had significantly ($P=0•02$) higher numbers of germinal centres than the bursectomized-irradiated chickens. Surgical bursectomy alone without whole body irradiation did not completely eliminate formation of germinal centres. However, the numbersof these centres were found to be markedly reduced by neonatal bursectomy and it was found that there were significantly ($P = 0•04$) higher numbers of germinal centres in the spleens of intact non-irradiated birds compared to thoseof the bursectomized non-irradiated chickens. Whole body irradiation also reduced the numbers of germinal centres in the spleen since larger numbers of germinal centres were found in the spleens of intact non-irradiated chickens than in the spleens of intact irradiated chickens; however, the difference between the numbers of these centres among these two groups of birds was found to be statistically insignificant ($P = 0•1$).

While there were large numbers of mature and immature plasma cells in the red pulp strands of the spleens of the intact birds, these cells were totally absent in the spleens of the bursectomized-irradiated chickens. The only pyroninophilic cell found in the red pulp strand of these bursaless birds were blast cells. These cells, however, were very few and scanty with very pale cytoplasm. The red pulp areas of the spleens of the bursectomized-irradiated birds, thus, were very thin and depleted (plate 3).

Surgical bursectomy alone without whole body irradiation reduced significantly the plasma cells and blast cells, but did not eliminate totally these series of cells in the spleen. However, the plasma cells and blast cells found in the spleen of these bursectomized chickens, had a very pale cytoplasm often with few empty vacuoles. In general, these cells were very old and showed signs of degeneration.

In the periphery of the white pulp of the chicken spleen there are the ellipsoids or the Schweigger - Seidel sheaths. A mantle of pyroninophilic cells were found around the ellipsoids in the spleens of all non-bursectomized chickens while these cells were totally absent around the ellipsoids in the spleens of the bursectomized-irradiated chickens.

The Thymus - chicken thymic lobes are located along the neck. These are 14 thymic lobes, 7 lobes in either side of the neck. Each lobe is composed of several rather large and elongated lobules. Each thymic lobule has two distinct zones, the medulla in the middle encircled by the cortex in which the cells are denser and stain much more deeply than that of the medulla.

In the periphery of the thymic cortex of the intact-irradiated and intact non-irradiated chickens, there were scattered pyronin

Plate 2

UP, x 150

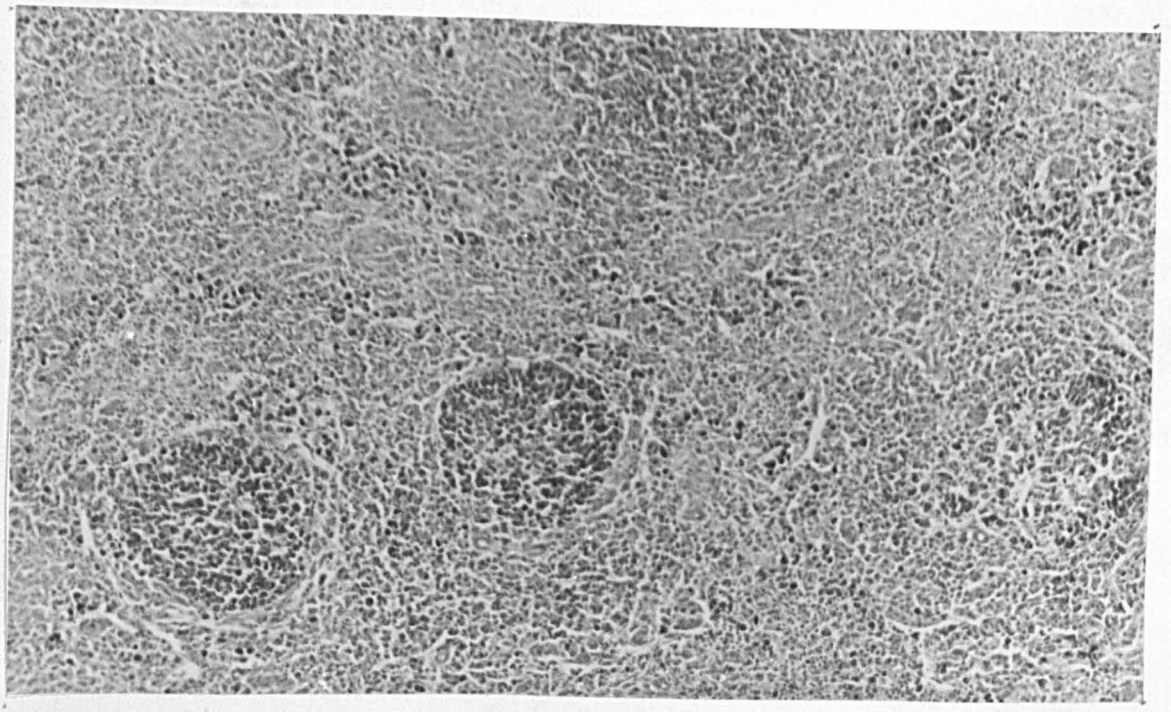
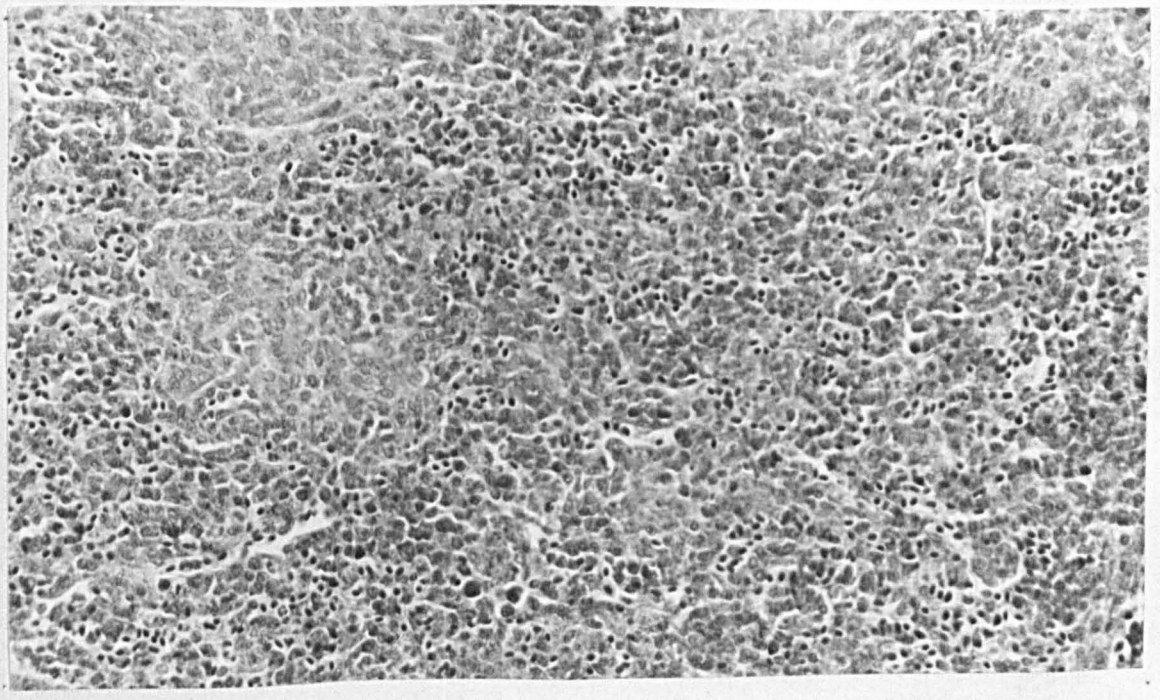


Plate 3

UP, x 350



Photomicrographs of sections of spleens of an intact-irradiated chicken (plate 2) and a bursectomized-irradiated chicken (plate 3) at 6 weeks of age. The chickens had been inoculated i.p. with bacteriophage ϕX174 eight days previously. There are two large germinal centres, one in the lower left and one in the lower centre of the plate 2. Note also pyroninophilic plasma cells in the same plate. Plate 3 shows total absence of germinal centre and complete depletion of plasma cell series of cells from the spleen.

positive blast cells among the small lymphocytes of the thymus. In the medulla, the pyroninophilic cells consisted of immature and mature plasma cells. These cells were few and scanty and located mainly in the cortico-medullary region. In certain lobules these plasma cells were absent.

In the thymic lobes of two chickens - one intact non-irradiated and one intact-irradiated, round structures similar to germinal centres were found located in the medulla. There were lymphocytes and blast cells in each centre (plate 4).

In the thymic lobes of the bursectomized-irradiated chickens, no blast cell was found in the cortex. There was no mature or immature plasma cells or germinal centre in any thymic sections of these bursaless birds (plate 5). Disappearance of pyronin positive cells was the only difference noticed in the thymus of the bursectomized-irradiated birds. There was no apparent depletion of lymphocytes nor any structual changes in the Hassall's corpuscles. In the thymus of the bursectomized chickens which had not been irradiated few immature plasma cells in the medulla and few scanty blast cells were found. No germinal centres were found in the thymic sections of these bursaless birds.

Tonsilia Caecalis (Caecal Tonsils) - In chicken, there are two caeca (appendices) which branch off from the junction of the small and large intestine. The beginning of each caecum is marked by an enlargement which is an aggregation of lymphoid tissues and has been appropriately named tonsilia caecalis (Fig.1). Large numbers of germinal centres were found in the sections of the tonsilia caecalis of the non operated birds. These centres had developed in the lamina propria specially under the submucosa. Large numbers of pyronin positive cells were also found in the sections of the caecal tonsils of non-operated birds. These

Plate 4
UP, x 102

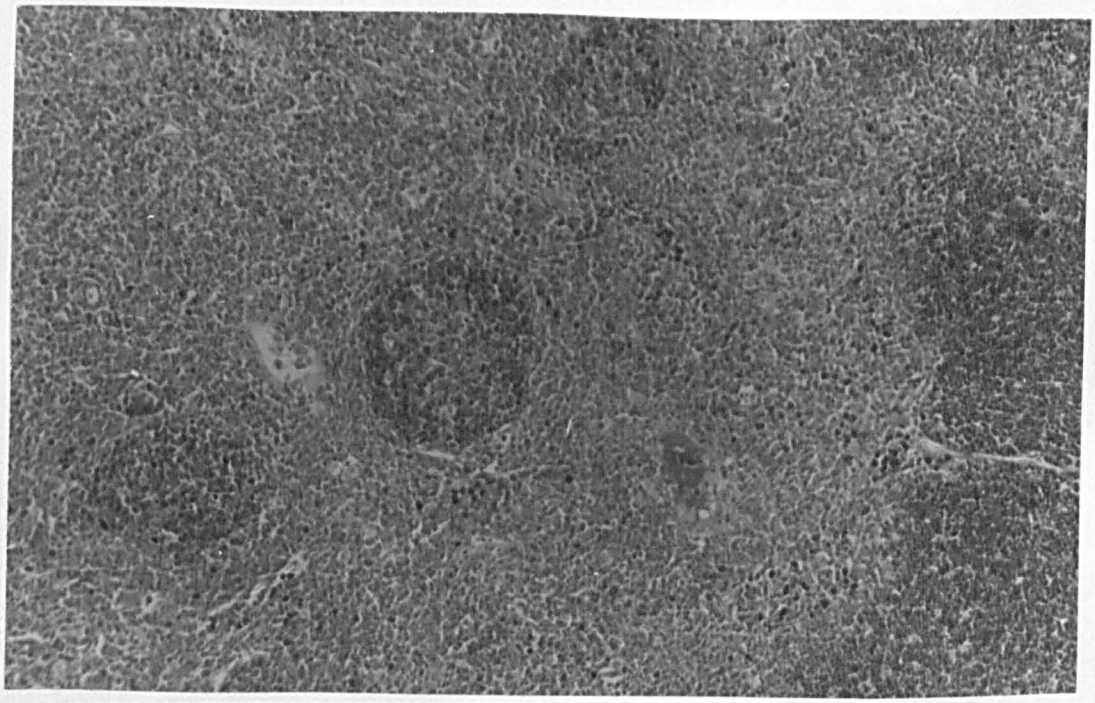
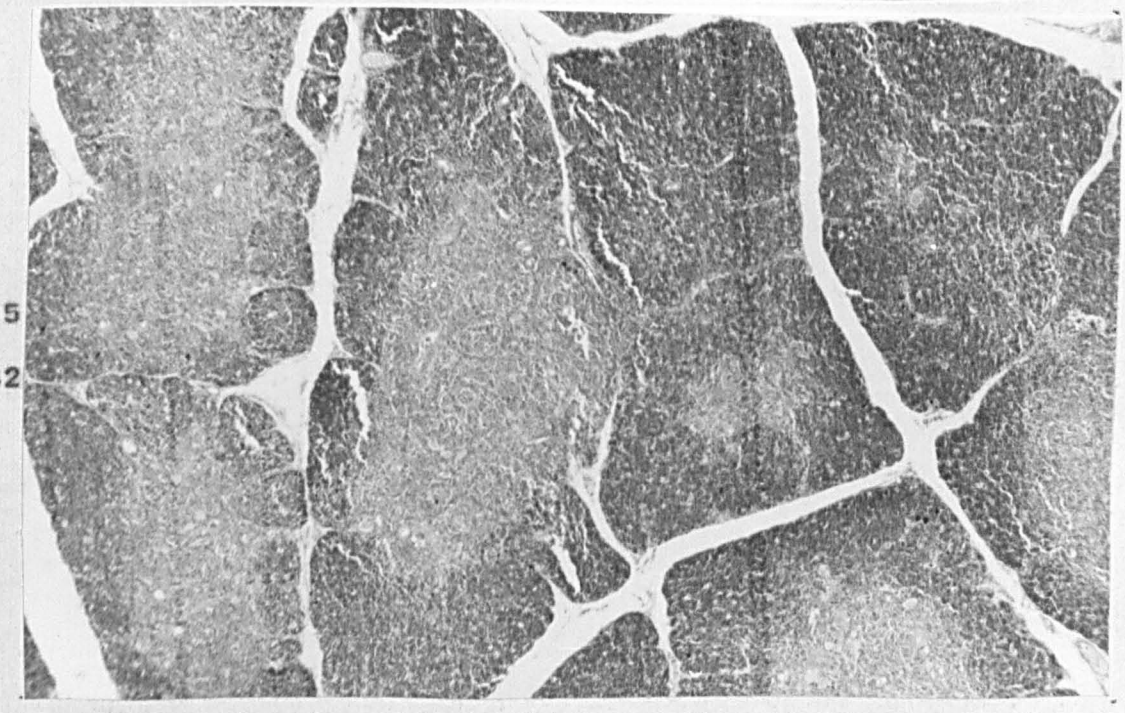


Plate 5
UP, x 42



Photomicrographs of sections of thymus of an intact-irradiated chicken (plate 4) and a bursectomized-irradiated chicken (plate 5) at 6 weeks of age. The chickens had been inoculated i.p. with bacteriophage ϕX174 eight days previously. There are two germinal centres and large numbers of pyroninophilic plasmablasts in the medulla of the thymic lobule of the intact-irradiated chicken. Germinal centres and pyroninophilic cells are completely absent in the thymic lobules of the bursectomized-irradiated chicken.

cells were chiefly located in the cores of the villi.

A thorough microscopical examination of the sections cut from the caecal tonsil of the bursectomized irradiated chickens revealed that germinal centres, immature and mature plasma cells had not developed in this gut associated lymphoid organ. Bursectomy without whole body irradiation significantly reduced, but did not eliminate totally the pyroninophilic cells in the caecal tonsil. Few germinal centres were also found in the sections cut from the caecal tonsil of one of the bursectomized non-irradiated birds.

The Bursa of Fabricius. There were no apparent histological differences between the bursa sections of the intact-irradiated birds with those of the intact non-irradiated birds.

Summary - The concept of whether the bursa of Fabricius is the only lymphoid organ responsible for development of humoral immunity in chicken was investigated.

BX-IR chickens failed to produce any detectable amount of specific antibody to 'phage ϕX174. Neonatal bursectomy followed by whole body irradiation suppressed development of plasma cells and formation of germinal centres in the lymphoid tissues. Peri-ellipsoidal areas in the spleens of BX-IR chickens were found particularly markedly depleted of pyroninophilic cells.

These results suggest strongly that the bursa of Fabricius is the only organ which contributes to formation of antibody to 'phage ϕX174. Furthermore, this lymphoid organ is responsible for development of plasma cells and formation of germinal centres in the lymphoid tissues.

Experiment 2 - The Effect of Bursectomy on Biphasic production of Antibody and Formation of Granuloma After A Single i.m. Injection of HSA in FCA.

Introduction - Production of antibody in chicken after i.m. inoculation of an albumin antigen in FCA is characterized by a high level of circulating antibody which lasts for several months. In addition, the antibody production appears to occur in two separate phases (French, Stark and White 1970). French and her co-workers found that chicken inoculated with HSA in Freund's complete adjuvant showed an initial rise of circulating antibody with a peak at day 8 - 12 followed by a gradual decline in antibody level. The circulating antibody level rose again by day 21 and production of antibody continued up to day 59 after the initial immunization. The antibody level in the second phase was found to be very much higher than that of the first phase. French and her co-workers further produced evidence that the bulk of antibody produced in the second phase came from the granuloma which had formed in the pectoral muscles at the site of inoculation of the antigen in FCA.

In this experiment the occurrence of such biphasic production of antibody in bursectomized chickens will be investigated with the view to determine whether the late production of antibody is bursa dependent or a humoral immune system independent of the bursa of Fabricius contributes to its formation. Furthermore, the contribution of the bursa of Fabricius in formation of granuloma at the site of inoculation of HSA in FCA will be investigated.

Brief Outlines of Materials and Methods (detailed accounts of the materials and methods were given in the "General Materials and Methods").

Birds - Thornber 808 chicks were used in this experiment.

Surgical Operation and Irradiation - The chicks were bursectomized on the day of hatching. The bursectomized chicks and their intact controls were subjected to whole body irradiation from a ^{60}Cobalt Source. The total amount of radiation given to each bird was 800 rads with a source skin distance of 70 cm.

Antigens and Immunization Schedule. The chicks were immunized at 4 weeks of age with HSA in FCA. The inoculum for each bird consisted of 4 mg HSA and 1 mg M. tuberculosis in 0·5 ml Bayol/ Arlacel and 0·5 ml saline. The site of inoculation was the right pectoral muscles.

The birds were bled through the wing vein on days 10, 15, 21 and 28 after immunization and anti HSA in their sera was estimated by Farr Test. After the last bleeding the chickens were killed and a piece of granuloma which had formed in the pectoral muscles was cut. The granuloma and spleen were histologically examined.

RESULTS

Antibody Production - Table 3 and Fig.IV show the serum antibody levels of the BX-IR and IN-IR chickens at different intervals after a single i.m. inoculation of HSA in FCA.

The initial rise of antibody levels in the sera of IN-IR birds on day 10 was followed by a decline in antibody levels on day 15. The circulating antibody levels, however, rose again by day 21 and continued to rise up to day 28. Thus antibody production in IN-IR birds occurred in two separate phases or cycles. The serum antibody levels in the second cycle of this biphasic antibody response showed greater magnitude than the first cycle.

BX-IR chickens failed to produce any detectable amount of antibody to HSA at any stage after i.m. inoculation of this antigen in FCA.

Table 3 _ Serum antibody levels (ABC_{30} ug/ml) in bursectomized-irradiated and intact-irradiated chickens at different intervals after i.m. inoculation of 4 mg HSA in Freund's complete adjuvant.

Chickens	Day 10		Day 15		Day 21		Day 28	
	No. of Chick-ens	ABC_{30} ug/ml Mean & Range	NO. of Chick-en s	ABC_{30} ug/ml Mean & Range	No. of Chick-ens	ABC_{30} ug/ml Mean & Range	No. of Chick ens	ABC_{30} ug/ml Mean & range
Intact-irradiated	6	8.94 3.84-12.6	6	5.1 1.02-11.10	6	17.65 1.86-66.0	2	54.9 16.8-93.0
Bursectomized irradiated	9	<0.06 <0.06-<0.06	8	<0.06 <0.06-<0.06	7	<0.06 <0.06-<0.06	2	<0.06 <0.06-<0.06

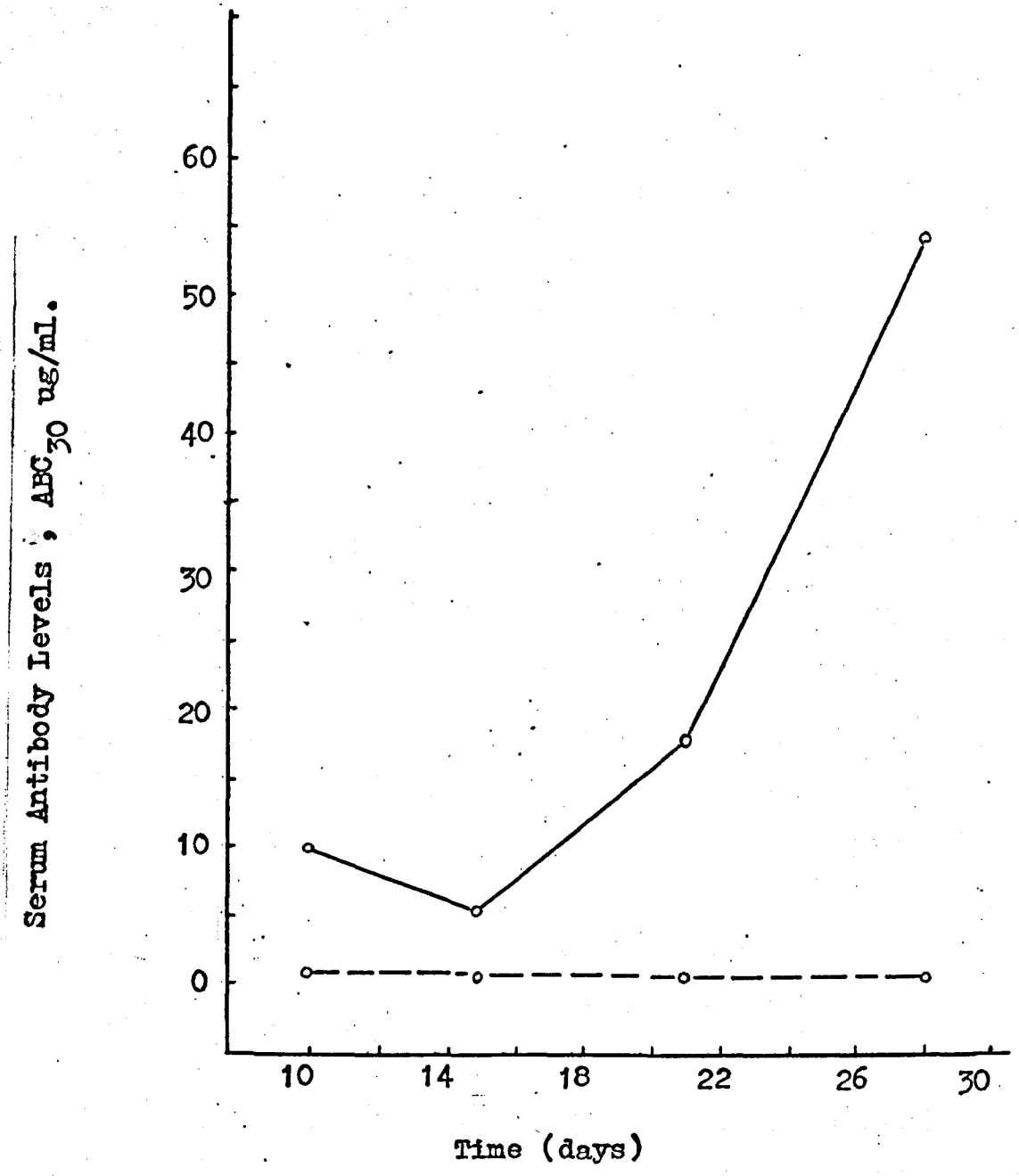


Fig.IV- Serum antibody levels in a group of intact-irradiated chickens (solid line) and a group of bursectomized-irradiated chickens (broken line) at different intervals after a single i.m. injection of 4 mg HSA in FCA. The intact- irradiated chickens produced antibody in two phases as antibody levels in the circulation showed two cycles. The bursectomized-irradiated chickens failed to produce any detectable amount of antibody up to 4 weeks after the initial immunization.

Histological Findings in the Granuloma - Histological sections of the granulomas of both groups of birds revealed that the muscle tissues had been infiltrated by a variety of cells not found normally in the pectoral muscles. The infiltration of cells to the area where the antigen in Freund's complete adjuvant had been deposited was so dense that the muscle tissues had been replaced by entirely different tissue whose main cellular components were macrophages, epitheloid cells and lymphocytes. In the granulomas of the non-bursectomized chickens immature and mature plasma cells were by and large the most numberous types of cells present all over the sections (plate 6) Epitheloid cells, lymphocytes and multinucleated giant cells were found chiefly around the tubercles and oil droplets.

In the granulomas of the bursectomized birds immature and mature plasma cells were completely absent throughout the entire sections (plate 7). The cellular elements which comprised the granulomas of the bursectomized chickens consisted mainly of epitheloid cells, giant cells and lymphocytes.

Histological Findings in the Spleen - Both plasma cells and germinal centres were found in the spleen sections of the intact-irradiated chickens which were killed four weeks after i.m. injection of 4 mg HSA in Freund's complete adjuvant. In contrast to the histological sections of the granulomas, there was, however, no indication of massive proliferation of plasma cells in the spleen. These pyroninophilic cells were in very moderate numbers and were found in the red pulp strands of the spleen.

There was complete absence of plasma cells and germinal centres in the spleen sections of all bursectomized-irradiated chickens.

Summary - The concept of whether the bursa of Fabricius is the only lymphoid organ in chicken responsible for development of humoral immunity was further investigated.

Plate 6

UP, x 400

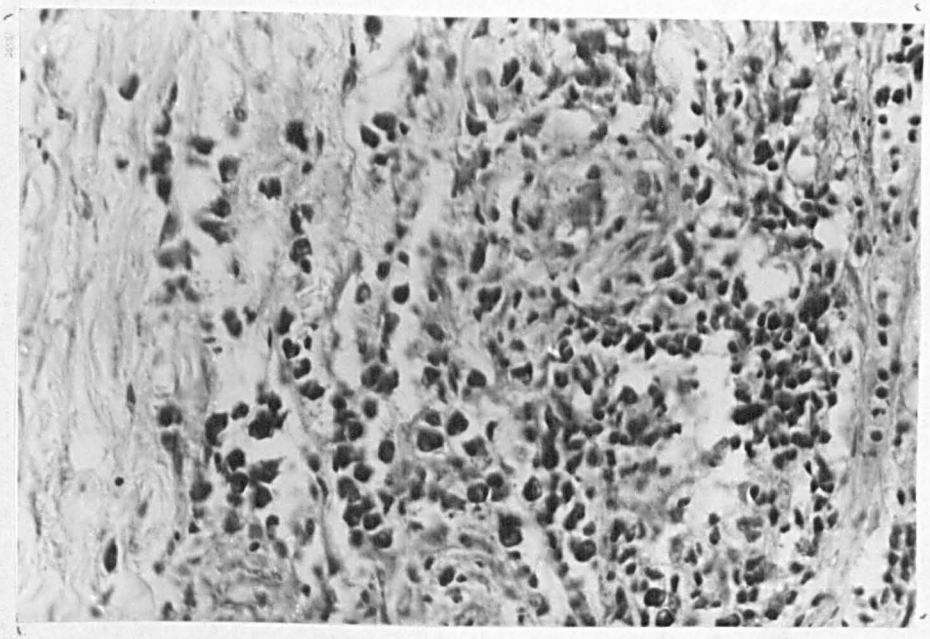
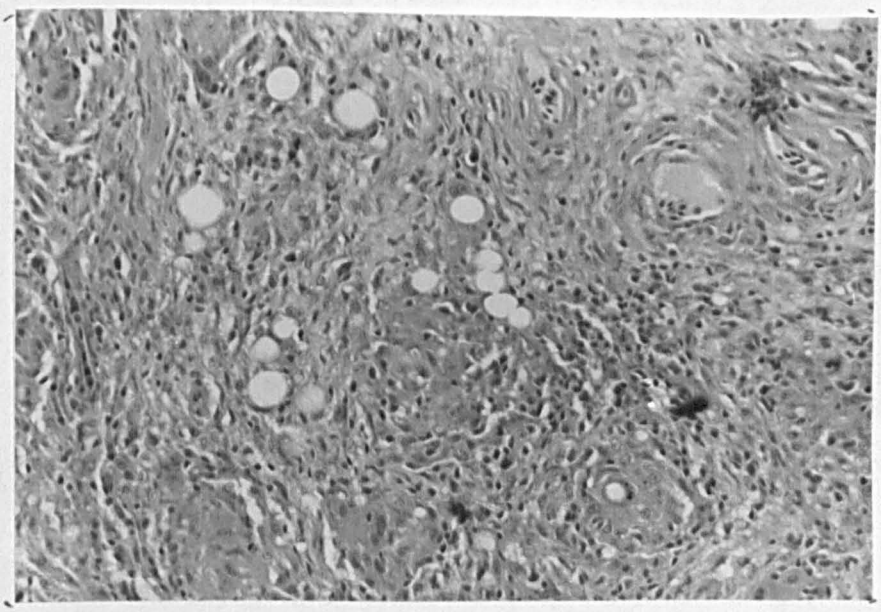


Plate 7

UP, x 250



Photomicrographs of sections of the granulomas which formed in the pectoral muscles of the intact-irradiated chicken (plate 6) and bursectomized-irradiated chicken (plate 7) at the site of inoculation of 4 mg HSA in Freund's complete adjuvant. The chickens were inoculated with the antigen-adjuvant mixture 28 days previously. There are large numbers of plasma cells in the section of the granuloma of the intact-irradiated chicken. Plasma cell series of cells are completely absent in the section of the granuloma of the bursectomized-irradiated chicken.

BX-IR chickens failed to produce any detectable amount of antibody up to 28 days after i.m. inoculation of 4 mg HSA in FCA. The IN-IR controls which were similarly inoculated with 4 mg HSA in FCA showed a biphasic antibody response with two major cycles of antibody in the circulation.

A granuloma was formed at the site of inoculation of HSA in FCA in both groups of birds. The granulomas of the BX-IR chickens were smaller than the controls and lacked the plasma cell series of cells.

The results suggest that the bursa of Fabricius is the only lymphoid organ which contributes to formation of antibody to HSA. The cells responsible for production of both phases of antibody are bursa dependent.

Experiment 3 - The Effect of Thymectomy on Biphasic Production of Antibody and Formation of Granuloma After a Single i.m. Injection of HSA in Freund's complete Adjuvant.

Introduction - Production of a high level of circulating antibody in chicken after i.m. inoculation of HSA in FCA is associated chiefly with the epitheloid granuloma which forms at the site of inoculation (French, Stark and White, 1970). There is ample evidence which suggests that epitheloid-granuloma is a cellular immune response with the characteristics of cell-mediated immunological reactions since it is transferrable by immune lymphoid cells but not by antiserum (Boros and Warren, 1973). Cell-mediated immune reactions in chicken are thymus-dependent (Jankovic et al. 1963b, Cooper et al. 1966) and it is likely that the thymus may also contribute to the formation of this type of granuloma, thereby

enhancing production of circulating antibody.

This experiment is therefore designed to determine if and to what extent neonatal thymectomy affects biphasic production of antibody and formation of epitheloid-granuloma in chicken after i.m. inoculation of HSA in Freund's complete adjuvant. Furthermore, spleen and the bursa of Fabricius of the thymectomized chickens are histologically examined to add further evidence on the role of thymus to the cellular developments of these lymphoid organs.

Brief Outlines of Materials and Methods (detailed accounts of the materials and methods were given in the "General Materials and Methods").

Birds - Thornber 808 chicks were used in this experiment.

Surgical Operation and Irradiation - The chicks were thymectomizd on the day of hatching. The thymectomized chicks along with their intact controls were subjected to whole body irradiation on their second day of life in order to eliminate the cells which had migrated from the thymus to other sites in the body during the embryonic period. The chicks were kept under the exposure of 800 rads of radiation from a ^{60}Cobalt source with source skin distance of 70 cm.

Antigen and Immunization Schedule - The chicks were immunized at 4 weeks of age with HSA in FCA. The inoculum for each bird consisted of 4 mg HSA and 1 mg M. tuberculosis suspended in 0·5 ml Bayol-Arlacel and 0·5 ml saline. The site of inoculation was right pectoral muscles.

The birds were bled through the wing vein on days 10, 15, 21, 28 and 38 after the immunization. One of the IN-IR chickens which was found to produce a high titre of anti HSA antibody was spared to obtain large amounts of chicken anti HSA antibody which was needed for another experiment. The remaining four IN-IR and

three TX-IR chickens were killed 5 minutes after i.v. injection of 0·5 ml indian ink.

The birds were weighed immediatly before they were killed. The areas along the jugular veins in the thymectomized birds were carefully examined in search of any thymic residue. The thymic lobes, spleen and bursa were removed from the birds and were immediately weighed. The pectoral muscles of each bird were also inspected for presence of granuloma at the site of inoculation of HSA in FCA. A piece of granuloma together with spleen and bursa were fixed in formol-saline for histological examination.

RESULTS

Antibody Production - Anti HSA antibody in the sera of the TX-IR and IN-IR chickens was measured with Farr test. The results are presented in table 4 and Fig. V.

In both groups of birds, the initial rise in the serum antibody levels, with its peak on day 10, after the initial immunization, was followed by a decline in the antibody levels. This decline in the serum antibody levels was detected on day 15. The serum antibody levels in both groups of birds subsequently rose again without further immunization and continued to rise up to day 38 after the initial immunization. The TX-IR birds, thus, like their IN-IR controls produced circulating antibody in two separate phases after a single i.m. injection of HSA in FCA.

Statistical analysis showed that the titre of anti HSA antibody (ABC_{30}) in the sera of IN-IR chickens on day 10 was significantly higher than that of the TX-IR chickens ($P = 0·04$) whereas the antibody titre of the TX-IR chickens on day 21 was significantly higher than that of the IN-IR birds ($P = 0·09$). The differences between the antibody titres in the sera of these two groups of birds on days 15, 28 and 38 after the immunization were not statistically significant ($p = 0·15$, 0·38 and 0·21 respectively).

Table 4 - Serum antibody levels (ABC_{30} ,ug/ml) in thymectomized-irradiated and intact-irradiated chickens at different intervals after a single i.m. injection of 4 mg HSA in Freund's complete adjuvant.

Chickens	Day 10		Day 15		Day 21		Day 28		Day 38	
	No. of Chick-ens	ABC_{30}ug/ml Mean & Range	No. of Chick-ens	ABC_{30}ug/ml Mean & Range	No. of Chick-ens	ABC_{30}ug/ml Mean & Range	No. of Chick-ens	ABC_{30} ug/ml Mean & Range	No. of Chick-ens	ABC_{30} ug/ml Mean & Range
Thymectomized -irradiated	4	1.2 0.09-3.3	4	0.45 0.5-0.9	4	10.55 1.7-24.6	3	40.6 25.8-66.0	3	109.4 34.2-204-0
Intact -irradiated	7	3.64 0.6-9.0	7	1.48 0.18-5.1	7	4.0 0.5- 9.6	5	128.86 20.3-384.0	5	851.7 45.6-3720.0

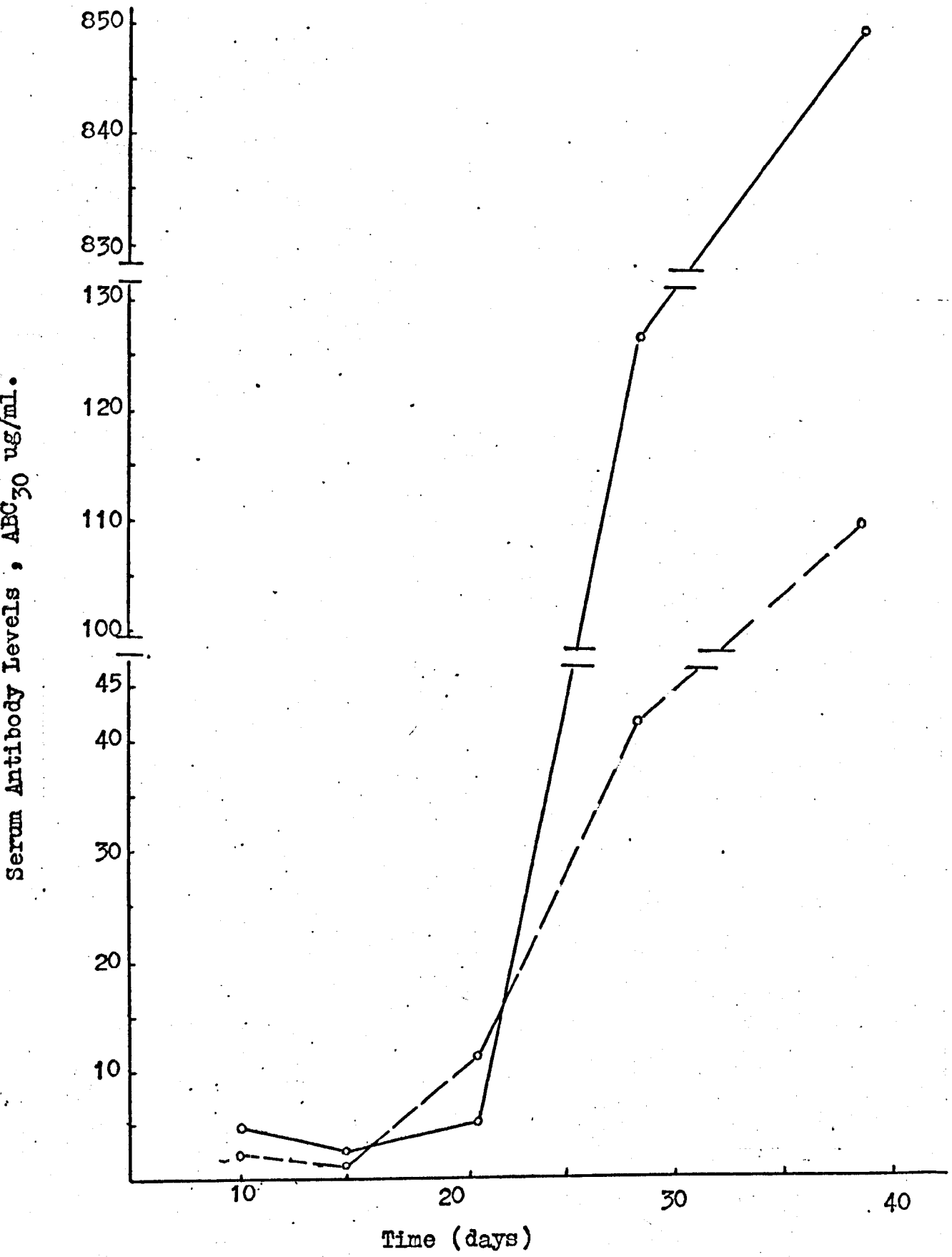


Fig.V- Serum antibody levels in a group of thymectomized-irradiated chickens(broken line) and a group of intact-irradiated chickens(solid line) up to 38 days after a single i.m. injection of 4 mg HSA in FCA. The thymectomized-irradiated chickens, like their intact-irradiated controls , produced antibody in two phases as antibody levels in the circulation showed two cycles.

Body Weights and the Weights of the Lymphoid organs - The weights of the spleen, bursa and the thymus as well as the body weights of TX-IR and IN-IR chickens are presented in table 5. The differences between the body weights and the weights of the spleens and the bursas in these two groups of birds were not statistically significant (P = 0·14, 0·15 and 0·47 respectively).

Table 5 - Body weights and weights of the lymphoid organs of the thymectomized-irradiated and intact-irradiated chickens at 10 weeks of age.

Chickens	No. of chicks	Body weight (g) mean & range	Spleen weight (mg) mean & range	Bursa weight (mg) mean & range	Thymus weight (mg) mean & range
Thymectomized irradiated	3	626·6 455-725	1329·0 725-1938	1275·6 656-1940	133·3 122-144
intact irradiated	4	753·7 625-945	1852·7 1260-2713	1300·5 1156-1502	5208 4138-6296

At autopsy all TX-IR chickens were found to have rudimentary or well-developed thymic lobes in the lower end of the neck. The weights of the thymic lobes in the IN-IR chickens and those found in the TX-IR chickens are presented in table 5. The difference between the thymus weights of the IN-IR chickens and those of the TX-IR chickens was statistically highly significant (P = 0·0001).

Histological Findings

The Granuloma - A granuloma had formed in the pectoral muscles of both groups of birds, where the antigen in FCA had been injected.

Plate 8

UP, x 144

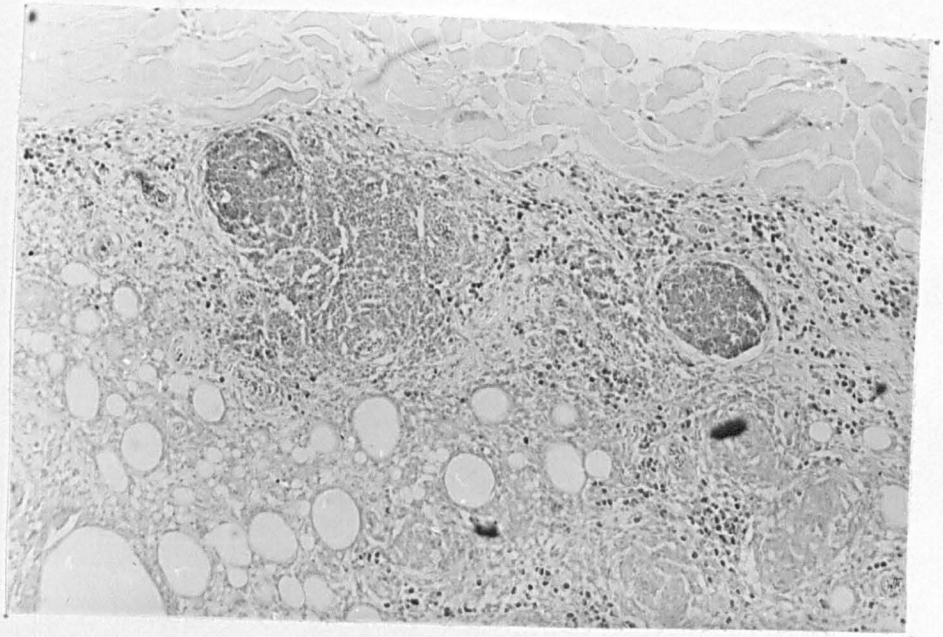
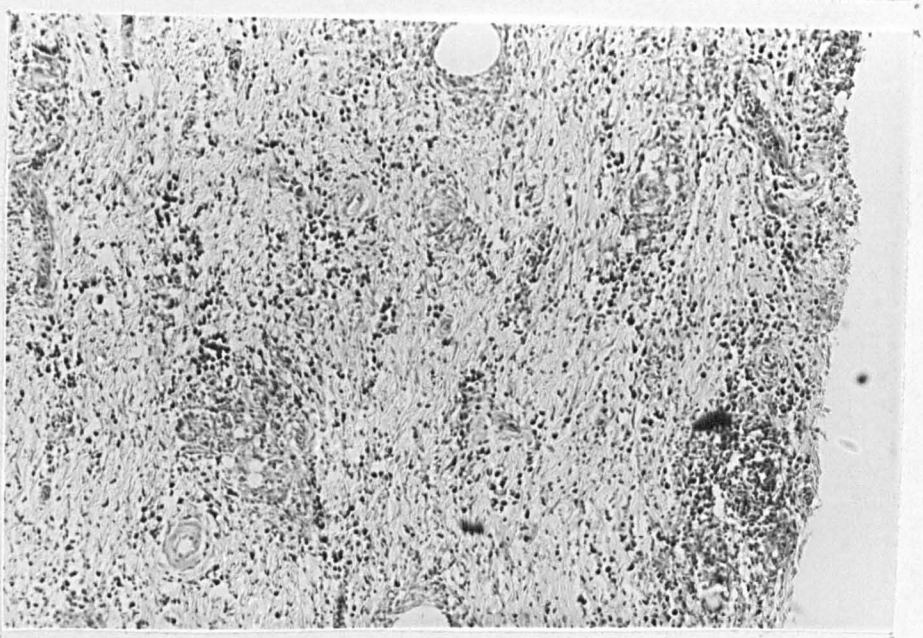


Plate 9

UP, x 102



Photomicrographs of sections of the granulomas which formed in the pectoral muscles of a thymectomized-irradiated chicken (plate 8) and an intact-irradiated chicken (plate 9) at the site of inoculation of 4mg HSA in Freund's complete adjuvant. There are large numbers of plasma cells in the granulomas of both intact and thymectomized chickens. Note also formation of two germinal centres in the granuloma of the thymectomized chicken. Epitheloid cells and lymphocytes in the granuloma of the thymectomized bird are very scarce. In contrast, the granuloma of the intact chicken shows massive infiltration of epitheloid cells and lymphocytes.

The TX-IR chickens had rather smaller granulomas than those of the IN-IR chickens whose granulomas had spread through almost all right pectoral muscles.

In histological sections, it was found that the muscles tissues had been replaced by a lympho-epitheloid tissue. Large numbers of immature and mature plasma cells were present in the granulomas of both groups of birds. In the histological section of the granuloma of one TX-IR bird two germinal centres were found indistinguishable from the germinal centres of the spleen (Plate 8). However, the histological sections showed clearly that there was higher infiltration of cells in the granulomas of the IN-IR birds in comparison to that of the TX-IR birds (Plate 9). There was no indication that neonatal thymectomy coupled with whole body irradiation had suppressed proliferation of plasma cell series of cells in the granuloma. However, it was very clear in the histological sections that infiltration of epitheloid cells, mulincleated giant cells and lymphocytes had bean considerably inhibited by neonatal thymectomy followed by whole body irradiation of the birds.

Spleen - Moderate numbers of plasma cells and germinal centres were found in the spleen sections of both IN-IR and TX-IR chickens. No plasma cell or germinal centre counts were carried out in any spleen section. However, there was no indication that neonatal thymectomy coupled with whole body irradiation had brought about a marked reduction in the numbers of plasma cells and germinal centres in the spleen.

In the spleen sections of the TX-IR chickens, there was a noticeable depletion of lymphocytes in the white pulp strand. The lymphocyte depletion was confined particularly to the peri-arterial sheaths of the white pulp (Plate 10). It was found that the degree of lymphocyte depletion in the white pulp of the spleen varied among the TX-IR birds. Marked lymphocyte depletion was found in

Plate 10

UP, x 384

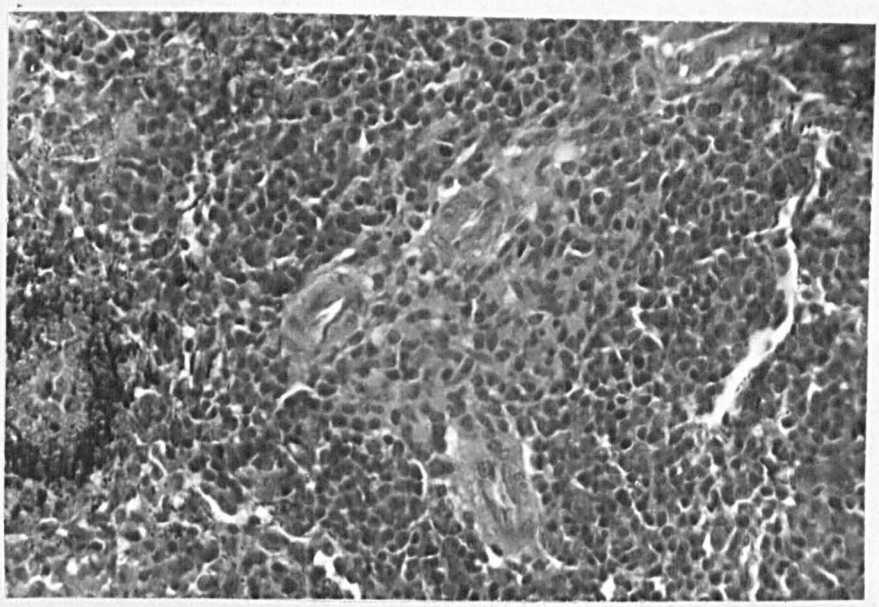
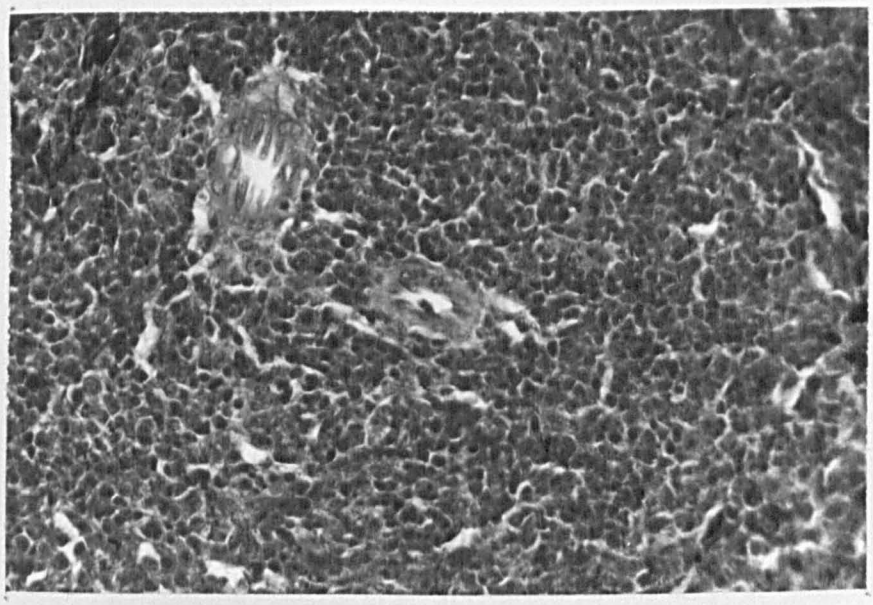


Plate 11

UP, x 384



Photomicrographs of sections of spleens of a thymectomized-irradiated chicken (plate 10) and an intact-irradiated chicken (plate 11). The chickens had been immunized with i.m. inoculation of 4 mg HSA in Freund's complete adjuvant 38 days previously and were injected intravenously with indian ink 5 minutes before they were killed. Note depletion of small lymphocytes in the peri-arteriole sheath of the white pulp in the spleen section of the thymectomized-irradiated chicken. There is no apparent cell depletion in the peri-arteriole sheath of the white pulp of the intact-irradiated chicken. The carbon-bearing cells are largely at the periphery of the ellipsoids and to a lesser extent in the white pulp of the spleens of both chickens.

the white pulp of the spleen of the TX-IR bird which had the smallest thymus residue.

The macrophages in and around the ellipsoids, in the spleens of both groups of birds were found loaded with the carbon particles of the indian ink. There was no difference between the pattern of localization or distribution of carbon particles in the spleens of TX-IR and IN-IR chickens.

Bursa of Fabricius - There were no apparent histological differences between the bursas of the TX-IR birds and those of their corresponding intact controls.

Summary - The role which thymus plays in enhancing production of antibody and formation of epitheloid granuloma at the site of inoculation of HSA in FCA was investigated.

Neonatally thymectomized and irradiated chickens were immunized, along with their intact-irradiated controls with i.m. injection of 4 mg HSA in FCA. Both groups of birds produced antibody in two major cycles or phases. Neonatal thymectomy coupled with whole body irradiation reduced the serum antibody levels in this biphasic antibody response.

Histological investigation showed that neonatal thymectomy followed by whole body irradiation suppressed infiltration of epitheloid cells, giant cells and lymphocytes in the granuloma and brought about a depletion of lymphocytes in the peri-arteriole sheaths of the white pulp of the spleen.

These results are discussed in relation to the role of the thymus in formation of the lympho-epitheloid granuloma which is a site where the bulk of antibody is formed during the second phase of the biphasic antibody response. The depletion of lymphocytes in the white pulp of the spleen is discussed in relation to migration of cells from the thymus to the spleen.

Experiment 4 - Study of the Effect of Thymus on the Ability of Freund's Complete Adjuvant (FCA) to Potentiate Antibody response of the Chicken to HSA.

Introduction - The results of the preceding experiment in which antibody production in thymectomized chickens given HSA in FCA was studied revealed that circulating antibody levels of the TX-IR chickens were lower than those of their corresponding intact controls. Several groups of workers have recently found that T - cells are required for potentiation of antibody formation when antigen is given in FCA. Allison et al. (1971) found that in mice depleted of T-cells antibody response to BSA in FCA is decreased. When mice are reconstituted with a thymus graft marked stimulation of antibody formation by the adjuvant is again obtained. Similar findings in mice were reported by Dresser (1972) using sheep RBC and pertussis adjuvant.

The aim of this experiment is to ascertain whether in chicken cooperation of the thymus is necessary for full effectiveness of FCA in enhancing production of antibody. The experiment is designed on the hypothesis that chickens depleted of T-cells, by neonatal thymectomy coupled with whole body irradiation, would produce the same amount of antibody when immunized to HSA in FCA as that of intact-irradiated chickens given HSA in water-in-oil emulsion.

Brief Outlines of Materials and Methods (detailed accounts of the materials and methods were given in the "General Materials and Methods").

Birds - Thornber 808 chicks were used in this experiment.

Surgical Operation and Irradiation - The chicks were thymectomized on the day of hatching. The thymectomized chicks along with their intact controls were subjected to whole body irradiation on the day after hatching. The chicks were kept under the exposure of 800

rads of radiation from a ^{60}Cobalt source with source skin distance of 70 cm.

Antigens and Immunization Schedule - At 4 weeks of age, the thymectomized-irradiated chickens and a group of intact-irradiated chickens were inoculated intramuscularly with HSA in FCA. The inoculum for each bird consisted of 4 mg HSA and 1 mg M. tuberculosis suspended in 0.5 ml Arlacel-Drakeol 6 VR and 0.5 ml saline.

The second group of intact-irradiated chickens were inoculated intramuscularly with HSA in water-in-oil emulsion. The inoculum for each bird in this group consisted of 4 mg HSA suspended in 0.5 ml Arlacel-Drakeol 6 VR and 0.5 ml saline (table 6). The site of inoculation in all groups of birds was right pectoral muscles.

The chickens were bled at different intervals after immunization and anti HSA antibody in their sera was measured by Farr test.

There was a high rate of mortality among the thymectomized-irradiated birds. Out of 16 TX-IR birds only 4 survived by the age of 4 weeks. Another two TX-IR birds died during the course of experiment. It was decided, however, to continue measuring the circulating antibody in the remaining TX-IR birds and all the intact-irradiated controls as long as possible to determine the peak and fall of antibody levels in the second phase of the biphasic antibody response.

All chickens were sacrificed at the age of five months and an autopsy was carried out on each bird. The site of inoculation of HSA in FCA or HSA in water-in-oil emulsion was dissected to determine the formation of granuloma in the area. The area along the jugular veins in the TX-IR birds was also carefully examined to determine whether all thymic lobes had been completely removed in the surgical operation which was carried out on the day of hatching.

RESULTS

Antibody Production - Anti HSA antibody in the sera of TX-IR and IN-IR chickens was measured with Farr test up to 113 days after the initial immunization. The results are presented in table 6 and Fig VI.

In all groups of birds, the initial rise in the serum antibody levels, with its peak on day 10, was followed by a decline in the antibody levels. This decline in the antibody levels was detected in the sera of all three groups of birds on day 15. The serum antibody levels in all groups of birds subsequently rose again without further immunization and continued to rise up to day 51 (in the IN-IR birds immunized to HSA in water-in-oil emulsion) or day 64 (in TX-IR and IN-IR birds immunized to HSA in FCA.) The serum antibody levels then showed an abrupt decline on day 64 (in IN-IR birds immunized to HSA in water-in-oil emulsion) or on day 92 (in TX-IR and IN-IR birds immunized to HSA in FCA).

With regard to the peak of the serum antibody levels in the first phase of antibody response, the IN-IR chickens immunized to HSA in FCA were found to have significantly higher titres of antibody (ABC_{30}) than the TX-IR chickens which were immunized similarly ($P = 0.029$). The difference between the antibody titres (ABC_{30}) in the sera of these two groups of birds on the peak of second phase of antibody response was not statistically significant ($P = 0.37$).

Post Mortem Findings - All IN-IR chickens which were inoculated with HSA in FCA had developed a large granuloma at the site of inoculation. The TX-IR chickens were also found to have granuloma at the site of inoculation. The granuloma in the TX-IR chickens were obviously smaller than thoseof the IN-IR chickens but the appearance of the cut surface of both were similar. No granulation

Table 6- Serum antibody levels (ABC_{30} ug/ml) in a group of thymectomized-irradiated and two groups of intact-irradiated chickens at different intervals after a single i.m. injection of 4 mg HSA suspended either in Freund's complete adjuvant (FCA) or in water-in-oil emulsion(WO).

Chickens	Day 10	Day 15	Day 21	Day 28	Day 38	Day 51	Day 64	Day 92	Day 113
Thymectomized-irradiated, immunized to 4 mg HSA in FCA	* 3.5 **0.25-5.0 *** 4	1.93 1.3-2.2 4	16.36 9.2-25.2 4	37.6 11.4-51.0 3	38.1 37.8-38.4 2	52.2 48.0-56.4 2	68.1 59.5-76.7 2	34.5 32.4-35.7 2	39.3 35.0-43.6 2
Intact-irradiated, immunized to 4 mg HSA in FCA	.* 8.2 **6.6-12.3 *** 6	3.25 2.4-2.7 6	6.64 2.5-13.2 6	28.2 9.0-58.8 6	51.8 12.6-70.8 6	52.4 27.0-66.0 6	100.9 28.2-246.9 6	49.7 9.1-133.8 6	53.7 23.7-145.1 6
Intact-irradiated, immunized to 4 mg HSA in wo	* 5.34 **1.5-11.7 *** 7	1.95 1.5-2.4 7	6.60 2.5-108 7	25.58 11.7-39.0 7	51.24 30.0-72.0 5	65.4 36.6-156.0 5	59.2 44.6-106.3 5	30.6 16.0-61.5 5	34.7 16.1-51.0 4

* Mean serum antibody levels (ABC_{30} ug/ml)

** Range of serum antibody levels (ABC_{30} ug/ml)

*** Numbers of chickens.

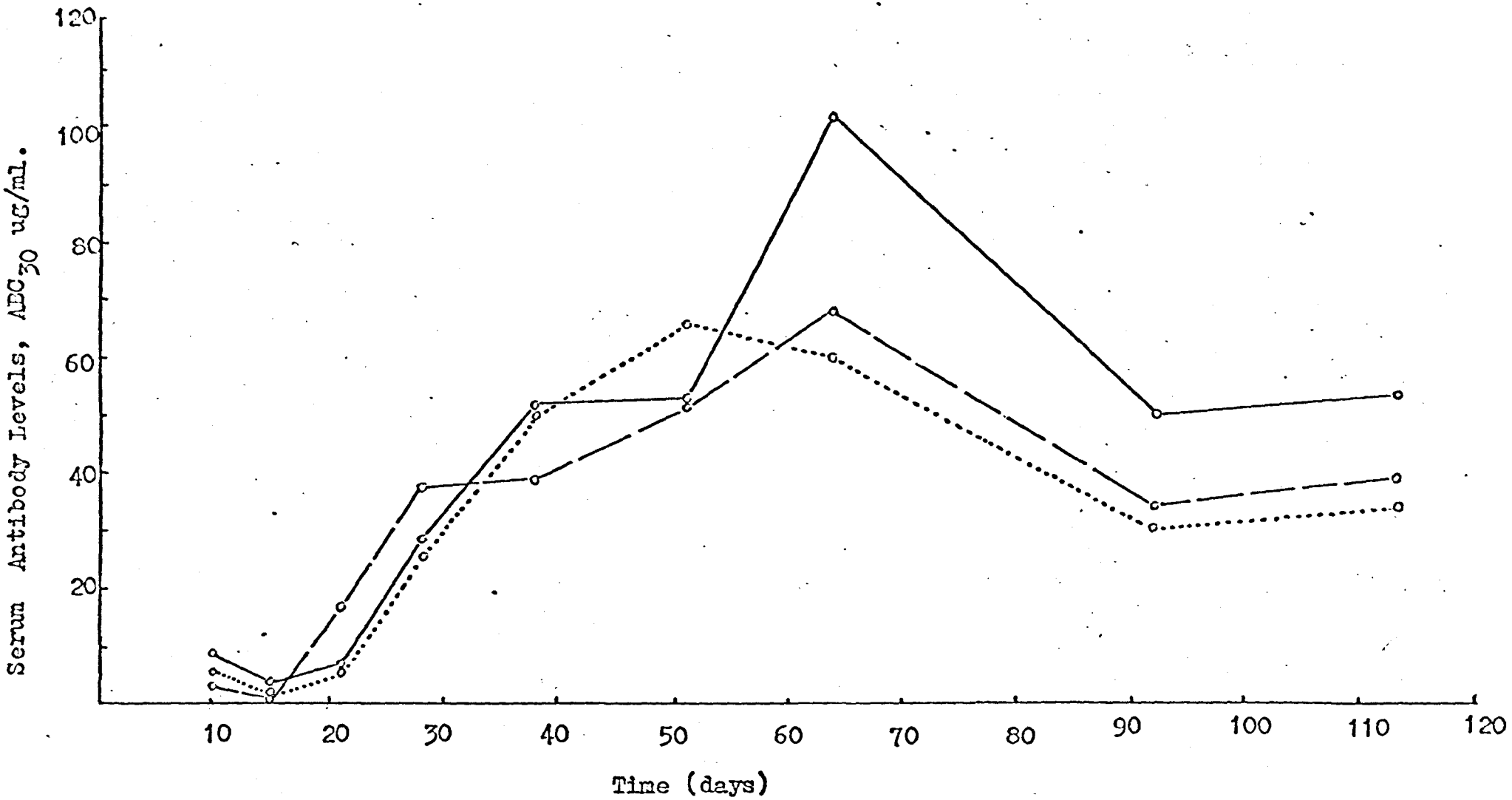


Fig.VI-Serum antibody levels in a group of thymectomized-irradiated chickens (broken line) and a group of intact-irradiated chickens (solid line) after a single i.m. injection of 4 mg HSA in FCA . The dotted line shows the serum antibody levels in a second group of intact-irradiated chickens after a single i.m. injection of 4 mg HSA in water-in-oil emulsion (WO).

tissue was obvious by naked eye inspection in the pectoral muscles of the IN-IR chickens which had been inoculated with HSA in water-in-oil emulsion.

Inspection of the areas along the jugular veins in the thymectomized-irradiated birds revealed that there was regeneration of thymic tissues in these birds. Each bird had two rudimentary thymic lobes which were located in the lower end of the neck. The thymic lobe in this area is difficult to remove and it is usually removed by bits and pieces. Any thymic tissue which is left, provided it has vascular connection, would regenerate.

Summary - The concept of whether cooperation of thymus is neccessary for full effectiveness of FCA in enhancing production of antibody was investigated.

A group of TX-IR chickens was given a single i.m. injection of 4 mg HSA in FCA. A group of IN-IR chickens was similarly given 4 mg HSA in FCA. A second group of IN-IR chickens was given a single i.m. injection of 4 mg HSA in water-in-oil emulsion.

Anti HSA antibody in the sera of all groups of birds was measured by Farr test at different intervals after immunization up to 113 days after the initial immunization.

Formation of antibody in all groups of birds occurred in two separate phases as there were two major cycles of antibody in the circulation.

With regard to the peaks of serum antibody levels in the first and second phase of antibody response, the IN-IR chickens immunized to HSA in FCA were found to have higher titres of antibody (ABC_{30}) than the TX-IR chickens which were immunized similarly with HSA in FCA. The antibody titres (ABC_{30}) of the TX-IR chickens were almost equal to those of the IN-IR chickens immunized to HSA in water-in-oil emulsion.

These results are discussed in relation to the part which the thymus

plays to bring about optimum effectiveness of FCA in enhancing antibody production.

Experiment 5 - The Effect of Bursectomy on Day 18th of Incubation of Chick Embryo on Cellular Development of the Thymus Gland and Production of Antibody to Sheep R.B.C.

Introduction - The bursa of Fabricius contributes to the development of the peripheral lymphoid tissues such as spleen and caecal tonsils by seeding these tissues with cells. In the young chick, bursa cells migrate to the peripheral lymphoid tissues as well as to the thymus gland (Linna et al. 1969, Hemmingsson et al. 1972a). The fact that neonatal bursectomy without whole body irradiation does not eliminate the entire population of plasma cells in the peripheral lymphoid tissues of the bursaless birds (Cooper et al. 1966) indicates also that migration of cells from the bursa to other sites in the body is well under way in the embryo.

It is not, however known whether the cells which migrate from the embryonic bursa to other lymphoid tissues represent a small population of cells or there is extensive cell migration from the bursa during the embryonic period. If there is extensive migration of cells from the bursa to other lymphoid tissues during the embryonic period, surgical ablation of bursa in the chick embryo or inhibition of the bursa in ovo by a steroid hormone is expected to bring about atrophy or marked depletion of cells in the spleen and thymus. Several groups of workers have in fact found that inhibition of the bursa of Fabricius in the embryo by steroid hormones brings about varying degrees of thymus atrophy (Warner et al. 1962b, Warner et al. 1964, Pierce et al. 1966). Thymus atrophy of the hormonally bursectomized chickens has been attributed to the effect of the steroid

hormone on the thymus. There is however no direct evidence on the inhibitory effect of the steroid hormones on the thymus and the possibility that deprivation of the thymus of the bursa cells and/or its humoral factor (if any) may bring thymus atrophy has not been ruled out.

In this experiment the bursa of Fabricius is surgically ablated on day 18th of incubation with the view to ascertain the part which the embryonic bursa plays in the cellular development of the thymus and spleen. Furthermore, the bursectomized chickens are immunized to sheep RBC to verify whether bursectomy in ovo on day 18th of incubation would completely abolish the humoral immune system which is responsible for production of specific antibody.

Brief Outlines of the Materials and Methods (detailed accounts of the materials and methods were given in the "General Materials And Methods").

Birds - Thornber 808 chicks were used in this experiment.

Bursectomy "In-ovo" - The fertile Thornber 808 eggs were put in the Westernette incubator. A random selection of embryonated eggs were taken out of the incubator on day 18th of incubation and the embryos were surgically bursectomized in ovo. The bursectomized embryos were again placed in the incubator until they hatched on the day 21st of incubation.

Antigen and Immunization Schedule - At eight weeks of age, the bursectomized chickens and the corresponding intact controls were each given an intravenous injection of 3×10^8 sheep RBC. The birds were bled on day 8 after the immunization and were killed 5 minutes after i.v. injection of 1 ml indian ink.

The birds were weighed immediately before they were killed. Spleen, bursa and all thymic lobes were carefully removed from the birds and were weighed. Spleen and thymus were then placed in

formol saline. Histological sections of these lymphoid tissues were cut and they were examined under the microscope.

Antibody to sheep RBC in the sera of the bursectomized and intact chickens was measured by haemagglutination test and serum IgG levels were determined by radial immunodiffusion test (Mancini test). The sera of both groups of birds were also analysed by immunoelectrophoresis.

RESULTS

Body Weights and Weights of the Lymphoid Organs - The body weights and weights of the lymphoid organs of the bursectomized and intact chickens are presented in table 7 .

Although the mean body weight of the intact chickens was higher than that of the bursectomized birds, the difference was not statistically significant ($P = 0{\cdot}30$). The difference between the spleen weights in these two groups of birds was also without statistical significance ($P = 0{\cdot}18$). The bursectomized chickens, however, were found to have very small thymic lobes and the difference between the weight of the thymus in bursectomized chickens with that of their corresponding control was statistically highly significant ($P = 0{\cdot}01$).

Antibody and IgG Levels in the Sera of Bursectomized and Intact Chickens

Table 8 shows the serum levels of haemagglutinin to sheep erythrocytes and the serum IgG levels.

A very low titre of haemagglutinin activity was detected in the sera of all bursectomized chickens. The haemagglutinin activity in the sera of the intact chickens was about 80 fold higher than that of the bursectomized chickens ($P = 0{\cdot}001$).

Immunoelectrophoresis showed the presence in the sera of bursectomized birds of precipitin arcs due to all classes of immunoglobulins. However, the quantitative determination of serum IgG by Mancini test showed that the level of this class of immunoglobulin in the sera of bursectomized chickens was three times lower than that of their

Table 7 - Body weights and weights of the lymphoid organs of the bursectomized and intact chickens at nine weeks of age.

Chickens	No. of Chickens	Body Weight (g) Mean & Range	Spleen Weight (mg) Mean & Range	Thymus Weight (mg) Mean & Range	Bursa Weight (mg) Mean & Range
Bursect-omized*	5	841·0 730-925	2303·4 1714-2786	3228·0 2560-4250	-
Intact	5	876·0 715-1020	2661·2 2031-3604	4890·8 3282-6014	3554·0 2434-4474

* These chickens were bursectomized <u>in ovo</u> on day 18th of incubation.

Table 9 - Numbers of germinal centres in four sections of the spleens of bursectomized and intact chickens 8 days after i.v. injection of 3×10^8 sheep RBC.

Chickens	No. of Chickens	Numbers of germinal centres in four sections of the spleen.	
		Mean	Range
Bursectomized*	5	18	0-47
Intact	5	164·8	36-369

* These chickens were bursectomized <u>in ovo</u> on day 18th of incubation.

corresponding intact controls ($P = <0.01$)

Table 8 - Antibody Titres and IgG Levels in the sera of bursectomized and intact chickens 8 days after a single i.v. injection of 3×10^8 sheep RBC.

Chickens	No. of Chickens	Reciprocal of antibody titres Mean & Range	IgG levels Mean & Range of the areas of the precipitin rings (mm^2)
Bursectomized*	5	5·6 4 — 8	78·96 39·34-113·41
Intact	5	460·8 256-512	233·20 200·96-254·34

* These chickens were bursectomized in ovo on day 18th of incubation.

Histological Findings

Spleen - In the spleen sections, the ellipsoids had been distinctively demarcated by the indian ink which was intravenously injected into the birds 5 minutes before they were killed. In the spleen sections of the intact birds, large numbers of macrophages bearing carbon particles were found in and around the ellipsoids as well as in the white pulp areas (Plate 12). Large numbers of germinal centres were found in the white pulp of the spleens of the intact birds (table 9). These germinal centres were free from carbon particles.

Spleen sections of the bursectomized chickens showed higher

Plate 12

UP, x 48

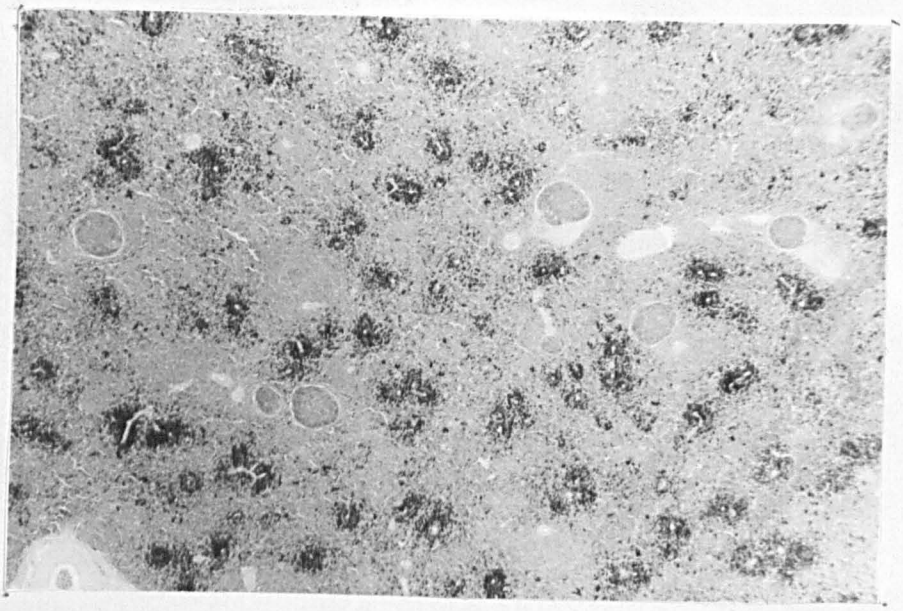
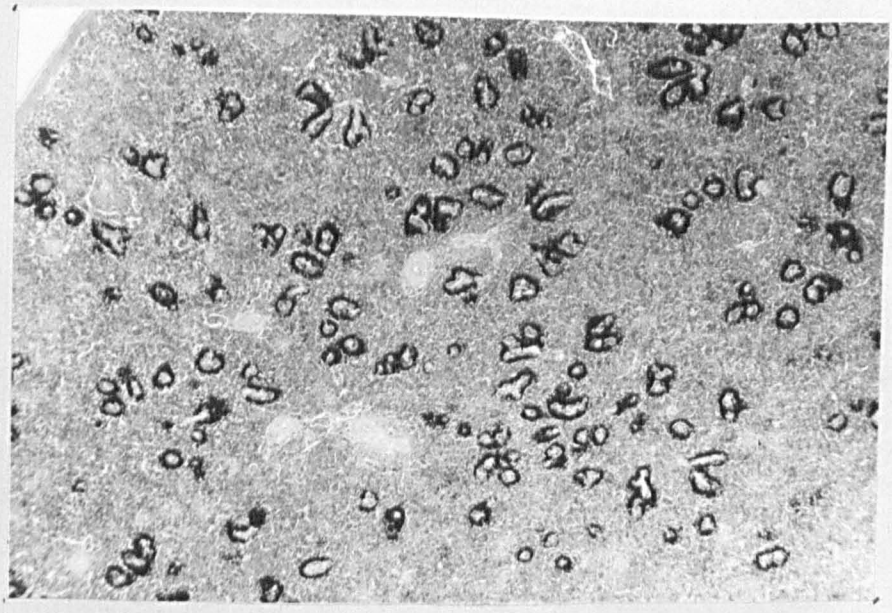


Plate 13

UP, x 48



Photomicrographs of sections of spleens of an intact chicken (plate 12) and a chicken which had been bursectomized <u>in ovo</u> on day 18th of incubation (plate 13). The chickens had been immunized with a single i.v. injection of 3×10^8 sheep RBC eight days previously and had been injected intravenously with indian ink 5 minutes before they were killed. Note large numbers of germinal centres in the plate 12 and total absence of these lymphoid nodules in the plate 13. The ellipsoids have been well demarcated by the carbon-bearing cells. Note that in the spleen section of the bursectomized chicken almost all carbon-bearing cells are confined to the peri-ellipsoidal zones, whereas in the spleen section of the intact control bird, large numbers of carbon-bearing cells are already in the white pulp strand.

concentration of the carbon particles in the ellipsoids, whereas the areas immediately around the ellipsoids as well as the white pulp strands were found almost free from carbon-bearing macrophages (Plate 13). Peri-ellipsoidal zones in the spleens of bursectomized chickens particularly were found markedly depleted.

In the spleen sections of 3 (60%) bursectomized birds, no germinal centre was found. However, in the spleen sections of the remaining bursectomized birds germinal centres were found in the white pulp strand with no apparent histological differences with those in the spleens of the intact birds. It was also quite evident that bursectomy in ovo on day 18th of incubation had substantially reduced the numbers of immature and mature plasma cells. However, these pyroninophilic cells were found scattered in the red pulp areas of all bursectomized chickens.

Thymus - The thymic lobules of the bursectomized chickens were found to be smaller than those of their corresponding controls. The medulla appeared particularly atrophied and depleted of cells (Plate 15 compare with Plate 14). Whereas there were large numbers of pyroninophic blast cells in the thymic medulla of the intact birds, these cells were totally absent from the medulla of the thymus of the bursectomized birds.

It was however quite obvious in the histological sections of the thymus, that there was also a depletion of lymphocytes in the thymus of the bursectomized chickens since pyroninophilic cells did not constitute a large proportion of the cells within the thymus of the control birds. It is thus unlikely that depletion of pyroninophic cells alone could cause a marked atrophy of the thymus in bursectomized chickens.

Summary - The effect of bursectomy on day 18th of incubation of chick embryo on cellular development of thymus, production of anti-

Plate 14

UP, x 42

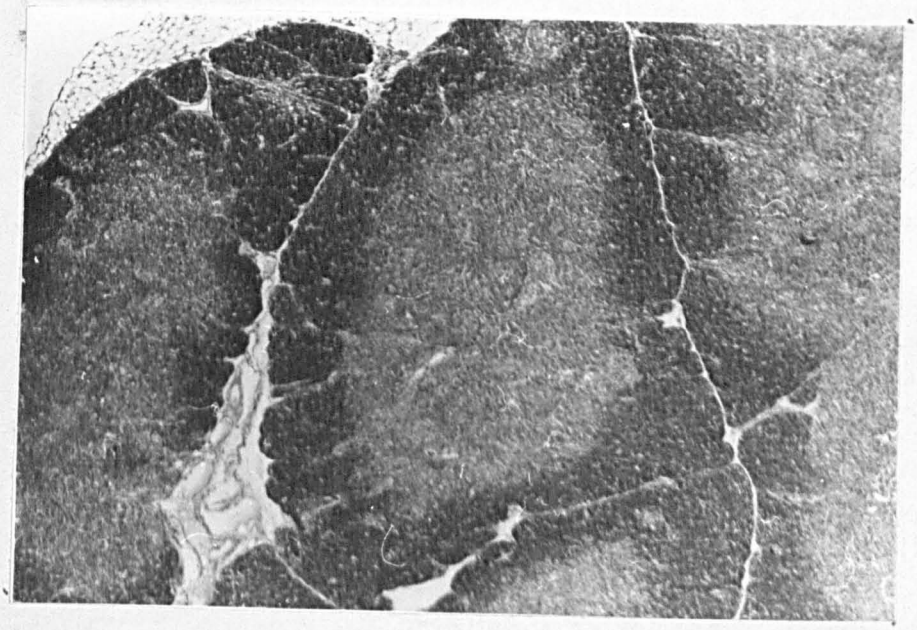


Plate 15

UP, x 42

Photomicrographs of sections of thymus of an intact chicken (plate 14) and of a chicken which had been bursectomized <u>in ovo</u> on day 18th of incubation (plate 15). The chickens had been immunized with a single i.v. injection of 3×10^8 sheep RBC eight days previously and had been injected intravenously with indian ink 5 minutes before they were killed. Note very small and atrophied thymic lobules of the bursectomized chicken in comparison to the large and well developed thymic lobules of the corresponding intact control chicken. Carbon-bearing cells are absent in the thymic lobules of both chickens.

body and immunoglobulin was investigated.

Bursectomy in ovo on day 18th of incubation brought about substantial reduction in the thymus weight. Histological examination of the thymic lobules revealed that both cortex and medulla had been markedly atrophied. Thymus atrophy in the bursectomized birds was found to be due to total absence of blast cells, plasma cells and partial depletion of lymphocytes.

Bursectomy in ovo on day 18th of incubation inhibited almost totally formation of germinal centres in the spleen but did not eliminate the entire population of plasma cells in this lymphoid organ.

Surgical ablation of the bursa in ovo on day 18th of incubation was found to suppress almost totally production of antibody to sheep RBC and reduced significantly circulating IgG levels.

These results suggest strongly that bursa of Fabricius contributes substantially to the cellular make up of the thymus. The findings in the spleen sections indicate that migration of cells from embryonic bursa to the spleen is well under way by day 18th of incubation. Furthermore, the results of this study provides additional evidence that development of the humoral immune system responsible for production of antibody to sheep RBC and IgG synthesis is bursa dependent.

Experiment 6 - Study on the Anatomical Relationship of the Umbilical Cord Residue with the Small Intestine and its Histological Structure in Bursectomized and Intact Chickens.

Introduction - The umbilical cord is a narrow stalk which connects the chick embryo with the yolk sac and includes all the germ layers:

an outer ectodermal layer, a double sheath of mesoderm and an endodermal lining. In addition it contains the blood vessels going to the yolk sac.

In the young and adult chickens although the yolk and the yolk sac disappear completely, part of the umbilical cord, however remains as a small vermiform appendix which is attached to the small intestine. The mammalian homologue of this umbilical residue is known as Meckel's diverticulum.

It has been shown that this vermiform appendix in chicken is composed mainly of lymphoid cells (Calhoun 1933). However, the relationships of the lymphoid cells of this appendix with the bursa of Fabricius has not been investigated. It is not known whether this appendix is populated with lymphocytes from the bursa of Fabricius in the same way as the peripheral lymphoid tissues such as spleen and caecal tonsils or the lymphoid cells of this appendix remains independent of the bursa of Fabricius.

This study was therefore undertaken to determine the relationship of this appendix with the small intestine and to investigate the cellular structure of this appendix in bursectomized and intact chickens with special reference to plasma cells and germinal centres.

Brief Outlines of the Materials and Methods (detailed accounts of the materials and methods were given in the "General Materials and Methods").

Birds - Thornber 608 chickens of both sexes were used in this study.

Post Mortem Examination - Day old and one week old chicks were killed with ether and nine weeks old chickens were killed with sodium pentabarbitone. A long mid line incision was made with scalpel in the skin and abdominal muscles. The skin and abdominal muscles on either sides were then laid open. The intestine was

exposed and it was disconnected from the mesentry, the gizzard and the cloaca by a pair of scissors. The intestine was then taken out from the body cavity and it was laid out on a flat surface in post mortem room. The small intestine was inspected for presence of the umbilical cord (in young chicks) or umbilical cord residue (in adult chickens) and their anatomical relationship with the yolk sac and small intestine was studied. In order to determine the exact position of the umbilical cord or umbilical cord residue in the small intestine, different parts of the small intestine in relationship to umbilical cord or its residue were carefully measured by a ruler.

In chicken, there is not a sharp demarcation between jejunum and ileum (Bradley, 1960). In this study, however, that part of the small intestine which lies between the doudenum and umbilical cord residue is referred to as jejunum and that part of the small intestine which lies between the umbilical cord residue and the beginning of large intestine is referred to as ileum.

The umbilical cord residue of nine weeks old chickens and that of their corresponding intact controls were cut for histological examination. These two groups of birds were those used in the previous experiment. One group consisted of 5 chickens which had been bursectomized in ovo on day 18th of incubation. Their controls consisted of 5 intact chickens. The chickens in both groups had each been given an i.v. injection of 3×10^8 sheep RBC eight days before they were killed.

RESULTS

Table 10 shows the measurements taken from the different segments of the small intestine, umbilical cord and umbilical cord residue. It appears from these results that the umbilical cord or its residue is attached to the small intestine almost mid way between

Table 10 - The length of the umbilical cord, umbilical cord residue (Meckel's diverticulum) and different segments of the small intestine in intact and bursectomized chickens.

Chicken	Age	No. of Chickens	Length of Duodenum mm (mean & range)	Length of jejunum mm (mean & range)	Length of umbilical cord or its residue* mm (mean & range)	Length of ileum mm (mean & range)
Intact	one day old	10	90·0 (70-110)	135·0 (120-160)	9·0 (8-10)	100·0 (90-115)
Intact	one week old	8	139·0 (130-150)	209·0 (200-225)	7·5 (6-8)	190·0 (160-210)
Intact	nine week old	5	223·0 (200-240)	402·0 (345-510)	5·5 (4-7)	399·0 (340-490)
Bursectomized	nine week old	5	200·0 (170-220)	380·0 (350-420)	4·0 (3-5)	376·0 (340-420)

* The figures in this column show the length of the umbilical cord in one day old and one week old chickens and in the case of nine weeks old chickens the figures in the column represent the length of the umbilical cord residue (Meckel's diverticulum).

the duodenum and large intestine.

The umbilical cord in day old and one week old chicks connects the yolk sac to the small intestine. The cord is a narrow stalk which is open at one end to the small intestine and at the other end to the yolk sac. (Plate 16)

In nine week old bursectomized and intact chickens, the yolk sac was not found in any bird. The umbilical cord residue in these birds appeared as a small blind vermiform appendix attached to the small intestine. The umbilical cord residue in nine week old birds was very much thicker and shorter than the umbilical cord in one day old and one week old chicks (plate 17,table 10).

Histological Findings - Histological sections of the umbilical cord residue in nine week old intact chickens showed structural similarity with that of the caecal tonsils. Beneath the outer serosa, a layer of circular and a layer of longitudinal smooth muscles respectively were found. The submucosa formed a thin layer. The lamina propria and the cores of the villi contained lymphoid cells - mainly small lymphocytes. Lieberkuhn glands with an irregular and variable sizes were embedded in the lymphoid tissues beneath the submucosa.

Germinal centres were found beneath the circular layer of muscles and between the Lieberkuhn glands (plate 18). These centres were packed with lymphocytes and blast cells. Large numbers of blast cells and plasma cells were present in the histological sections of the umbilical cord residue of the intact chickens.

In the histological sections of the umbilical cord residue of the bursectomized chickens, germinal centres were completely absent. Plasma cells and blast cells were also either totally absent or they had been substantially reduced by bursectomy in ovo on day 18th of incubation (plate 19)

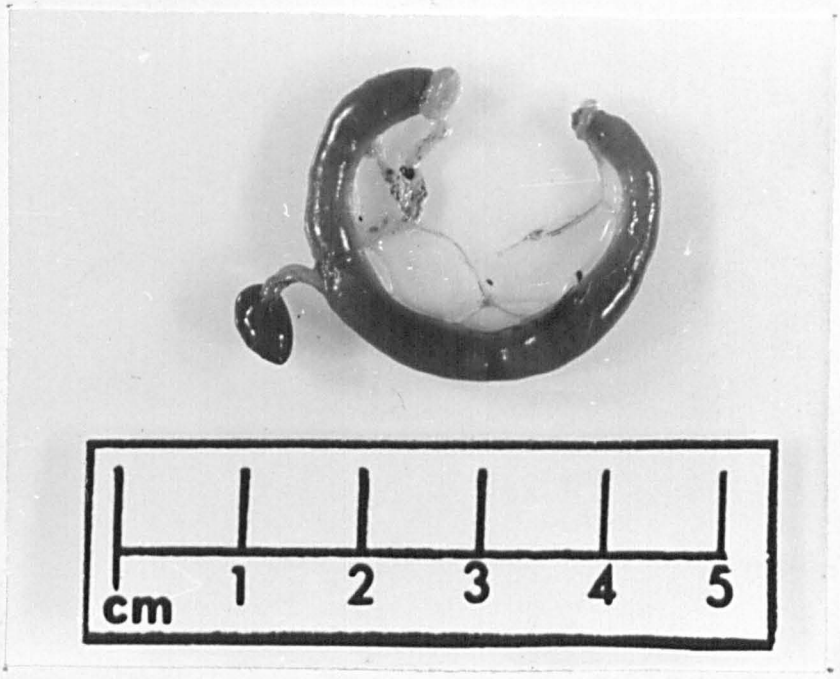


Plate 16 - The umbilical cord, yolk sac and part of the small intestine of one-week old chick. The umbilical cord of one-week old chick like that of the chick embryo connects the yolk sac to the small intestine and opens at one end into the yolk sac and at the other end into the intestine.

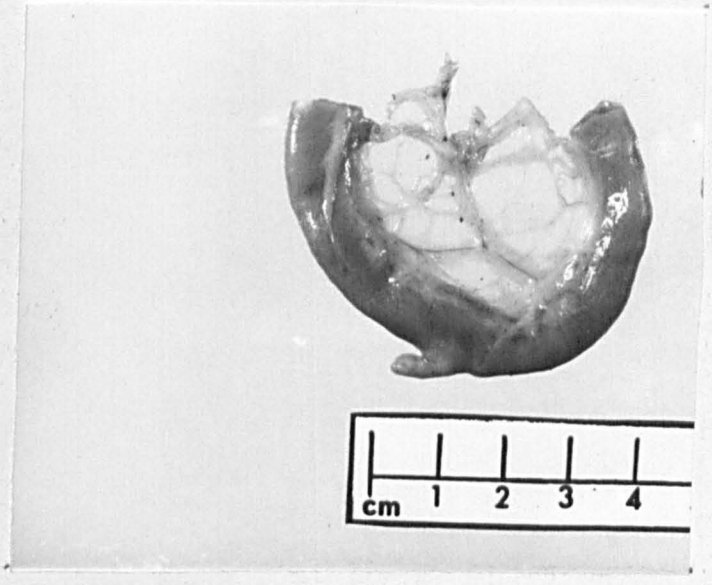


Plate 17 - The umbilical cord residue (Meckel's diverticulum) and part of small intestine of a nine-week old intact chicken. Note that the umbilical cord residue remains in chicken as a vermiform blind appendix which is attached to the small intestine.

PLATE 18

UP, x 102

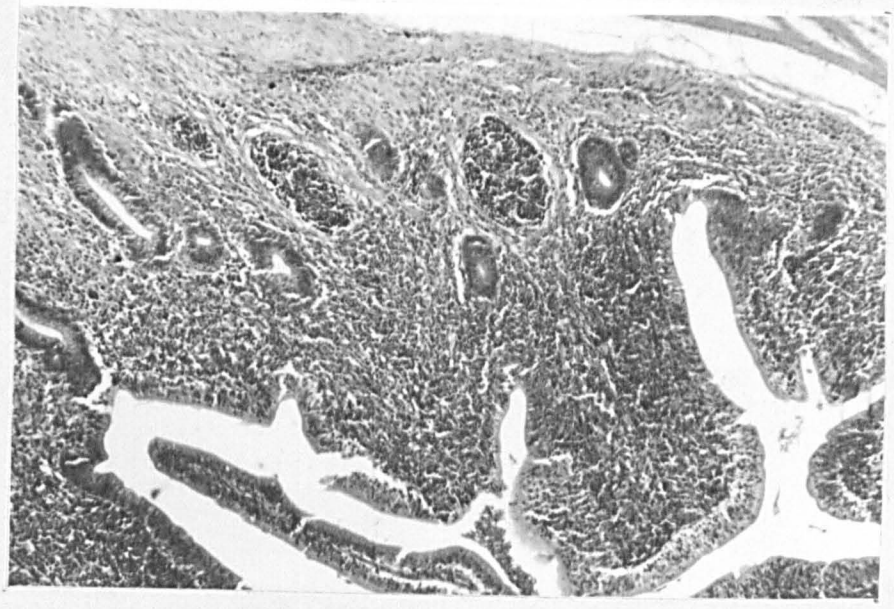
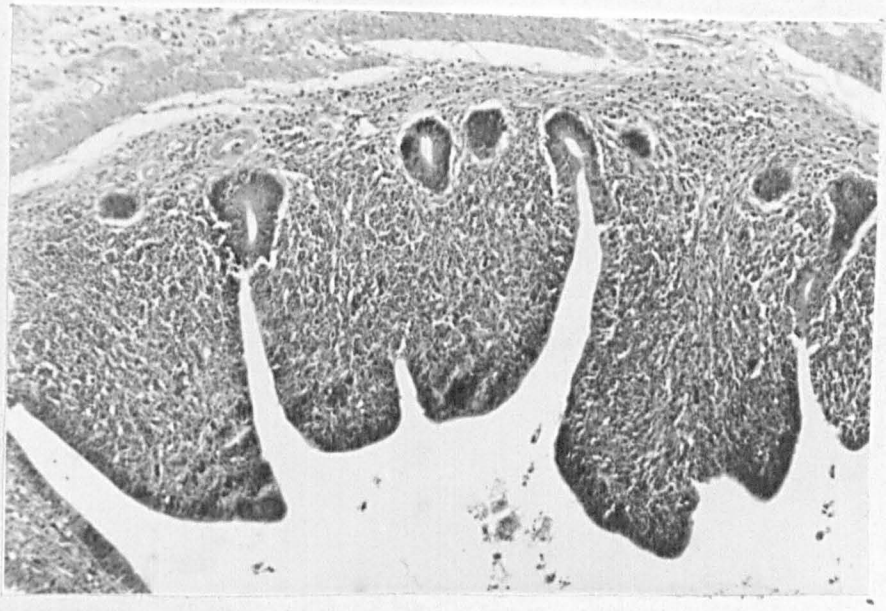


Plate 19

UP, x 102



Photomicrographs of longitudinal sections of the umbilical cord residues of an intact-chicken (plate 18) and a bursectomized chicken (plate 19) eight days after intravenous injection of 3×10^8 sheep RBC. There are two oval germinal centres between the Lieberkuhn glands in the longitudinal section of umbilical cord residue of the intact chicken. In the section of umbilical cord residue of the bursectomized chicken germinal centres are absent and there is depletion of plasma cell series of cells in the core of the villi.

Summary - The anatomical relationship of the umbilical cord residue with the small intestine and its histological structure in bursectomized and intact chickens were studied.

The umbilical cord residue was found as a vermiform blind appendix attached to the small intestine almost mid way between the duodenum and large intestine. Histologically this diverticulum resembles the caecal tonsils and it is composed almost entirely of lymphoid cells. Germinal centres were completely absent in the histological sections of the umbilical cord residue of the bursectomized chickens. Plasma cells were also either absent or they had been substantially reduced by bursectomy *in ovo* on day 18th of incubation.

This study revealed that the umbilical cord residue in chickens remains as a gut associated lymphoid organ in which its plasma cells and germinal centres are bursa dependent.

DISCUSSION

1 - Antibody Production in Bursectomized and Thymectomized Chickens.

One of the objects of the present study was to verify whether there is a bursa-independent humoral immune system in chicken which participates in antibody production. Some investigators (Lerner et al. 1971 and Bryant et al. 1973) found that bursectomy reduced production of antibody in chickens, nevertheless a low level of specific antibody was made by the immunized bursectomized birds. Accordingly these workers suggested that a system independent of the bursa of Fabricius was responsible for production of antibody.

The general approach to elucidate the role of the bursa of Fabricius in antibody production has been surgical ablation of this organ soon after hatching or its inhibition early in the embryo by administration of a steroid hormone such as 19 - nortestosterone. Such birds were challenged later in life with an antigen and their sera were tested for presence of specific antibody.

In the present study administration of a steroid hormone into the chick embryo as a measure to inhibit development of the bursa of Fabricius was dismissed as these hormones cause atrophy of the thymus (Warner et al. 1962b, Mueller et al. 1962 and Pierce et al. 1966). Neonatal bursectomy without subsequent whole body irradiation was also considered as inadequate for total elimination of bursa dependent lymphoid cells in the bird since by the time of hatching extensive perilipherazation of cells from the bursa has already occurred (Cooper et al. 1965) Neonatal bursectomy followed by whole body irradiation or surgical bursectomy performed in ovo on day 18th of incubation were the two methodsused in the present study.

It was shown clearly that chickens bursectomized neonatally and whole body irradiated were unable to produce specific antibody 8 days after a single i.p. injection of 'phage ØX174 at 6 weeks

of age (table 1). The bursectomized-irradiated chickens which were completely deficient in circulating antibody were found to have no bursa residue. Furthermore plasma cells and germinal centres were totally absent in the spleens, caecal tonsils and thymus of these birds (Plates 3 and 4). These results clearly indicate that in the absence of bursa of Fabricius there appears to be no lymphoid organ in chicken which contributes to the development of humoral immune response to 'phage ϕX174.

Some workers, however found that specific antibody was still made by the bursectomized chickens which appeared slowly 2 - 3 weeks after immunization (Bryant et al. 1973). The occurence of such delayed antibody production in bursectomized chickens was investigated up to 4 weeks after initial immunization. To potentiate production of antibody, the antigen (HSA) was inoculated i.m. with Freund's complete adjuvant in bursectomized-irradiated chickens at 4 weeks of age. The sera of the immunized birds were tested for presence of specific antibody with a highly sensitive test - the Farr test on days, 10, 15,21 and 28 after the initial immunization. The results presented in table 3 and Fig IV showed conclusively that bursectomized-irradiated chickens are completely unable to produce detectable amount of specific antibody as long as 28 days after immunization. This finding is taken to indicate that in the absence of bursa system in chicken, there is highly unlikely any organ which participates in production of antibody to this antigen.

Production of antibody in bursectomized chickens occurs usually in the chicken in which the organ itself has been ablated but the cells which had migrated from bursa to other sites in the body have not been eliminated. Cooper et al.(1966) showed that the effective method to eliminate the migrated bursa cells is to irradiate the neonatally bursectomized birds.

In the present study it was also shown that surgical ablation of the bursa of Fabricius on the day of hatching or on day 18 of incubation without whole body irradiation does not bring a total deficiency in the immunological system of chicken for production of a low level of circulating antibody to phage ΦX174 (table 1) or to sheep RBC was obtained (table 8). The fact that plasma cells and germinal centres were found in the lymphoid tissues of these bursectomized birds indicates clearly that bursa cells had migrated to other sites in the body before the extirpation of the organ. It is conceivable thus, these bursa-derived cells were the cells which responded to these antigens and produced the low levels of circulating antibody. Evidence on cell migration from bursa to other lymphoid tissues is presented in another chapter of this thesis.

Having established that in complete absence of bursa dependent lymphoid cells in chicken, detectable amount of specific antibody is not produced by other tissues, the possibility that the thymus may in fact participate in the humoral immune response with the cooperation of the bursa was next investigated. This investigation was carried out in thymectomized-irradiated chickens inoculated with 4 mg HSA in Freund's complete adjuvant. French, Stark and White (1970) showed that normal chickens inoculated intramuscularly with HSA in Freund's complete adjuvant produced an early phase of antibody response with a peak on day 12 followed by a rapid decline of circulating antibody on day 15. The circulating antibody level subsequently rose again, without further immunization, to a greater level and longer duration than that of the early antibody response. The fact that the bulk of antibody in the second phase was made in the epitheloid granuloma which formed at the site of inoculation suggests that thymus may contribute to the production of antibody in the second phase. There was however, no experimental evidence

on this point thus the present study was undertaken to clarify the part which thymus plays in cooperation with bursa in antibody production. The biphasic antibody response in chicken is primarily bursa dependent since bursectomized irradiated chickens inoculated i.m. with 4 mg HSA in Freund's complete adjuvant failed to produce detectable amount of antibody during and up to 28 days after the initial immunization (table 3 Fig.IV). Neonatal thymectomy did not abolish early or late phase of the biphasic antibody response. However, the antibody levels in the sera of the thymectomized irradiated chickens inoculated i.m. with 4 mg HSA in Freund's complete adjuvant were found to be considerably lower than those of their corresponding intact controls (table 4). These results suggested that thymus may cooperate with bursa in production of antibody to HSA.

The concept of cooperation of thymus with bursa in antibody production was further investigated in order to determine whether Mycobacterium tuberculosis was the main component of the adjuvant which evoked cooperation of thymus in antibody production. It appeared that omission of M. tuberculosis from the adjuvant reduces the antibody levels in the sera of the intact-irradiated chickens almost to the same levels as those in the thymectomized-irradiated birds inoculated with HSA in Mycobacterial adjuvant (table 6 Fig. VI). These results suggest strongly that cooperation of thymus with bursa in enhancing antibody production is induced by the Mycobacterium in the antigen-adjuvant mixture. A Similar finding has been reported in mice in which it has been shown that Freund's complete adjuvant potentiates antibody production with the cooperation of the T-lymphocytes (Allison et al. 1971b, Dresser 1972). Dresser showed that the lower the concentration of antigen, the greater is the dependence of the Freund's complete adjuvant on the cooperation of T-

lymphocytes. In the original study of French, Stark and White (1970) due to larger amount of Mycobacteria (5 mg) and smaller amount of HSA (40 µg) which were used for immunization of the birds the antibody level in the sera of the chickens inoculated with HSA in FCA was far higher than that of chickens given the same amount of antigen in water-in-oil emulsion. The thymectomized chickens used in the present study were not entirely free of T-lymphocytes as it was found that they all had a small thymus residue (table 5). It is conceiveble that complete elimination of the T-lymphoctyes might have brought greater suppression of antibody production in these birds.

In one experiment antibody production by the chickens immunized to HSA in Freund's complete adjuvant or in water-in-oil emulsion was followed up to 113 days after the initial inoculation of the antigen. There was no indication that the antibody production in the immunized birds would soon diminish as the antibody levels in the sera of all birds on day 113 of the primary immunization were considerably high. I.D. Aitken (personal communication) found that normal non-irradiated chickens given a single i.m. injection of BSA in FCA or in water-in-oil emulsion produced a high titre of specific antibody 18 months after the initial immunization. Herbert (1968) also found that mice given a single subcutaneous inoculation of ovalbumin in water-in-oil emulsion continued to produce antibody for at least a year. He showed that the long lasting antibody production is due to slow release of small quantities of the antigen from the site of inoculation over a long period of time. It has recently been shown (White 1973) that biphasic production of antibody in chicken is also due to slow release of the antigen from the site of inoculation as daily i.m. injection of HSA in saline produced a biphasic antibody response similar to that produced by chickens immunized with HSA in water-in-oil emulsion.

Serum Immunoglobulin levels in Chickens Bursectomized "In Ovo" or on Day of Hatch. - As seen in table 8 chickens bursectomized in ovo on day 18 of incubation were found at nine weeks of age to have serum IgG levels which were considerably lower than those of their intact controls. Reduction in the levels of circulating immunoglobulins of the bursectomized chickens has been found by several workers (Van Meter et al. 1968, Cooper et al. 1969, Aitken et al. 1972). Immunoglobulin deficiency of the bursectomized chickens thus is taken as an indication that production of immunoglobulin is restricted chiefly to a population of lymphoid cells which originates from the bursa of Fabricius. Production of immunoglobulin by the cells of the bursa of Fabricius has been shown in chick embryo on day 18 (Thorbecke et al. 1968) and on day 14 of incubation (Kincade et al. 1971). The serum immunoglobulin which was detected at nine weeks of age in chickens bursectomized in ovo is unlikely to be the immunoglobulin which formed by the bursa cells in situ before surgical ablation of this organ on day 18th of incubation. This is supported by the fact that the half-lives of IgG and Ig M in neonatally bursectomized chickens are 3·4 and 1·5 per day respectively and they would diminish rapidly (Frommel et al. 1970). There is growing evidence which suggests that bursa cells migrate to peripheral lymphoid tissues where they differentiate into plasma cells and participate in immunoglobulin synthesis (Linna et al. 1969, Hemmingsson et al. 1972a). Histological examination of the spleens of the chickens bursectomized on day 18 of incubation showed that this lymphoid organ was not entirely free from plasma cells. It appears, therefore, that by day 18 of incubation already migration of cells from the bursa to other sites in the body had begun. These bursa-derived cells are most probably the cells responsible for immunoglobulin synthesis in the bursectomized birds.

Whole body irradiation of the neonatally bursectomized chickens has been used by various workers to eliminate the cells which had migrated from the bursa to other sites in the body. This treatment effectively eliminates the plasma cells and deprives the birds of ability produce any detectable amount of specific antibody. Whole body irradiation of the neonatally bursectomized birds, however, does not bring a total deficiency of immunoglobulin synthesis (Alm et al. 1969, Ivanyi et al. 1969). The results of the present study are in accord with those of above-mentioned workers as it was found that bursectomized-irradiated chickens were totally deficient in circulating antibody to 'phage ΦX174 whereas immuno-electrophoresis of the sera of these birds at 6 weeks of age revealed that over 57% of the BX-IR birds had immunoglobulin of all classes (plate 1). It is conceivable that the cells which migrate from the bursa to the peripheral lymphoid tissues before hatching are not all killed soon after whole body irradiation of the neonatally bursectomized birds. Certain numbers of these cells probably survive for several weeks or longer and participate in immunoglobulin synthesis. Inability of the bursectomized irradiated chickens to produce specific antibody at 6 weeks of age, is therefore an indication that at this age all uncommitted B-lymphocytes had diminished. According to this view bursectomized irradiated chickens would also become totally deficient in circulating immumoglobulins at later age. There is indication that, in fact, total immunoglobulin deficiency awaits bursectomized irradiated chickens as it has been found that 59% of chickens neonatally bursectomized and whole body irradiated the day after operation showed complete lack of both IgG and IgM at 8 weeks of age (Van Meter et al. 1969).

Histological Findings in the Lymphoid Tissues of Chicken After Various Procedures on Surgical Ablation of the Bursa of Fabricius - Neonatal bursectomy followed by whole body irradiation caused a

cellular depletion in the lymphoid tissues which was very marked in the red pulp and periellipsoidal zones of the spleen, in the medulla of the thymus and in the villi of the caecal tonsils. The cell depletion was apparently due to the total absence of plasma cell series of cells and germinal centres (plates 3 and 5). There is ample evidence which suggests that plasma cells as well as blast cells of the germinal centres are descendants of the lymphocytes of the bursa of Fabricius. Studies on lymphocyte migration in chicken, has shown that bursa cells migrate to the spleen, caecal tonsils and the thymus (Linna et al. 1969 Hemmingsson et al. 1972a). Plasma cells are consistently absent within the follicles of the bursa of Fabricius. However, the finding that neonatal ablation of the bursa of Fabricius results in depletion of plasma cells in the spleen and other lymphoid tissues is taken as an indication that the cells which migrate from bursa to other lymphoid tissues transform into immature and mature plasma cells.

Neonatal bursectomy without whole body irradiation reduced but did not eliminate all plasma cells and germinal centres in the peripheral lymphoid tissues. Bursectomy in ovo on day 18th of incubation brought about even greater depletion of plasma cells and germinal centres in the spleen and thymus than neonatal bursectomy. These results strongly suggest that migration of cells from the bursa to other sites in the body is well advanced before hatching. Histological examination of the spleens of chickens bursectomized in ovo on day 18th of incubation showed that some bursa cells had populated this lymphoid organ before day 18 of incubation as indicated by the presence of small numbers of plasma cells and germinal centres (plate 13 and table 9).

Bursectomy in ovo on day 18 of incubation brought about far greater depletion of cells from the thymus than the spleen.

Depletion of plasma cells and germinal centres in the spleens of these bursectomized birds did not cause a significant reduction in the weight of this organ. However, the thymus of the chickens bursectomized *in ovo* on day 18th of incubation showed complete absence of plasma cells and germinal centres, a very pronounced atrophy of the medulla and cortex and a significant reduction in the weight of this lymphoid organ at 9 weeks after hatching (plate 15 and table 7). Atrophy of the thymus has also been found in chickens treated with steroid hormones during the embryonic period (Warner *et al.* 1962, Warner *et al.* 1964, Pierce *et al.* 1966). The present findings on the thymus atrophy of the chickens bursectomized surgically *in ovo* on day 18 of incubation suggests strongly that normal development of the thymus depends partly on the bursa of Fabricius. It is conceivable that atrophy of the thymus of the chickens bursectomized *in ovo* surgically or by treatment *in ovo* with steroid hormones is due to deprivation of thymus, during the embryonic period, from the cells and/or humoral factor of the bursa of Fabricius.

The thymus of the chicks bursectomized *in ovo* on day 18th of incubation consistently showed absence of plasmablasts and plasma cells especially from the medulla. These pyroninophilic cells, however, did not constitute a large proportion of the cells within the thymus gland of the corresponding intact controls. Thus it is suggested that atrophy of the thymus of the chickens bursectomized *in ovo* on day 18th of incubation is due to absence within this lymphoid organ of the pyroninophilic blast cells and plasma cells as well as a population of lymphocytes. Presence within the thymus of a high population of bursa-dependent cells has also been shown by Zucker *et al.* (1973). These workers found that neonatal bursectomy reduced the total lymphocytes of the chicken thymus by 15% at 16

weeks of age.

It should be mentioned that neonatal bursectomy coupled with whole body irradiation also eliminates plasmablasts and plasma cells from the thymus but does not bring about a marked atrophy of the thymus (plate 5). It appears therefore that bursa of Fabricius contributes to the cellular development of the thymus particularly during the embryonic period.

The contribution of the bursa of Fabricius to the cellular development of the umbilical cord residue (Meckel's diverticulum) had not been investigated previously. In the present study, this diverticulum was found histologically very similar to that of the caecal tonsils. Absence of plasma cells and germinal centres in the Meckel's diverticulum of the bursectomized chickens indicates strongly that this vermiform appendix, like caecal tonsils is also populated with the cells of the bursa of Fabricius. This view is substantiated by the demonstration that labelled bursa cells home in this gut associated lymphoid tissue after in situ labelling of the bursa with 3H-thymidine. This evidence will be presented in part two of this thesis.

Histological Changes in the Lymphoid Tissues of Chickens After Neonatal Thymectomy - Neonatal thymectomy followed by whole body irradiation did not suppress proliferation of plasma cells and formation of germinal centres in the spleen. However, spleens of the thymectomized-irradiated chickens showed considerable cell depletion affecting only the lymphocytes. In the spleen, there was histological evidence of lymphocyte depletion in the peri-arterial sheaths of the white pulp (plate 10). There has been a number of studies which demonstrate migration of cells from the thymus to the spleen (Linna et al. 1969, Hemmingsson 1972). Further evidence on migration of cells from the thymus to the peripheral lymphoid tissues will be

presented in part two of this thesis. It is therefore suggested that cell depletion in the spleens of thymectomized-irradiated chickens is due to absence within this lymphoid organ of a population of lymphocytes which originate from the thymus. Neonatal thymectomy cuts off migration of thymocytes to the spleen, consequently certain specific areas within this organ remain depopulated. This view is substantiated by the fact that thymocytes labelled in vitro with 3H - adensosine home preferentially in the areas of the spleens of the intact chickens which correspond to the depleted areas in the spleens of the neonatally thymectomized chickens.

There was apparently no histological evidence of cell depletion in the bursa of the chickens thymectomized on the day of hatching. This finding is taken to indicate that the bursa of Fabricius of young birds is not populated by the thymic lymphocytes. Studies on migration of cells from the thymus to other lymphoid tissues have in fact shown that thymic cells do not home to the bursa of Fabricius in significant numbers (Hemmingsson, 1972).

It should be mentioned that neonatal thymectomy in chicken is a difficult operation. It is particularly difficult to remove the two thymic lobes which are deeply located in the upper part of the thoracic cavity. The thymectomized-irradiated chickens which were used in the present study were all found at post mortem examination to have thymus residue (table 5). It is likely therefore that the spleens of these thymectomized-irradiated chickens showed only a partial cell depletion.

Histological Findings in the Granuloma - There was complete absence of plasma cells in the epitheloid-granuloma which formed in the pectoral muscles of the bursectomized-irradiated chickens after i.m. injection of HSA in FCA (plate 7). Neonatal thymectomy followed by whole body irradiation did not suppress proliferation

of plasma cells in the epitheloid granuloma but inhibited infiltration of epitheloid cells, multinucleated giant cells and lymphocytes in the granuloma which formed at the site of inoculation of HSA in FCA (plate 11).

It appears therefore that in chickens i.m. inoculation of HSA and M. tuberculosis suspended in water-in-oil emulsion (FCA) bring to the site of inoculation several populations of cells which are mobilized either by the bursa of Fabricius or by the thymus gland.

The findings that formation of the epitheloid-granuloma in the pectoral muscles of the immunized BX-IR and TX-IR chickens was considerably inhibited can be taken to indicate that it is basically the lymphoid tissue of the immunized animal which is responsible for formation of the epitheloid-granuloma. The stimulus for formation of this type of granuloma is therefore immunological. It is likely therefore that the infiltration and proliferation of cellular elements of humoral and cell-mediated immunity in the epitheloid-granuloma can enhance considerably the severity of the immunological reactions to the test antigens. Several groups of workers have in fact found that there is a correlation between the size of the granuloma and the severity of the delayed hypersensitivity reaction to the test antigen in the granuloma (Wilkinson & White 1966, Wilkinson 1966, Reid & McKay 1967, Aiyedun 1971). Similarly, epitheloid-granuloma has been shown to contribute to the production of specific antibody. Thus White, Coons & Connolly (1955) demonstrated large numbers of antibody-containing cells in the granuloma. French, Stark and White (1970) who studied the biphasic production of antibody in chickens immunized to HSA in FCA found that the bulk of antibody in the second phase of the antibody response came from the granuloma which formed at the site of inoculation. In the present study, histo-

logical findings in the granuloma which formed at the site of inoculation of HSA in FCA in IN-IR and TX-IR chickens also suggest that the large numbers of the pyronin positive plasma cells in the granulomas of these two groups of birds are most likely responsible for production of antibody to HSA.

The cellular elements of the granuloma which formed at the site of inoculation of HSA in FCA consisted of both lymphoid and non-lymphoid cells. The non-lymphoid cells of the granuloma consisted of macrophages, epitheloid cells and multinucleated giant cells. It is not clear whether the bursa and the thymus are also responsible for infiltration and proliferation of non-lymphoid cells of the granuloma. This aspect of the granuloma can be studied in chickens in which both the thymus and the bursa of Fabricius have been neonatally ablated.

It has been shown that M. tuberculosis has a chemotactic action on macrophages (Wilkinson et al. 1973). Wilkinson and his co-workers have suggested that the chemotaxtic factors may play an important part in recruiting cells from the circulation to the area in which mycobacterial adjuvant has been deposited. It is likely that migration of the macrophages from the site of inoculation of antigen-adjuvant mixture is then inhibited by soluble factors of the lymphocytes. Production of migration inhibition factor (MIF) by the lymphocytes which prevent migration of macrophages has been shown in several species (Reviewed by Pick and Turk).

CONCLUSIONS

The immunological functions of the bursa of Fabricius and the thymus gland were studied with special reference to two immunological parameters: circulating antibody and cellular elements of the lymphoid tissues. The following conclusions have been reached.

1. Development of immunological system necessary for production of antibody to 'phage ϕX174, HSA and sheep RBC in chicken is exclusively bursa dependent.

2. In the chicken, cooperation of thymus is essential for the adjuvant effect of mycobacterium tuberculosis in water-in-oil emulsion in order to induce formation of a higher amount of antibody than that produced by chickens immunized to HSA in water-in-oil emulsion.

3. The bursa of Fabricius contributes to the development of plasma cells and germinal centres in the spleen, caecal tonsils, Meckel's diverticulum and the thymus. It is accepted that lymphocytes of the bursa of Fabricius migrate to the peripheral lymphoid tissues and the thymus where they transform into blast cells (plasmablasts) and plasma cells. Histological evidence indicates that migration of cells from the bursa to other sites in the body is well under way in the chick embryo. This migration begins before day 18th of incubation.

4. The thymus gland contributes to the development of the lymphocytes in the peri-arterial sheaths of the white pulp of the spleen. It is accepted that thymocytes migrate from the thymus to the white pulp of the spleen.

5. In the chicken thymus, the plasmablasts, plasma cells and probably a sub-population of lymphocytes are bursa dependent.

6. The bursa of Fabricius and the thymus gland both contribute to the cellular development of the granuloma which forms in chicken at the site of inoculation of HSA in Freund's complete adjuvant.

Thus, the plasma cell series of cells in the granuloma are bursa dependent. The small lymphocytes and the epitheloid and giant cells in the granuloma are the cells which appear to be thymus dependent.

7. The umbilical cord residue (Meckel's diverticulum) remains in the young and adult chickens as a vermiform appendix attached to the small intestine mid way between the jejunum and ileum. It is composed almost entirely of lymphoid cells. Histologically, this gut-associated lymphoid tissue is very similar to caecal tonsils. Its plasma cells and germinal centres are bursa dependent.

PART II

MIGRATION OF CELLS FROM THE BURSA OF FABRICIUS AND THE THYMUS TO OTHER SITES IN THE BODY.

One of the early studies which led to the concept of cell migration from the bursa of Fabricius to other sites in the body was that of Mueller et al. (1960). Mueller and his co-workers found that chickens bursectomized at one week of age produced significantly less antibody than their intact controls, whereas bursectomy later in life did not bring about such a marked suppression in the antibody response. These results were interpreted in terms of a progressive migration of cells from the bursa to other lymphoid tissues during the first few weeks of life. They suggested that the bursa of Fabricius is not a site for antibody production but a place where lymphoid cells are sensitized prior to migration to other parts of the body (Mueller et al. 1960, 1962). The finding that neonatal bursectomy depletes the lymphoid tissues of plasma cells and germinal centres provided additional support for the concept of cell migration from the bursa to the peripheral lymphoid tissues (Carey et al. 1964, Isakovic et al. 1964, Cooper et al. 1965). There was also an indication that the thymus gland populates spleen and possibly other lymphoid tissues with lymphocytes since neonatal thymectomy resulted in depletion of lymphocytes from the white pulp of the spleen (Cooper et al. 1966).

Direct evidence on transport of cells from the bursa and the thymus to the peripheral lymphoid tissues came from the studies by several groups of workers using radio-isotope tracing techniques. One of the earliest studies on cell migration in chickens involved in vitro labelling of the bursa and thymus cells from 2 - 3 months old chickens with 3H - uridine and transferring the labelled cells into two-week old allogeneic chickens. Both labelled bursa and thymus cells were found to home selectively in

the spleen (Warner 1965). It was shown later that the bursa cells labelled *in vivo* with *3H-thymidine* migrate to the spleen as well as to the thymus and caecal tonsils. Within 48 hours after *in vivo* labelling of the bursa cells, the labelled cells were found mainly in the white pulp of the spleen but not in the germinal centres (Linna *et al.* 1969).

The question of whether migration of cells from the bursa of Fabricius to other lymphoid tissues continues in the young as well as in the adult chickens was investigated by Hemmingsson et al. (1972a). The bursa cells were labelled *in vivo* with *3H - thymidine* and the birds were killed 48 hours later. Autoradiographs of the lymphoid tissues showed that transport of cells from the bursa to the spleen, thymus and caecal tonsils occurred in newly hatched, 9 day old and 6 week old chickens, but not in 14 week old chickens. In the 6 week old birds, the labelled bursa cells were found in the medulla of the thymus and among the pyronin-positive cells in the red pulp of the spleen. The labelled cells were not found in the germinal centres of the spleen or in the bone marrow. Similar study on thymic cell migration (Hemmingsson 1972) showed that thymus cells migrate to the spleen, caecal tonsils and bone marrow in 9 day old chickens. The transport of cells from the thymus to the spleen continued in 6 week old chickens but the rate of cell migration decreased in the older chickens. In the spleen, the labelled cells were found between the red and white pulp. No labelled cell was found in the germinal centres of the spleen or in the bursa of Fabricius.

The experiments which follow were designed to add further evidence on migration of cells from the bursa and thymus to other sites in the body. In particular, one of the main objects of the present

study is to determine whether the labelled bursa and thymus cells would home preferentially in the areas of the spleen corresponding to the depleted areas in the spleens of the bursectomized and thymectomized chickens. Furthermore, the distributions of the bursa and thymus cells in different micro-compartments of the spleen is determined with the view to ascertain whether the distributions of these two populations of lymphocytes differ in the spleen.

Experiment 1 - The Fate of Bursa cells Labelled *in vivo* with *3H - thymidine*.

In this experiment, the bursa of Fabricius is labelled *in situ* with *3H - thymidine*. The fate of those bursa cells which take up the radio-isotope *in vivo* and migrate to other lymphoid tissues will be investigated by the technique of autoradiography.

Studies which have been carried out on transport of radio-isotope labelled cells to the spleen have consistently failed to demonstrate presence of labelled cells within the germinal centres of the spleen (Linna *et al*. 1969, Miller III, 1969, Hemmingsson *et al*. 1972a). Since germinal centres are lymphoid nodules which begin to form three days after antigenic stimulation (White, 1963) it was decided to immunize some of the birds with HSA two days before *in situ* labelling of the bursa to determine whether immunization would induce formation of new germinal centres in the spleen with labelled bursa cells part of their cellular composition.

Brief Outlines of Materials and Methods (detailed accounts of the materials and methods were given in the "General Materials and Methods").

Birds - Thornber 808 chicks were used at the age of five weeks.

Antigen and Immunization Schedule - 5 chicks were immunized with intravenous injection of 5 mg HSA 48 hours before in situ labelling of the bursa of Fabricius. There are also 4 chicks in this experiment which were not immunized and serve as non-immunized controls.

"in-vivo" Labelling of the Bursa Cells - The bursa was exposed through an incision mid way between the cloacal vent and the base of the tail. A volume of 0.1 - 0.2 ml of PBS containing 40 UCi of 3H - thymidine was injected slowly into the substance of the bursa. To prevent the re-utilization of the breakdown products of the labelled cells, 0.5 ml unlabelled thymidine solution containing 0.5 mg thymidine was flushed immediately over the surface of the bursa and 2.5 mg of the unlabelled thymidine was also injected intraperitoneally into the bird.

Histology and Autoradiography - The birds were killed 24 hours after in situ labelling of the bursa. Spleen, thymus, bursa, caecal tonsils and umbilical cord residue (Meckel's diverticulum) were removed from each bird. These lymphoid organs were fixed in formol saline and histological sections were cut with microtome. The sections were coated with Ilford photographic emulsion. The coated sections were exposed for 4 weeks at 4°C. They were then developed, fixed and stained through the emulsion by the method of Unna Pappenheim.

RESULTS

Bursa of Fabricius - Autoradiographs showed that the bursa which had been injected with 3H - thymidine had preserved its normal cellular architecture and it was free from necrosis. Labelled cells were sharply localized to the areas of injection. They included cells in the cortex and medulla as well as scanty epithelial cells and cells in the sub-epithelial connective tissue.

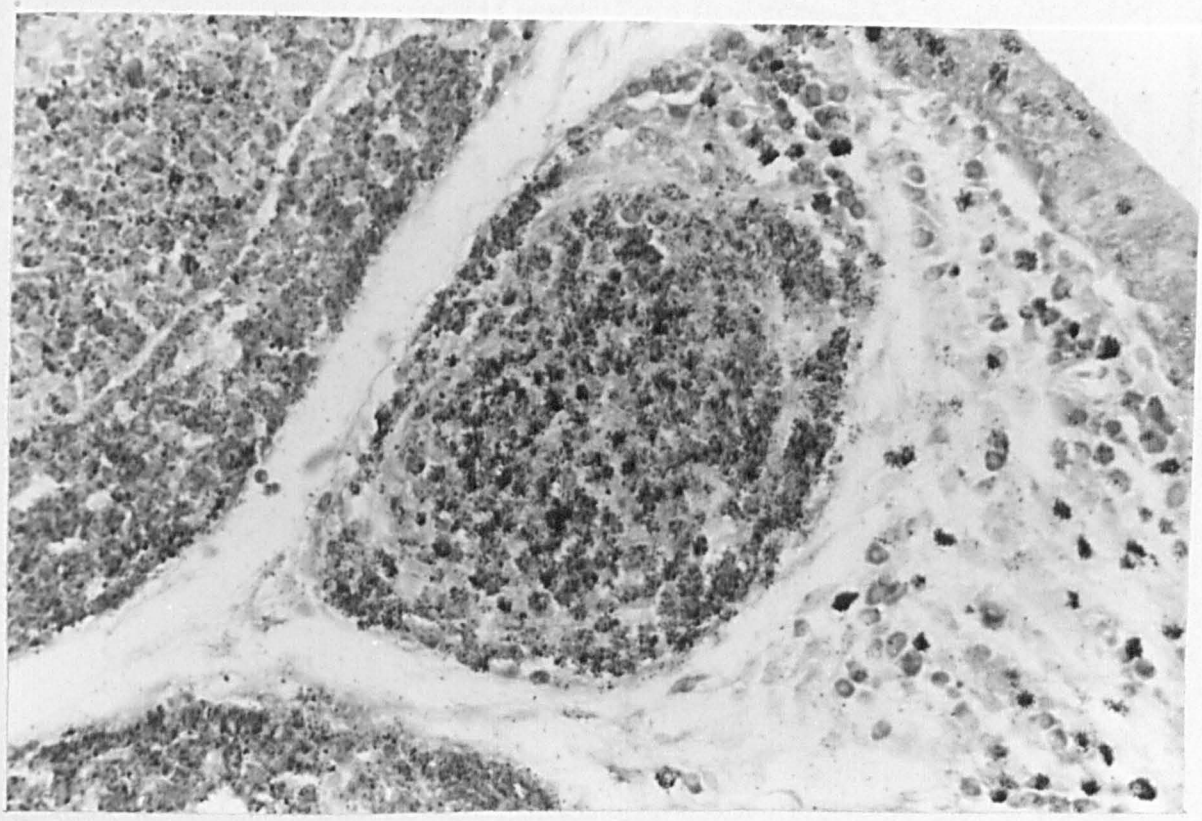


Plate 20 - Photomicrograph of the section of the bursa 24 hours after *in situ* labelling of this lymphoid organ with ^{3}H-thymidine. Large numbers of cells in the medulla and cortex of the follicles have been labelled. Some of the cells in the tunica propria (top right) have also been labelled. Note also that there are large numbers of heavily labelled cells in the area between the tunica propria and the bursa follicles. The cells in this area are mainly blast cells (plasmablasts). UP X600

A lesser proportion of the cells in the medulla than in the cortex had been labelled. The medulla contained a higher number of large lymphoid cells with a lower grain count per cell than those of the cortical small lymphocytes (plate 20).

Autoradiographs of the small intestine which were prepared at the same time in order to find out the possibility of re-utilization of the 3H - thymidine showed no evidence of uptake of the radio-isotope by the epithelial cells.

Spleen - Autoradiographs of the spleens were surveyed under the microscope to determine the location of the labelled bursa-derived cells in each of the five splenic micro-compartments: red pulp and white pulp including the ellipsoids, peri-ellipsoidal zone and germinal centres. It should be mentioned that labelled cells could not always be located exactly into these zones. The peri-ellipsoidal zone, in particular was sometimes difficult to define. It was better delineated in that part of its extent which is contiguous with the red pulp than the white pulp. It was also found that the width of this zone differed markedly in the spleen sections of different birds.

As seen from table 11 large numbers of labelled cells were found in the spleen of birds given an intra-bursal injection of 3H - thymidine. Prior administration of antigen did not appear to increase the total numbers of labelled bursa cells found in the spleen sections or alter the pattern of their distribution.

A major proportion of the labelled cells were found within the peri-ellipsoidal zone (plate 21). Lesser numbers of labelled cells were found in the red pulp and white pulp areas. Very few lightly labelled cells were found within the outline of the germinal centres.

Thymus - Labelled bursa cells were found mainly in the medulla of

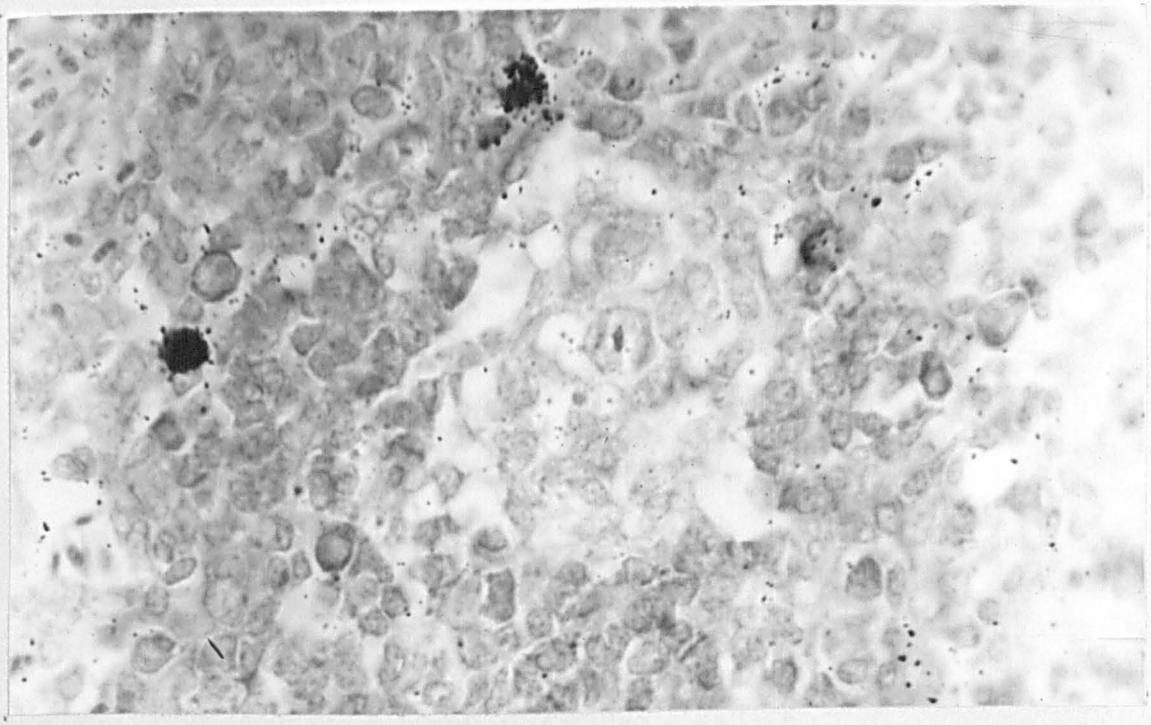


Plate 21 - Photomicrograph of section of the spleen of chicken 24 hours after *in situ* labelling of the bursa of the same chicken with ^{3}H-thymidine. The chicken had also been immunized with i.v. injection of 5 mg HSA seventy two hours previously. Note the heavily labelled bursa-derived cells in the peri-ellipsoidal zone of the spleen. UP, X1140

the thymic lobules. Scanty labelled cells were also present at the cortico - medullary junction.

Caecal Tonsils and Umbilical Cord Residue - Moderate numbers of labelled cells were found in the sections of the tonsilla caecalis and umbilical cord residue. These cells appeared in the diffuse lymphoid tissue of the lamina propria and sub-mucosa. No labelled cell was seen in any germinal centre.

Table 11 - Distribution of Labelled bursa cells in different lymphoid organs 24 hours after in situ labelling of the bursa with 3H - thymidine. The chicks marked with * had been given an i.v. injection of 5 mg HSA 72 hours previously. The figures in this table show the total number of labelled cells counted in individual sections.

Chick No	Spleen RP	Spleen PEZ	Spleen E	Spleen WP	Caecal Tonsil	Um. Residue	Thymus Cor	Thymus Med
*1	0	12	0	8	10	1	-	-
*2	4	4	0	7	4	-	0	9
*3	11	17	2	5	7	-	0	21
	15	25	3	9				
*4	29	57	0	18	14	4	0	8
*5	30	52	0	21	49	-	0	32
6	17	88	0	12	35	10	4	8
7	27	97	0	31	25	5	1	11
8	40	88	5	31	20	-	0	24
9	28	33	0	13	21	-	0	11

RP = Red pulp
PEZ = peri-ellipsoidal zone
E = Ellipsoid
WP = White pulp
cor = cortex
med = medulla
um = Umbilical cord

Summary - This experiment is summarized with next experiment on page 110

Experiment 2 - The Fate of Bursa cells and Thymus cells labelled *in vitro* with *3H - adenosine*.

In this experiment, the migration of both bursa cells and thymus cells to various lymphoid organs of the chickens are investigated after intravenous injection of either labelled bursa cells or labelled thymus cells. These two populations of lymphocytes differ from each other with regard to their origins as well as functions. In order to determine whether they also show different distribution patterns in the spleen, the migration of these two populations of cells are investigated under similar experimental conditions. Thus both populations of cells are labelled with the same isotope, using the same technique. The birds are injected intravenously with their own bursa or thymus cells which had been labelled *in vitro*. They are then killed at different time intervals after i.v. injection of the labelled cells. It is clear that in this experiment, the two groups of birds can each serve as a control group as well as experimental group.

Brief Outlines of the Materials and Methods (detailed accounts of the materials and methods were given in the "General Materials and Methods").

Birds - Thornber 808 chicks were used at the age of two weeks.

Radio-isotope Labelling of the Bursa and Thymus Cells - Bursa and thymus cells were labelled *in vitro* with *3H - adenosine*.

The whole bursa was removed from each bird and bursa cell suspension was prepared from the organ as described in details in the General Materials and Methods. In another group of birds,

five to six thymic lobes were removed from each bird and thymic cell suspension was similarly prepared using the thymic lobes.

Each cell suspension was then incubated with 5u uci 3H-adenosine at a concentration of 1u UCi/ml. After incubation, the cells were washed in Eagle's medium to remove any amount of 3H-adenosine which had not been utilized by the cells. The viability of the cells was determined by trypan blue exclusion method. The labelled cells were then injected intravenously into the birds in volume of 0·1 - 0·5 ml. Each bird was injected into the wing vein its own bursa or thymus cells.

Histology and Autoradiography - The birds were killed at different time intervals after intravenous injection of labelled bursa or thymus cells (table 12). Spleen, thymus, bursa (where applicable), caecal tonsils, liver and lung were removed from each bird. These organs were fixed in formal-saline and histological sections of these organs were cut with microtome. The sections were coated with photographic emulsion. The coated sections were exposed for 4 weeks at 4°C. They were then developed, fixed and stained through the emulsion with UP.

RESULTS

1 - The Fate of 3H - adenosine Labelled Bursa Cells After Intravenous injection. One chicken died during the intravenous injection of the bursa cells (chick No.10). Autoradiographs of the various lymphoid organ of this bird revealed that large numbers of the labelled cells were in the lung. Labelled cells were also present in the spleen at this early time. The majority of the labelled cells were in the red pulp, but a considerable number (28%) were either in the ellipsoid's lumen or among the cells immediately surrounding the lumen. Moreover, it was found that the cells present in the ellipsoid's lumen or among the cells

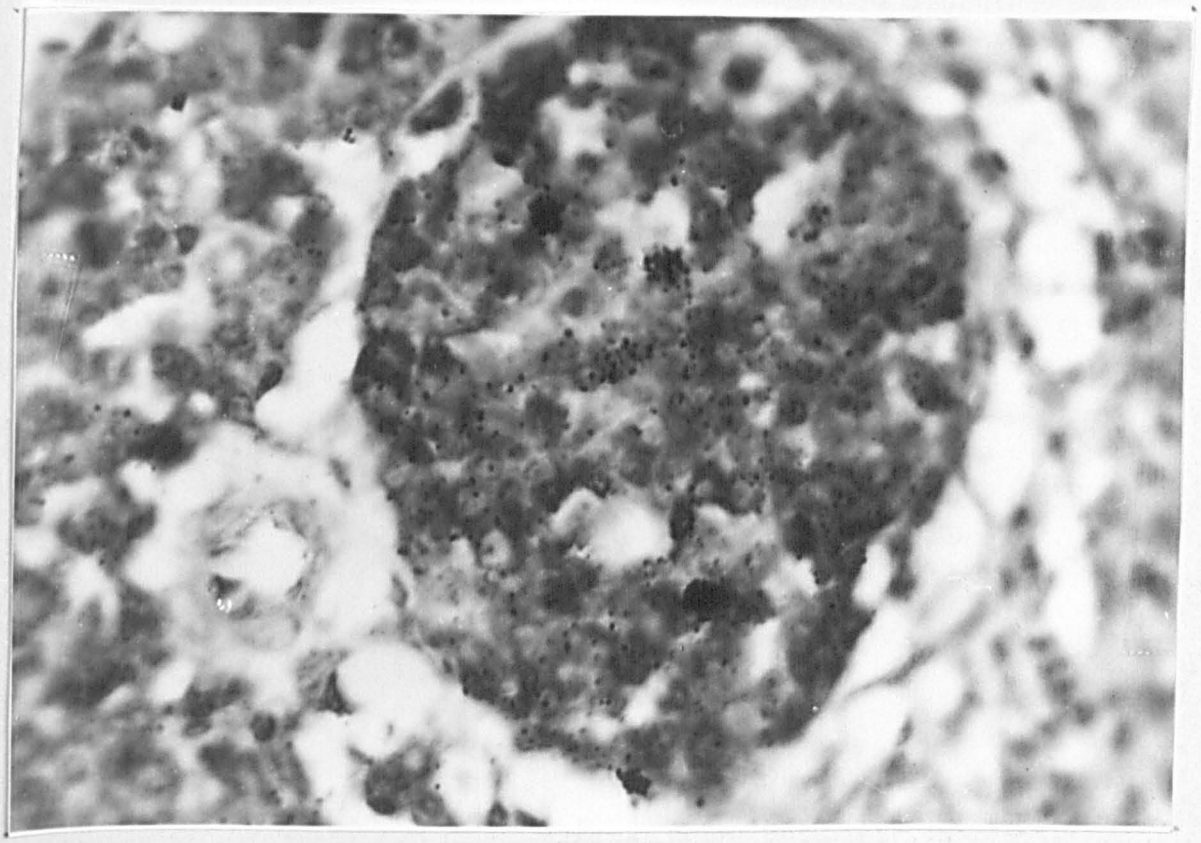


Plate 22 - Photomicrograph of section of the chicken spleen 24 hours after i.v. injection of ^{3}H-adenosine labelled autologous bursa cells. Four heavily labelled bursa cells have taken part in formation of a germinal centre in the white pulp of the spleen

UP, X1140

of the ellipsoid wall were much smaller than the majority of the labelled cells in the red pulp.

Autoradiographs of the birds which were killed one hour after i.v. injection of the labelled bursa cells showed that large numbers of labelled cells were still present in the liver and lung, very few in the thymus or caecal tonsils. In the spleen, most labelled cells were present in the red pulp, although in one or two birds a small percentage were already found in the peri-ellipsoidal zone.

Labelled bursa cells were also found in the germinal centres of the spleens 24 hours after intravenous injection (table 12). In each centre, 2 or 3 cells were found very heavily labelled (plate 22). However, the numbers of germinal centres having labelled cells were never very high.

In general, the distribution of the bursa cells labelled <u>in vitro</u> with <u>3H - adenosine</u> in the spleen at 24 hours was similar to the distribution of cells derived from the bursa 24 hours after <u>in situ</u> labelling of the bursa with 3H - thymidine (Fig. VII). The labelled cells appeared to predominate in the zone surrounding the ellipsoids in the spleens in which this zone could be clearly delineated.

<u>Thymus and Caecal Tonsils</u> - At 24 hours, the numbers of labelled bursa cells found in the thymus and caecal tonsils increased considerably. In the thymus, all labelled cells were present in the medulla, either in the substance of the medullary lymphoid tissue or in connection with medullary vessels. In the caecal tonsils, the cells were found in the diffuse lymphoid tissue of the lamina propria and sub-mucosa. A few labelled bursa cells were also found within the germinal centres of caecal tonsils in one bird (chick no. 16).

Table 12 - Distribution of the 3H-adenosine labelled bursa cells in different organs after intravenous injection. The figures in this table show the total number of cells counted in individual sections. The figures within the brackets represent numbers of labelled cells found in germinal centres.

Chick No.	No. of cells injected	Time *	Thymus Cor	Thymus Med	Liver	Caecal Tonsil	Spleen RP	Spleen PEZ	Spleen E	Spleen WPS
10	2×10^7	0-1min	0				71	13	32	0
11	1.5×10^7	1h	0	1	125	2	113	6	4	1
12	1.5×10^7	1h	0	1	131	1	68	24	2	3
							56	3	0	1
							57	15	2	9
13	6×10^6	1h	0	1	80	0	31	12	0	0
14	6×10^6	24h	0	43	2	8	48	18	0	7
							18	62	0	30(4)
15	7.5×10^6	24h	0	3	13	5	22	69	0	6(1)
							16	26	0	2
16	8.5×10^6	24h	0	5	8	15(3)	73	79	0	41(2)
							34	63	0	12(7)
							44	31	0	8

* Time intervals after i.v. injection of labelled bursa cells

cor = cortex PEZ = Peri-ellipsoidal zone

Med = Medulla E = Ellipsoid

RP = Red pulp WP = White pulp

2 - The Fate of 3H - adenosine Labelled Thymus Cells After Intravenous Injection - In the chicks killed at 10 - 15 minutes or 1 hour after i.v. injection of the labelled thymus cells, large numbers

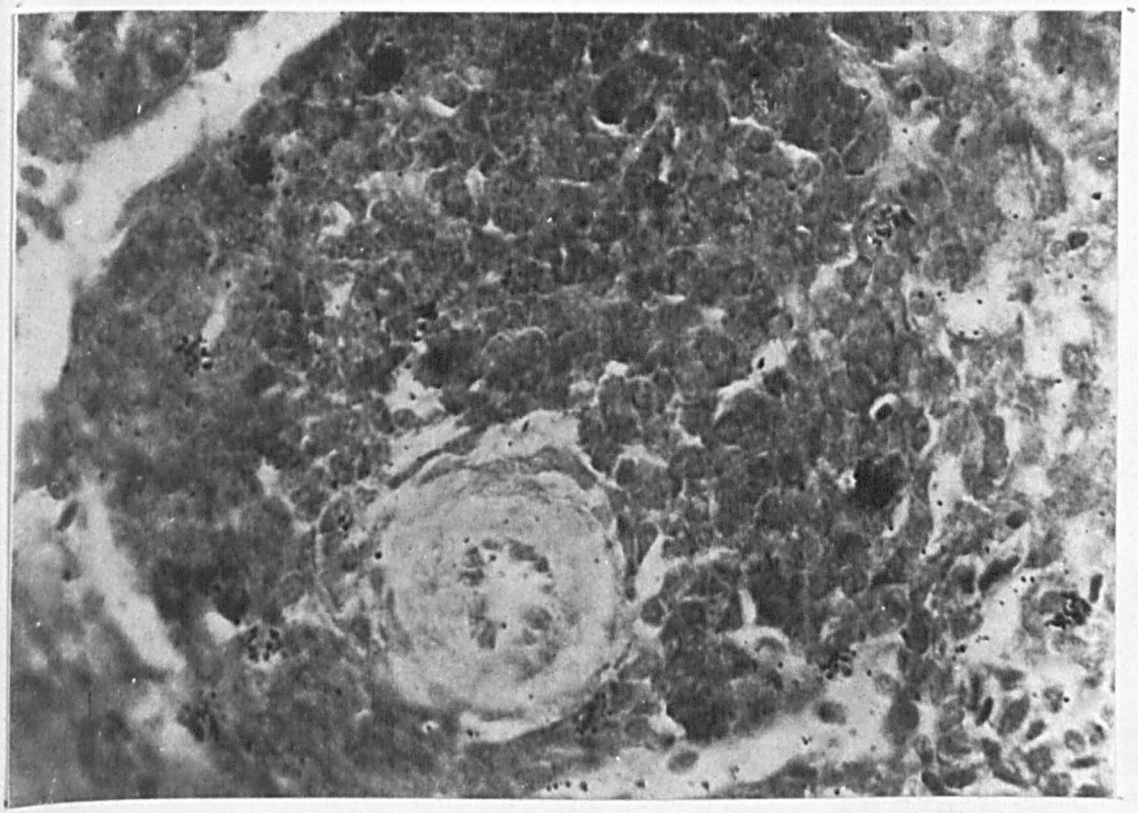


Plate 23 - Photomicrograph of section of chicken spleen 24 hours after i.v. injection of 3H-adenosine labelled autologous thymus cells. Note heavily labelled thymus cells in the peri-arteriole sheath of the white pulp. UP, X1140

Table 13 - Distribution of ^{3}H-adenosine labelled thymus cells in different organs after intravenous injection. The figures in the table show the total numbers of cells counted in individual sections.

Chick no.	No.of cells injected	Time *	Thymus Cor	Thymus Med	Bursa c/m	Liver	Caecal Tonsils	Spleen RP	Spleen PEZ	Spleen E	Spleen WP
17	7×10^{6}	10-15min	0	2	2	196	0	205	5	0	7
18	$1{\cdot}5\times10^{7}$	10-15min	0	8	12	668	1	156	4	1	2
19	4×10^{7}	1h	1	11	6	338	-	900	0	0	47
20	4×10^{7}	1h	0	2	3	241	1	819	17	0	57
21	3×10^{7}	1h	2	6	3	-	-	361	2	0	49
22	2×10^{7}	24h	1	46	1	-	12	58	14	0	228
23	2×10^{7}	24h	1	24	1	35	44	108	11	0	105
								29	0	0	10
24	2×10^{7}	24h			7	10	-	59	0	0	54
								118	5	0	176

* Time intervals after i.v. injection of labelled cells.

Cor = Cortex

Med = Medulla

c/m = cortico-medullary junction

RP = Red pulp

PEZ = peri-ellipsoidal zone

E = Ellipsoids

WP = White pulp

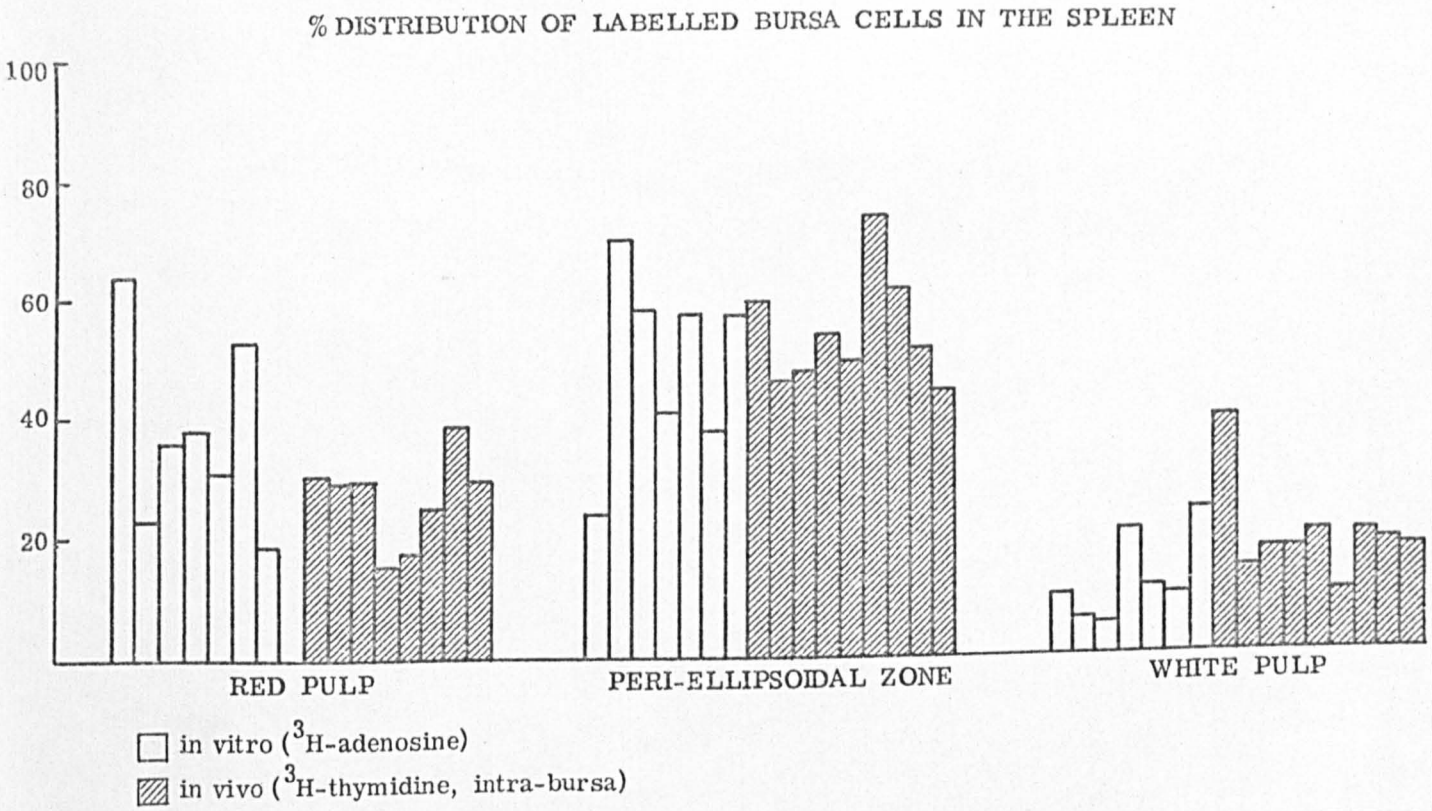


Fig. VII Distribution patterns of labelled bursa cells in chicken spleen 24 hours after <u>in situ</u> labelling of bursa with <u>3H-thymidine</u> and at 24 hours after i.v. injection of bursa cells labelled <u>in vitro</u> with <u>3H-adenosine</u>. Each bar represents the per centage of the total bursa cells in the individual spleen sections. Note that the use of two different techniques for labelling the bursa cells and the use of two different radio-isotopes did not alter the per centage distributions of the bursa cells in different parts of the spleen.

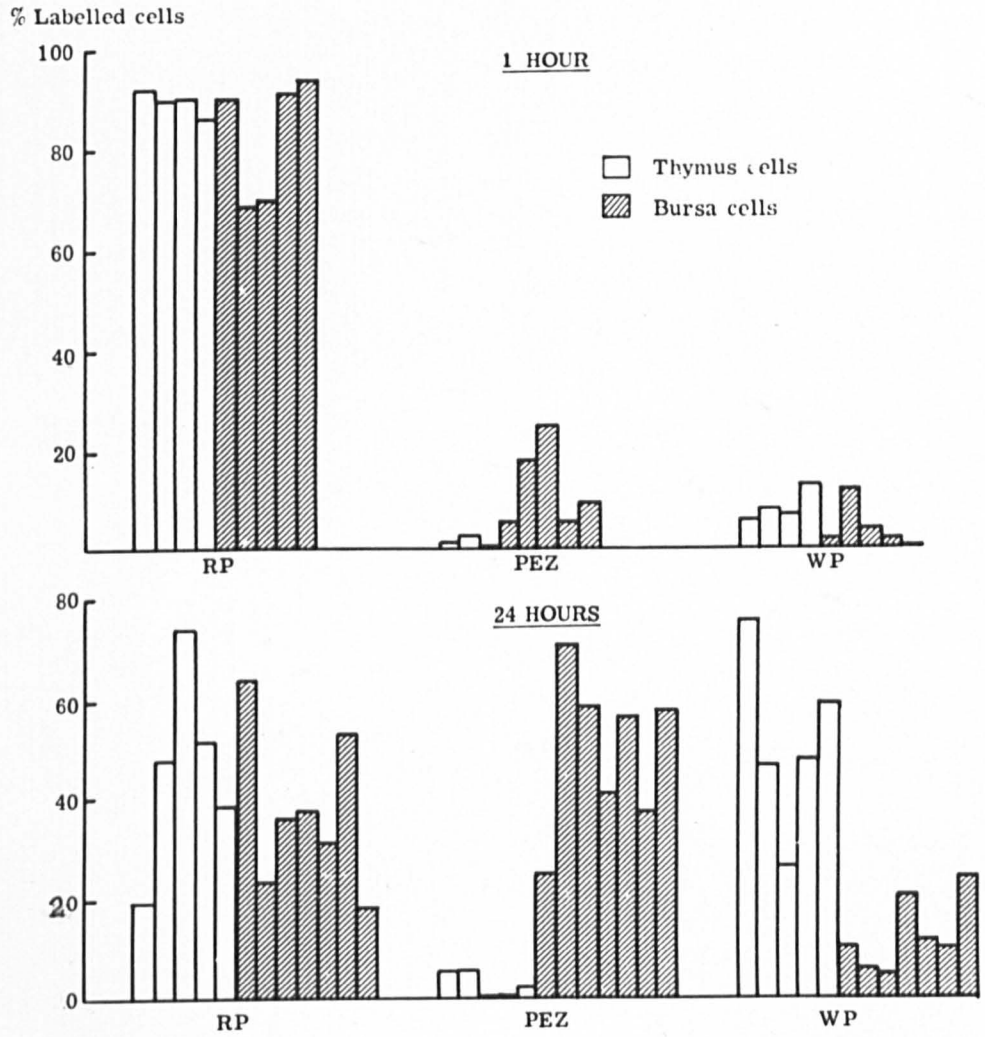


Fig. VIII Distribution of 3H-adenosine labelled bursa and thymus cells in the chicken spleen at 1 hour and 24 hours after i.v. injection. Each bar represents the per centage of the total labelled bursa or thymus cells in the individual spleen sections. Note that distribution patterns of these two populations of cells in the spleen show distinct differences which are very marked particularly at 24 hours after i.v. injection. A large per centage of the thymus cells homed in the WP (white pulp), whereas the PEZ (peri-ellipsoidal zone) was an area in the spleen which was homed by a high per centage of bursa cells. Almost equal per centages of labelled bursa and thymus cells homed in the RP (red pulp).

of labelled cells were found in the autoradiographs of the lung, liver and spleen (table 13). In the spleen, at 10 - 15 minutes, practically all labelled thymus cells were found in the red pulp. At 1 hour, the proportion of labelled cells that migrated to the white pulp had increased and in the birds killed at 24 hours, a considerable percentage of the total numbers of cells were found in the white pulp of the spleen (Fig VIII plate 23). No labelled cell was found in any germinal centre of the spleen. The distribution pattern of the labelled thymus cells in the spleen thus differs from that of the bursa cells.

At 24 hours, few labelled cells were present in the liver. Scanty labelled thymic cells were present at all times in the bursa of Fabricius. These labelled cells were clearly located at the junction between cortex and medulla of the bursa follicles.

Significant numbers of the labelled cells were found in the thymic medulla and in the caecal tonsil's diffuse lymphoid tissues.

Summary to the experiment 1 and 2 - Migration of labelled bursa and thymus cells to different lymphoid organs in chicken was studied by the technique of autoradiography. The distribution patterns of the labelled bursa and thymus cells in the spleen after i.v. injection of 3H-adenosine labelled bursa and thymus cells and after in situ labelling of the bursa with 3H-thymidine were also determined.

Large numbers of heavily labelled cells were found in the spleen at 1 hour and 24 hours after i.v. injection of 3H-adenosine labelled bursa cells and 24 hours after insitu labelling of the bursa with 3H-thymidine. Considerable numbers of labelled bursa cells were also found in the thymus, caecal tonsils and to a lesser extent in the umbilical cord residue. Few germinal centres were found to contain heavily labelled bursa cells.

Spleen was also populated by a large numbers of heavily labelled cells at 1 hour and 24 hours after i.v. injection of 3H-adenosine labelled thymus cells. Caecal tonsil was another lymphoid organ which was found to contain labelled thymus cells.

Distribution patterns of the labelled bursa and thymus cells in the spleen showed distinct differences which were particularly very marked 24 hours after i.v. injection of these labelled cells. A high per centage of the labelled bursa cells in the spleen were found in the peri-ellipsoidal zone. A high per centage of the labelled thymus cells which populated the spleen, were found in the peri-arterial sheath of the white pulp.

These results are discussed in relation to specific migration of bursa and thymus cells to other lymphoid organs. Distribution pattern of the labelled bursa and thymus cells in the spleen showed that the bursa and thymus cells home preferentially to the areas of the spleen which correspond respectively to the depleted areas in the spleens of bursectomized and thymectomized chickens.

DISCUSSION

DISCUSSION

Large numbers of heavily labelled cells were found in the lymphoid tissues of the chickens following *in situ* labelling of the bursa with *3H-thymidine* and i.v. injection of *3H-adenosine* labelled autologous bursa and thymus cells.

Spleen appeared to be the main target organ for both bursa and thymus cells since far higher numbers of labelled cells were found in this lymphoid organ than in caecal tonsils or in the thymus.

The portal entry of the labelled cells into the spleen is difficult to envisage. It is likely that the labelled cells take the arterial route and emerge from the ellipsoids of the spleen. Soon after entry into the spleen, the labelled bursa and thymus cells localize chiefly in the red pulp since a high per centage of the labelled bursa and thymus cells in the spleen were found in the red pulp at 10 minutes - 1 hour after i.v. injection.

The chickens which were killed at 1 hour after i.v. injection of the labelled cells received higher numbers of either labelled bursa cells or thymus cells than the chickens killed 24 hours after i.v. injection of these labelled cells. Yet the numbers of the labelled bursa cells found in the peri-ellipsoidal zone and the numbers of thymus cells found in the white pulp * of the spleens of chickens killed 24 hours after i.v. injection of the labelled cells were considerably higher than those found in the corresponding areas of the spleens of the chickens killed 1 hour after i.v. injection of these labelled cells (table 12 and 13).

* The term "white pulp" in this context is used to denote only the peri-arteriolesheath of the white pulp and does not include the ellipsoids, peri-ellipsoidal zone or germinal centres.

It is unlikely that the increase in the numbers of heavily labelled bursa cells (in the peri-ellipsoidal zone) and thymus cells (in the white pulp) 24 hours after i.v. injection of these labelled cells resulted from multiplication of the labelled cells in these areas of the spleen. Mitotic division of the radio-isotope labelled cells often leads to considerable decrease in the amount of radio-isotope in the cell as the 3H-adenosine labelled RNA and DNA molecules are distributed between the daughter cells. Such cells have low grain counts in the autoradiographs and they either become undetectable or because of low grain counts they are not considered to be truly labelled. It is therefore likely that during the time interval between 1 hour and 24 hours after i.v. injection of the labelled cells, the spleen was populated by more labelled bursa and thymus cells. This view, however, remains speculative since it is difficult to determine whether the total numbers of cells which homed in the spleen increased at 24 hours after i.v. injection as the birds killed at 1 hour and those killed at 24 hours were injected different numbers of labelled cells. The possibility that the increase in the numbers of labelled bursa cells (in the peri-ellipsoidal zone) and thymus cells (in the white pulp) by 24 hours after i.v. injection resulted from migration of labelled bursa and thymus cells from other areas of the spleen to the peri-ellipsoidal zone and the white pulp respectively cannot be ruled out.

The distribution pattern of the 3H-adenosine labelled bursa cells in the spleen 24 hours after i.v. injection was similar to that of the labelled cells derived from the bursa 24 hours after in situ labelling of the bursa with 3H-thymidine (Fig. VII). These results indicate clearly that distribution of these cells in different parts of the spleen is not random but it is very selective. Thus the use of two different techniques for labelling

of the bursa cells with two different radio-isotopes did not alter the distribution pattern of the bursa cells in the spleen.

With regard to distribution patterns of labelled bursa and thymus cells in the spleen, these two populations of lymphocytes showed distinct differences which were very marked particularly 24 hours after i.v. injection of these labelled cells. A high per centage of the labelled bursa cells in the spleen were found in the peri-ellipsoidal zone. The per centage of the labelled thymus cells in the spleen was very low in peri-ellipsoidal zone whereas a high per centage of the labelled thymus cells in the spleen were found in the white pulp areas. The red pulp of the spleen remained an area which contained a high per centage of both labelled bursa and thymus cells (Fig.VIII). These results indicate that the labelled bursa and thymus cells home preferentially in the areas of the spleen which correspond respectively to the depleted areas in the spleens of bursectomized and thymectomized chickens. Thus the peri-ellipsoidal zone remains a bursa-dependent area whereas the peri-arterial sheath of the white pulp remains a thymus-dependent area.

Germinal centres in the spleen and caecal tonsils were also found to contain 3H-adenosine labelled bursa cells at 24 hours after i.v. injection of these cells (plate 22). However, the numbers of germinal centres found in the spleen or caecal tonsils with labelled bursa cells were very low (table 12). Intravenous injection of 5mg HSA 48 hours before *in situ* labelling of the bursa with 3H-thymidine did not increase the numbers of labelled cells in the germinal centres or in other areas of the spleen at 24 hours after *in situ* labelling of the bursa.

There are several lines of evidence which indicate strongly that bursa-derived lymphocytes contribute to formation of germinal centres in the spleen and other lymphoid tissues. These lymphoid

nodules are consistently absent in the peripheral lymphoid tissues of the neonatally bursectomized chickens (Cooper et al. 1966, Alm et al. 1969). Electron microscope examination of the lymphocytes within the germinal centres has shown that they are morphologically identical with the lymphocytes of the bursa of Fabricius as indicated by presence of numerous polyribosomes in these lymphocytes. The lymphocytes of the thymus contain only small numbers of individual ribsomes (Clawson et al. 1967). Presence of immunoglobulin on the surface of the lymphocytes, which is characteristic of the B-lymphocytes, has also been demonstrated on the surface of lymphocytes in the germinal centres of the chicken spleen (French et al. 1969). Thus the failure of the labelled bursa cells to take part in formation of larger numbers of germinal centres in the spleen and caecal tonsils is probably due to the fact that the lymphoid tissues of the chickens used in the present study had already been populated by the non-labelled bursa cells. Hence it is conceivable that the bursa-derived lymphocytes which contributed to formation of germinal centres were mainly those non-labelled bursa cells which had previously been migrated to the spleen and caecal tonsils. It is also likely that larger numbers of the labelled bursa cells take part in formation of germinal centres three days after their entry into the spleen or caecal tonsils since these centres are formed normally 3 days after immunization (White, 1963).

Autoradiographs of the liver showed that large numbers of labelled cells were present in this organ at 10 minutes - 1 hour after i.v. injection of the 3H-adenosine labelled bursa and thymus cells. At 24 hours after i.v. injection, very few labelled cells were found in this non-lymphoid organ (table 12 & 13). This is in sharp contrast with the spleen in which very large numbers of labelled bursa and thymus cells were found both at 1 hour and at

24 hours after i.v. injection of these labelled cells. These findings are taken to indicate that a high per centage of the labelled bursa and thymus cells which were found in the spleen represent the bursa and thymus lymphocytes which migrated specifically to this lymphoid organ.

Both labelled bursa and thymus cells were found in the caecal tonsil 24 hours after i.v. injection of 3H-adenosine labelled bursa and thymus cells. In situ labelling of the bursa with 3H-thymidine also resulted in homing of labelled bursa-derived cells in the caecal tonsil. Caecal tonsil is one of the gut-associated lymphoid organs which is depleted of plasma cells and lymphocytes following neonatal bursectomy and neonatal thymectomy respectively (Cooper et al. 1966). The labelled bursa and thymus cells in this lymphoid organ thus are likely to represent the bursa and thymus cells which preferentially homed to this lymphoid organ. Homing of the bursa and thymus cells in the caecal tonsil has also been shown 48 hours after in vivo labelling of the bursa and thymus with 3H-thymidine (Hemmingsson et al. 1972, Hemmingsson, 1972). These findings substantiate the concept that there is a specific migration of cells from the bursa and thymus to the caecal tonsil.

Labelled cells were also found in the umbilical cord residue (Meckel's diverticulum) 24 hours after in situ labelling of the bursa with 3H-thymidine. It was shown, in part one of this thesis, that plasma cells and germinal centres are depleted from this lymphoid organ following surgical ablation of the bursa on day 18th of incubation (plate 19). Homing of the labelled bursa cells in the umbilical cord residue thus indicates that bursa cells migrate to this gut-associated lymphoid organ where they take part in development of plasma cells and formation of germinal centres.

A considerable number of labelled cells were found in the thymus 24 hours after i.v. injection of 3H-adenosine labelled bursa cells and 24 hours after in situ labelling of the bursa with 3H-thymidine. Thymus in chicken is the central lymphoid organ for cell-mediated immunity (Warner et al. 1962). However, this lymphoid organ in intact chickens contains a variable number of cellular elements of humoral immunity including plasmablasts and plasma cells. Germinal centres which are bursa-dependent lymphoid nodules may also develop in the medulla of the thymic lobules (plate 4). The pyroninophilic plasmablasts and plasma cells and garminal centres are completely absent in the medulla of thymus of the bursectomized chickens (plate 5 & 14). In this study the labelled bursa cells were found mainly in the medulla of the thymic lobules. These findings, thus, are taken to indicate that labelled bursa cells migrated specifically to the thymus and homed preferentially in the medulla of the thymic lobules.

In the present study, scanty labelled thymus cells were found in the cortico-medullary junction of the follicles of the bursa of Fabricius 24 hours after i.v. injection of 3H-adenosine labelled thymus cells. Hemmingsson (1972), who studied migration of thymus cells to other lymphoid organs in chicken, found that the bursa of Fabricius was free from labelled cells 48 hours after in vivo labelling of the thymus with 3H-thymidine. Moreover, neonatal thymectomy does not bring an apparent depletion of cells in the bursa of Fabricius (Cooper et al. 1966) Thus it is conceivable that thymus cells do not migrate to the bursa in considerable numbers.

CONCLUSIONS

Migration of bursa and thymus cells to the different lymphoid organs of the chickens was studied by the technique of autoradiography. The fate of bursa cells was determined within 24 hours after intravenous injection of 3H-adenosine labelled bursa cells and at 24 hours after *in situ* labelling of the bursa with 3H-thymidine. The fate of thymus cells was also determined within 24 hours after intravenous injection of the 3H-adenosine labelled thymus cells. The following conclusions have been reached:

1 - Bursa cells migrate to the spleen, thymus, caecal tonsils and umbilical cord residue.

2 - Thymus cells migrate to the spleen and caecal tonsils.

3 - The bursa and thymus cells populate the areas of the spleen which correspond respectively to the depleted areas in the spleens of bursectomized and thymectomized chickens. Thus bursa cells home preferentially to the peri-ellipsoidal zone, germinal centres and the red pulp, whereas the thymus cells home preferentially to the peri-arteriol sheath of the white pulp and to the red pulp.

4 - Bursa cells do not form germinal centres within 1 hour after they home to the spleen or the caecal tonsils and very few bursa cells take part in formation of germinal centres in the spleen or caecal tonsils within 1 - 24 hours after they home to these lymphoid organs. It is likely that the bursa cells have to remain in these peripheral lymphoid organs for longer periods in order to become competent in formation of germinal centres.

PART III

THE EFFECT OF BURSECTOMY ON LOCALIZATION OF NATIVE HSA , AGGREGATED HSA AND HSA-ANTI HSA COMPLEXES TO THE DENDRITIC CELL.

Antigens are substances, often proteins, which are able to elicit a specific immune response when they are introduced into the body of an animal. Study of the events which take place in the lymphoid tissues of an immunized animal leading to formation of antibody are of great importance in understanding the phenomenon of humoral immune response. To determine where in the body the antigen ends up and what types of cells are involved in handling the antigen a practical approach is to search for the localization and distribution of the antigen in different tissues of the immunized animal. White (1963) using fluorescent antibody technique studied the fate of HSA in the spleen of chicken after i.v. injection. He found that the antigen localization appeared 30 hours after the initial injection. The antigen had been localized to certain elongated, spindle shaped cells which he designated dendritic macrophages. He drew attention to the fact that the pattern of localization differed from that exhibited by normal macrophages in that the injected material appeared to be on the surface of the fine dendritic cell processes. He showed that the cells migrate through the white pulp to end within the germinal centres, where the antigen may persist for as long as three weeks. Stark (1969) made a similar study in chicken using ^{125}I-HSA. He showed that the antigen bearing cells had been concentrated along the length of the penicillary arteries and in the germinal centres of the spleen 88 hours after i.v. injection. Nossal and his co-workers (Nossal, Ada and Austin 1964, Ada, Nossal and Austin 1964) studied the localization of antigen in the lymphoid tissues of the rat. They used flagellin antigen from Salmonella adelaide which had been labelled with ^{131}I. When the labelled antigen was injected into the foot-pads of rats, autoradiographs of the sections of the lymph nodes showed that heavily

labelled cells were present in the medullary sinuses within 5 minutes after injection. Twenty four hours after injection, the radioactive labelled antigen was detectable in the primary lymphoid follicles on the cells with dendritic processes extending between the lymphoid cells. While antigenic materials localized to both medullary macrophages and lymphoid follicles, non-antigenic substances such as carbon particles were taken up by medullary macrophages but did not localize to the lymphoid cells. These workers suggested that the differences between distributuon of antigen and non-antigenic substances in the lymph nodes were related to the ability of dendritic cells to recognize "foreigness" due to association of the antigen with opsonizing factors.

On the question of mechanism of binding the antigen to the dendritic cells, White et al. (1966) showed that early synthesized antibody by the chicken immunized to HSA is the main factor in the localization of the antigen over the dendritic cells. They showed that with fluorescein labelled anti-chick γ-globulin it was possible to reproduce the pattern of antigen localization to the dendritic cells in the germinal centre, indicating that γ-globulin is present with the antigen at the surface of dendritic cells. Furthermore, they showed that chickens rendered specifically tolerant to HSA were unable to localize the antigen.

Since at the time of first appearence of HSA localization, already antibody containing plasma cell precursors are present in the red pulp of the spleen, French et al. (1969) and White et al. (1970) concluded that antibody necessary for the antigen localization was provided by these cells. The antigen at the surface of the dendritic cells was accompanied by immunoglobulin in the form of antigen-antibody complexes. Balfour and Humphrey (1967) also found that localization of antigen in the germinal centres coincided

with appearance of specific antibody. Lang et al. (1967a, 1967b) showed that if HSA was combined with anti-HSA antibody and injected into the foot-pad of a rat, the complex was taken up by the macrophages more readily and the amount taken up by these cells increased by a factor of about 10 compared to HSA alone. In the absence of specific antibody, HSA showed no tendency for follicular localization.

Experiment 1 - The Effect of Bursectomy on Localization of HSA to the Dendritic Cells

Introduction - Localization and persistence of HSA to the dendritic cells of the spleen was first shown by means of fluorescent antibody technique (White 1963). The dendritic cells were also found to have a major role in formation of germinal centres since all antigen bearing dendritic cells were found to aggregate into groups or collections of cells which together with B-cells formed germinal centres in the white pulp of the spleen (White et al. 1966, White et al. 1970).

Neonatal bursectomy in chicken prevents formation of germinal centres in the spleen. Since germinal centres are formed, in the spleens of normal birds, only after an antigen is localized to the dendritic cells, failure of the bursectomized chickens to form germinal centres in the spleen may be due to the deficiency of these birds to localize antigens to the dendritic cells.

The aim of the present experiment is, thus, to determine if and in what manner bursectomy affects localization of HSA to the dendritic cells.

Brief Outlines of the Materials and Methods (detailed accounts of the materials and methods were given in the "General Materials and Methods")

Birds - Thornber 808 chicks were used in this study.

Surgical Operation and Irradiation - The chicks were bursectomized on the day of hatching. The bursectomized chicks and their intact controls were subjected to whole body irradiation on their second day of life. Each bird was subjected to 840 rads of γ-rays from ^{60}Cobalt with source skin distance of 70 cm.

Antigen and Immunization Schedule - At 12 weeks of age each bird was inoculated intravenously with 10 mg HSA. The chickens then were bled and sacrificed on day 4, 5 or 6 after immunization (table 14). The spleen was removed immediately after the bird had died. Frozen sections of the spleens were cut in a cryostat and were stained with fluorescein labelled rabbit anti HSA antibody (single and double layer techniques). The sections then were examined under a u.v. microscope. The antibody in the sera collected from the birds was measured with Farr Test.

RESULTS

Serum Antibody Levels in Bursectomized and Intact Chickens - Anti HSA antibody in the sera of BX-IR and IN-IR chickens was measured with Farr test. The results are presented in table 15 .

No antibody activity was detected in the sera of BX-IR chickens on day 4, 5 or 6 after the i.v. injection of 10 mg HSA. The sera of all IN-IR chickens, however, contained measureable amounts of specific antibody. IN-IR chickens killed on day 6 after the immunization had produced considerably higher amounts of antibody (ABC_{30}) than the chickens killed on day 4 or 5 after the immunization.

Localization of HSA in the Spleens of Intact-irradiated and Bursectomized-irradiated Chickens - In the frozen sections of the spleens treated with a single layer of fluorescein labelled anti HSA antibody, the antigen was detected as a granular line of bright fluorescence on the dendritic cells in the white pulp of the spleens of

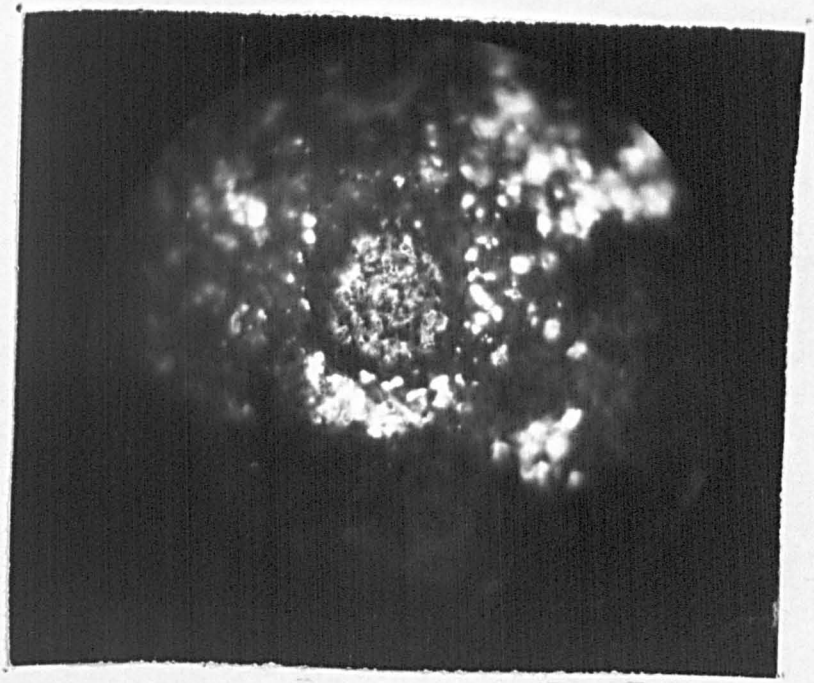


Plate 24 - Fluorescence photomicrograph of frozen section of spleen of an intact-irradiated chicken 5 days after i.v. injection of 10 mg HSA. The section was first treated with fluorescein-labelled anti HSA antibody and then with fluorescein-labelled anti chicken IgG. There is one germinal cnntre in the white pulp which contains HSA-bearing dendritic cells. The antigen has localized on the cell membrane of the individual dendritic cells. Note also IgG-containing cells at the periphery of the germinal centre. (X 300)

the intact-irradiated chickens killed on day 4 after immunization. The HSA bearing cells appeared as large spindle-shaped cells with fine cytoplasmic extensions. The dendritic cells loaded with the antigen were chiefly confined to the white pulp strands or within the germinal centres, the red pulp and peri-ellipsoidal zones had remained completely free from antigen bearing cells. Germinal centres were found in various stages of formation. Some appeared as a group of antigen-bearing dendritic cells, spread widely in the white pulp and had no well defined margins. However, germinal centres with definite margins had already been formed in juxtapositions to white pulp arterioles and each contained between 10 to 20 antigen-bearing dendritic cells.

Spleen sections of the bursectomized irradiated chickens killed 4 days after i.v. inoculation of 10 mg HSA were also stained with a single layer of fluorescein labelled anti HSA, under identical conditions as those of the intact controls. There was however, no evidence of localization of the antigen on any cell in the spleen sections of the bursectomized birds.

In the spleen sections of the intact-irradiated birds killed on day 5 after i.v. injection of HSA, dendritic cells loaded with this antigen were found mainly within the germinal centres of the spleen. There were very few antigen-bearing dendritic cells outside the territory of the germinal centres at this stage of immune response. Larger numbers of well-developed germinal centres were evident in each spleen section in comparison to those found in the spleen sections of the intact birds killed on day 4 after immunization. The cellular elements of these germinal centres consisted of antigen-bearing dendritic cells as well as smaller cells with the morphology of small lymphoctyes. These cells, however, neither had antigen on their cell membranes nor within their cytoplasm (plate 24).

Table14 Time scales for the localization of HSA in the spleens of bursectomized-irradiated and intact-irradiated chickens on day 4, 5 or 6 after an intravenous injection of 10 mg HSA. The numbers of chickens killed on each day is referred to in the brackets.

Chickens	Day 4 E. W. G.	Day 5 E. W. G.	Day 6 E. W. G.
Bursectomized-irradiated	- - - (2)	- - - (3)	- - - (2)
Intact-irradiated	- + + (2)	- + + (3)	- - + (2)

+ = Localization of HSA
- = Lack of localization of HSA

E = Ellipsoids
W = White pulp
G = Germinal centres

Table 15-Serum antibody levels (ABC_{30} ug/ml) in bursectomized-irradiated and intact-irradiated chickens on day 4, 5 or 6 after an intravenous injection of 10 mg HSA.

Chickens	Day 4		Day 5		Day 6	
	No. of chicks	ABC_{30}, ug/ml mean & range	No. of chicks	ABC_{30} ug/ml mean & range	No. of chicks	ABC_{30}, ug/ml mean & range
Bursecto-mized-irradiated	2	<0.06 (<0.06-<0.06)	3	<0.06 (<0.06-<0.06)	2	<0.06 (<0.06-<0.06)
Intact irradiated	2	1·83 (1·56-2·1)	3	5·73 (2·28-10·11)	2	13·5 (13·2-13·8)

No antigen-bearing cell was found in any spleen sections of the bursectomized-irradiated chickens given an intravenous injection of 10 mg HSA five days previously.

In the spleen sections of the intact irradiated chickens killed 6 days after immunization with HSA, dendritic cells bearing this antigen were found only within the germinal centres. There appeared to be no antigen-bearing dendritic cell outside the territory of the germinal centres. Bursectomized-irradiated chickens killed 6 days after i.v. injection of 10 mg HSA, like the bursectomized chickens killed on day 4 and 5 after immunization showed a complete lack of response regarding the localization of HSA. There was a total absence of antigen-bearing cells in the spleen sections of these bursectomized birds.

Antibody Containing Cells in the Spleens of Intact and Bursectomized Birds - Antibody-containing cells in the spleen sections were visualized by treating the sections first with a dilute solution of HSA in 0·15 M saline and then with a layer of fluorescein labelled anti HSA antibody. The anti HSA antibody, thus, appeared as an area of very bright fluorescence in the cytoplasms of immature and mature plasma cells. These antibody-containing cells were found in the spleen sections of intact irradiated chickens killed on days 4, 5 and 6 after i.v. injection of 10 mg HSA. These cells were confined to the redpulp of the spleen especially at the peripheries of the arteries (plate 25).

Frozen sections of the spleens of the bursectomized-irradiated chickens were also stained, under identical conditions as those of the intact controls, to demonstrate presence of antibody containing cells in the spleen. There was however, no antibody containing cell in any spleen section of the bursectomized-irradiated chickens killed on day 4, 5 and 6 after i.v. injection of 10 mg HSA.

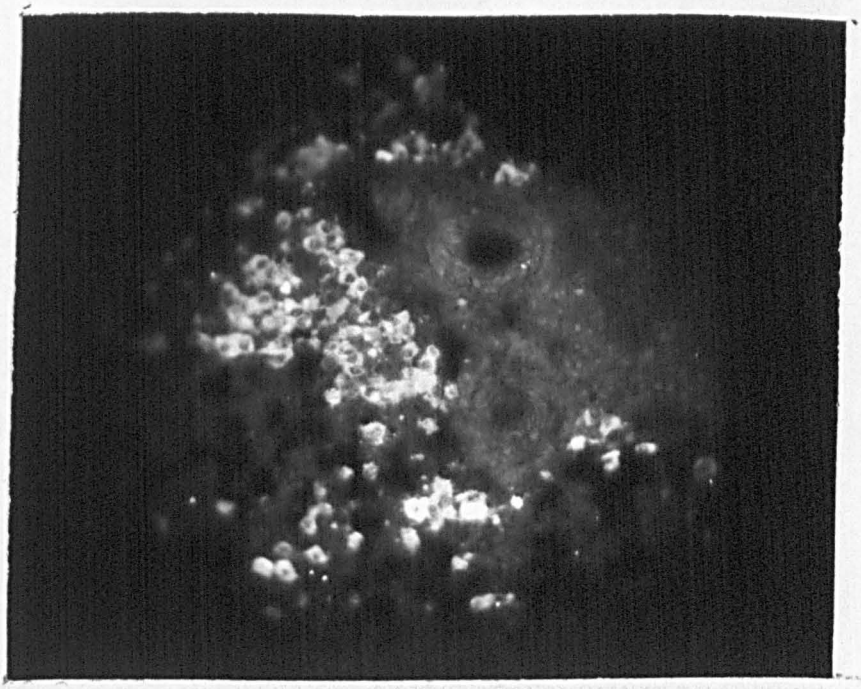


Plate 25 -Fluorescence photomicrograph of frozen section of the spleen of an intact-irradiated chicken 6 days after i.v. injection of 10 mg HSA . The section was first treated with a solution of HSA and with fluorescein-labelled anti HSA antibody . There are large numbers of antibody-containing plasma cells in the red pulp. The white pulp is completely free from antibody-containing cells. (X 300)

Summary - This experiment is summarized with next two experiments on page 139.

Experiment 2 - The effect of Bursectomy on Localization of Aggregated HSA on Dendritic Cells.

Introduction - It was shown, in the previous experiment, that the use of native HSA as an antigen in bursectomized chickens does not result in localization of this antigen on the dendritic cells. Localization of an antigen on the cell membrane of a cell depends largely on chemical groupings or receptors on the molecule of that antigen which permit attachment of the antigen to the cell surface. Some of the receptors of an antigen are normally buried within the molecule, hence they do not come in close contact with the cell membrane. Denaturation of native proteins by gentle heating results in opening up and extension of the coiled polypeptide chains of the native molecule, thus formation or disappearance of some chemical groupings in the molecule. It has been shown that denatured BSA is taken up by macrophages with greater avidity than the native BSA (Thorbecke et al. 1960). The lymphoid cells also show an enhanced response to the heat denatured albumins as indicated by higher levels of circulating antibody in rabbits immunized to denatured BSA (Hirata et al. 1966).

The object of this experiment is to determine whether aggregation of native HSA by gentle heating would similarly bring a change in the molecules of this antigen which facilitates its localization on the dendritic cells in the spleens of bursectomized-irradiated chickens.

Brief Outlines of Materials and Methods (detailed accounts of the materials and methods were given in the "General Materials And Methods").

Birds - Thornber 808 chicks were used in this experiment.

Surgical Operation and Irradiation - The chicks were bursectomized on the day of hatching. The bursectomized chicks along with their intact controls were subjected to whole body irradiation using ^{60}Cobalt. The total amount of radiation given to each bird was 840 rads with a source skin distance of 70 cm.

Antigen and Immunization Schedule - The bursectomized-irradiated chickens and their intact-irradiated controls were each given an intravenous injection of 10 mg heat aggregated HSA at 7 - 10 weeks of age. Some of the birds were also injected intravenously each with 0·5 ml indian ink one minute after the inoculation of aggregated HSA. The birds were then killed at different intervals after the immunization (table 16). Spleen, bursa and thymus were removed from the birds immediately after the bird had died. Frozen sections of these organs were cut in a cryostat and were stained with fluorescein labelled rabbit anti HSA antibody (single layer and sandwich technique) to demonstrate localization of aggregated HSA or presence of antibody containing cells in the frozen sections of these lymphoid organs. The sections were examined under the U.V. microscope.

RESULTS

Localization of Aggregated HSA and Distribution of Carbon Containing Cells in the Spleen - In the frozen sections of the spleens treated with a single layer of fluorescein-labelled anti HSA, aggregated HSA was found in association with the cells at the periphery of the ellipsoids in the spleens of both bursectomized and intact birds killed 5 minutes after i.v. inoculation of 10 mg aggregated HSA. The antigen had localized on the cell surfaces as a granular bright line, outlining each individual cell (plate 26). Colloidal carbon (Indian ink) which was intravenously injected one minute before the birds were killed was also found in the ellipsoids

Plate 26- Fluorescence photomicrograph of spleen section of a bursectomized-irradiated chicken after i.v. injection of aggregated HSA and indian ink. The aggregated HSA and indian ink were injected respectively 5 minutes and one minute before the bird was killed. The spleen section was stained with a single layer of fluorescein-labelled anti-HSA antibody. This plate shows three ellipsoids where the aggregated HSA and carbon particles of the indian ink have localized. The localization of aggregated HSA can be seen as bright lines on the surface of the cells at the periphery of the ellipsoids. The cells in the inner layer of the ellipsoids have localized the carbon particles of the indian ink. (X 570)

of the spleens of both groups of birds. The carbon-containing cells, however, were confined within the surrounding sheath of the ellipsoids. The white and red pulp of the spleens of both groups of birds, thus, were found completely free from antigen-bearing or carbon-containing cells.

In the spleen sections of the intact-irradiated birds killed 24 hours after i.v. injection of aggregated HSA and colloidal carbon, no antigen-bearing cell was found in the ellipsoids or any other parts of the spleen. The ellipsoids also appeared to be completely free from carbon-containing cells. The carbon-containing cells, however, were found scattered in the white pulp areas.

Spleen sections of the bursectomized-irradiated chickens killed 24 hours after i.v. inoculation of aggregated HSA and colloidal carbon showed evidence of localization of the antigen on the cells in the peri-ellipsoidal zones of the spleen. The concentration of the antigen on individual cell, however, was considerably less than that of the cells of the chickens killed 5 minutes after inoculation of this antigen. The localization of the antigen did not appear as a continuous granular bright line of fluorescence on the cells but as bright spots of fluorescence on the cell well isolated from each other. The white pulp of the spleen appeared completely free from antigen-bearing cells.

The cells bearing the colloidal carbon were found mainly in the white pulp strand. There were also few cells in each ellipsoid which contained colloidal carbon particles.

In the spleen sections of the intact-irradiated birds killed 48 hours after immunization with aggregated HSA, dendritic cells bearing this antigen were found in the white pulp areas. The aggregated HSA, like native HSA, had localized on the cell body as

well as cytoplasmic extensions of the dendritic cells. In places where the white pulp arterioles appeared longitudinally, the antigen-bearing dendritic cells were found to lie along these arterioles. There was, however, no evidence of localization of aggregated HSA in the spleens of the bursectomized chickens killed 48 hours after i.v. inoculation of this antigen.

Spleen sections of the intact birds killed 72 hours after i.v. inoculation of aggregated HSA showed a high numbersof antigen bearing dendritic cells in the white pulp of the spleen. These cells had aggregated into groups or collections of cells along the penicillary arterioles of the white pulp. Germinal centres with antigen-bearing dendritic cells were also found at this stage of immune response in different stages. Some of these centres were merely a collection of antigen-bearing dendritic cells with no well-defined outline. (Plate 27) There were however, few germinal centres with well demarcated outline, which had formed in juxtaposition to an arteriole.

A detailed examination, under the U.V. microscope, of the spleen sections of the bursectomized chickens did not show any antigen-bearing cells in any parts of the spleen.

Antigen-bearing dendritic cells in the spleens of intact-irradiated birds killed 96 hours after i.v. injection of aggregated HSA were confined chiefly within the germinal centres of the spleen. There were very few antigen-bearing dendritic cells outside the territory of germinal centres at this stage of immune response.

No antigen-bearing dendritic cell was found in any spleen sections of the intact-irradiated birds killed 96 hours after i.v. injection of this antigen. Carbon-containing cells were found scattered in the white pulp of the spleens of both groups of birds killed 96 hours after i.v. injection of indian ink. Some of the carbon-containing cells were found at the periphery of the germinal centres in the spleens of intact-irradiated birds. These cells,

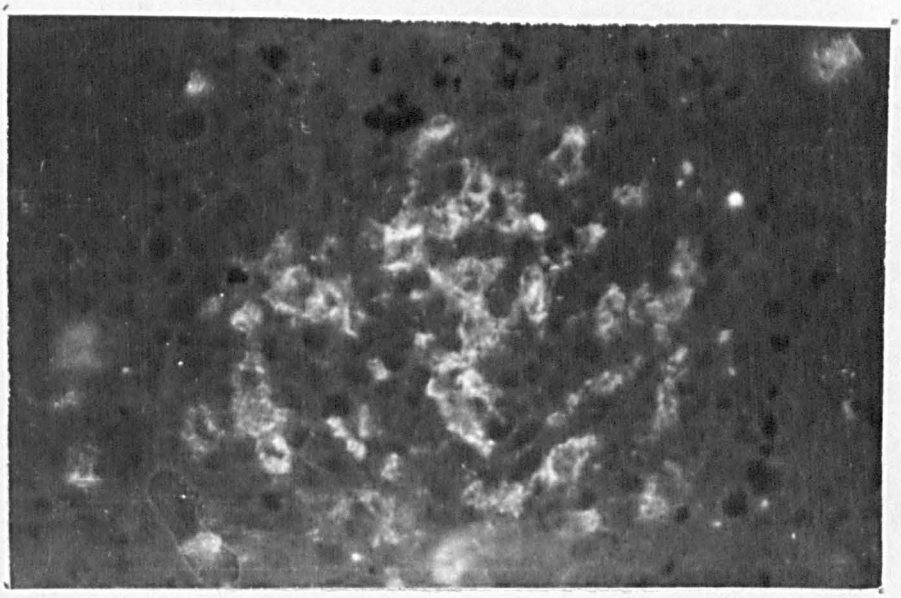


Plate 27 - Fluorescence photomicrograph of spleen section of an intact-irradiated chicken 72 hours after i.v. injection of aggregated HSA and indian ink. The frozen section of the spleen was stained with a single layer of fluorescein-labelled anti HSA antibody. There are large numbers of antigen-bearing dendritic cells within the irregular outline of a germinal centre. Note that the carbon-bearing cells have not entered within the territory of the germinal centre. (X 630)

however, were only rarely found within the territory of some germinal centres. The colloidal carbon particles were never found in association with the cell membrane or cytoplasm of dendritic cells. The carbon particles were found in macrophages in the spleens of both groups of birds as a mass or a large black spot inside the cell.

Table 16 - Time scales for the localization of aggregated HSA in the spleens of bursectomized-irradiated and intact-irradiated chickens at different intervals after an intravenous injection of 10 mg aggregated HSA. The numbers of chickens killed at each stage after immunization is referred to in the brackets.

Chickens	5 minutes E. W. G.	24 hours E. W. G.	48 hours E. W. G.	72 hours E. W. G.	96 hours E. W. G.
Bursectomized-irradiated	+ - - (2)	± - - (2)	- - - (3)	- - - (3)	- - - (2)
Intact-irradiated	+ - - (2)	- - - (2)	- + - (3)	- + + (3)	- + + (2)

+ = localization of aggregated HSA

- = lack of localization of aggregated HSA

± = weak localization of aggregated HSA

E. = Ellipsoids

W. = White pulp

G. = Germinal centres

Antibody-Containing Cells in the Spleen - Frozen sections of the spleens of both intact-irradiated and bursectomized-irradiated chickens killed at different intervals after i.v. injection of

10 mg aggregated HSA were also stained by sandwich technique in an attempt to demonstrate the presence of antibody-containing cells in the spleen sections.

No antibody-containing cell was evident in any spleen section of intact irradiated birds killed 24 hours after immunization. Clusters of antibody containing cells were found in the spleen sections of intact irradiated birds killed 48 hours after immunization. These cells showed characteristic morphology of plasmablasts and immature plasma cells. Due to combination of fluorescein labelled rabbit anti HSA antibody with HSA-anti HSA complexes in these cells, an area of very bright fluorescent became visible within each cell which covered almost the entire cytoplasm. Spleen sections of the intact-irradiated birds killed 72 and 96 hours after immunization showed considerably larger numbers of antibody containing cells. These cells were all confined to the red pulp strands and were not found in the white pulp or within the germinal centres.

No antibody containing cell was found in the spleen sections of the bursectomized irradiated birds killed 24, 48, 72 and 96 hours after i.v. injection of 10 mg aggregated HSA.

Antigen-Bearing and Antibody-Containing Cells in the Bursa and Thymus

Frozen sections of the thymus of both groups of birds and frozen sections of the bursa of intact-irradiated birds were stained with single layer technique as well as sandwich technique to detect antigen-bearing and antibody-containing cells in these two central lymphoid organs. The frozen sections of the thymus and bursa were stained along with the frozen sections of the spleens, under identical conditions.

There was, however no evidence of localization of aggregated HSA in the frozen sections of the thymus of IN-IR and BX-IR chickens killed at different intervals after i.v. injection of this antigen.

The frozen sections of the bursa of the IN-IR birds similarly did not show any antigen bearing cells.

Antibody containing cells were also completely absent from the frozen sections of the thymus and bursa of the IN-IR birds and from the frozen sections of the thymus of the BX-IR birds killed 48,72 and 96 hours after i.v. injection of 10 mg aggregated HSA.

Carbon containing cells were found in the medulla of the thymic lobules of both groups of birds and in the medulla of the bursa follicles of the IN-IR birds, killed at different intervals after i.v. injection of indian ink. The numbers of cells bearing the carbon particles in the thymic lobules of both groups of birds were very few and did not exceed 4 - 8 cells per lobules. There were fewer carbon bearing cells in the bursa follicles in the comparison with the thymic lobules.

Summary - This experiment is summarized with experiments 1 and 3 of this part of the thesis on page 139.

Experiment 3 - The Effect of Bursectomy on Localization of HSA-Anti HSA Complexes to the Dendritic Cells.

Introduction - In the preceding two experiments we established clearly that neonatal bursectomy followed by whole body irradiation results in total deficiency of the BX-IR birds to localize native or aggregated HSA to the dendritic cells of the spleen. It is highly unlikely that the failure of the BX-IR chickens to localize HSA is due to inavailability of dendritic cells in the spleens of these birds since bursectomy does not bring depletion of non-lymphoid cells in the spleen. There is, however, ample evidence suggesting that neonatal bursectomy eliminates the pool

of B-lymphocytes including the plasma cells, consequently such birds fail to produce specific antibody (Cooper et al. 1966, Alm et al. 1969). Moreover, it has been well documented that intact chickens rendered immunologically tolerant to HSA are incapable of localizing this antigen on the dendritic cells (White et al. 1966) Thus it is suspected that the inability of bursectomized chickens to localize HSA on the dendritic cells is due chiefly to failure of these birds to produce antibody.

In this experiment the bursectomized chickens are inoculated intravenously with HSA-anti HSA complexes in an attempt to determine whether such birds passively provided with specific antibody become competent regarding localization of HSA on the dendritic cells.

Brief Outlines of the Materials and Methods (detailed accounts of the materials and methods were given in the "General Materials and Methods".)

Birds - Thornber 808 chicks were used in this experiment.

Surgical Operation and Irradiation - The chicks were bursectomized on the hatching day. The bursectomized chicks and their intact controls were subjected to whole body irradiation on their second day of life. Each chick was subjected to 840 rads of X-rays from ^{60}Cobalt with source skin distance of 70 cm.

Antigen and Immunization Schedule - The chickens were inoculated intravenously with HSA-anti HSA complex at 7 - 9 weeks of age. The inoculum for each bird consisted of 1 ml antigen-antibody complexes containing anti HSA antibody precipitated by 1 mg HSA, where HSA and its corresponding antibody were in optimal proportions. The complex, then, had been disolved by addition of 10 mg HSA.

The chickens were killed at different intervals after i.v. injection of the complex (table 17). Some birds were also injected intravenously each with 0.5 ml indian ink one minute before they

were killed. Spleen, thymus, bursa (where applicable) were removed from the birds immediately after they had died. Frozen sections of these three organs were cut in a cryostat and were stained with fluorescein labelled rabbit anti-HSA antibody (single layer and sandwich techniques) in an attempt to demonstrate localization of the HSA-anti HSA complex to the dendritic cells or presence of antibody containing cells in these lymphoid organs. Thymus and bursa (where applicable) sections were also treated with a single layer of fluorescein labelled anti chicken immunoglobulin to demonstrate immunoglobulin containing cells in these two central lymphoid organs.

RESULTS

Localization of HSA - anti HSA Complex in the Spleen - In the spleen sections of the chickens killed 5 minutes after intravenous injection of HSA-anti HSA complex, the cells loaded with the complex were found at the periphery of the ellipsoids in the spleen sections of both BX-IR and IN-IR birds. In general, the localization of the complex in the peri-ellipsoidal zones of the spleen resembled that of the localization of the aggregated HSA. The complex on the surface of the cells, however, showed considerably brighter fluorescence due to higher concentration of the HSA - anti HSA complexes on the individual cell (plate 28). Very few cells loaded with the complex were also found in the white pulp areas at this early stage of the immunization.

In the spleen sections of the both IN-IR and BX-IR chickens killed 24 hours after i.v. injection of the HSA-anti HSA complex, the ellipsoids and peri-ellipsoidal zones were found completely free from complex-bearing cells. The dendritic cells bearing the complex were, however, found scattered in the white pulp areas of the spleens of both BX-IR and IN-IR birds. There were obviously

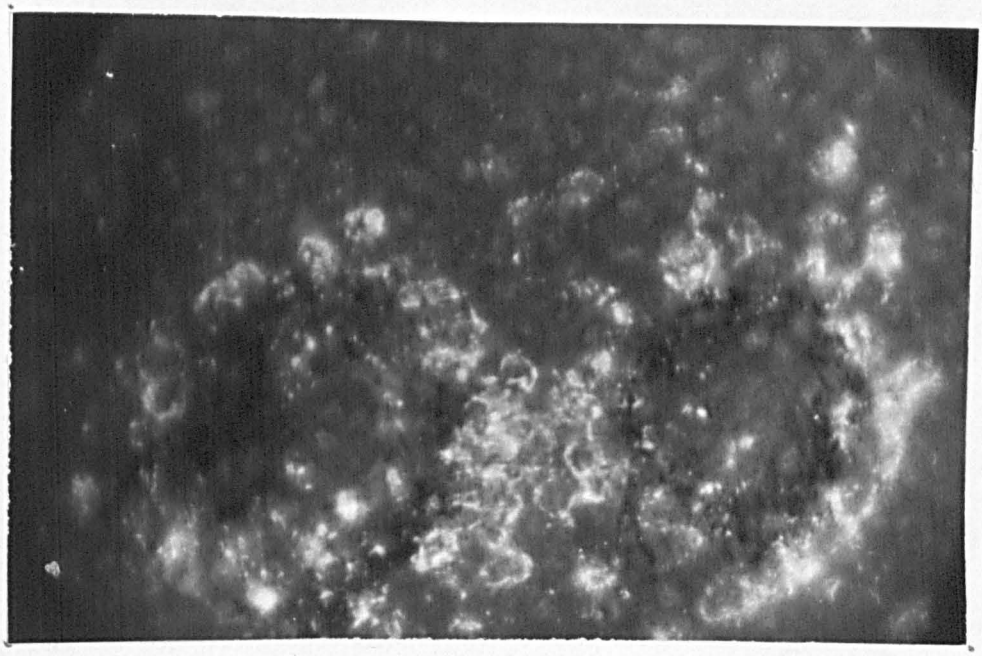


Plate 28 - Fluorescence photomicrograph of spleen section of a bursectomized-irradiated chicken after i.v. injection of HSA-anti HSA complexes and indian ink. The HSA-anti HSA complexes and indian ink were injected respectively 5 minutes and one minute before the bird was killed. The frozen section of the spleen was stained with a single layer of fluorescein-labelled anti HSA antibody. This plate shows two ellipsoids where the Ag/Ab complex and carbon particles of the indian ink have localized. The Ag/Ab complex has localized on the cells and appears as bright granular lines on the cells at the periphery of the ellipsoids. The cells of the inner layer of the ellipsoids have localized the carbon particles of the indian ink (X 630)

more complex-bearing cells in the spleen sections of the IN-IR birds in comparison to BX-IR birds. There was no evidence of formation of a germinal centre by the complex-bearing dendritic cells in any spleen section.

In the spleen sections of the IN-IR birds killed 48 hours after i.v. injection of the complex, dendritic cells bearing the complex were found as groups or collections of cells consisting of 4 or 8 cells along the penicillary arteries of the white pulp. Germinal centres were also found at this early stage of immune response which contained dendritic cells bearing the complex. These germinal centres had formed in juxtaposition to a white pulp arteriole and some contained up to 25 complex bearing dendritic cell (plate 29).

Dendritic cells bearing the complex were also found in the spleen sections of the BX-IR chickens killed 48 hours after i.v. inoculation of HSA-anti HSA complex. These cells were found along the white pulp arterioles singly, in pairs or as groups of cells consisting not more than 6 or 8 cells. However, detailed examination of the spleen sections of the bursectomized-irradiated chickens did not show any evidence of formation of a germinal centre in any section.

In the spleen sections of the intact birds killed 72 hours after i.v. injection of HSA-anti HSA complex, the majority of dendritic cells bearing the complex were found within the fully developed germinal centres. There were very few complex bearing dendritic cells outside the territory of germinal centres at this stage.

The distribution of the complex bearing cells in the spleen sections of the bursectomized birds killed 72 hours after i.v. inoculation of HSA-anti HSA complex did not differ very much from that of the bursectomized birds killed 48 hours after inoculation

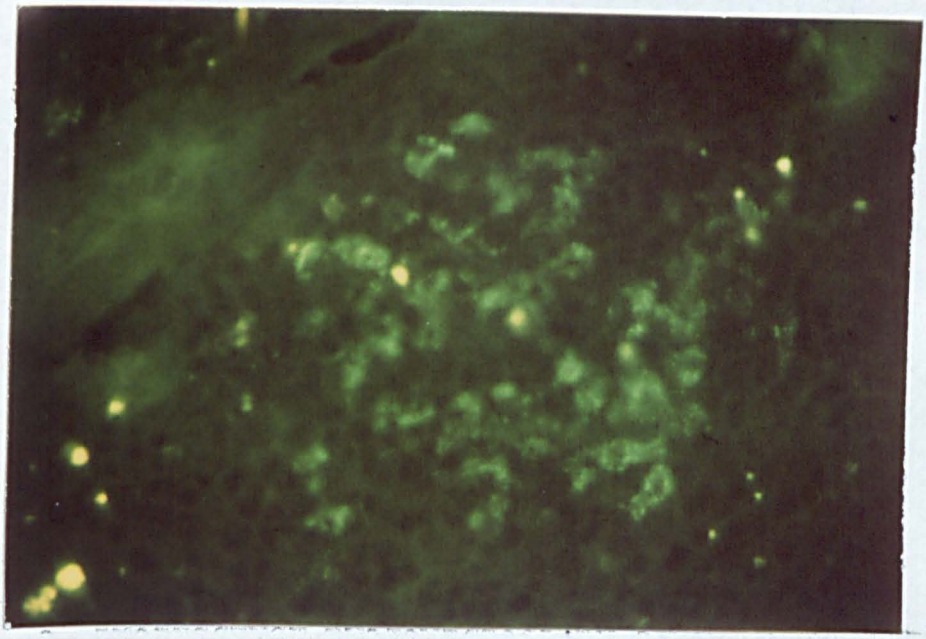


Plate 29 - Fluorescence photomicrograph of spleen section of an intact-irradiated chicken 48 hours after i.v. injection of HSA-anti HSA complexes. The frozen section of the spleen was stained with a single layer of fluorescein-labelled anti HSA antibody. There are large numbers of complex-bearing dendritic cells within the irregular outline of one germinal centre. Provision of specific antibody as HSA-anti HSA complexes speeded up formation of this germinal centre by one day. (X 630)

of this complex. There was no evidence of formation of germinal centre by the dendritic cells bearing the complex in any section. Spleen sections of the bursectomized birds contained considerably fewer complex bearing cells in comparison to those of their corresponding intact control.

Table 17 - Time scales for the localization of HSA-anti HSA complex in the spleens of bursectomized-irradiated and intact-irradiated chickens at different intervals after i.v. injection of HSA-anti HSA complex. The numbers of chickens killed at each stage after immunization is referred to in the brackets.

Chickens	5 minutes E. W. G.	24 hour E. W. G.	48 hour E. W. G.	72 hour E. W. G.
Bursectomized-irradiated	+ - - (3)	- + - (3)	- + - (4)	- + - (4)
Intact-irradiated	+ - - (3)	- + - (3)	- + + (4)	- + + (4)

+ = Localization of the complex
- = Lack of localization of the complex
E. = Ellipsoid
W. = White pulp
G. = Germinal centre

Antibody-containing Cells in the Spleen - Frozen sections of the spleens of the BX-IR and IN-IR chickens killed 72 hours after i.v. injection of HSA-anti HSA complex were also stained by sandwich technique in an attempt to demonstrate presence of antibody - containing cells in the spleen. Large numbers of plasma cells containing anti HSA antibody were found in the spleen sections of the IN-IR chickens. These cells were confined to the red

pulp strand and were not found near the ellipsoids or germinal centres. Spleen sections of the BX-IR chickens stained under identical conditions as those of their corresponding intact controls did not show presence of any antibody-containing cells in any section.

Antigen-bearing cells, Antibody-containing cells and IgG-containing cells in the Bursa and Thymus - In the frozen sections of the bursa and thymus treated with a single layer of fluorescein-labelled anti HSA antibody, no evidence of localization of complex was detected in the thymus of the BX-IR or IN-IR chickens killed at 5 minutes, 24, 48 and 72 hours after intravenous injection of the HSA-anti HSA complex. There was also total absence of complex-bearing cells in the bursa of the IN-IR birds killed at similar intervals after intravenous injection of HSA-anti HSA complex.

Frozen sections of the thymus and bursa were also stained by sandwich technique, using fluorescein-labelled anti HSA antibody, in an attempt to demonstrate presence of antibody-containing cells in these two central lymphoid organs. There was, however complete absence of antibody-containing cells in the thymus of both groups of birds and in the bursa of the IN-IR birds killed at different intervals after intravenous injection of HSA-anti HSA complexes.

Frozen sections of the thymus of the BX-IR and IN-IR chickens and those of the bursa of the IN-IR chickens were also treated with a single layer of fluorescein-labelled rabbit anti chicken IgG to demonstrate presence of IgG-containing cells in these two lymphoid organs. The frozen sections of the thymus and bursa of 3 intact non-immunized, non-irradiated Thornber 808 chickens which were eight weeks old were also similarly stained to serve as controls.

Plate 30

× 60

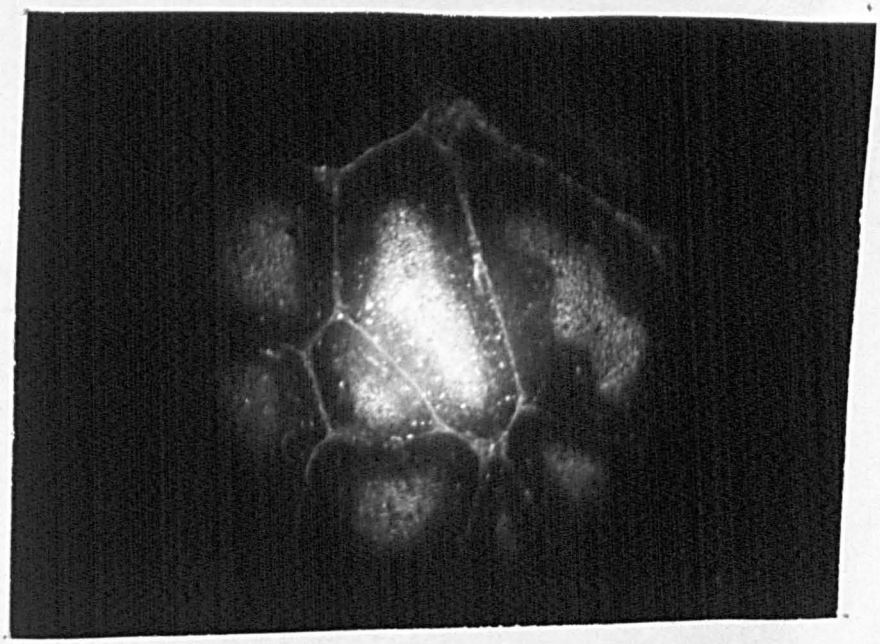
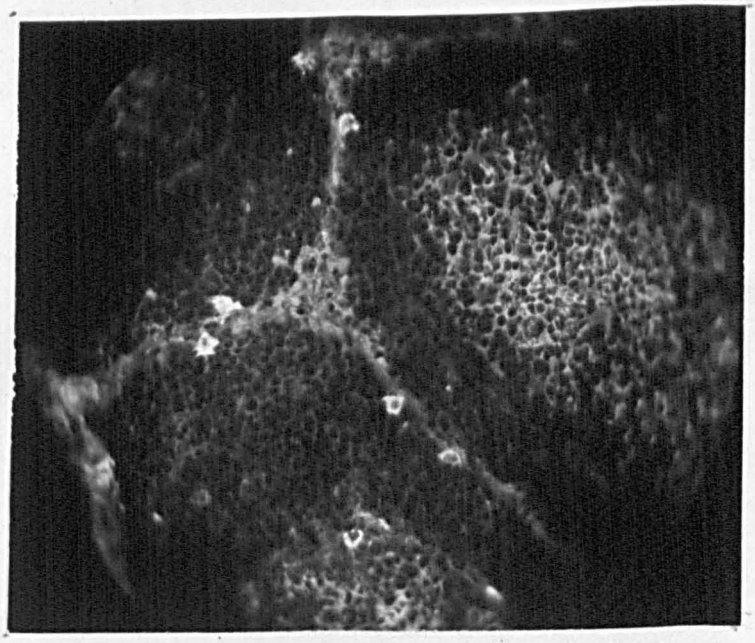


Plate 31

× 300



Fluorescence photomicrographs of sections of the bursa of Fabricius of an intact-irradiated chicken at 8 weeks of age. The sections were stained with a single layer of fluorescein-labelled anti chicken IgG.

There are large numbers of IgG-containing cells in the medulla of the bursa follicles. Note also few individual IgG-containing cells at the margins of the bursa follicles.

Large numbers of IgG-containing cells were found in the frozen sections of the bursa of the IN-IR birds which had been killed at different intervals up to 72 hours after intravenous injection of HSA-anti HSA complexes. The frozen sections of the bursa of the intact non-irradiated non-immunized birds also showed presence of IgG-containing cells in the medulla of the bursa follicles. Almost all bursa follicles in both groups of intact birds showed presence of IgG-containing cells. Thus there was no indication that immunization of one group of birds with HSA-anti HSA complexes had increased the numbers of bursa follicles which showed IgG-containing cells.

The IgG-containing cells were confined almost exclusively to the medulla of the bursa follicles. The cortex of the follicles thus remained as a dark zone surrounding the very bright central regions (plates 30 and 31).

The thymic sections of all groups of birds were also treated, under identical conditions as bursa sections, with a single layer of fluorescein-labelled rabbit anti chicken IgG. There was, however, total absence of IgG-containing cells in all thymic sections.

Summary to the Experiment 1, 2 and 3 - The effect of bursectomy followed by whole body irradiation on localization of native and aggregated HSA to the dendritic cells was investigated by means of fluorescent antibody technique.

Dendritic cells bearing the native HSA were detected in the spleens of intact-irradiated chickens on days 4, 5 and 6 after i.v. injection of 10 mg native HSA. Localization of the aggregated HSA to the dendritic was also detected in the spleens of the intact-irradiated chickens at 48, 72 and 96 hours after i.v. injection of 10 mg aggregated HSA. Antigen-bearing dendritic cells were found to take part in formation of germinal centres in the white pulp of the spleen.

Presence of antigen-bearing dendritic cells in the white pulp of the spleens of the intact-irradiated chickens was coincided with formation of antibody-containing cells in the red pulp of the spleen.

Bursectomized-irradiated chickens failed to localize native and aggregated HSA to the dendritic cells of the spleen. Antibody containing cells were also completely absent in the spleens of the bursectomized-irradiated chickens.

Provision of specific antibody as HSA-anti HSA complexes restored localization of HSA to the dendritic cells in the spleens of bursectomized-irradiated chickens. However, the antigen-bearing dendritic cells in the spleens of bursectomized-irradiated chickens failed to form any germinal centre.

These results clearly indicate that failure of the bursectomized-irradiated chickens to localize native or aggregated HSA is due chiefly to deficiency of these birds in production of specific antibody. The finding that provision of specific antibody, as HSA-anti HSA complexes to the bursectomized-irradiated chickens did not restore formation of germinal centre in the spleen is taken to indicate that B-lymphocytes as well as antigen-bearing dendritic cells are necessary for formation of germinal centres.

Aggregated HSA and HSA-anti HSA complexes did not localize in the thymus of the bursectomized-irradiated and intact-irradiated chickens. The bursa of the intact-irradiated chickens was also found completely free from aggregated HSA and HSA-anti HSA complexes.

Lack of localization of antigen in the thymus and bursa after intravenous injection of aggregated HSA and HSA-anti HSA complexes is discussed in relation to the fine vascular systems of these organs which considerably reduce the inflow of the circulating antigens in these two central lymphoid organs.

DISCUSSION

1 - Localization of Antigen and Antigen-Antibody Complexes in the Spleen - Both aggregated HSA and HSA-anti HSA complexes appeared on the cells at the periphery of the ellipsoids in the spleens of intact-irradiated and bursectomized-irradiated chickens 5 minutes after intravenous injection (plates 26 & 28). Localization of aggregated HSA and HSA-anti HSA complexes in and around the ellipsoids suggests that these materials had emerged from the ellipsoidal sheath and they had been taken up by a net of macrophages in these areas. Carbon particles of the indian ink were also found to segregate rapidly in the ellipsoids soon after intravenous injection. Clearance of the particulate materials from the circulation by the macrophages in and around the spleen ellipsoids is, thus immunologically non specific since there appears to be no difference between the antigens and non-antigenic materials in this respect and both intact-irradiated and bursectomized-irradiated chickens show similar pattern of localization.

Localization of aggregated HSA in the spleens of bursectomized-irradiated and intact-irradiated chickens 24 hours after i.v. injection was next investigated. There was no evidence of localization of aggregated HSA on the cells at the periphery of the ellipsoids or in any other parts of the spleens of the intact-irradiated birds 24 hours after intravenous injection of this antigen. It is unlikely that the cells which initially localized this antigen on their cell membranes, subsequently released it into the circulation. It is however, conceivable that following localization of the antigen on the cell membrane, the antigen was transported into the cytoplasm where it was broken down by the proteolytic enzymes. The antigen fragments within the cell became indetectable by the immunofluorescent technique. This view is supported by the fact that ^{131}I-HSA is readily detected in the cells at the periphery of the ellipsoids of the chicken spleen 72 and 88 hours after intravenous

injection (White et al. 1970). The technique of autoradiography permits detection of the radio-isotope labelled HSA within the cell.

A small amount of aggregated HSA was found on the surface of cells at the periphery of the ellipsoids in the spleens of bursectomized-irradiated chickens. With regard to localization of carbon particles, chickens bursectomized in ovo on day 18th of incubation were also found to retain carbon particles on their cells at the periphery of the ellipsoids longer than their corresponding intact controls (plate 13). It is possible that the rate of phagocytosis of aggregated HSA and carbon particles in the spleen are lower in the bursectomized chickens than the intact chickens. With regard to clearance of particulate substances such as carbon particles of the indian ink, bursectomized-irradiated chickens do not show any deficiency (Aiyedun 1971). Phagocytosis of the foreign particles by the macrophages normally takes place in two distinct stages. Firstly adhesion of the particles to the cell membrane and secondly transport of the particles into the cytoplasm. The present findings suggest that bursectomized chickens are probably deficient in the second stage of phagocytosis. It is well established that antibodies and immunoglobulins are opsonins which facilitate phagocytosis (Humphrey and White 1970). The deficiency of the bursectomized chickens in endocytosis of the aggregated HSA and carbon particles is probably due to the deficiency of these birds in production of antibodies and immunoglobulins. This concept is supported by the fact that provision of specific antibody as HSA-anti HSA complexes restores the normal function of the macrophages at the periphery of the ellipsoids in the spleens of the bursectomized-irradiated chickens as indicated by the total absence of the antigen-antibody complexes on the surface of these cells 24 hours after intravenous injection of the complex.

Aggregated HSA was subsequently found to have localized on the surface of the dendritic cells in the white pulp of the spleens of the intact-irradiated chickens 48 hours after immunization. The localization of this antigen on the dendritic cells in the spleens of intact-irradiated chickens coincided with appearance of antibody-containing cells in the red pulp strands of the spleen. White and his co-workers (White, French and Stark, 1966) have produced ample evidence which suggests that specific antibody is an essential factor for localization of the antigen to the dendritic cells of the spleens of the intact birds. They showed that immunologically tolerant intact birds which do not produce detectable amount of specific antibody are totally unable to localize HSA to the dendritic cells of the spleens. Furthermore, by the use of fluorescein-labelled rabbit anti chicken immunoglobulin they showed that the antigen at the surface of dendritic cells is in the form of antigen-antibody complexes (French, Wilkinson and White 1969). Lack of localization of aggregated HSA on the dendritic cells during the first 48 hours after immunization is therefore considered to correspond to the lag in production of antibody which normally occurs for similar length of time after the primary antibody response.

Bursectomized-irradiated chickens did not localize the native HSA to the dendritic cells of the spleen on day 4, 5 and 6 after intravenous injection of this antigen. The localization of aggregated HSA was also completely absent from the spleens of bursectomized-irradiated chickens at 48, 72 and 96 hours after intravenous injection of this antigen. Antibody containing cells were totally absent in the spleens of the bursectomized-irradiated chickens inoculated intravenously with 10 mg native HSA or 10 mg aggregated HSA. The sera of the bursectomized-irradiated birds immunized to native HSA did not contain any detectable amount of

specific antibody.

Provision of specific antibody in the form of HSA-anti HSA complexes resulted in localization of the antigen to the dendritic cells in the spleens of the bursectomized-irradiated chickens. These results rule out the possibility that the deficiency of bursectomized-irradiated chickens in localization of native or aggregated HSA is due to the lack of dendritic cells in the spleens of these birds. The competency of the dendritic cells to localize antigen-antibody complexes also does not diminish after neonatal bursectomy followed by whole body irradiation. It is therefore suggested that the inability of the bursectomized-irradiated chickens to localize native or aggegated HSA is due entirely to inability of these birds to produce specific antibody.

The role of antibody is to provide a link between the antigen and the dendritic cells since some antigens such as human gamma globulin (HGG) is readily localized to the dendritic cells in the spleens of antibody deficient bursectomized chickens (White, Henderson, Eslami and Nielsen 1975). White and his co-workers showed that localization of HGG to the dendritic cells occurs by means of a receptor in the Fc portion of the molecule since HGG treated with pepsin failed to localize to the dendritic cells in the spleens of bursectomized-irradiated chickens. Failure of native and aggregated HSA to localize to the dendritic cells in the absence of antibody, in the spleens of bursectomized-irradiated chickens, is therefore due to the lack of the appropriate receptors which could link these antigens directly to the dendritic cells.

Provision of specific antibody as HSA-anti HSA complex to the dendritic cells accelerated the events leading to formation of germinal centres in the white pulp. Thus in the spleens of the intact-irradiated chickens germinal centres with complex-bearing dendritic cells were found at 48 hours after intravenous injection of the complex (plate 29). The earliest formation of

germinal centres in the spleens of the intact-irradiated birds immunized to native or aggregated HSA was 72 hours after immunization. Thus, the time scale for formation of germinal centres in the spleens of intact-irradiated birds had been shortened by 24 hours due to passively administered specific antibody.

The dendritic cells loaded with the complex in the spleens of bursectomized-irradiated chickens did not form germinal centres even 72 hours after intravenous injection of the complex. It has been suggested that formation of germinal centre is due to a progressive capture and aggregation of lymphocytes by the antigen-bearing dendritic cells (White et al.1970) Neonatal bursectomy followed by whole body irradiation depletes the spleen and other lymphoid tissues from B-lymphocytes. The present finding on the inability of the complex bearing dendritic cells to form germinal centre in the spleens of bursectomized-irradiated chickens thus suggests that the bursa derived cell (B-lymphocyte) is an essential cellular element for formation of germinal centres.

2 - Localization of Antigen in the Bursa and Thymus - There was no evidence of localization of aggregated HSA and HSA-anti HSA complexes or presence of antibody-containing cells in the frozen sections of the bursa of the intact-irradiated birds killed at 5 minutes, 24, 48, 72 and 96 hours after a single intravenous injection of these antigens.

The frozen sections of the thymus of the bursectomized-irradiated and intact-irradiated chickens which had similarly been given an i.v. injection of aggregated HSA or HSA-anti HSA complexes and had been killed at similar intervals after immunization were also found completely free from aggregated HSA, HSA-anti HSA complexes and antibody-containing cells.

Frozen sections of the thymus of both intact-irradiated and bursectomized-irradiated chickens also showed complete absence of

IgG-containing cells. In contrast, the frozen sections of the intact-irradiated birds showed large numbers of IgG-containing cells which were located chiefly in the medulla of the bursa follicles (plates 30 and 31).

These results clearly demonstrate that, with regard to localization of antigen and formation of antibody-containing cells, the central lymphoid organs of chicken differ markedly from the spleen in which both localization of the antigen and formation of antibody-containing cells readily develop following i.v. injection of native or aggregated HSA.

Unlike the peripheral lymphoid organs such as spleen, the bursa and the thymus begin to involute in chicken with the onset of sexual maturity at 4 – 4½ months of age (Ackerman and Knouff, 1959). Lack of localization of the antigens and formation of antibody-containing cells in the bursa and the thymus, however, can not be attributed to the involution of these two organs since the birds used in the present study were 7 - 10 weeks old. The cellular architecture of the thymus, thus, was normal and did not apparently show any sign of atrophy. Bursa of Fabricius also showed no sign of atrophy as indicated by the follicles packed densely with cells and presence of large numbers of IgG-containing cells in the medulla of the follicles (plates 30 and 31).

Several groups of workers have also found that the bursa and the thymus do not show a marked cellular reaction following intravenous injection of an antigen. Thus antibody-containing cells were found absent following i.v. injection of sheep RBC (Dent et al. 1965, Jankovic et al. 1967). Although bursa of Fabricius is the lymphoid organ which contributes to the formation of germinal centres in other lymphoid tissues; these centres never develop in the bursa of Fabricius. Formation of germinal centres in the thymus after a single i.v. injection of an antigen

is also a very rare cellular reaction.

One of the early studies which was carried out to establish the conditions in which antigen localization may occur in the thymus was that of Marshall and White (1961). They showed that i.v. injection of diphtheria toxoid in rat and guinea pig does not bring any histological changes in the thymus whereas direct inoculation of the same antigen into the thymic lobes results in localization of the antigen in the Hassell's corpuscles and formation of germinal centres in the medulla of the thymus. These workers accordingly introduced the concept of blood-thymus barrier which suggests that lack of reactivity of thymic tissues to circulating antigen is due to a barrier against the entry of the antigen into the thymus or a lack of suitable phagocytic mechanism to segregate the antigen in this lymphoid organ.

In chicken, the bulk of intravenously injected colloidal carbon of the indian ink is taken up by the liver, spleen and lung since there is a large volume of blood flow as well as numerous phagocytic cells in these organs (Aiyedun, 1971). Bursa follicles and thymus lobules have very small arteries and veins. The fine vascular systems of the bursa and thymus considerably reduce the inflow of circulating antigens into these lymphoid organs. It is also likely that dendritic cells and macrophages are less prominent in the thymus and bursa. There is no report regarding the presence of dendritic cells in the thymus and bursa of Fabricius. However, the fact that germinal centres formed in the medulla of the chicken thymus following multiple immunization with sheep RBC (Jankovic et al. 1966) or after a single i.p. injection of 'phage ΦX174 (plate 4) can be taken to indicate that the antigen had entered the thymus and had localized to the dendritic cells.

Presence of carbon-containing cells in the medulla of the

thymic lobules and in the medulla of the bursa follicles also indicates that macrophages are not absent in these two central lymphoid organs. However, the numbers of the cells bearing the colloidal carbon of the indian ink in the thymus and bursa of Fabricius were very few. This finding substantiates the concept that liver, lung and spleen primarily take the bulk of antigen and colloidal carbon from the circulation, the bursa and thymus remain free from a high concentration of these materials. It is therefore likely that multiple intravenous injection of an antigen may lead to localization of the antigen in the thymus and bursa of Fabricius.

CONCLUSIONS

The effect of bursectomy on localization of native HSA, aggregated HSA and HSA-anti HSA complexes to the dendritic cells was studied using fluorescent antibody technique. The following conclusions have been reached.

1. The early localization of aggregated HSA (at 5 minutes after intravenous injection) to the cells at the periphery of the ellipsoids does not depend on the availability of specific antibody as both intact and antibody deficient bursectomized-irradiated chickens show similar pattern of localization.

2. Failure of the bursectomized-irradiated chickens to localize native and aggregated HSA to the dendritic cells is due chiefly to the inability of these birds to produce antibody since provision of specific antibody as HSA-anti HSA complex restored localization of HSA to the dendritic cells in the spleens of the bursectomized-irradiated chickens.

3. The fact that provision of specific antibody to the bursectomized-irradiated chickens, as HSA-anti HSA complexes, did not restore formation of germinal centres in the spleen is taken to

indicate that the B-cells (bursa derived lymphocytes) as well as antigen-bearing dendritic cells are necessary for formation of these centres.

4. Lack of localization of aggregated HSA and HSA-anti HSA complexes in the bursa and thymus after a single intravenous injection of these antigens is probably due to the fine vascular system in these organs which reduce considerably the inflow of the circulating antigen in these two central lymphoid organs.

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