GERMINAL CENTRE INDUCTION

IN NEONATAL GERM-FREE CHICKENS

A thesis

by

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Summary of Ph.D. thesis J.C. Anderson

The hypothesis was that the cellular architecture of the lymphoid tissue was determined by antigenic stimulation. In order to test that hypothesis chickens were produced and maintained in an environment as free from antigens as possible. The chickens were then challenged with an antigen and the cellular changes in the lymp tissue, especially the spleen, examined.

The thesis thus falls into three sections. In Section I, after the concept of germ-free life was introduced, the practical problems of producing and maintaining germ-free chickens were discussed in relation to the needs of the present work. A detailed description then followed of the method by which germ-free chickens were produced and maintained in this study. The use of the germicide "Portex D.C.R." was described for sterilizing the surface of fertile eggs in order to obtain germ-free chickens.

In Section II the lymphoid tissue of the conventional chicken was described. In order to form a base-line or norm for the study of the effect of antigens in germ-free chickens the lymphoid tissue of the four week old unstimulated germ-free chick was studied. Serum proteins from these birds were immunoelectrophoresed. It was shown that in the unstimulated four week old germ-free chicken no germinal centres were present in the spleen and that there were fewer cells of the plasmacellular seri when compared with four week old conventional chickens. The level of immuno-

globulins was lower in germ-free chickens than in conventional chickens.

Having established this norm it was then possible (Section III) to challenge the germ-free chickens with an antigen in an attempt to induce germinal centre for tion and to study the way in which germinal centres were formed. The tissues from the germ-free chickens stimulated with a known antigen were examined using conventional histology and immunofluorescence; serum antibodies were estimated a serum proteins were immunoelectrophoresed. In experiment I an attempt was made induce germinal centre formation in conventional birds using Shigella flexneri as antigen. In the next two experiments a soluble protein antigen (Human serum albu was administered to seven day old germ-free chickens (expt. 2) and to seven day ol conventional chickens (expt. 3) to induce germinal centre formation. In the remaining three experiments a staphylococcus isolated from a chicken was used as antigen. Germ-free chickens were given the staphylococcus as a primary injectio at seven days old (expt. 4) and at twenty-one days old (expt. 5) and as a secondary injection at twenty-one days old (expt. 6) following a primary at seven days old.

From these experiments it was concluded that the cellular architecture of th lymphoid tissue and in particular the spleen, was dependent upon immunogenic stimulation. However the response to different antigens administered at the same was not the same. In germ-free chickens both the soluble protein antigens (HSA) and the particulate antigen (staphylococcus) induced proliferation of cells of the plasmacellular series but germinal centres were only found in the spleen following stimulation with staphylococcus. Further, the response to the same dose of the sa antigen (staphylococcus) varied with the age of the chicken during the neonatal pe

A greater number of germinal centres and a greater proliferation of cells of the plasmacellular series was induced following injection of staphylococcus into 21 day old germ-free chickens than into 7 day old germ-free chickens.

A secondary challenge with staphylococcus at 21 days old in germ-free chick following a primary injection at 7 days old induced a greater number of germinal centres than a primary injection at 21 days old.

The germinal centre appeared to be formed in the spleen not by rapid multiplication of a small focus of cells but by aggregation of haemocytoblasts in the periarterioler lymphocyte sheath. Lymphocytes did not appear to be incorporated in germinal centre formation but seemed to be formed from haemocytoblasts within the germinal centre.

No cellular changes were induced in the thymus or bursa of Fabricius follow antigenic stimulation of germ-free chickens.

It was clear from the experimental work that the cellular architecture of the spleen of the chicken was dependent upon immunogenic stimulus.

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CONTENTS

GERMINAL CENTRE INDUCTION IN NEONATAL GERM-FREE CHICKENS

Introduction

The association of antibody production with lymphoid tissue has resulted in a fresh interest in this type of tissue. It is known that an antigenic stimulus brings about a series of cellular changes in a lymph node or in the spleen and the effect of a number of such antigenic stimuli is to produce a node or spleen which is "reactive". A number of cellular reactions, each at a different stage, are superimposed in the organ to produce a complicated histological picture.

Thus it seems that much of the cellular architecture of the spleen could be antigen dependent. However, an important question is - how much of the cellular structure of the spleen is due to inherent gene controlled development and how much is due to extraneous or foreign stimulus? In particular, do the germinal centres of the spleen arise independently of antigenic stimulus, as a normal growth change or are these centres induced by immunogenic stimuli? The hypothesis is that the cellular architecture of the lymphoid tissue and of the spleen in particular is dependent less on genetic constitution than on the stimulus of a foreign environment. The stimulus for the development of the lymphoid tissue is provided by proteins and polysaccharides in the diet, bacteria

and dust in the air, contact with bedding and housing, and probably most important, bacteria, viruses, protozoa and fungi which are present in the gut of the animal. This last group is most important because they are self-replicating, they can multiply in the gut and invade the tissues of the animal.

In order to test the hypothesis that the lymphoid architecture is built up by repeated antigenic challenge it was decided to attempt to cut down the stimulus provided by the environment by rearing the chickens under sterile conditions. By rearing them in this sterile environment contact with living self-replicating micro-organisms would be prevented. This would remove a major source of stimuli although it would not be possible to remove the non-replicating macromolecules - the proteins and polysaccharides - of the diet. Having produced and reared the chickens under this sterile or germ-free environment it would then be possible to stimulate the lymphoid tissue with a single antigen and study the cellular changes produced by that antigen. It would be possible to determine if germinal centres could be induced by an antigen.

The thesis thus falls into three sections. In Section I the method of producing and maintaining germ-free chickens is described. In Section II the unstimulated lymphoid tissue of the germ-free chicken is characterized in order to form a base-line or norm for the third section in which the reaction produced by the injected antigen is studied. The results are then discussed.

SECTION I GERM-FREE CHICKENS

Concept of germ-free life

The study of organisms by themselves or in association with other known species in the absence of all other demonstrable living organisms is known as gnotobiology. An organism is said to be germ-free if it exists in the absence of all demonstrable living micro-organisms. A gnotobiote is an organism living free from contamination or in association only with organisms known to the investigator.

Two or more individuals of the same species living together do not violate the condition of germ-freeness. In the same way a germ-free mouse and a germ-free rat can live together without violating the germ-free state. It is argued that the presence of the one species has very little biological effect on the other. However, the existence of germ-free lice on chickens or worms (Toxocara canis) in dogs would mean that the chickens or the dogs were not germ-free. Since, although they are distinct species, the relationship is intimate. Thus the germ-free animal becomes contaminated when the contaminant is living and in intimate contact with the original germ-free animal. This distinction is important since it means that if an intracellular virus is found in a "germ-free" animal that animal ceases to be germ-free. But at the same time, this allows the existence of, for example, bacteria in a corner of the isolator which, though living, are not in intimate contact with the germ-free animal and do not, therefore, destroy its germ-freeness. If, however, the animal escapes from its cage and

picks up the bacteria then the animal would cease to be germ-free.

Perhaps the concept can be clarified by an example. An isolator containing only rats and chickens would contain two germ-free species. If that system were inoculated with Lactobacillus acidophilus there would be no germ-free animals and three kinds of gnotobiotes.

Germ-free conditions in Nature

The true germ-free state in which a single organism exists in isolation is rare in nature. However, it is known that Thiobacillus thio-oxidans is one of the few organisms which can grow in 5% H_2SO_4; and Lactobacillus bifidis is the predominant (99.9% pure) organism in the gastro-intestinal tract of infants fed only colostrum. Blue-green algae are known to be the first organisms to invade the sterile soil of volcanos.

In nature situations exist where an organism is in isolation except for the cellular environment of its host. For example, intracellular bacteria, rickettsia and viruses fall into this category. They can exist in their intracellular environment free from other species excepting the host. In the same way helminths and protozoa can exist in the sterile tissues of their host. This is illustrated by the intramuscular larvae of Toxocara canis, or the intra-erythrocyte location of Babesia divergens.

Of more practical value is the germ-free state within an animal. The blood of a healthy animal is sterile and would therefore yield germ-free or sterile blood cells. The fertile egg of a healthy bird contains an embryo which is free of all micro-organisms; and the foetus of a healthy woman is in utero

completely isolated from all detectable micro-organisms. These facts are of value in gnotobiology for it is from these germ-free situations that germ-free animals are derived.

General historical development of gnotobiology

The idea of rearing germ-free animals was first expressed by Pasteur in 1885 in a note following the presentation of the work of his student, Duclaux, to the French Academy of Science. The note ran as follows:-

"In presenting this note of Duclaux, I take the liberty to offer an idea for an experiment which comes not only from the evidence which I give to the Academy in his name, but also from work, no less distinguished, which he has already done on the role of bacteria in digestion.

For several years during discussions with young scientists in my laboratory, I have spoken of an interest in feeding a young animal (rabbit, Guinea pig, dog or chicken) from birth with pure nutritive material. By this expression I mean nutritive products which have been artificially and totally deprived of the common micro-organisms.

Without affirming anything, I do not conceal the fact that if I had the time, I would undertake such a study, with the preconceived idea that under these conditions, life would be impossible.

If this work could be developed simply, one should be able to study digestion by the systematic addition to the pure food, of one or another single micro-organism or diverse micro-organisms with well defined relationships.

A chicken egg could be used without serious difficulty for this type of experiment. Before the chick is hatched the exterior could be cleaned of all living organisms; then the chick would be placed in a cage without any kind of micro-organisms. In this cage where pure air is given, the chick would also be supplied with sterile food (water, milk and grains).

Whether the result would be positive and confirm my preconceived view, or whether it would be negative, in other words, that life would be easier and more active, it would be most interesting to perform the experiment." (Pasteur, 1885).

It was perhaps fortunate that Pasteur had stated his preconceived idea for it was opposed by Nencki (1886) who argued that life was possible without bacteria. Thus the early attempts to obtain germ-free animals and therefore the development of the techniques were spurred on by the question which had been raised - was life possible without bacteria?

It was not till 1895 - 96 that the first germ-free animals were obtained. Nuttall and Thierfelder (1895 - 96) placed Caesarean delivered guinea-pigs in a sterile bell jar adapted for the purpose and fed them aseptically drawn and sterilized milk. The guinea-pigs were germ-free and appeared healthy. They were sacrificed at eight days of age. It was concluded that the presence of bacteria in the intestinal tract was not essential for life. This was Nuttall and Thierfelder's answer to Pasteur's remark of 1885.

After Nuttall and Thierfelder's first experiment the idea of gnatobiology

was taken up by other workers and the techniques developed. The fields of application fanned out; the techniques were applied to different animals. Thus germ-free fish (Shaw, 1957), insects (House, 1959), amphibia (Wollman, 1913), protozoa (Dougherty, 1959) and tissues (Paul, 1959) were obtained as well as small laboratory animals (Pleasants, 1968), large mammals (Whitehair, 1968) and chickens and quail (Coates, 1968).

It must be added however, that all the early workers with guinea-pigs were not as successful as Nuttall and Thierfelder (1895 - 96) and the controversy continued for many years. It was not until 1959 that healthy guinea-pigs were reared through a complete generation under germ-free conditions (Teah, 1959, cited by Luckey, 1963).

Particular historical development of germ-free eggs

Nuttall and Thierfelder (1897) confirmed Pasteur's contention that the chick embryo was sterile while the surface of the egg was not sterile. However, they were not able to successfully sterilize the surface of the egg. Schottelius (1899) verified that the embryo can be sterile; he was able to sterilize the egg surface and obtained germ-free chickens. Schottelius' germ-free chickens did not gain weight at the same rate as conventional chickens and became moribund. He concluded that intestinal bacteria were necessary for adequate nutrition of the animal. Later Schottelius (1902) using a glass partition divided the germ-free cage into two compartments in each of which were two chickens. To one of the compartments he added 20 gm of nutritive broth inoculated with fresh excreta

from conventional chickens. The inoculated group gained weight; the germ-free group died. Schottelius concluded that bacteria were necessary for adequate nutrition of the animal. Modern reviewers (Reyniers et al. 1949a) attribute Schottelius' lack of success to the detrimental effect of heat sterilization on the diet.

Cohendy (1912) learned the technique of producing germ-free chickens from Schottelius and using his own diet he produced germ-free chickens which grew and gained weight. He was able to observe that germ-free chickens resembled conventional chickens as far as their gross morphology, development and appearance were concerned.

From 1912 till 1937 there was no published work on germ-free chickens.

In 1937 Balzam (1937) studied the effect of the intestinal flora of the chicken on B-vitamin requirements and reached the conclusion that the intestinal flora gave practically no protection against B-vitamin deficiency.

Reyniers began work on germ-free chickens at the Lobund Laboratory, Notre Dame, Indiana in 1943 and in 1949 reported the first egg-chicken-egg-chicken cycle (Reyniers et al. 1949b). In that same year the Lobund Report No. 2 appeared (Reyniers et al. 1949a) in which the techniques for rearing germ-free chickens were standardized. The publication of this report established first of all that chickens could live in the germ-free state; secondly, it outlined methods of producing germ-free animals which are still followed to this day; and thirdly, it began the work of characterizing germ-free animals - which has been the task of many workers since.

Germ-free chickens - theoretical considerations

In order to obtain germ-free animals it is necessary

a) to obtain animals devoid of micro-organisms, and

b) to maintain these animals under germ-free conditions, and at the same time supply them with all they need to live and, if required, to reproduce. Now it is quite clear that the environment in which we live is full of innumerable micro-organisms. Therefore, it is necessary to confine the animals to an enclosure which has previously been sterilized. This enclosure is called an isolator, or unit. Further, this isolator must be an effective barrier against micro-organisms while at the same time allowing a route of entry and exit to the animals. To achieve this situation the isolator must be thoroughly sterilized; the mechanical barrier must be effective; and scrupulous attention must be paid to sterile techniques. The point cannot be overemphasized since the entry of one micro-organism can result in the contamination of the isolator and the ruin of an experiment.

Types of isolator

Isolators can be divided into three main groups:

1 Glove-box isolators That is, isolators in which manipulations are carried out by an operator who remains outside the sterile area. The hands and arms enter the isolator by means of gloves which form part of the isolator wall.

The isolator designed by Reyniers (1959) - the Reyniers isolator - is cylindrical in shape and is made of stainless steel. Entrances are made through a

small autoclave which is placed at one end and forms an integral part of the isolator. A viewing part is incorporated in the wall along with the gloves and other necessary tubes. The stainless steel is sufficiently strong to withstand steam at 121°C which is passed into the isolator; in this way it is sterilized. It is really a modified autoclave.

The isolator described by Miyakawa (1959) is similar to the Reyniers isolator except that instead of working through gloves the manipulations are made using mechanical pick-up arms which are operated outside the isolator.

The Gustafson isolator was first described by Gustafson in 1948 (Gustafson, 1948). It is made of light-weight stainless steel which can be autoclaved. Since the pressure outside and inside the isolator is in equilibrium a large pane of glass can be incorporated in the wall. Entry is usually made to the isolator through a germicidal tank, but Gustafson (1959) added a small side autoclave for easy entry.

Phillips et al. (1962) described an isolator constructed of rigid acrylic sheet. This isolator is sterilized chemically.

A flexible isolator made of thin (0.5 - 2 mm) polyvinyl sheeting was described by Trexler and Reynolds (1957), Trexler and Barry (1958), Trexler (1959a) and Trexler (1960). The polyvinyl film can be easily worked and heat sealed into any desired shape. The flexible isolator is maintained in positive pressure which tends to counteract the entrance of micro-organisms. The isolator is sterilized with 2% peracetic acid. This sterilizing agent acts rapidly on all known micro-organisms and destroys spores. Entrances are made to the isolator through a lock

which is sterilized with peracetic acid.

Lev (1962) described an isolator which is made of autoclavable nylon. Entry is made to this isolator through a germicidal tank.

II Sterile Rooms The isolator is of the dimensions of a room and the operator enters after dressing in a diving suit the outside of which has been sterilized. The walls of the sterile room described by Trexler (1959b) were made of plastic which was resistant to peracetic acid. The diving suit for entering the room was sterilized with formaldehyde, quaternary ammonium compounds or peracetic acid. The difficulties of successfully sterilizing the suit has meant that the technique is little used.

III Jacket Isolator This type falls between the glove-box and the sterile room and is so designed that the operator can enter the unit from the waist up by means of a half-suit which forms part of the isolator wall, and is attached to it through a circular opening in the floor. This type of isolator (Trexler, 1961) is the largest isolator that can be used without a diving suit.

The glove-box isolator described by Lev (1962) seemed to be best suited to the needs of the projected experiments. The unit could be constructed cheaply and quickly and a large autoclave was available to sterilize the unit.

Obtaining germ-free animals

The most reliable method of obtaining germ-free animals is from other germ-free animals. However, other germ-free animals of the required species may not be available and it is not always practicable to maintain colonies of germ-free animals. At any rate, it is quite clear that the original animals must

have been obtained from a source other than existent germ-free animals. For this original source of germ-free animals it is necessary to turn to the germ-free situations found in nature. As was pointed out the embryo in the uterus of a healthy female is sterile and the embryo in the egg of a healthy bird is sterile. These situations became the respective sources of germ-free mammals and chickens.

Germ-free mammals Briefly, since this study is on germ-free chickens, not germ-free mammals, germ-free mammals are obtained by Caesarean delivery from the pregnant female into a sterile environment at term.

Germ-free chickens Germ-free chickens are obtained by sterilizing the surface of the shell at the 19th day of incubation and completing the incubation in sterile conditions. The chickens which hatch should be germ-free. Given that the unit is effectively sterilized and is an effective barrier, the germ-freeness of the chickens depends on the effective sterilization of the shell and the absence of living organisms in the egg.

Sterility of the avian embryo

It is known that avian infectious encephalomyelitis, infectious bronchitis, lymphoid leukosis and Newcastle disease all caused by viruses; chronic respiratory disease and infectious synovitis both caused by mycoplasma; and fowl typhoid, pullorum disease and tuberculosis all caused by bacteria, can be transmitted through the egg (Payne, 1967). Non-pathogenic bacteria may infect the egg in the reproductive tract of the hen (Harry, 1963a). Viruses of low pathogenicity - the chicken-embryo-lethal-orphan (CELO) virus and the Gallus adeno-like (GAL) virus - have been shown to be present in avian embryos (Yates and Fry, 1957;

Kohn, 1962). Extra-genital infection of the egg may occur from contamination of the shell with faeces or bedding. Pathogenic organisms such as salmonellae or non-pathogenic organisms such as pseudomonas, proteus or fungi may gain entrance to the egg at this stage (Harry, 1963b).

There is, therefore, considerable evidence against the idea that the embryo in the egg is germ-free. However, despite this, studies of the actual infection rate of newly laid eggs show that this is in fact low. Harry (1957) puts this infection rate at 2.5% and Reyniers et al. (1949a) at 5%. The antibacterial action of the yolk is probably important in the control of this route of infection.

In practical terms, fertile eggs to be used for producing germ-free chickens should only be taken from healthy birds thus eliminating the transmission of pathogenic organisms. The healthy birds should be housed in clean premises so that the eggs may be laid and collected as free from contamination as possible. In this way the risk of using eggs which are not sterile is reduced to a minimum.

Sterilization of egg surface

As has been pointed out, if the surface of the fertile egg can be successfully sterilized and the incubation completed under sterile conditions the resultant chickens will be germ-free. Many workers since Nuttall and Thierfelder (1897) have attempted to find a germicide for this purpose. Such a germicide must be capable of killing spores, yet at the same time it must not damage the developing embryo. Mercuric chloride was used by Nuttall and Thierfelder (1897), the method was evaluated and standardized by Reyniers et al. (1949a) and mercuric chloride

is still the most commonly used germicide (Coates, 1968). Mercuric chloride however, often results in a lower hatch rate and further it is known that mercuric chloride does not kill all spores (Luckey, 1963). Detergents have been used in combination with mercuric chloride without any real improvement.

A new disinfectant has been described for sterilizing the surface of eggs in order to obtain germ-free chickens (Anderson, 1969). This disinfectant - "Pertex D.C.R." - is a solution of dipenidam, a brand of "Rhudane". It is described by the manufacturers (Brocades Ltd., Westbyfleet, Surrey) as "a charge-transfer complex of an alkylaminosulphonic acid, iodine and a non-ionic surfactant." It is active against Gram-positive and Gram-negative organisms, spores and fungi. Pertex D.C.R. is not an iodophor since it does not give a positive starch reaction. There is therefore no free iodine, however, dipenidam has a charge complex from which iodine is made available to act upon the organism.

The advantage of this disinfectant seemed to be its bactericidal and sporicidal action together with its low toxicity. The manufacturers claimed 100% kill of vegetative forms of Bacillus subtilis (NCTC 3610) at 1:400 dilution in 2 minutes at 18 - 20°C and 100% kill of spores of Bacillus subtilis and Bacillus stearothermophilus at 1:400 dilution in 5 minutes at 18 - 20°C. At the same time a low toxicity was claimed. Intravenous injection of 2 ml of 2% Pertex D.C.R. failed to cause death in six male rats and 100% Pertex administered orally at 2 g/Kg failed to kill six male rats. On the basis of these claims the disinfectant was used at 1:400 dilution for 2 minutes at 37.5°C (incubation temperature) P

sterilize the surface of eggs. Since a good hatch rate was obtained and no contamination was found which could be traced to inadequate sterilization of the egg shell the disinfectant was considered to be of value in producing germ-free chickens.

Microbial testing

For a germ-free system to be of any value it must be known to be germ-free. Now the idea of germ-freeness is really a negative concept, since it is based on the failure to demonstrate living organisms from the sample. Further, it is worth observing that an organism will not be found which is not looked for: an animal cannot be said to be free of rickettsia if rickettsia are not looked for. Thus the routine of microbial testing can become an elaborate and exacting task. Wagner (1959) has described and evaluated sterility tests and his recommendations are still in use.

Theoretically, samples should be taken from food, water, bedding, and cage surfaces as well as from the body surface, excretions and secretions of the animals. These samples should be inoculated into common and selective media which are incubated at 25°C, 37°C and 55°C aerobically and anaerobically. At the same time steps must be taken to detect viruses, mycoplasma, rickettsia, protozoa and fungi. Direct films are stained by Gram's method and examined microscopically. Special stains such as Fontana and Ziehl Neelsen may be used. The animals should also be observed macroscopically for evidence of clinical infection.

In practice, a microbial testing routine must be found which is not a

study in itself yet is a reliable indication of the microbial status of the animals.

Sterilization of air.

Most air sterilization systems consist of a prefilter and a filter. Nuttall and Thierfelder (1897) passed the air through two cotton filters. The air was carried between these two filters by a platinum tube which was heated to red heat by a Bunsen burner. Cohendy (1912) and Balzam (1937) passed the air through a cotton filter and then bubbled it through mercuric chloride. Reyniers et al. (1949a) pumped prefiltered air through long glass wool filters. These filters were sterilized by steam. Trexler and Reynolds (1957) used four layers of glass wool filter (PF-105). This filter was also sterilized by steam. Trexler (1959) and Phillips et al. (1962) used FG-50 glass wool filter down and this has proved to be an effective filter. Lev (1964), using FG-50 filter down, designed a filter which could be easily constructed and sterilized by steam. The filter was then dried in the hot air oven. Lev (1964) tested the efficiency of the filter by dispersing 10^6 phage particles of size 0.1 x 0.001 μ in 10 ml of saline as a fine spray in front of the filter while in use (5 - 10 minutes). Phage particles were not detected in the air after passage through the filter. Further, a sodium chloride cloud (particle size c. 0.5 μ) was completely retained by the filter. It was decided to use this filter to sterilize the air.

Sterilization of the diet

The early workers sterilized the diet by steam and this still remains one of the main ways of sterilizing the diet. However, it is known that in steam

sterilization there is a loss of nutritive value of proteins particularly through its effect on lysine, methionine and cysteine, loss of certain B vitamins and also of vitamins A and E (Reddy et al. 1968).

Ethylene oxide has been used (Porter and Lane-Petter, 1965) but again some reduction in the B vitamin content was experienced.

Sterilization of the diet by gamma irradiation has proved to be a successful method. Coates et al. (1963) found that an enriched natural-type diet failed to support normal growth after autoclaving, but the same diet irradiated at 5 Mrads from a ^{60}Co source supported better than conventional growth. Although vitamin K is one of the most radiosensitive nutrients and germ-free chickens are especially sensitive to a deficiency of this vitamin, the irradiated diet permitted normal clotting times. Chemical analyses of the diet showed that even the radiosensitive vitamin E and carotene suffered only 50% loss from irradiation, while thiamine was reduced only 32%.

Because of the proven value of irradiation as a method of sterilizing the diet for germ-free chickens it was decided to adopt that method of sterilization. The diet was therefore sent to Wantage Research Laboratory, U.K.A.E.A., Wantage, Berkshire where it was irradiated at 4 Mrads from a ^{60}Co source. It was found that the dose could be reduced to 4 Mrads without altering the sterility of the diet.

The diet used was Baby Chick Crumbs (British Oil and Cake Mills, Ltd.) with the following ingredients - Herring meal, Soya meal, Decorticated groundnut meal, Sunflower meal, Decorticated cotton cake, Barley, Oats, Maize, Milo,

Wheatfeed and Molasses, and the following minerals and vitamins:-

Limestone	1 3/4%
Dicalcium phosphate	3/4%
	Grammes per ton
Magnesium sulphate	224
Zinc oxide	45
Salt	1250
Potassium iodide	2
Topanol	128
Calcium pantothenate	20
Folic acid	1
Vitamin K	2
Vitamin B_1	2
	Mgm per lb
Vitamin B_2	2.23
Vitamin B_{12}	2.06
	m.i.u. per ton
Vitamin A	7.5
Vitamin D_3	1.5
Vitamin E	0.015

However, while the diet was sterile, that is, free of all self-replicating macromolecules it still contained non-replicating macromolecules in the form of proteins and polysaccharides. In other words it was still potentially antigenic.

PRODUCTION AND MAINTENANCE OF GERM-FREE CHICKENS

Introduction

It was decided that germ-free chickens should be produced by the method originally described by Lev (1962). This decision was reached because, first, a large autoclave was available and secondly, funds were not available for the purchase of expensive equipment. The original apparatus and method were modified and adapted where necessary.

Materials and Methods

Construction of the Unit The unit consisted of a tube of autoclavable nylon (Portland Plastics, Ltd., Hythe, Kent) supported by a steel frame (Handy Angle Ltd., Hayes, Middlesex). The frame was 36" long and 18" wide. It rose to 18" at the back and the top consisted of a 12" horizontal piece which was joined to a 10" perpendicular from the front by a 10" piece of steel. This made five junctions in each end part of the frame and the corresponding junctions of each end were joined by steel strips 36" long. A 7' length of autoclavable nylon of 76" circumference and 0.002" thickness was drawn over the frame. One end was led through a 5" diameter ring and clamped to the top of the germicidal tank. The other end was used as an entrance until the unit was loaded when it was closed using a Jubilee clamp.

Entrances and exits were made to and from the unit via the germicidal tank.

Fig. 1a. Diagram of front of germ-free unit.

Fig. 1b. Diagram of end of germ-free unit.

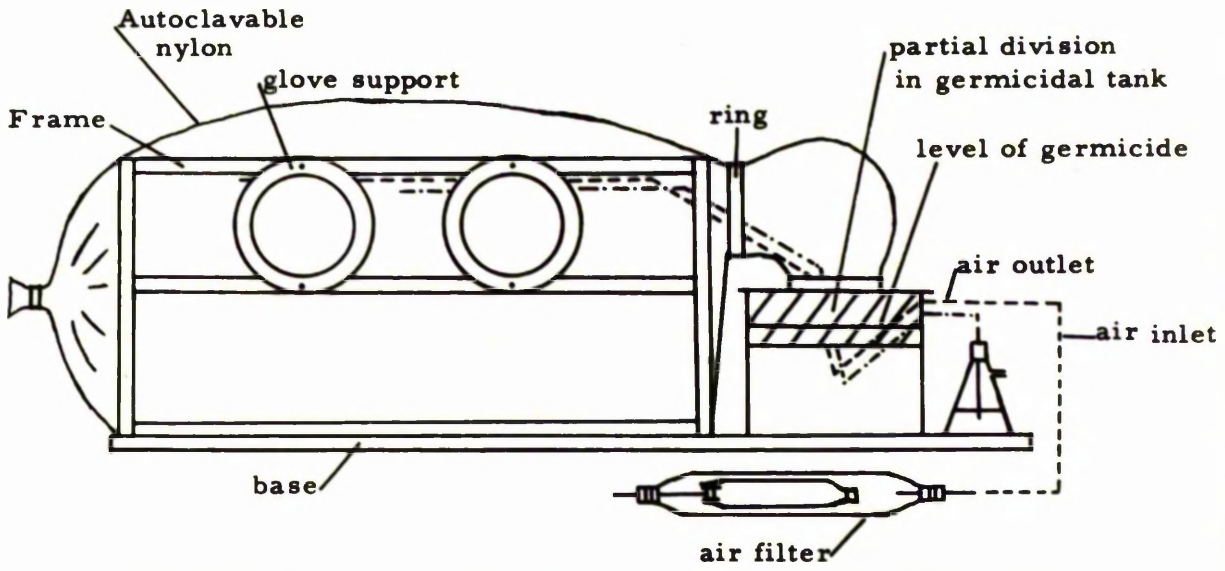
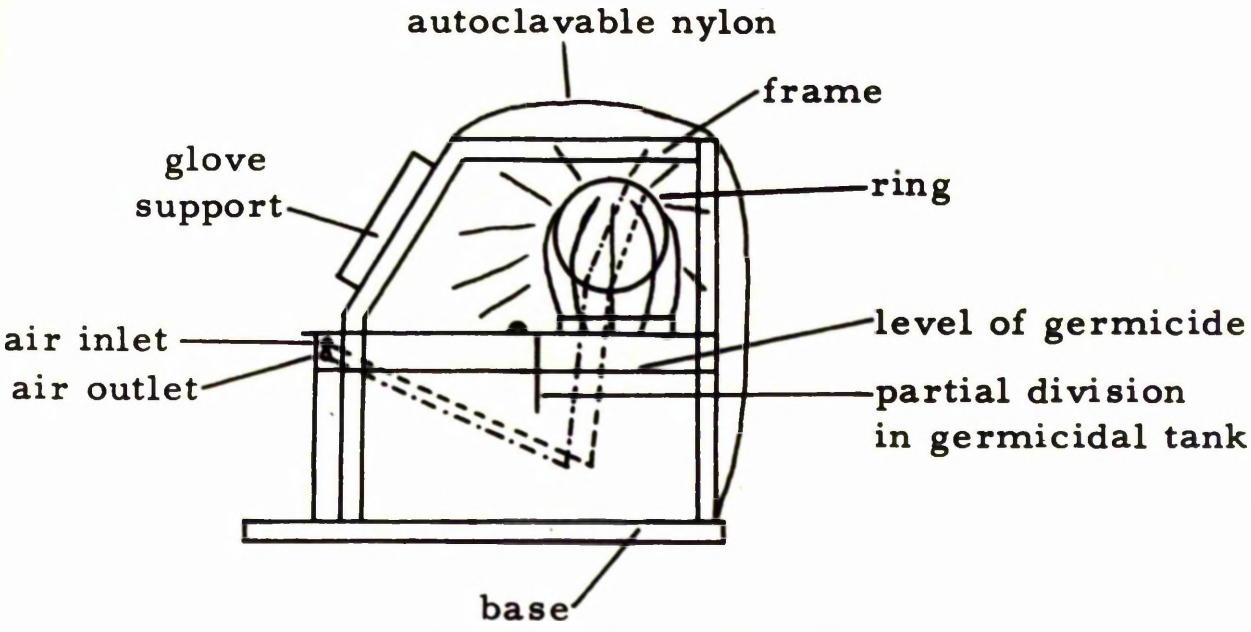

Autoclavable
nylon
glove support
Frame
ring
partial division
in germicidal tank
level of germicide
air outlet
air inlet
base
air filter

Fig 1a.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support
ring
air inlet
air outlet
level of germicide
partial division
in germicidal tank
base

Fig 1b.

The germicidal tank was made of stainless steel and measured 20" x 10" x 10". The rear half of the top was fitted with a sleeve 6" in diameter and 1" high to which the end of the nylon was clamped. The front portion of the top consisted of a lid hinged at mid-line. Internally the tank was partially divided by a steel plate which ran from just behind the hinge for a depth of 4".

The glove support was made by joining a cylinder of stainless steel (2.5" long by 6" diameter) to a stainless steel disc (7.5" outer diameter by 6" internal diameter). Two such supports were bolted to the front of the frame and two holes were made in the nylon so that the nylon fitted tightly round the glove supports.

The gloves (Industrial gauntlets, Medium grade) were then stretched over the glove supports and finally clamped with a Jubilee clamp. It was found that a piece of cloth between the support and the glove and between the glove and the clamp reduced the wear; this being a vulnerable part.

Air was supplied by a compressor pump (Model EB3, Edwards High Vacuum Ltd., Crawley, Sussex) and sterilized by passing through a filter made of FG-50 filter-down media (American Air Filter (G.B.) Ltd., Bassington Industrial Estate, Cramlington, Northumberland. The filter was made as described by Lev (1964) except that the air was passed in in the opposite direction from that indicated by the author; the sterilized air was then passed into the unit by a rubber tube (1/4" in diameter) which passed through the germicidal tank. The air was passed out by a rubber tube (1/4" in diameter) which again passed through the germicidal tank. The end of the outlet tube was immersed in 10% "Roccal" (Benzalkonium chloride) in a conical flask. The unit was maintained in positive pressure by controlling the

volume of air input and the depth of the outlet tube under "Roccal" in the conical flask (Fig. 1a and b).

Two cages 14" x 7.5" x 9.5", standing on plastic trays were placed on the floor of the unit. Cotton-wool, scissors, thermometer (250°C), stainless steel water bowls and feeding troughs, nasal swabs, a Coplin jar, tin foil, and 12 x 500 ml bottles of sterile water were all added before the end of the nylon tube was finally closed.

Sterilization of the Unit After construction and loading, the unit was placed in the autoclave. For this purpose the unit was built on a large tray (4' x 2') which formed the top of a trolley; this trolley was made of Handy Angle. The air filter was sterilized separately and the tubes from the unit were sealed with short lengths of cotton-wool filled glass tubing. The autoclave did not have a tap by which the germicide could be run into the germicidal tank immediately after sterilizing. This problem was overcome by filling the germicidal tank with water before autoclaving and adding the concentrated "Roccal" immediately after opening the autoclave door. Water was lost from the germicidal tank during autoclaving but this did not fall below the level of the partial division. The final concentration in the germicidal tank was 1% "Roccal" (Benzalkonium chloride).

The autoclave was evacuated to -5" Hg and steam was passed in and a pressure of 20 lbs/sq.in. was held for 25 - 30 minutes. The pressure was reduced and a second vacuum was drawn and held for 10 minutes. Air was allowed to

enter the autoclave through a filter overnight and the unit was dry by the morning. The unit was then removed from the autoclave on to the trolley and taken to the gnotobiotic room. The filter was wrapped in Kraft paper after the ends had been plugged with cotton-wool, then sterilized at 20 lbs/sq.in. for 20 minutes and thoroughly dried. The filter was connected to the air source and then connected to the intake tube of the unit taking all sterile precautions. The outlet tube was connected to the conical flask.

Equilibration of the Unit The unit thus set up and inflated was heated by suspending three infra-red lamps above. The temperature in the unit was regulated by the height of the lamps above the unit. If the eggs were exposed to the direct heat of the lamps they would be heated by radiation as well as by convection. The temperature of the eggs would thus be higher than the air temperature. This was obviated by placing a sheet of aluminium foil between the lights and the eggs. The foil was stretched over the roof of each cage, and put in position during the loading process and so sterilized. The temperature in the unit was maintained at 37.5°C. The humidity was controlled by bubbling air through water. A Coplin jar was suitable for holding the water and a small piece of rubber tubing (3/16" in diameter) was led from the Coplin jar through the germicidal tank and connected by a second filter to a small pump (Type MU119/26 pump, Austen Pumps Ltd., Byfleet, Surrey). The Coplin jar and the rubber tube were added during the loading process and sterilized in the autoclave. The second filter was sterilized in the same way as the first.

Operation for obtaining and rearing germ-free chickens Clean eggs (Thornber 606) were incubated in a conventional incubator and at day 18 were candled and the clear eggs discarded. The surface of each 19 day egg to be passed into the unit was sterilized by immersing and gently massaging in 1/400 "Portex D.C.R." (Brocades Ltd., Westbyfleet, Surrey), for 2 minutes at 37.5°C. Fresh disinfectant was used after every four eggs. Each surface sterilized egg was next passed through the germicidal tank and placed in one of the cages under the shade of the aluminium foil. A total of twelve eggs was introduced.

The eggs did not need to be turned during the two remaining days of incubation but they were not allowed to stick to the cotton wool on the floor of the cage. The chickens hatched at day 21 and the shells were removed as soon as possible. Water was poured into the bowl when the chickens were dry and food was also given at this stage. Baby chick crumbs (BOCM Ltd.) were made up in 100 gm lots, double wrapped in polythene bags and heat sealed. They were then irradiated at 4 Mrads (Wantage Research Laboratory, UKAEA, Wantage, Berkshire). The double-wrapped pack was surface sterilized with 2% peracetic acid for 30 minutes; the outer wrapper was removed, the inner pack was dropped into the germicidal tank and handed through to the operator. For this purpose as for all entrances armlength polythene gauntlets sterilized with 2% peracetic acid were worn. After the chickens had hatched the humidity was dropped by withdrawing the rubber tube and stopping the small pump. The temperature was gradually reduced to 20°C by the fourth week of life.

Assessment of sterility Rectal swabs were taken at the end of the first week of life. This was found to be the most sensitive indicator of infection. A smear was stained by Gram's method and examined microscopically. The swabs were streaked on to blood agar plates and McConkey's medium and incubated aerobically at 37°C for 10 days. Blood agar plates were incubated anaerobically at 37°C for 3 days. Swabs were also inoculated into Robertson's cooked meat medium and incubated at 37°C for 10 days. Sabouraud's medium was inoculated for the detection of fungi and mycoplasma were looked for using a mycoplasma culture medium (Hayflick, 1965). Swabs were also taken from the food, cages, water and bedding to check their sterility and pieces of shell were incubated in Robertson's cooked meat medium. If no organisms were found by these methods then the chickens were considered germ-free.

Results

Germ-free chickens have been produced by this method on a number of occasions and kept for up to four weeks. When the temperature and humidity within the unit are optimal a hatch rate as high as 83% can be obtained. On one occasion a hatch rate of 100% was obtained. A more usual hatch rate, given optimal conditions, is 75%. The hatch rate under the same conditions, but using mercuric chloride to sterilize the surface of the eggs, is 58%.

The average weight of 12 germ-free birds was 166.25 gm at 4 weeks while the average weight of 11 conventional birds was 162.27 gm, at the same age.

Some batches of eggs became contaminated. When this happened, every

effort was made to find the source of contamination. A Gram positive spore-bearing organism (anthracoid) usually gained access through the germicidal tank. A contaminated filter generally resulted in contamination of the chickens by cocci and Gram negative rods as well as spore bearers. A hole in the gauntlets resulted in the entrance of staphylococci. Contamination was never traced to bacteria present on the egg shell.

Discussion

A number of adaptations were made to Lev's method. The availability of autoclavable nylon lay-flat tubing 36" wide made construction of the unit much easier. A length of tubing was used once then discarded. The partial division of the germicidal tank internally made access easier without increasing the susceptibility to contamination. In earlier experiments latex gauntlets were used. They allowed great manoeuverability within the unit which meant that one operator could both pass in and receive eggs. These gauntlets were, however, easily punctured. In later experiments heavy duty rubber gauntlets were used. These gauntlets, though not ambidexterous, were less easily punctured.

The variation in the methods of heating and humidifying the unit resulted in a greater control of these factors. In the end it is these factors which determine the success of the technique, since the hatch rate is dependent on temperature and humidity, given live embryos. The germicidal tank is not intended to sterilize articles which enter the unit; the tank merely acts as a barrier against air-borne organisms. All articles to be passed into the unit must already be sterilized by

some other means. The most valuable modification to Lev's method was the introduction of "Portex D.C.R." as the germicide for sterilizing the surface of the egg. This proved to be an effective sporicide while at the same time allowing a good hatch rate.

The inflated unit, and the interior of the unit is seen in Plate 1a and b.

Plate 1a. Front of germ-free unit.

Plate 1b. End of germ-free unit.

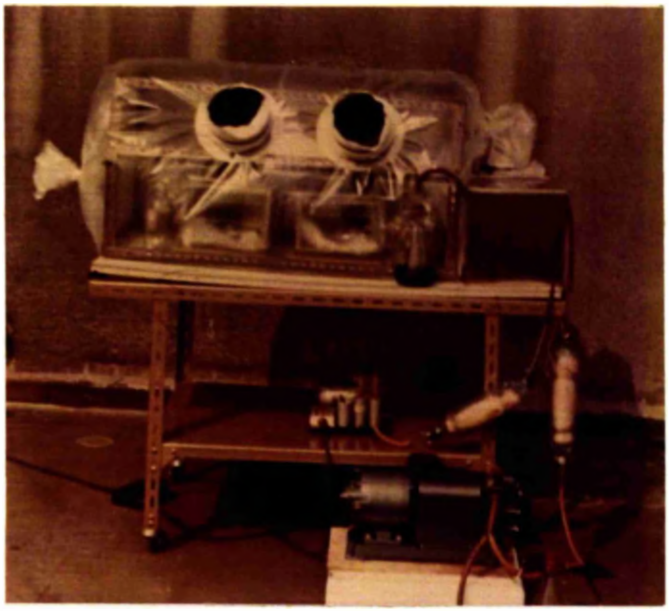


Plate 1a.

Plate 1b.

SECTION II LYMPHOID TISSUE IN THE GERM-FREE AND CONVENTIONAL CHICKEN

Lymphoid tissue in the chicken

Gallinaceous birds and pigeons differ from other birds in that they have no definitive lymph nodes (Yoffey and Courtice, 1956). Lymph nodes are present in water, marsh and shore birds (Further, 1913). Usually two pairs of nodes are found: the cervicothoracic nodes and the lumber nodes. However, despite the lack of lymph nodes the chicken possesses a rich plexus of lymph-vessels, the largest of which are the right and left thoracic ducts. These run forwards from the abdominal cavity on each side of the vertebral column and open into the cranial venae cavae (Bradley, 1960).

Although the chicken has no lymph nodes it is not lacking in lymphatic tissue. The thymus, spleen and the bursa of Fabricius are lymphoid organs. The chicken also possesses aggregations of lymphoid cells distributed along its intestinal tract. These are particularly concentrated in the caecae. The chicken has two caecae which join the alimentary tract at the junction of the small and large intestine. The first part of each caecum is rich in lymphoid tissue. Biggs (1957) has described lymphoid tissue in association with the lymph vessels in the chicken. These he calls "mural lymphoid nodules" and they are found in the lymph vessels accompanying the veins in the limbs, especially the hind limbs. Jordan (1936) has demonstrated lymphoid tissue in the bone marrow and in the periportal region in the liver of the chicken. Bang and Bang (1968) have described

lymphoid tissue in the paraocular and paranasal regions of the chicken. Lymphoid tissue has been reported in the skin (Billingham and Silvers, 1959), and in this laboratory lymphoid tissue has been seen in the ckicken lung. Thus, the lymphatic system in the chicken is extensive despite the lack of lymph nodes.

The Spleen The chicken spleen is situated in the angle formed by the junction of the ventriculus and the proventriculus. It lies slightly to the right of mid-line and its hilus is opposite the hilus of the liver. Blood vessels enter the spleen at several points along its hilus.

The splenic capsule is thin and contains many reticulum fibers as well as fibroblasts. There are no trabeculae from the capsule into the spleen. The substance of the spleen is divided into white and red pulp though in the chicken the division is not very definite (Plate 2a). The pulp, both red and white, is supported by a reticular framework (Plate 3b).

The structure of the spleen is best explained by describing the course of an artery from its penetration into the spleen (Fig. 2). The artery, immediately on entering the spleen, branches to enter the pulp and is supported by its own adventitia. Many of the smaller arteries and arterioles are ensheathed partly or completely by lymphocytes (periarteriolar lymphocytic sheath, Cheville, 1967). This is the non-nodular lymphatic tissue of Jankovic and Isakovic (1964). At other points along the artery or arteriole lymphoid cells form aggregations or nodules which are surrounded by a fibrous capsule. These nodules are called germinal centres.

The cells within the germinal centre are not a homogeneous population.

Plate 2a. Spleen of the chicken. Two germinal centres (GC) are prominent and one of the ellipsoids (E) has been indicated. The connective tissue capsule (C) is seen and a periarterioler lymphocyte sheath (PALS) is present. There is no clear division between red and white pulp.

Giemsa x 60.

Plate 2b. Spleen of chicken five minutes after intravenous injection of indian ink. The ellipsoids are outlined by the ink but no ink is associated with the germinal centre (GC).

Haematoxylin and Eosin x 60.

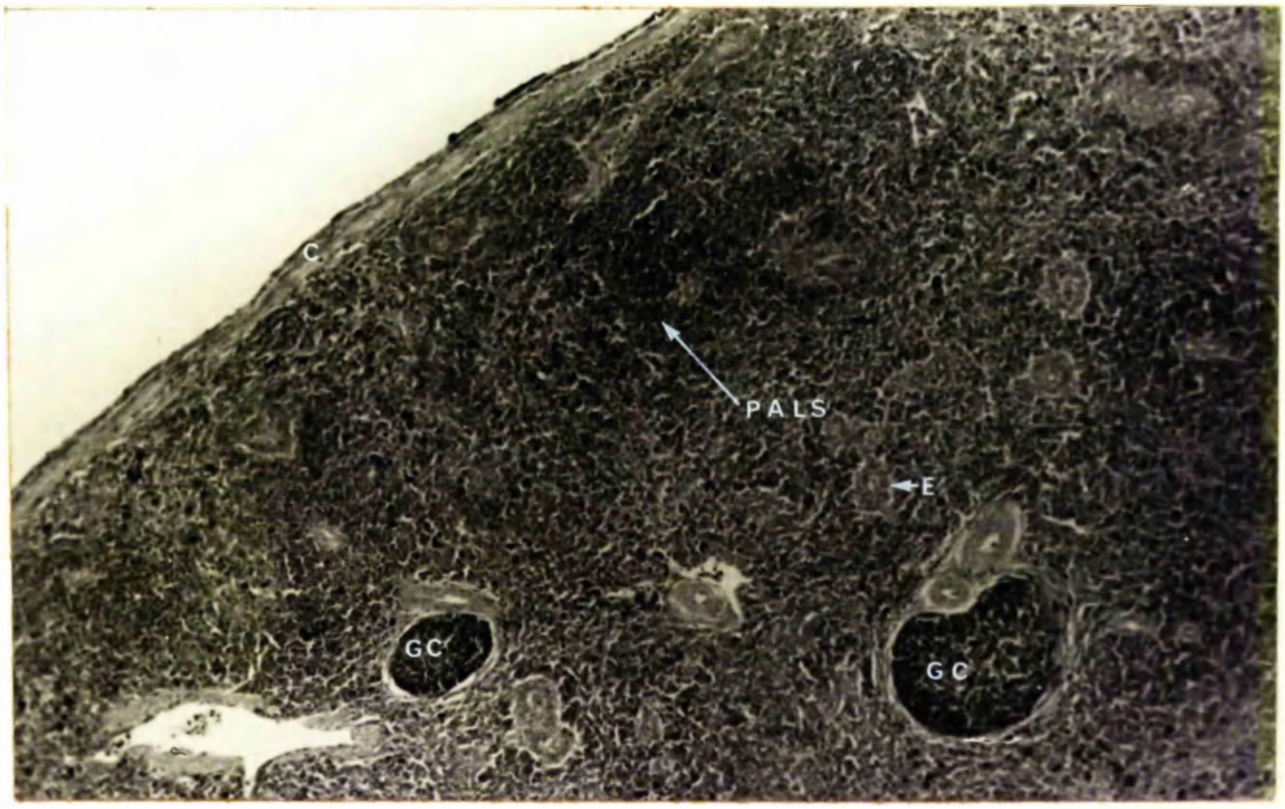


Plate 2a.

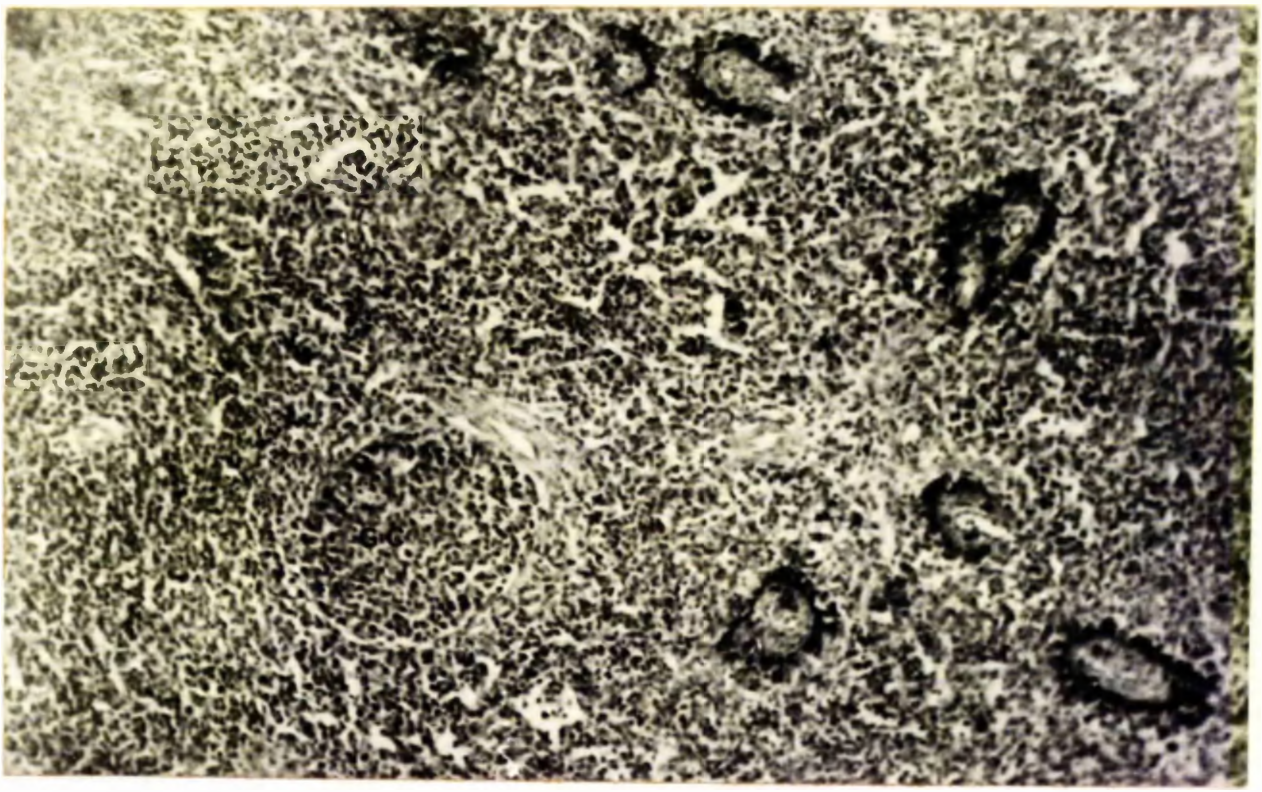


Plate 2b.

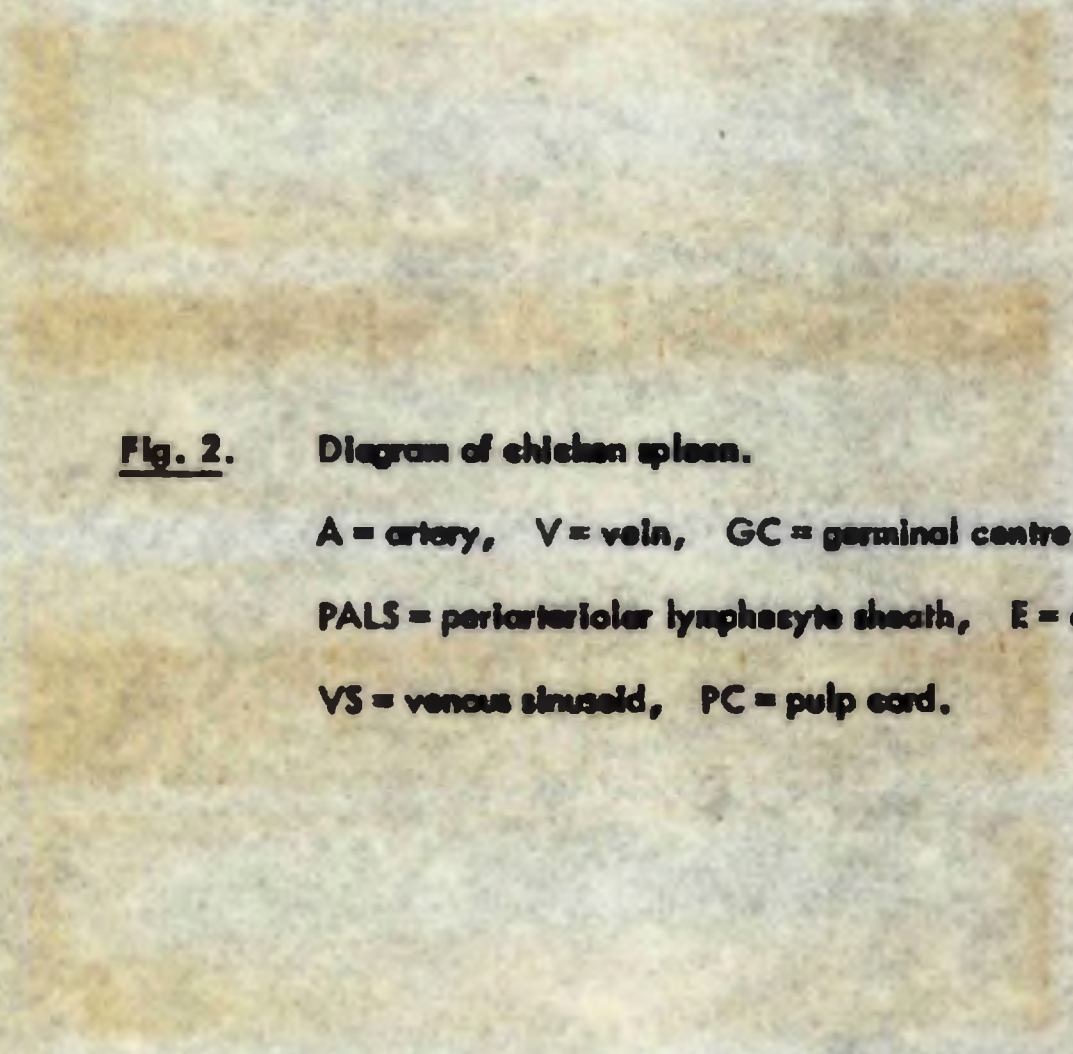


Fig. 2. Diagram of chicken spleen.

A = artery, V = vein, GC = germinal centre,

PALS = periarteriolar lymphocyte sheath, E = ellipsoid,

VS = venous sinusoid, PC = pulp cord.

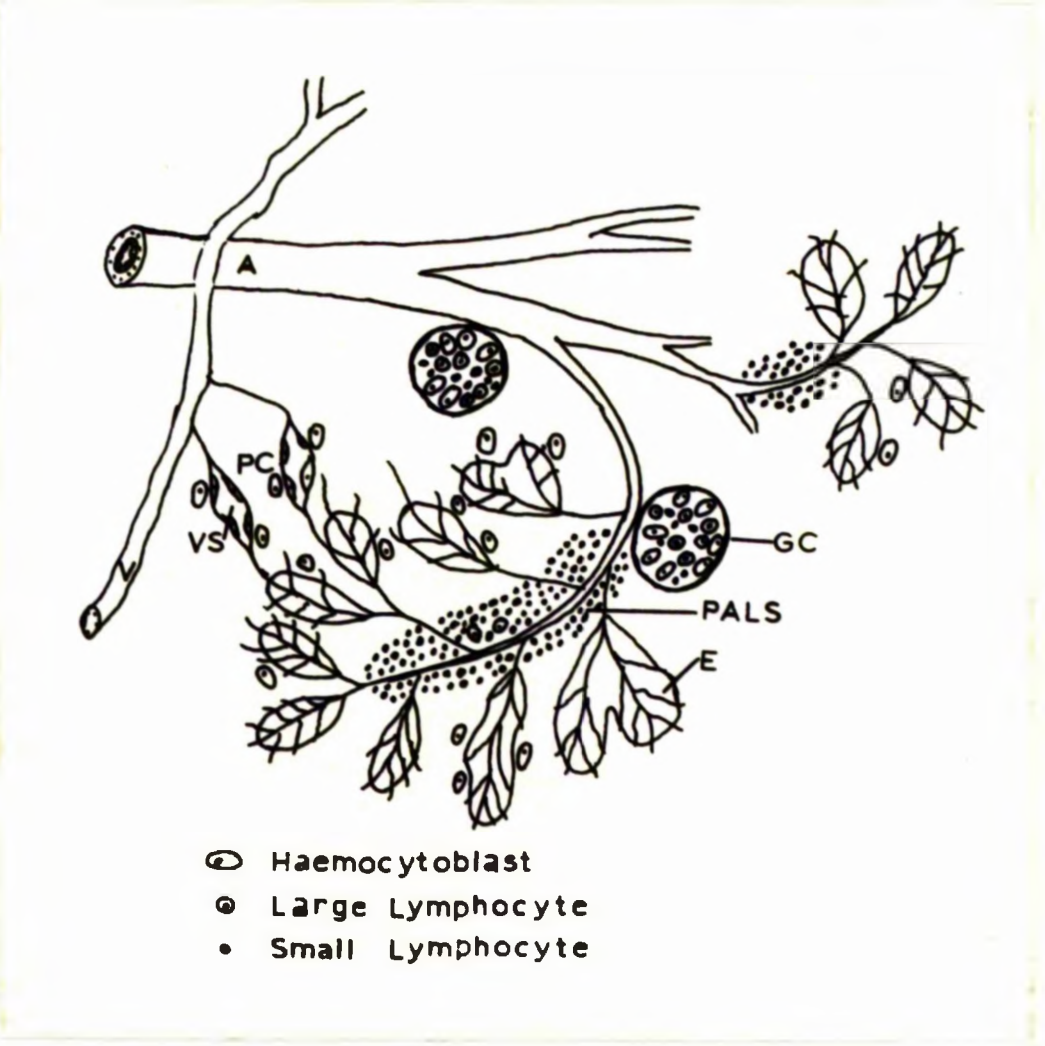

A
PC
VS
GC
PALS
E
Haemocytoblast
Large Lymphocyte
Small Lymphocyte

Fig 2.

They consist of large and small lymphocytes, haemocytoblasts and phagocytes. Mitotic figures are frequently seen in the centre and the tingible body macrophages occur but are not common in the chicken germinal centre. Unlike the mammalian germinal centre, the chicken germinal centre is not surrounded by a dense circle of small lymphocytes. Usually small lymphocytes are found only on the side of the germinal centre adjacent to the artery. Thus the white pulp - nodular and non-nodular - is distributed along the smaller arteries.

The artery further decreases in size to become an arteriole, a capillary, and then enters an ellipsoid (Schweigger-Seidel sheath). The ellipsoid is "a condensation of reticular fibres and reticuloendothelial cells around an arteriole" (McNee, 1931). In the chicken the ellipsoids are a prominent feature and can be readily demonstrated in the spleen of a chicken killed five minutes after an intravenous injection of indian ink. The indian ink is seen at the periphery of the ellipsoids (Plates 2b and 3a). Only rarely is the indian ink seen in the cells of the ellipsoid at this time. The chicken ellipsoid has a central lumen bordered by endothelial cells. These endothelial cells stand on a basement membrane which is rich in reticular fibres (Plate 3b). The central lumen is then surrounded by concentric lamellae of phagocytic cells. No muscle fibres are seen. In most ellipsoids a second ring of reticular fibres is seen about two cells distant from the basement membrane. The phagocytic nature of these cells can be demonstrated by intravenous injection of indian ink. By 72 hours indian ink is visible in the cytoplasm of the peripheral cells of the ellipsoids.

The framework of the red pulp consists of a mesh of reticular fibres.

Plate 3a. Ellipsoids of spleen five minutes after injection of Indian ink. Four ellipsoids are seen in the centre of the field with Indian ink at their periphery.

Methyl-green-pyronin x 300.

Plate 3b. Reticular framework of the spleen. The four ellipsoids show the characteristic two bands of reticulum; one below the endothelium, the other about two cells distant.

Laendrum's method for reticulum fibres counterstained with 1% neutral red. x 300.

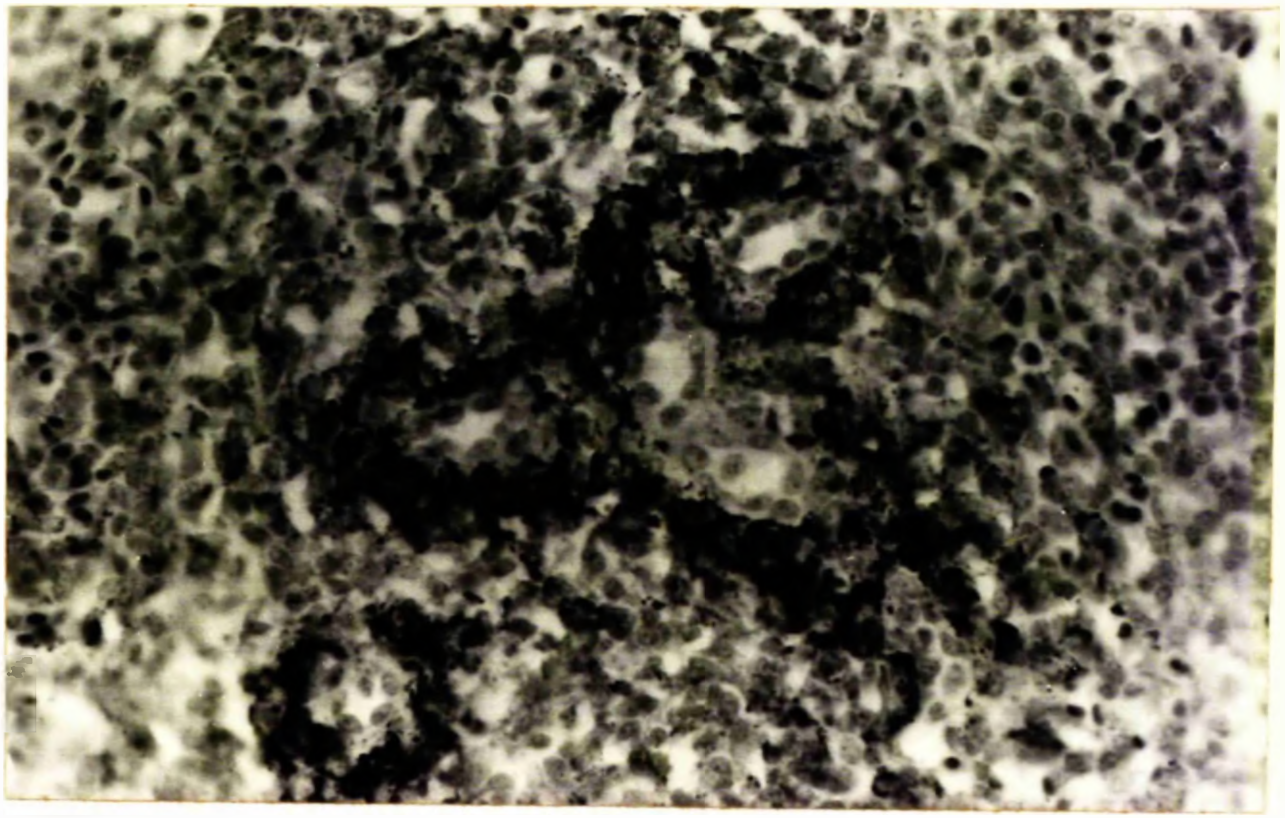


Plate 3a.

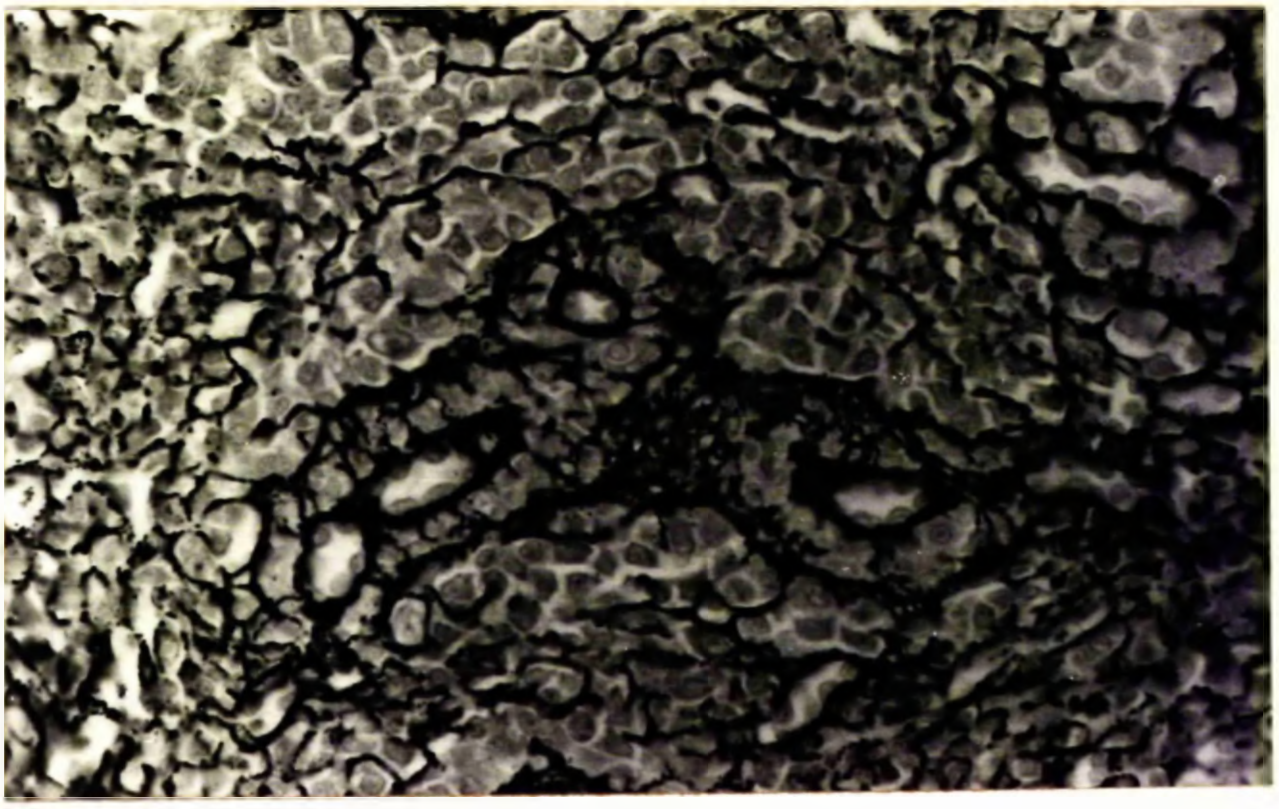


Plate 3b.

Within this meshwork are passageways which drain into the veins and are thus called venous sinusoids. Reticulo-endothelial cells form the walls of these sinusoids though the wall is not complete for cells can pass between the processes of the reticulo-endothelial cells into the pulp cords which lie between the sinusoids. All the cell types seen in the circulating blood are to be found in the red pulp. Because of the terminal nature of ellipsoids they are usually surrounded by red pulp.

The spleen is of mesodermal origin (Danchakoff, 1916). Erythrocytopoiesis begins at about day 7 of incubation and continues only for a few days. Granulocytopoiesis, especially production of heterophils, begins about day 12 of incubation, reaches a peak at day 16 or 17 and decreases in post-hatching life. Lymphocytes appear in the spleen at day 14 - 15 of incubation (Danchakoff, 1916). Germinal centres do not appear till the second week of post-hatching life.

As well as cells of the circulating blood, cells of the plasmacytic series are found in the spleen. These cells will now be described in detail.

Many workers agree that the plasma cell precursor is the fixed reticulum cell (Fagraeus, 1948; Marshall and White, 1950; Parsons, 1943; Ringertz and Adamson, 1950; Thiery, 1960). The primitive reticular cell (fixed reticulum cell) is found in the red pulp cords. In haematoxalin and eosin stained sections the cell is about 8μ in diameter with a lightly staining nucleus which contains dots of condensed chromatin. There are no nucleoli. The outline of the cell is indistinct and the cytoplasm is only slightly basophilic. The primitive reticular cell is not a phagocyte. It is a fixed cell and appears to be connected to its neighbours by

syncytial processes. As it matures it becomes a free cell and develops to a haemocytoblast.

The haemocytoblast (White, 1963) corresponds to the transitional cell of Fagraeus (1948), the activated reticulum cell of Marshall and White (1950), the plasmoblast of Schooley (1961) and the immature lymphoid cell of Ehrich et al. (1949). The haemocytoblast, found in germinal centres and in the red pulp, is a large cell with a leptochromatic nucleus greater than 10 μ in diameter. One or two nucleoli are prominent in the nucleus. The cytoplasm shows some basophilia, sometimes as a rim at the cell wall, sometimes as scattered particles. The haemocytoblast is not a phagocytic cell.

The plasmablast is a large cell 15 - 20 μ in diameter. The nucleus is central in position and has a fine chromatin pattern. One or two nucleoli are present. The cytoplasm is basophilic, though not uniformly basophilic.

The immature plasma cell has an eccentrically placed nucleus which shows some condensation and thickening of the chromatin strands. A nucleolus is usually present. The cytoplasm is basophilic but a clear vacuole 3 - 5 μ in the long diameter is present in the cytoplasm adjacent to the nucleus. The cytoplasmic basophilia is as intense as that of the plasmablast and is uniformly distributed. These cells are found in the red pulp but not in the germinal centres.

The plasma cell is the end cell in the series and is found in the red pulp and the pulp cords. It has the typical eccentrically placed nucleus with clumps of chromatin arranged in the nucleus in a "cart-wheel" or "clock-face" fashion. The cytoplasm is uniformly basophilic except for a small clear area at one pole

of the nucleus. This represents the Golgi apparatus.

The cells of the plasmacytic series take up the stain pyronin in proportion to the RNA in the cytoplasm. This stain, methyl-green-pyronin, is therefore of value in studies involving plasmacytic cells. The haemocytoblast has a large area of cytoplasm which stains strongly red (pyroninophilic). The nucleoli stand out as red dots against the blue nuclear background. The immature plasma cell has a smaller nucleus than the haemocytoblast with some condensation of the chromatin; usually a nucleolus is present. The nucleus is slightly off-centre and the cytoplasm is uniformly red except for the vacuole on the cytoplasmic side of the nucleus. The mature plasma cell is smaller than the immature plasma cell and has the characteristic eccentrically placed nucleus with clumps of chromatin arranged round the periphery. No nucleolus is present. The cytoplasm is uniformly red except for the vacuole which is usually smaller than in the immature plasma cell.

The Thymus The thymus of the chicken most often consists of 14 separate lobes, seven on each side of the neck. The lobes are associated with the large blood vessels, the most distal lobes entering the thoracic cavity.

Each lobe is pinkish-grey in colour and is surrounded by a thin capsule of connective tissue. This extends into the substance of each lobe to form septa and divide the lobes into lobules. The septa, on penetrating into the substance of each lobe, meet and fuse with other septa but not extensively enough to surround the thymic tissue of each lobule completely; hence the thymic tissue of each lobule is continuous in the more central part of each lobe with that of other

lobules. The concentrated lymphocytes which form the cortex are disposed toward those borders of each lobule that abut on the capsule or an interlobular septum. The medulla is less rich in lymphocytes and is continuous with the medullary tissue of other lobules. The medulla is richly supplied with blood and lymphatic vessels (Plate 4).

Lymphocytes are so numerous in the cortex that they obscure the reticular cells on which they lie. The greatest number of cells showing mitosis is found in the subcapsular region. The predominant lymphocyte is the small lymphocyte.

Although lymphocytes are numerous in the medulla they are not so densely packed as to obscure the reticular cells. Epithelial cells also occur in the medulla as do Hassal's corpuscles. Haemocytoblasts are seen both in the cortex and in the medulla and plasmablasts are also present, though plasma cells and germinal centres are not normally seen in the thymus.

The thymus is of endodermal origin, arising from the third and fourth pharyngeal pouch (Schrier and Hamilton, 1952), though Hammond (1954) presents evidence favouring the ectodermal origin of the thymus epithelium. Auerbach (1961) showed that the lymphocytes only develop from the endodermal epithelium when mesenchyme is present even though separated by a filter. This dependence on mesenchyme appears to be true for the induction of lymphoid transformation in the chicken thymus (Good et al., 1966).

The Bursa of Fabricius The bursa of Fabricius occurs on the dorsal wall of the terminal part of the cloaca and is connected to the cloaca by a short hollow

stalk. It is a hollow unpaired organ, with cleft-like diverticula or plicae in its walls. These plicae are thick and are composed of polyhedral masses of mononuclear cells - the follicles of Stannius. The bursa reaches its maximum size at puberty (4 - 5 months) then begins to regress. It is surrounded by a thin circular layer of smooth muscle beneath which is a connective tissue zone containing vessels. Trabeculae pass inward from this zone to form the stroma and to invest each follicle. The lumen of the bursa is lined by columnar epithelium which becomes pseudostratified at the cloaca.

The follicles of Stannius show two zones upon section. An outer darkly staining cortex and a lighter medulla separated from the cortex by a basement membrane. A scanty reticulum net runs throughout the follicle (Plate 5).

The cortex consists of haemocytoblasts, lymphocytes and rare macrophages. The medulla consists of haemocytoblasts, small and medium sized lymphocytes, undifferentiated epithelial cells and primitive reticular cells.

The bursal follicle is derived from a bud of endodermal epithelial cells (Ackerman and Knouff, 1959). According to Ackerman and Knouff (1964) as the epithelial bud enlarges, the cellular constituents in the nodule undergo a series of changes. A row of undifferentiated epithelial cells which is continuous with the epithelial bud, is seen on the medullary side of the basement membrane. These cells have a slightly to moderately basophilic cytoplasm and a somewhat polyhedral contour. They often show mitotic figures perpendicular to the basement membrane. They give rise to daughter cells which assume a more central location in the medulla.

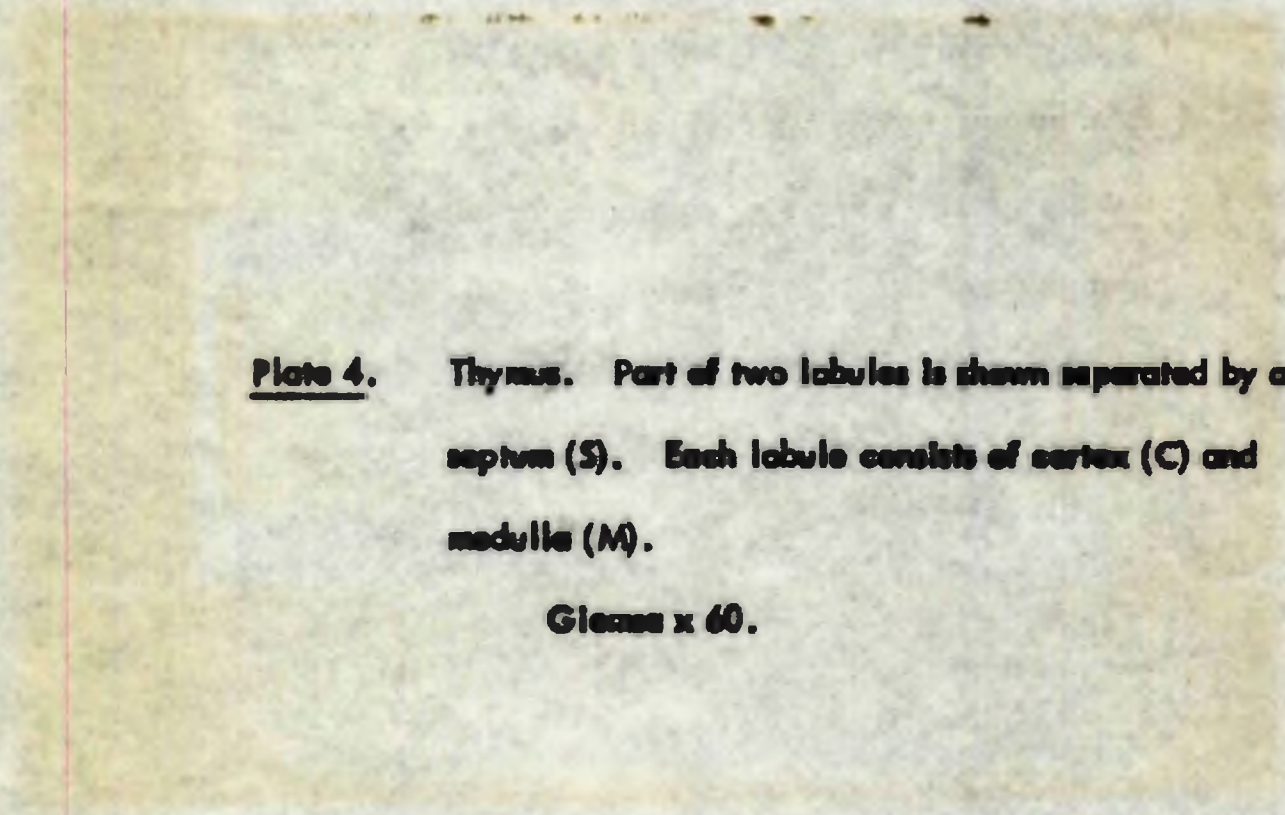


Plate 4. Thymus. Part of two lobules is shown separated by a septum (S). Each lobule consists of cortex (C) and medulla (M).

Giemsa x 60.

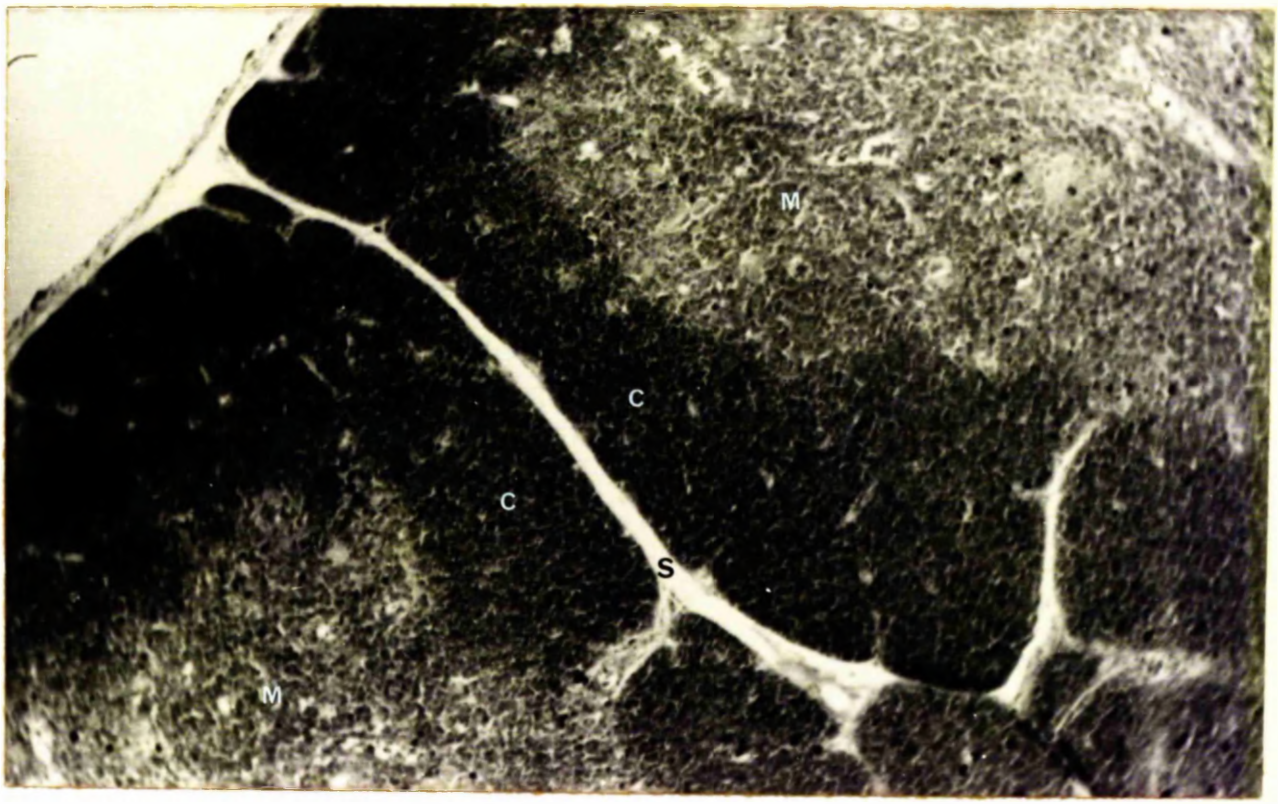


Plate 4.

Plate 5. Bursa of Fabricius. Part of two plicae is shown separated by the lumen (L). Each plica is made up of a number of follicles (follicles of Stannius) each of which has a cortex (C) and a medulla (M). The cortex and medulla is separated by a basement membrane. The epithelial bud (EB) is the point from which a new follicle develops.

Giemsa x 60.

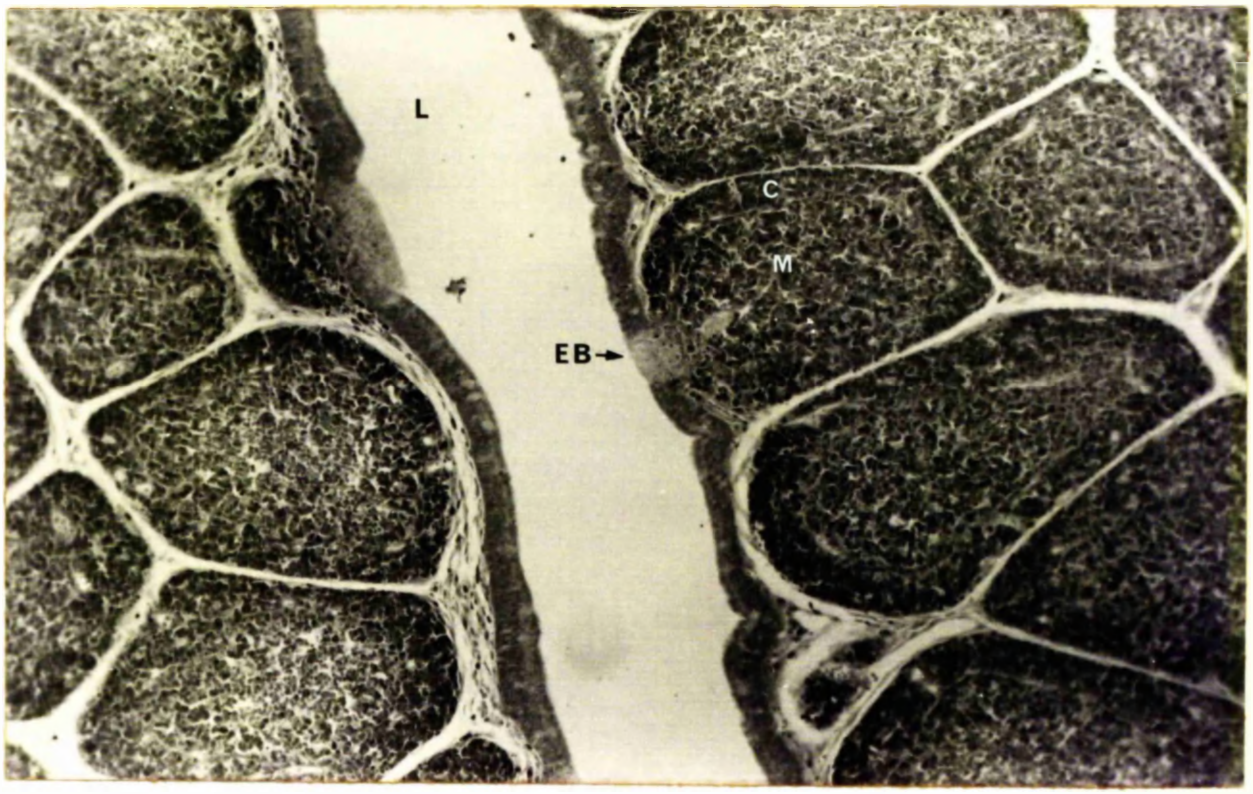


Plate 5.

Plate 6. Caecum. Several germinal centres are seen in the lamina propria three of which have been indicated.

Giemsa x 60.

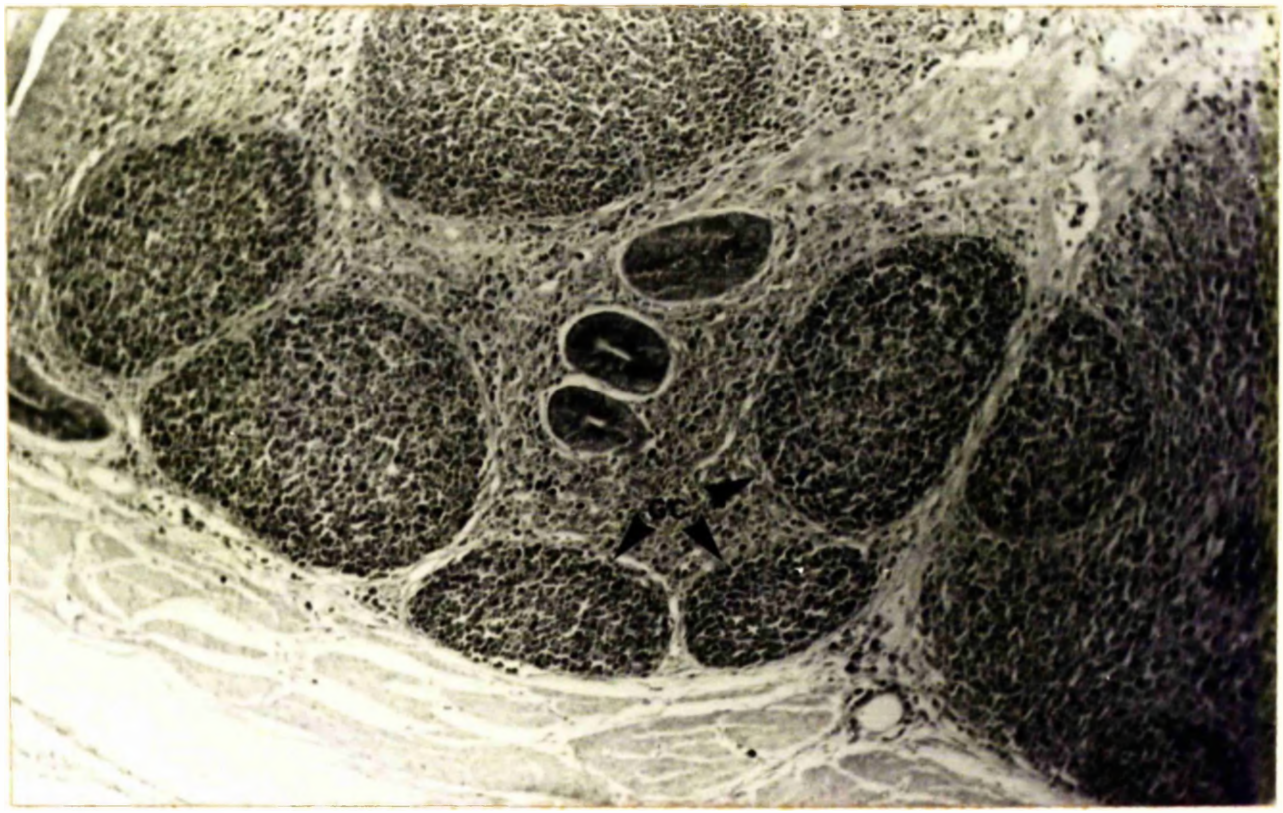


Plate 6.

The daughter cells enlarge, become intensely basophilic, show nucleoli and are identified as haemocytoblasts. The haemocytoblasts proliferate and undergo further differentiation giving rise to a developmental series of lymphocytes. These lymphocytes migrate into the cortex as do some of the haemocytoblasts.

Thus the medulla develops first. Bud formation occurs at 11 - 12 days of incubation, haemocytoblast transformation at 14 - 15 days of incubation and at 15 - 16 days of incubation all stages of lymphocytopoiesis may be observed in the medulla although the definitive condition is not reached until about the end of the first week post hatching.

Mature plasma cells are not found either in the medulla or the cortex of the bursa.

A mesenchymal element also exists in the bursa and it seems possible that the endodermal epithelium only gives rise to lymphocytes in the presence of the mesenchyme as Auerbach (1961) has shown to occur in the morphogenesis of the thymus.

Lymphoid tissue in the Caeca The location of the lymphoid tissue in the caeca, which is often called the caecal tonsil, has been described. Lymphoid tissue also occurs in smaller aggregates along the chicken's intestine. These are referred to as Peyer's patches.

Histologically the caecal tonsil consists of masses of lymphoid cells and many germinal centres in the lamina propria. The germinal centres are histologically identical to those seen in the spleen. The lymphoid mass surrounding the germinal

centres is made up of lymphocytes, predominantly small, and cells of the plasmacytic series. The first lymphocytes make their appearance in the caecal tonsil at the 17th day of incubation but germinal centres do not occur until the second week of post hatching life (Plate 6).

Peyer's patches are histologically similar to the caecal tonsil.

Immunological responsiveness in the chicken

No study of immune responses in the chicken would be complete without some mention of the concept of dissociation of immunological responsiveness. This idea was first put forward by Warner et al. (1962) following the finding that antibody production was depressed in bursectomized chickens (Glick et al., 1956). The concept is that antibody production, cells of the plasmacytic series and germinal centres are dependent on an intact bursa and that delayed type hypersensitivity reactions, homograft reactions and circulating lymphocyte numbers are dependent on an intact thymus (Warner et al., 1962; Cooper et al., 1966).

The importance of the work in this field to the present study is twofold. First, a connection is established between cells of the plasmacytic series and germinal centres. Bursectomy coupled with irradiation resulted in a reduction in the number of plasmacytic cells and germinal centres (Cooper et al., 1966) and this was especially so when bursectomy was performed in embryonic life (Cooper et al., 1969). The absence of germinal centres is attributed to lack of bursal cells. Secondly, evidence has been presented that bursal cells seed to the

peripheral lymphoid tissue (Woods and Linna, 1965) and that germinal centres and plasmacytic cells can be restored in irradiated-bursectomized chickens by intraperitoneal injection of autologous bursal cells (Cooper et al., 1966b). This seeding from the central lymphoid tissue - thymus and bursa - to the peripheral lymphoid tissue - lymphoid tissue other than thymus and bursa - is thought to be antigen independent, (Miller, 1968).

Avian Blood Cells

The blood cells of the bird differ from those of mammals in several respects. The erythrocytes are nucleated. The thrombocyte is a nucleated cell with a clear cytoplasm which contains at least one pink granule. The heterophil (Plate 7a) corresponds functionally to the polymorphonuclear leucocyte of mammals and has numerous strongly eosinophilic rods in the cytoplasm. The cells in avian blood are described in detail by Lucas and Jamroz (1961).

The lymphocytes (Plate 7b) in the chicken are divided into small and large lymphocytes (Lucas, 1959). The small lymphocyte differs morphologically from the large lymphocyte in being smaller and having more condensed nuclear chromatin than the large lymphocyte.

After studying many Leishman stained blood films from chickens, especially young chickens, it became apparent that another type of lymphocyte existed. This cell was called a "type 1 lymphocyte" (Plate 7a).

The type 1 lymphocyte as seen in Leishman stained smears is slightly larger in diameter than the large lymphocyte i.e. about 8.5 μ. The nucleus is large and often oval or rod-shaped, as opposed to the round nucleus of the large

and small lymphocyte. The nuclei of the small and large lymphocyte is characteristically filled with dense chromatin, especially in the small lymphocyte but in the type 1 lymphocyte chromatin lumps are less dense and less frequent. The lumps are connected by fine chromatin threads but often the nucleoplasm between the chromatin lumps is almost clear. No nucleolus is seen. The nucleo-cytoplasmic ratio is low in the type 1 lymphocyte compared to the large lymphocyte. A rim of faintly basophilic cytoplasm is seen all round the nucleus. No distinguishing features are seen in the cytoplasm, no Magenta bodies and no hyaline mantle as in the monocyte (Plate 7c). The cell outline is often very faint.

On the basis of its size, nuclear shape, chromatin pattern and nucleo-cytoplasmic ratio this type of cell could be identified and counted independently of the large and small lymphocytes. The recognition of this cell as morphologically distinct from other lymphocytes was only reached after studying all the artefacts and abnormal cells described by Lucas and Jamroz.

The origin or function of the type 1 lymphocyte is unknown but the hypothesis is that this cell is associated in some way with the bursa of Fabricius.

Plate 7a. Nucleated avian erythrocytes with a thrombocyte (T), a heterophil (H) (a polymorphonuclear leucocyte) and three type I lymphocytes.

Leishman x 600.

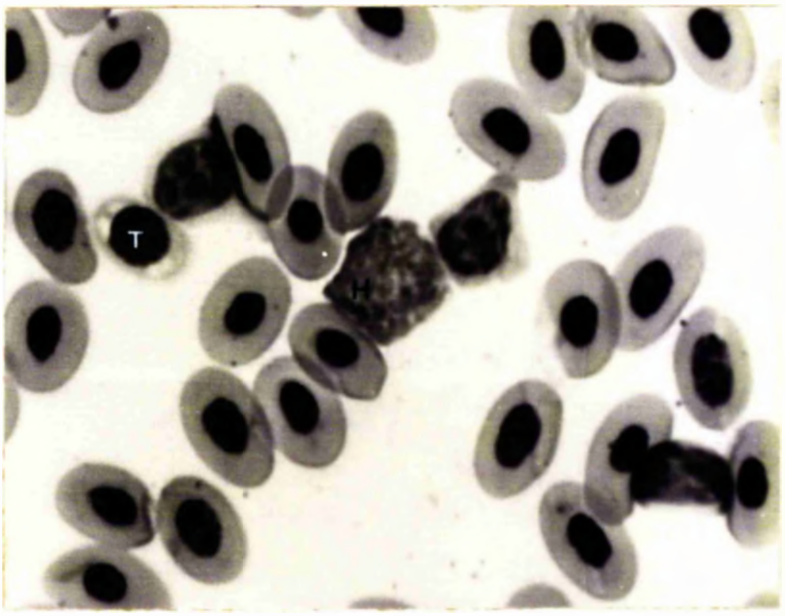
Plate 7b. Erythrocytes with a large lymphocyte and a small lymphocyte (lower cell).

Leishman x 600.

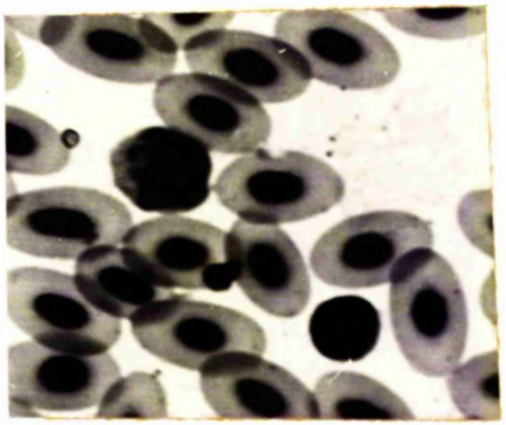
Plate 7c. Erythrocytes with a monocyte.

Leishman x 600.

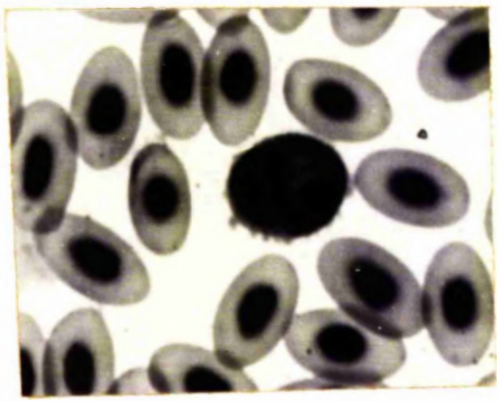
Plate 7.



a.



b.



c.

A COMPARISON OF THE LYMPHOID TISSUE IN GERM-FREE AND CONVENTIONAL CHICKENS AT 4 WEEKS OF AGE

Introduction

The lymphoid tissue of germ-free and conventional chickens has been compared by Thorbecke et al. (1957); Gordon (1959); Thorbecke (1959) and Reyniers et al. (1960). Thorbecke et al. (1957) and Thorbecke (1959) found that germ-free chickens generally showed less development of the lymphoid tissue, fewer plasma cells and germinal centres, and a lower gamma-globulin content in the serum than the conventional chickens. They found no histological difference in the thymus and bursa between germ-free and conventional chickens. Gordon (1959) and Reyniers et al. (1960) both reported a low number of circulating lymphocytes per cubic mm. of blood in germ-free chickens. The present study was necessary to verify the major findings of these authors and to form a norm with which experimentally stimulated germ-free chickens could be compared.

Materials and Methods

Germ-free chickens (Thornber 606) were obtained, reared and tested for sterility as described in Section I. At four weeks of age six germ-free and an equal number of conventional chickens (Thornber 606) were weighed. Blood was taken from the radial vein for haematological study and the birds killed and bled out. Each serum was separated from the blood clot and stored in the frozen state (-20°C) until required.

Histological preparations were made from the thymus, the spleen, the bursa of Fabricius and the lymphatic portion of the caecum. The organs were fixed in 10% formol saline and stained by haematoxalin and eosin and by methyl-green-pyronine.

The spleens were weighed before fixing and the weight corrected for 100 gm. body weight.

Haematology a. Total Red Cell Count The radial vein was punctured with a needle as it passes over the carpal joint. Blood was sucked into the standard red cell pipette up to the 1 mark. The pipette was then filled to the 101 mark with a diluting fluid of the following composition (Darcel, 1951):-

Sodium chloride	0.66 gm
Sodium citrate	1.11 gm
Neutral formalin	5 ml
Distilled water	95 ml

The pipette was then rotated to mix the diluted blood. The coverglass was pushed firmly over a Neubauer counting chamber so that the glass adhered and Newton's coloured rings appeared. Both sides of the chamber were filled with diluted blood from the red cell pipette. The number of red cells in 80 small squares on each side of the chamber was counted (i.e. 5 large squares each consisting of 16 small squares were counted on each side of the chamber). The values were substituted in the following formula to give the number of red cells per cubic millimeter of blood:-

$$\frac{\text{Sum of red cells in 5 large squares on each side of the chamber}}{4} \times 10^4$$

b. Total White Cell Count This was made by an indirect method. From the stained blood film the number of leucocytes per 1,000 red blood cells was counted. This was done by placing a cardboard window in the eye-piece of the microscope which circumscribed 100 blood cells when the film was evenly spread and the cells almost touching each other. 50 x 100 cells were counted and an average value for the number of white cells per 1,000 red blood cells obtained.

c. Differential White Cell Count A blood film was made from the radial vein blood. The film was dried in the air and stained with Leishman's stain (British Drug Houses Ltd.). A differential white cell count was made counting 100 cells.

Agar-gel Immunoelectrophoresis a. Preparation of Plates A clean sheet of glass 20 x 10 cm was siliconized on its upper surface and placed in a sandwich box on a horizontal bench. Two filter paper wicks (20 x 5 cm) were cut from Whatman No. 3 paper. They were dampened in barbitone buffer (0.05M. pH 8.6) and placed parallel to the long edges of the glass plate so that they overlapped it by about 1 cm. Ionagar 0.8% containing 0.05M barbitone buffer and 1:10.000 merthiolate was kept in 100 ml aliquots. One bottle of ionagar was melted and the contents poured over the glass plate until the thickness of the agar on the plate was about 1 - 1.5 mm. The agar was left for 30 minutes to set *in situ* and was then transferred to the 4°C refrigerator overnight for use the following day.

b. Electrophoresis The central glass plate with its filter paper wicks was cut from the agar and lifted free. Troughs and wells were cut in the agar using a No. 1 cork borer, a scalpel and a ruler taking great care not to damage the surface of the agar. The well had a diameter of 0.4 cm and was separated from the troughs on either side by 0.4 cm of agar. Each trough was 0.4 cm wide. A drawing of this was made on graph paper and the glass plate superimposed on this diagram so that the wells and the troughs could be cut out. The agar plate was placed over the central compartment of the Shandon tank. The filter wicks were immersed in 0.05M barbitone buffer in the compartments on either side. Barbitone buffer 0.05M pH 8.6:-

Sodium barbitone	10.3 gm
Barbitone	1.84 gm
Distilled water	1 litre

The sera to be electrophoresed were placed in the wells. A small amount of bromphenol blue was incorporated in one of the serum. This attached itself to the albumin and indicated the rate of protein separation. The current was switched on and a current of 30 - 35 mA or a voltage of 150 - 200 V was selected (Vokam power pack). The current was switched off when the blue spot reached the edge of the plate (4 - 5 hours). The troughs were then filled with the appropriate antisera. The filter wicks were cut away and the plate left in the chamber for approximately 36 hours to allow the precipitin lines to develop. The plates were then washed for 24 hours in saline and for at least 24 hours in water to get rid of buffer and diffusible proteins present in the agar.

c. Staining The precipitin lines were stained with Chlorazol black 0.2% made up as follows:-

Chlorazol black E (Gurr) 200 mg

Ethylene glycol 25 ml

Distilled water 75 ml

Make up in ethylene glycol first. When dissolved add water and filter.

The moist agar plate was immersed in the stain for 1 - 3 hours depending on the age of the stain (longer times for older stains). Decolourising was achieved by washing with running tap water overnight. The agar was then dried in the 37°C incubator.

Antisera a. Rabbit anti-chicken globulin 5 mg of 30% ammonium sulphate precipitated normal chicken serum in complete Freund's adjuvant was injected intramuscularly into both hind legs.

The Freund's adjuvant contained:-

0.1 ml antigen in saline

0.1 ml arlacel

0.3 ml bayol

5.0 mg Myco. tuberculosis

An emulsion was made and the dose divided between the two hind legs. Four weeks later 2.5 mg of 30% ammonium sulphate precipated normal chicken serum in 0.5 ml of aluminium phosphate was administered. Half the dose was injected intravenously and the remainder was given subcutaneously. The animal

was bled 10 days later and the serum obtained stored in the frozen state (-20°C).

b. Rabbit anti-chicken IgM IgM was eluted from a DEAE cellulose chromatography column with 0.15 M phosphate buffer, pH 7.5. Precipitin arcs were obtained by immunoelectrophoresis against rabbit anti-chicken globulin and the agar containing the arc formed by the reaction of the IgM and the rabbit anti-chicken globulin was excised taking care not to include any contaminating protein. This agar was washed, broken up and homogenized in Freund's complete adjuvant mixture. It was then injected bilaterally into the popliteal lymph nodes of the rabbit (Goudie et al. 1966). A second injection of the same antigen-antibody precipitate was given in saline 4 weeks later by intramuscular and intravenous injection. The animal was bled 10 days later. The antiserum thus produced formed a precipitin line against chicken 19S immunoglobulin. A precipitin line was also formed against 7S immunoglobulin due to the sharing of light chain antigens. Dr. P. C. Wilkinson prepared and provided this antiserum (Wilkinson and French, 1969).

Histological examination All sections were carefully examined. Using MGP stained sections of spleen and caecum a value was found for the number of haemocytoblasts, immature plasma cells and mature plasma cells per high power field (x 600). This was done by counting the haemocytoblasts, immature plasma cells and mature plasma cells in each of 50 high power fields and calculating average values.

The number of germinal centres per splenic section was counted. The area of the section was calculated by placing it against mm square graph paper

and the number of germinal centres corrected for 25 sq. mm. of spleen. As many sections of each spleen as possible were examined leaving an interval of 125 μ between each section and an average number of germinal centres/25 sq. mm. obtained.

Results

1. Weighings. The total body weights and the corrected spleen weights are presented in Table 1. There was no significant difference between the total body weight of germ-free and conventional birds, and no significant difference in the weight of the spleens of germ-free and conventional chickens.

2. Immunoelectrophoresis. The serum from germ-free chickens was electrophoresed and rabbit anti-chicken globulin was put in the trough. Conventional chicken serum was also put up against rabbit anti-chicken globulin (Plate 8). This revealed the presence of IgG in germ-free serum but the precipitin line was considerably weaker than the equivalent line formed by conventional chicken serum.

In order to determine whether IgM was present in germ-free serum or not, the germ-free serum was immunoelectrophoresed against rabbit anti-chicken IgM. At the same time conventional chicken serum was set up against rabbit anti-chicken IgM. The serum from the conventional chickens produced two lines against rabbit anti-chicken IgM, the inner line being the IgM line and the outer line being formed against the common light chains of IgG. The germ-free serum produced a faint IgG (light chain) line and also an extremely faint IgM line.

Plate B. Immunoelectrophoresis. Serum from germ-free chickens (four weeks old) in all the wells except the centre well (C) which contains pooled serum from conventional chickens. All the troughs contain rabbit anti-chicken gamma globulin. The IgG line is arrowed in the serum from the conventional and one of the germ-free chickens.

C.

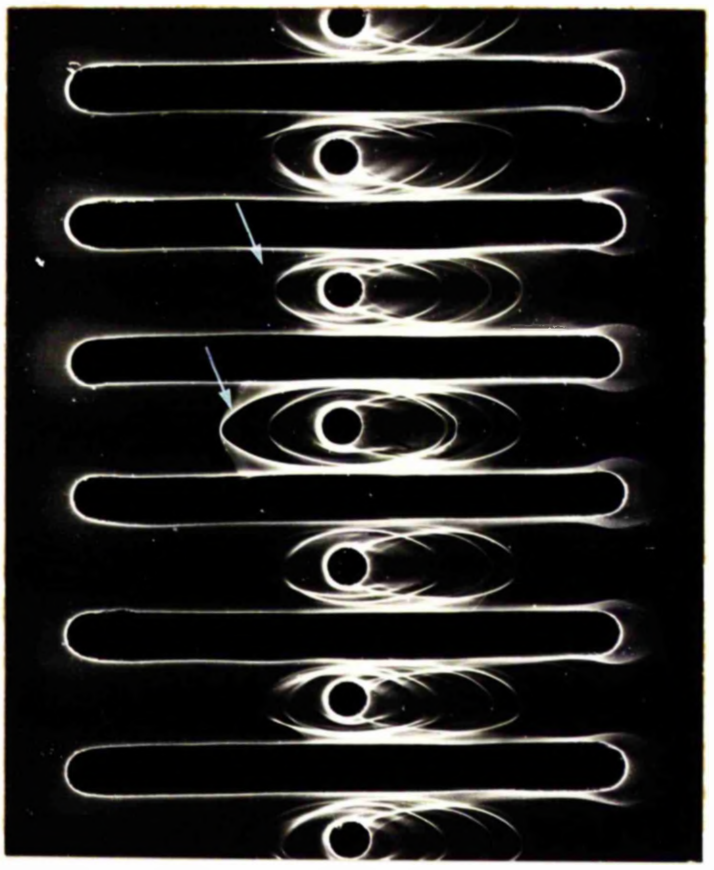


Plate 8.

Thus the germ-free chicken serum contains both IgG and IgM though in far less amount than in conventional chickens.

3. Haematology. From the differential white cell count and the total white cell count the total number of heterophils and large, small and type 1 lymphocytes was obtained. These are presented in the form of a histogram (Table II) and individual values in Table V.

From the averages the number of type 1 lymphocytes in the germ-free chickens was found to be 2.5 times as great as the number of these cells in conventional birds. On the other hand, the number of small lymphocytes in the germ-free birds was approximately 1/5 that in conventional birds. There were more circulating heterophils in the germ-free birds than in the conventional birds. However, when the "Student's" t test (Bailey, 1959) was applied to the values in Table V no significant difference was found between the same cell type in each group.

The total number of white blood cells in the germ-free birds was slightly lower than that in the conventional birds (conventional - 14,716 wbc/mm^3; germ-free - 13,626 wbc/mm^3). The total number of red blood cells in each group was almost the same (Table II).

4. Histology. Spleen The spleens of germ-free chickens differed from those of the conventional chickens in two respects. The germ-free spleens lacked germinal centres and contained very few cells of the plasmacytic series. The number of germinal centres in sections of germ-free and conventional spleens is presented in Table III. Five of the germ-free birds contained no germinal

centres in the sections examined and the sixth bird had one germinal centre in the eleven sections examined. The difference in the number of cells of the plasmacytic series was apparent on observation, however values are presented in Table IV. There was a significant difference in the number of cells per high power field in each of the three cell types counted and the number of mitotic figures was significantly lower in the germ-free spleens.

Immature lymphoid cells were seen in both groups. These cells were most prominent round the ellipsoids but were also seen in the red pulp cords. The immature lymphoid cell was smaller than the haemocytoblast i.e. about 8 μ in diameter. The nucleus was prominent and was oval rather than round. On haematoxalin and eosin stained sections the nucleoplasm was colourless with delicate chromatin threads which formed condensed dots at their intersections. No nucleolus was seen. The cytoplasm was indestinct and showed no basophilia. The cell outline was ill-defined. Using methyl-green-pyronin as stain the cell showed a narrow faintly staining rim of pyroninophilic cytoplasm. The nucleus stained a faint blue and again contained no nucleolus.

Periarteriolar lymphocytic sheaths were present in the spleens of both germ-free and conventional chickens. Ellipsoids were equally prominent in both groups.

Caecum Like the spleen, the caecal tissue contained fewer of each of the cells of the plasmacytic series counted; all sections of caecum from germ-free chickens contained at least one germinal centre. All the conventional caeca contained germinal centres. Fewer mitotic figures were seen in the germ-

free caeca.

Bursa of Fabricius No difference could be detected between the bursa of conventional and germ-free chickens. The bursa in both groups had normally developed follicles with a clear cortex and medulla with haemocytoblasts on the medullary side of the basement membrane.

Thymus Again no difference could be detected between the germ-free and the conventional thymus. Cortex and medulla were equally distinct in each group. Haemocytoblasts were seen at the edge of the cortex and Hassal's corpuscles were present in both groups. Very few immature plasma cells and no mature plasma cells were seen in the thymus of either group.

Discussion

It is generally agreed that the lymphoid tissue of the germ-free chicken is under-developed - in terms of lack of proliferation of cells of the plasmacytic series, lack of germinal centres and low level of gamm-globulin in the serum (Thorbecke et al. 1957; Thorbecke, 1959; Gordon, 1959). The same conclusion is reached in this study.

Thorbecke et al. (1957) evaluated the plasma cells and germinal centres with an arbitrary scale ranging from 0 - 4. A value of 0 meant that no such elements were present; a value of 1 that they were only scarcely present; and so on. This was an extremely subjective method which gave no information about the frequency of germinal centres. In this present study haemocytoblasts, immature plasma cells and mature plasma cells were counted in the spleen of germ-free and conventional chickens. From these figures it has been shown that all three cell

types are significantly reduced in germ-free chickens.

Because of the importance of germinal centres in this study it was necessary to have a reliable and reproducible method of counting germinal centres. Thorbecke et al. (1957) applied their arbitrary scale. This method is not reproducible and in their tables gives a misleading idea of the frequency of germinal centres. Ideally the total number of germinal centres in a spleen would have been the best measure but no method of reaching this figure could be devised. It was possible to count the number of germinal centres on a cross-section of spleen but because some sections were large and some were small the figure was meaningless. However, the area of the section could be calculated either from the microscope stage or be placing the section on top of a piece of mm. square graph paper. The number of germinal centres could then be expressed as a number per unit area - the area taken was 25 sq. mm. This then was really a measure of the density of germinal centres per unit area in that particular section. It did not take into account the germinal centres in any other part of the spleen. Another part of the spleen may have been particularly rich in germinal centres. Therefore, other sections had to be looked at but these sections had to be sufficiently far apart that the same germinal centre was not cut and counted twice. From other work on germinal centre dimensions it had been found that the germinal centre measured $91.9 \pm 23.5\ \mu \times 75.0 \pm 19.7\ \mu$ (based on measurement of long and short axis of 100 germinal centres). An interval of 125 μ was therefore allowed between each section examined and as many sections as possible were examined from the piece of spleen available. The number of germinal centres in each section was then corrected for 25 sq. mm. and an

average obtained for the spleen. In the end this did not measure the total number of germinal centres in a spleen but it did give a measure of germinal centre frequency which is reproducible and, given that the spleens are of the same size, comparable.

Using this method no germinal centres were found in the spleens of five germ-free chickens and in the sixth chicken one germinal centre was found in one of the eleven sections examined. Germinal centres were seen in all the sections from the spleens of conventional chickens (Table III).

Gordon (1959) and Reyniers et al. (1959) recorded that the number of lymphocytes per unit volume of blood was lower in germ-free chickens but gave no values for the different types of lymphocyte. In this study the number of circulating lymphocytes per unit volume of blood was slightly lower in germ-free chickens than in conventional chickens though the difference was not significant. The lymphocytes were also counted separately as type 1 lymphocytes, large and small lymphocytes. The findings are equivocal. From the average values there appears to be considerable difference between the numbers of type 1 lymphocytes and small lymphocytes in the two groups. Yet because of the variation in individual values (Table V) there is, in fact, no statistical difference between the same cell type in each group when the 't test' is applied. This disparity suggests that further work must be done in order to determine whether the germ-free state has a real effect on the number of each circulating cell type.

It would seem that the major stimulus for the development of the

lymphoid tissue is the intestinal flora, since the germ-free chickens lacked this flora. Yet, despite the fact that the amount of gamma globulin is lower and that there are fewer germinal centres and cells of the plasmacytic series, these entities do exist in the germ-free chickens. In order to account for this it must be remembered that although the germ-free chickens are free of all living micro-organisms they are not antigen free. The diet contains macro-molecules which may be antigenic and the dust from the food, bedding and the chickens themselves may be antigenic. It has also been established that dead bacteria are present in the diet and have been seen in Gram stained films from rectal swabs. Reyniers et al. (1960) was unable to demonstrate antibodies to dietary proteins - casein, gelatin, yeast extract and hog protein - in germ-free chickens, however he was able to demonstrate so-called natural antibodies in unstimulated germ-free chickens but this was related to the presence of dead bacteria in the diet. In the present study the existence of germinal centres in the caeca and the presence of more plasma cells in the caeca than in the spleen of germ-free chickens (Table IV) would support the idea that an antigen in the diet is the main stimulus in germ-free chickens.

The low level of IgM and IgG would be consistent with this low grade antigenic stimulus. Van Meter et al. (1969) have shown that all maternally derived IgG is eliminated from the circulation of normal chickens by the third week of life and that thereafter the IgG level rises to a level of 80% of an adult reference pool by the fourth or fifth week of life. IgM, none of which is derived from the hen, rises sharply after the first week of life and reaches a peak level

of 78% of the reference plasma level by the third week of life. Thus it seems that the IgG in the germ-free chickens (4 weeks old) cannot be attributed to the hen, but that both the IgG and the IgM must have been synthesized by the germ-free chickens.

The lack of difference between the thymus and the bursa of germ-free chickens and the thymus and bursa of conventional chickens is consistent with the idea of these organs as central lymphoid organs which are antigen insensitive (Miller, 1968). Further, the lack of germinal centres in the spleen and the reduced number of cells of the plasmacytic series in the germ-free chickens coupled with the low level of immunoglobulin support the theory that these structures are associated with antibody production (Cooper et al., 1966).

Having established the degree of lymphoid development in the unstimulated neonatal germ-free chicken it is now possible to study the reaction in the lymphoid tissue when the neonatal germ-free chicken is stimulated by an antigen. This is done in Section III.

Table I

Body weights and spleen weights (corrected for 100 gms body weight) of conventional and germ-free chickens at four weeks old

Body weights (gms)		Spleen weights (gms)	
Conventional	Germ-free	Conventional	Germ-free
195	185	.140	.066
155	200	.202	.094
135	140	.275	.108
175	150	.146	.110
155	155	.08	.121
210	160	.150	.104
Average			
170.8	165	.166	.101

No significant difference between the body weights in each group.
No significant difference between the spleen weights in each group.

Table II. Comparison of circulating blood cells in conventional and germ-free chickens at four weeks of age.

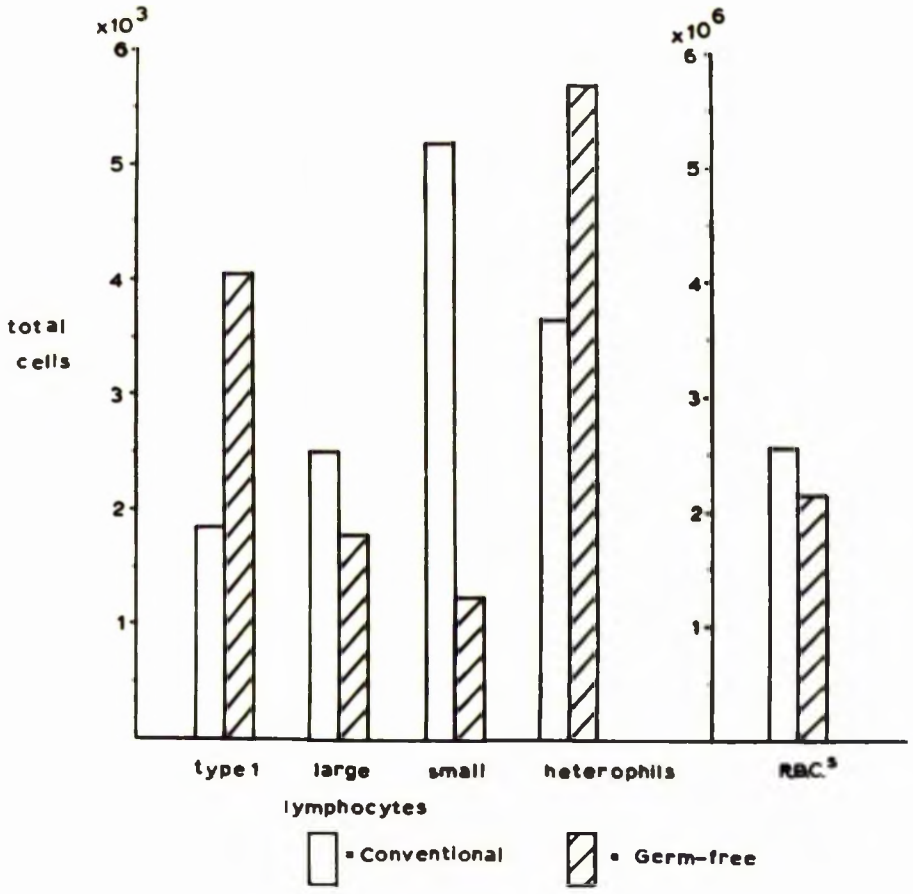

x10^3
6
5
4
3
2
1
total cells
x10^6
6
5
4
3
2
1
type 1
large lymphocytes
small
heterophils
RBC.s
= Conventional
= Germ-free

Table II.

Table III

Number of germinal centres per unit area in conventional and germ-free chickens at 4 weeks old

Chicken	No. of sections	No. of GC's per 25 sq. mm.
Conventional		
10	5	13.1
11	6	12.3
12	7	15.6
Germ-free		
31	11	0
32	22	0
33	11	0
34	22	0
35	11	0.55
36	15	0

Table IV

Average number of each cell type and mitotic figures per high power field (x 600) in conventional and germ-free chickens at four weeks old

Spleen	Conventional	Germ-free	P
Haemocytoblasts	2.3	1.1	< 0.001
Immature plasma cells	1.0	0.4	< .01 > .002
Plasma cells	0.3	0.05	< .01 > .002
Mitoses	0.5	0.1	< .002 > .001

Caecum	Conventional	Germ-free	P
Haemocytoblasts	2.6	0.8	< 0.001
Immature plasma cells	1.3	0.4	< .01 > .002
Plasma cells	0.2	0.05	< .05 > .02
Mitoses	0.4	0.1	< 0.001

Table V

The number of type 1, large and small lymphocytes and heterophils per cubic mm of blood in conventional and germ-free chickens at four weeks old

	Conventional					Germ-free			
Bird	Type 1	Large	Small	Hetero	Bird	Type 1	Large	Small	Hetero
10	303	3631	6808	3631	31	3389	1762	1762	5288
11	649	1168	6882	3765	32	1383	2152	3074	8147
12	1578	3156	7101	2524	33	4532	1762	1259	3777
13	5347	2970	2822	2376	34	7943	1528	0	4736
14	2724	2594	3373	3373	35	5152	1472	588	6477
15	607	1670	4102	6227	36	1842	2187	806	5985
Average	1868	2531	5181	3649		4060	1810	1248	5735

No significant difference between the same cell type in each group ("Student's" t test).

SECTION III INDUCTION OF GERMINAL CENTRES IN GERM-FREE AND CONVENTIONAL CHICKENS

Introduction

The germinal centre was first discovered and described in mammals by Flemming (1885). Because of the number of mitotic figures which it contained he argued that daughter cells produced by these divisions were pushed out as the result of a slow centrifugal-pressure mechanism to form the peripheral layer of small lymphocytes. These small lymphocytes subsequently entered the blood stream. Flemming's opinion was that the germinal centre was a site of lymphocytopoiesis of the body though it must be added that he did not consider that the germinal centre was the only site of lymphocytopoiesis of the body.

In the 1920's Hellman (see Ehrich, 1929) on the basis of a number of observations, criticized Flemming's theory. Hellman noted that the marginal zone did not grow along with the growth of Flemming's germinal centre and that the marginal zone of the small centres did not surpass that of the large centres. No relation between mitotic figures and the size of the marginal zone could be found. Transitional stages from the centre cells to the small lymphocytes were never seen, neither in the centre itself nor towards the marginal zone. There were often many macrophages and nuclear fragments in the germinal centre. Hellman further observed that in humans germinal centres appeared only some time after birth although in the last months of embryonic life a considerable number of lymphocytes were produced. Hellman arrived at the working hypothesis

that the secondary nodules were "reaction centres" against foreign irritants entering the lymphatic tissue. A small amount of toxic substance stimulated productive processes and a large amount lead to necrobiotic changes. Thus two theories of germinal centre function existed; either they were centres of lymphocyte production (Flemming) or they were sites of lymphocyte destruction (Hellman). Histologically, Flemming's germinal centre consisted of an outer dark staining zone of lymphocytes surrounding a paler staining centre of medium and large lymphocytes many of which were undergoing mitosis, scattered reticular cells and a few macrophages; Hellman's reaction centre also consisted of an outer dark staining zone of lymphocytes surrounding a paler staining centre containing few lymphocytes with only rare mitotic figures, scattered reticular cells and numerous macrophages and cellular debris.

Ehrich (1929b) argued that if Hellman's theory was correct then it should be possible to induce the development of germinal centres experimentally so that they could be studied in all stages of their development. Ehrich (1929b) therefore injected staphylococcus of low virulence subcutaneously into rabbits and studied the changes in the draining lymph node and the changes in the number of circulating lymphocytes. He found that germinal centres were formed but that at the time when lymphocytes in the blood were highest the germinal centres were absent. The converse was also true; at the time of the highest development of germinal centres no increase in the number of lymphocytes in the blood could be found. He concluded that the original concept of Flemming that the germinal centre is a site of lymphocytopoiesis could not be accepted. Ehrich (1929c)

reached the same conclusion after intravenous injection of staphylococcus in rabbits.

However, in an anatomical study of secondary nodules based on experimentally unstimulated rabbits and humans Ehrich (1929a) described various forms of the secondary nodule which he said represented different stages in germinal centre transformation. In this sequence he described a lymphocytopoietic germinal centre yet he denied the lymphocyte producing function of the germinal centre. In the same way Sjovall and Sjovall (1930) and Hellman and White (1930) though they described and illustrated lymphocytopoietic centres agreed with Hellman that the function of the centre was the destruction of lymphocytes. Thus it is quite clear that the proliferative activity of the germinal centre as described by Flemming does exist. Maximow (1928) in an impartial assessment describes the lymphocytopoietic centre as being in the active phase.

The objection that no germinal centres are present in the neonatal animal though lymphocytes are present in large numbers would only be valid if it were insisted that the germinal centre was the only site of lymphocytopoiesis. This was never Flemming's contention. And Ehrich's observation that a lymphocytosis occurs in the blood before the development of new germinal centres does not disprove the proliferative activity of the centre.

It was left to Conway (1937) to explain the contradictory opinions on the structure and function of the nodules of the lymphatic tissue. She injected B. monocytogenes intravenously into rabbits and killed the rabbits at close intervals after the time of injection. Using this method Conway (1937) discovered that

primarily the nodule was the centre of a rapidly proliferating focus of cells - a germinal centre - and that only secondarily did it become a site of destruction and phagocytosis - a reaction centre. She emphasized the need to realize that the lymphatic tissue was very labile and that its appearance at any given time was only a reflection of its function at that time. Thus the spleen from an animal recently exposed to a toxin would contain many germinal centres whereas the spleen from an animal killed some time after exposure to a toxin would contain fewer germinal centres but more reaction centres. Conway (1937) suggested that the lymphatic nodule underwent a cycle which began with a lymphocytopoietic phase (Flemming) or active phase (Maximow) and regressed to a depleted, phagocytic phase - the reaction centre of Hellman. When this "life cycle" of the lymphatic nodule is appreciated Hellman's objections to Flemming's original theory become understandable.

Conway (1937) considered that new germinal centres could develop anywhere in the lymphatic tissue of the lymph node or in the white pulp of the spleen dependently or independently of pre-existing centres. Two or more cycles of proliferation could succeed each other in the same nodule and a nodule could contain both a lymphocytopoietic and a reaction centre. The nodule could revert to diffuse or loose lymphatic tissue and nodules could arise in diffuse or loose lymphatic tissue. Although Conway did not in the end explain the function of the germinal centre, apart from lymphocytopoiesis, her insistence on the dynamic, labile, nature of the germinal centre, though perhaps too dynamic, was an important contribution to the understanding of the germinal centre.

However, Hellman's observations cannot be dismissed simply by saying that he was looking at the terminal stages of a cyclical reaction. The contribution that Hellman and his students (Ehrich, 1929b and c; Hellman and White, 1930; Sjovall and Sjovall, 1930) made to the study of germinal centres was the finding that germinal centres were formed following challenge with bacteria or bacterial toxins. Other workers (Osterlind, 1938; Ringertz and Adamson, 1950; Congdon and Makinodan, 1961) made the same discovery and the effect of this was to associate the germinal centre with the immune response. Gilmstedt's (1936) finding that germinal centres were absent from germ-free guinea-pigs supported the idea that the germinal centre was part of the cellular response to an immunogenic stimulus.

In general terms it was agreed that following the intravenous injection of bacteria or bacterial toxin the first reaction in the spleen was a lymphoid hyperplasia in which some authors (Ehrich, 1929b and c; Ehrich and Harris, 1942; Congdon and Makinodan, 1961; Hanna, 1964) described the breaking down or dissociation of existing germinal centres so that they merged with the surrounding lymphoid tissue. An increase in the number of circulating lymphocytes occurred at this time and was followed by the appearance of new germinal centres in the spleen. It was also established that part of the cellular response to an antigen was the proliferation of plasma cells (Bjorneboe and Gormsen, 1943; Fagraeus, 1948) and that these plasma cells produced antibody (Fagraeus, 1948b; Ehrich et al., 1949).

Now, while there is considerable evidence to support the hypothesis that the formation of germinal centres is a cellular response dependent upon the immunogenicity of the injected stimulus these experiments were unsatisfactory because of the existence and the effect of antigens other than the test antigen. The experiments were complicated by the immunogenic effect of an intestinal flora and a contaminated environment. This meant that germinal centres at various stages of development and numerous cells of the plasma cellular series were already present when the animals were challenged with the test antigen. These changes obscured the changes induced by the test antigen.

The effect of antigens other than the test antigen can be eliminated by administering the test antigen to an animal which is maintained in a germ-free environment - and that is what was done in this study. It must be added, however, that even this germ-free environment is not completely satisfactory since it is not antigen free: the diet contains non-replicating antigens.

In Section II it was shown that these non-replicating antigens are of minimal effect. The spleen of the four week old unstimulated germ-free bird does not contain any germinal centres and contains significantly fewer cells of the plasmacytic series than the four week old conventional bird. Thus these non-replicating antigens can be ignored and the test antigen studied in a spleen which is virtually unstimulated.

The situation may be stated thus: the spleen of the newborn chicken contains no germinal centres; germinal centres are present in the conventional chicken at four weeks of age but are absent in four week old germ-free chickens.

The hypothesis is that germinal centres are induced by extraneous antigens and that under experimental germ-free conditions germinal centres can be induced by administration of a specific antigen. Further, by examining the spleen at different times after the initial injection it was hoped to reveal something of the cellular events involved in the formation of the germinal centre.

A series of experiments follows in which an attempt was made to induce germinal centre formation. In experiment 1 an attempt was made to induce germinal centres in conventional birds using Shigella flexneri as antigen. In the next two experiments a soluble protein antigen (human serum albumin) was administered to 7 day old germ-free chickens (expt. 2) and to 7 day old conventional chickens (expt. 3) to induce germinal centre formation. In the remaining three experiments a staphylococcus isolated from a chicken was used as antigen. The staphylococcus was given as a primary injection at 7 days old (expt. 4) and at 21 days old (expt. 5) and as a secondary injection at 21 days old (expt. 6), the primary injection having been given at day 7.

Materials and Methods

Chickens Germ-free chickens were produced and maintained as described in Section 1. All chickens, whether germ-free or conventional, were Thornber 606.

Antigens

a) Staphylococcus

Isolation and identification. A staphylococcus was isolated from the rectum of a chicken and grown in pure culture on blood agar. This organism showed haemolysis, it was catalase positive, and coagulase positive. It was sensitive to penicillin

(1.5 units), ampicillin (2 μg), tetracycline (10 μg), erythromycine (10 μg), cloxacillin (5 μg), and chloramphenicol (10 μg). The staphylococcus was non-typable with the following staphylococcus phages - Phage gp I, 29, 52, 52A, 79, 80. Phage gp II, 3A, 3C, 55, 71. Phage gp III, 6, 42E, 47, 53, 54, 75, 77, 83A, 84, 85. Phage gp IV, 42D. Not allotted, 81, 187. at Routine Test Dilution (RTD) and at 1000 RTD.

Preparation of Standard Suspension. 5 ml of saline was added to a 24 hour culture of the organism on blood agar and the culture suspended using a sterile bent glass rod. The suspension was transferred to a sterile Universal and killed by heating at 60°C for one hour. The suspension was washed three times in saline and finally suspended in 5 ml 0.15 M NaCl. Using Brown's Opacity Tubes the suspension was found to contain 12,000 million organisms per ml. The sterility of the suspension was checked by attempting to grow a loopful of the suspension on blood agar. No bacteria grew. Using this method several aliquots of staphylococcus suspension (12×10^9 orgs/ml) were prepared.

Preparation of rabbit anti-staphylococcus serum. An adult rabbit was injected intravenously with 0.5 ml of the Standard Suspension (6×10^9 orgs). This was followed at weekly intervals for three weeks by 0.5 ml of the Standard Suspension subcutaneously. The rabbit was bled 4 days after the last boost. The blood clot was allowed to retract and the serum removed. The titre of antibody against staphylococcus was measured by an agglutination test. A titre of 1/512 was obtained.

Agglutination Test. Serial dilution (1/2 - 1/1024) of antiserum was made in saline to a final volume of 0.25 ml. To this 0.25 ml of antigen was added. The antigen for the agglutination test was prepared by making a 1/25 dilution of the Standard Suspension in saline (i.e. 4.8×10^8 orgs/ml). The tubes were incubated at 37°C for two hours then placed in the cold room (4°C) for 20 hours and read with the aid of a hand lens (x 5) at room temperature. A known positive, a known negative, antigen in saline and serum in saline were also set up as controls.

b) Shigella flexneri

The culture used was an acetone dried Shigella flexneri serotype 1a (NCTC 3) prepared as follows. The purity of the organism was checked by morphological, biochemical and serological tests and its smoothness confirmed by colonial appearance and salt stability before and after culture. The organism was grown on C.C.Y. agar (Gladstone and Fildes, 1940), containing 0.5% (w/v) lactose for 18 hours at 37°C. The harvested material was homogenized, filtered and acetone dried.

This antigen was provided by Dr. D.A.R. Simmons.

The agglutination test for Shigella was performed as for the staphylococcus agglutination test except that the antigen was used at 200 µg/ml in 0.15M NaCl.

c) Human Serum Albumin (HSA)

Crystaline human serum albumin, "purified" Behringwerke AG, was injected in 0.15M NaCl without adjuvant. Batches 3202 and 2653 were used.

Introduction of HSA to the germ-free unit

The HSA (120 mg) was dissolved in 6 ml of 0.15M NaCl and divided into two equal lots. The solution was sterilized by a Hemmings filter using a cellulose

acetate membrane (Oxoid Ltd.). The Hemmings filters were spun at 2,000 rpm until all the solution passed through the membrane. The filtered HSA was checked for sterility by removing a loopful under sterile conditions, streaking on to a blood agar plate and incubating at 37°C. If no growth was obtained after 72 hours the solution was considered to be sterile.

The outside of the HSA containers was sterilized by placing in an aqueous solution of 2% peracetic acid (v/v) for 20–25 minutes. The containers were then passed through the germicidal tank into the germ-free unit using a sterile gauntlet. The outside of the plastic container of a 2 ml pre-sterilized (gamma irradiation) syringe (Gillette) and of a pre-sterilized needle (Gillette 21G x 1.5) were also sterilized in 2% aqueous peracetic acid (v/v) for 20–25 minutes then passed into the unit.

Introduction of staphylococcus to the germ-free unit

A volume (2 ml) of the Standard Suspension was transferred to a sterile Bijou bottle. The suspension was checked for sterility by culturing a loopful on blood agar at 37°C. If no growth was obtained after 72 hours then the suspension was considered to be sterile.

The outside of the Bijou, the outside of the container of a pre-sterilized 2 ml syringe and the outside of the container of a pre-sterilized needle (25G x 15/16) were sterilized by placing in 2% aqueous peracetic acid (v/v) for 20–25 minutes. The items were then passed through the germicidal tank into the unit using a sterile gauntlet.

Microbial Testing

The chickens were assessed for sterility as described in Section I. A rectal swab was taken from each bird before it was removed from the unit in addition to the routine microbial testing.

Removal of a chicken from the germ-free unit

When the unit was set up for sterilization in the autoclave a sufficient number of autoclavable nylon bags was placed in the unit. These bags were made from autoclavable nylon lay-flat tubing (Portland Plastics Ltd., Hythe, Kent) heat sealed to form bags 12 ins long by 5 ins broad.

The chicken to be removed was placed at the bottom of a bag and passed into the germicidal tank where it was received by an assistant wearing a sterile gauntlet. The chicken was thus removed without destroying the sterility of the unit.

Each chicken removed was swabbed per rectum, weighed and killed by decapitation after stunning. Blood was removed to obtain serum for antibody estimation. The bursa, a lobe of thymus, caecum and half of the spleen were used for conventional histology and half of the spleen was used to obtain frozen sections.

Phosphate Buffered Saline

This was prepared by dissolving 17.532 gm NaCl in 400 ml of M/20 $Na_2HPO_4.2H_2O$ plus 100 ml of M/20 KH_2PO_4 and adding distilled water to 2 litres. The pH was 7.2 with a concentration of 0.15M NaCl.

Preparation of Antisera

1) Antiserum against HSA. An adult rabbit was injected with 0.5 ml of a water-in-oil emulsion containing 5 mg HSA and 1 mg of heat killed human type

Mycobacterium tuberculosis (complete Freund-type adjuvant). This was divided and given in two equal doses subcutaneously in each hind leg. A second injection of 5 mg HSA in 0.5 ml of aluminium phosphate was given partly subcutaneously and partly intravenously four weeks later. The rabbit was bled 7-14 days later and the level of antibody estimated by a precipitin test using doubling dilutions of antiserum. The antigen was used at 100 µg/ml in 0.15M NaCl.

2) Antiserum against chicken gamma globulin. This was prepared as described previously (p 43).

Preparation of fluorescent anti-HSA

20 ml of a serum showing a high antibody level to HSA was precipitated at 4°C with a final concentration of 50% ammonium sulphate. The mixture was stirred during this process and the stirring continued for 15 minutes. The mixture was spun in a refrigerated centrifuge at 3,000 rpm for 15 minutes and the supernatant discarded. 20 ml of half saturated ammonium sulphate was added to the precipitate and the precipitate broken up with a glass rod. The mixture was allowed to stand for a further 15 minutes and then spun down as before. The precipitated globulin was dissolved in the minimum volume of 0.15M NaCl and dialysed against several changes of 0.15M NaCl in a cellophane sac until no sulphate ions appeared in the dialysate (i.e. failure to precipitate barium sulphate when a few drops of 2% barium chloride is added to 2 ml of acidified dialysate).

The protein concentration of the globulin was evaluated by spectrophotometry at 280 mµ using UV light and enough antiserum to contain 100 mg of protein was used for conjugation.

The antiserum was placed in a small beaker provided with a mechanical stirrer and surrounded by an ice bath. The globulin was diluted, if necessary, with 0.15M NaCl to 8.5 ml and 1.5 ml of freshly prepared 0.5M of carbonate/bicarbonate buffer was added (buffer - 3.7 gm $NaHCO_3$ plus 0.6 gm Na_2CO_3(anhydrous) plus distilled water to 100 ml. pH 9.0). The mixture was vigorously stirred to yield a vortex without frothing. 5 mg of finely ground fluorescein isothiocyanate (FITC) (G.T. Gurr, Ltd.) was added in small quantities to the stirred, cooled, buffered globulin mixture. The stirring was continued overnight at 4°C in a cold room.

The free fluorochrome was removed by passing the mixture through a Sephadex G-25 column. 10 gm of dry Sephadex G-25 was suspended in PBS and after swelling the grains were poured into a glass tube 50 cm x 1.0 cm (internal diameter). When this had settled and was washed through with PBS the fluorochrome-globulin mixture was carefully pipetted on to the top of the column. Two bands appeared and the first band to come through the column was collected, this being the FITC-linked globulin.

The non-specific fluorescence was removed by passing the FITC-linked globulin through a column of di-ethylaminoethyl cellulose (DEAE) (Whatman). The column was prepared using 4 gm of DEAE cellulose powder suspended in PBS in a 50 x 1 cm glass tube. When the column had settled and was equilibrated using at least 400 ml of PBS the FITC-linked globulin (5 ml) was added to the top of the column. As it passed down the column it separated into a fast moving band with a low fluorescein: protein ratio and a slow moving band with a high fluorescein:protein ratio. The fast band has minimal non-specific fluorescence and was collected.

Finally the conjugate was reduced to a convenient volume, 3 ml, by dehydration against Carbowax 20 m (G.T. Gurr, Ltd.) and a drop of 1% merthiolate was added as preservative.

Preparation of fluorescent anti-chicken gamma globulin

This conjugate was prepared by the method described for the preparation of fluorescent anti-HSA.

Preparation and immunofluorescence staining of tissue

Each chicken was killed by decapitation after stunning and the spleen removed. It was divided into two and half fixed in 10% formol saline for conventional histology; the other half was lodged on the wall of a thin walled glass tube, sealed with a rubber bung, and rapidly frozen by immersion in a freezing mixture at -70°C. Sections 5μ thick were cut in a cryostat at -20°C, placed on a glass microscope slide and melted and dried in a stream of warm air. The sections were then fixed in absolute methanol at room temperature for 15 minutes. After being rapidly dried in a current of warm air the sections were stained in one of the following ways.

i) To demonstrate the presence of antigen (HSA or chicken gamma globulin)

The sections were hydrated by briefly dipping in PBS. Fluoresecin labelled anti-HSA (or anti-chicken gamma globulin) was then applied as a drop directly over the section and the section left in a moist chamber at room temperature for 30 minutes. The sections were then washed in PBS with intermittent shaking for 10 minutes before mounting in PBS.

ii) To demonstrate the presence of antibody to HSA

A solution of HSA (2 mg/ml in 0.15M NaCl) was first applied to the hydrated

section for 30 minutes. After a 5 minute wash in PBS fluorescein labelled anti-HSA was added for 30 minutes. The sections were finally washed and mounted in PBS.

The "sandwich" technique also detects the presence of antigen in the section but when two adjacent sections are stained by each method, antibody can be differentiated from antigen by its presence only in the section stained by the two layers of the "sandwich" technique.

Fluorescence microscopy

The tissue sections were examined microscopically using a Wild M20 microscope fitted with an Osram HBO 200 high pressure mercury arc, oil immersion objectives and a cardioid bi-reflecting dark ground condenser. The exciter filter was a UG1 or a BG12 and the barrier filter (Schott) was a GG13 or an OG1 respectively.

Isotope labelling of the antigen

Human serum albumin (HSA) was trace labelled with radioiodine by the direct oxidation technique of Hunter and Greenwood (1962) using chloramine-T and thiosulphate-free isotope (IBS3 Iodine-131 from the Radiochemical Centre, Amersham, England).

HSA (5 mg) was dissolved in 0.5 ml of phosphate buffer (0.1M, pH 7.5). The top of the isotope vial containing 0.5 mc Iodine I-131 was removed with forceps and 0.2 ml of the HSA solution together with 0.25 ml of chloramine-T (1 mg/ml in phosphate buffer) was added to the vial and the contents allowed to interact for 1-3 minutes. Metabisulphite solution (0.1 ml of 2.4 mg/ml in phosphate buffer) was added to convert any free iodine to iodide; 0.2 ml potassium iodide (10 mg/ml in phosphate buffer) was added to act as carrier iodide.

The labelled protein was then separated from the free iodide ions by passing through a Sephadex G-25 column. Fractions were collected in 2 ml amounts and the radioactivity and the protein concentration of each aliquot determined in a well counter and spectrophotometer respectively and the intensity of labelling expressed as $\mu c/\mu g$.

The labelled HSA was provided by Dr. J.M. Stark.

Estimation of serum antibody level

Quantitative estimation of antibody to HSA was carried out by a modification of the method of Farr (1958). The trade labelled HSA was used at a concentration of 1 μg/ml. The serum was used neat (0.1 ml) and serum from conventional and germ-free chickens four weeks old were used as controls (2 tubes of 0.1 ml conventional chicken serum, one for negative control and one for trichloroacetic acid (TCA) control; same for germ-free serum). To all tubes 0.2 ml of antigen (1 μg/ml) was added and after mixing the tubes were left overnight in the cold room. Saturated ammonium sulphate (0.2 ml) was added to all tubes except TCA controls to which 0.3 ml of 10% trichloroacetic acid (TCA) was added to precipitate all the protein present. The tubes were mixed and left for one hour. After spinning for 30 minutes at 3,000 rpm the supernatant was removed and discarded from all the tubes except the TCA controls and the negative controls. The supernatant from these tubes was transferred to clean tubes. The precipitates were then washed by the addition of 0.5 ml of 40% ammonium sulphate to all tubes except TCA controls to which 0.6 ml of 10% TCA was added. The tubes were again spun and the supernatant discarded except for TCA controls and negative controls which were again kept. The

precipitates were finally resuspended in 0.5 ml of 0.15M NaCl and the radioactivity in the tubes measured in a well-type sodium iodide crystal scintillation counter.

This method precipitates all the globulin but only that amount of antigen attached to antibody globulin. The radioactivity of the precipitate is then proportional to the amount of antigen present and this is assumed to be linearly proportional to the amount of specific antibody.

Because of the very low level of antibody present the results were expressed as a percentage of the total antigen bound by .1 ml of serum.

The count for the negative control was subtracted from the TCA control to find the count range. In experiments with germ-free chickens under four weeks old, serum from unstimulated four week old germ-free chickens was used in the controls: in experiments with neonatal conventional chickens, serum from unstimulated conventional four week old chickens was used in the controls. The count for each individual tube was arrived at by subtracting the appropriate negative control. Each tube could then be expressed as a percentage of total antigen used.

Histology

Half of the spleen together with the thymus, caecum and the bursa of Fabricius from each bird was placed in 10% formol saline and paraffin sections prepared. Staining was done by haematoxylin and eosin, Leishman or Giemsa and methyl-green-pyronin.

The half of the spleen used to provide sections for immunofluorescent staining was also used to provide sections for Leishman staining. These sections were cut with an interval of at least 125 μ.

Germinal centre counts and cell frequencies were calculated as before.

Immunoelectrophoresis

Immunoelectrophoresis was carried out as described previously.

Experiments

1. Induction of germinal centres after intraperitoneal injections of Shigella flexneri

Experimental design

Twelve conventional 3 week old chickens were given 10 μg of Shigella flexneri in 0.15M NaCl intraperitoneally. This was followed by a further 50 μg 3 days later and a final 20 μg 4 days after the second dose. The second and third doses were also given by the peritoneal route.

Twelve control birds were given 0.5 ml saline intraperitoneally when the test birds were given Shigella flexneri.

Seven days after the last injection (i.e. at 5 weeks old) all the birds were weighed, killed, the spleens removed, weighed and sections made. Serum was obtained from the blood for a Shigella agglutination test.

Results

Weight of birds (average ± standard deviation)

Shigella group	Control group
274.5 ± 13.2 gms	276.6 ± 24.6 gms

No significant difference.

Weight of spleens (average ± standard deviation, each spleen corrected for 100 gm body weight)

Shigella group	Control group
0.331 ± 0.095 gms	0.283 ± 0.057 gms

No significant difference.

Germinal centres (average number ± standard deviation per 25 sq. mm. of spleen)

Shigella group	Control group
11.48 ± 5.2	13.56 ± 8.3

No significant difference.

Agglutination test

All the sera of the Shigella group gave a positive agglutination test. The titres varied from 1/4 to 1/256. The sera from the control group were all negative.

Summary

The number of germinal centres per unit area in the spleens of conventional chickens injected intraperitoneally with a total of 80 µg Shigella flexneri at 3-5 weeks of age did not differ significantly from the number of germinal centres per unit area of spleen in unstimulated control birds. Antibody to Shigella flexneri was detected in the serum of stimulated birds. No antibody was found in the serum of unstimulated birds.

2. Induction of germinal centres after intraperitoneal injection of a soluble protein antigen (HSA) at day 7 in germ-free chickens

Experimental design

Germ-free chickens were injected intraperitoneally with 10 mg HSA at 7 days of age. One chicken was removed at day 7 without having been injected and of the remainder one was removed at days 2, 4, 6, 8, 10, 12 and 14 after injection.

Results

	Day 0	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12	Day 14
wt of bird (gms)	40	62	95	90	100	110	130	160
wt of spleen (for 100 gm body wt)	0.06	0.089	0.07	0.082	0.092	0.083	0.14	0.13

Histology

The frequencies of the haemocytoblast, the immature plasma cell and the mature plasma cell in the spleen are presented in Plate 9.

From the methyl-green-pyronine (MGP) stained sections the following features were observed. At day 0 (7 days old) before stimulation, the ellipsoids were the prominent feature of the chicken spleen. They stood out due to the lack of other elements. The pulp between the ellipsoids consisted of supporting reticulo-endothelial cells and blood cells; immature lymphoid cells could also be seen (Plate 10a). These cells which were also seen in the four week old unstimulated germ-free spleen and described in detail (p 47) had a large lightly staining nucleus and a thin rim of pyroninophilic cytoplasm. No nucleolus was seen. A few haemocytoblasts were seen and occasionally a cell was seen which had the characteristics of an immature plasma cell.

Plate 9. Graph of frequency of different cell types in the spleen per high power field following stimulation (day 0) of 7 day old germ-free chickens with human serum albumin. The antibody produced is shown as % antigen bound.

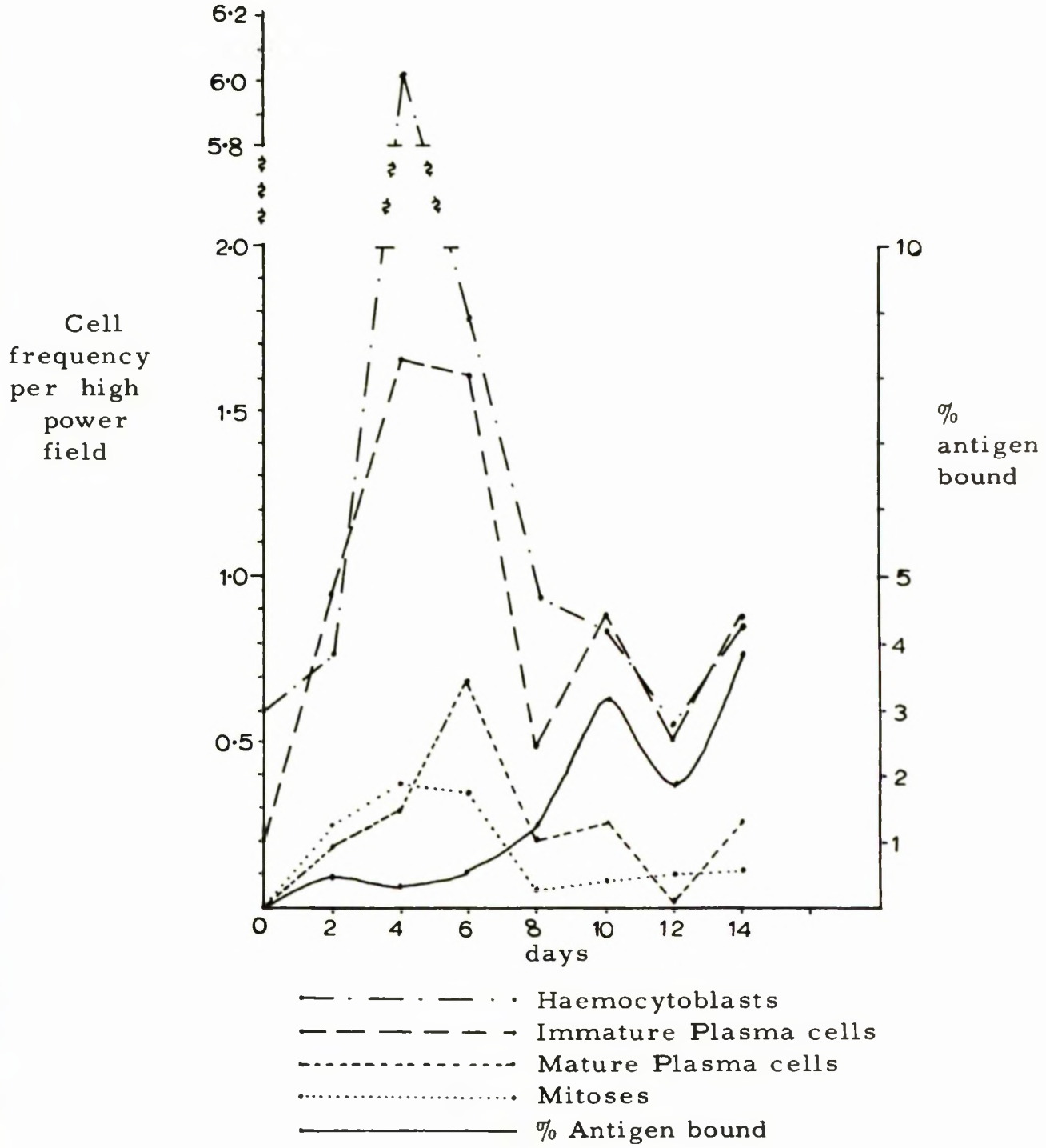

Cell frequency per high power field
6·2
6·0
5·8
2·0
1·5
1·0
0·5
10
5
4
3
2
1
% antigen bound
0
2
4
6
8
10
12
14
days
Haemocytoblasts
Immature Plasma cells
Mature Plasma cells
Mitoses
% Antigen bound

Plate 9.

At day 2 after injection of HSA more haemocytoblasts and immature lymphoid cells were present in the spleen than at day 0 (Plate 9). Plasma cells were also more numerous. Many immature lymphoid cells could still be seen round the ellipsoids.

At day 4 after injection of HSA the peak histological reaction was seen (Plate 9). The reaction was characterized by a considerable increase in the number of haemocytoblasts. These cells were arranged round the periphery of the ellipsoids at the junction with the white pulp (Plate 10b). The haemocytoblasts were not found in the ellipsoids nor were they found associated with the periarteriolar lymphocytic sheath. The haemocytoblasts were mainly confined to this pri-ellipsoid site. The number of immature plasma cells and mature plasma cells was also increased and there were many mitotic figures.

At day 6 the haemocytoblasts were less obviously arranged round the ellipsoids. By this time the cellular reaction was taking place in the pulp cords. There were fewer haemocytoblasts than at day 4, about the same number of immature plasma cells but more mature plasma cells.

At day 8 all three cell types were less frequent in occurrence (Plate 9) and the cells were again seen in the pulp cords and sinusoids.

On the following six days the number of each cell type decreased but never reached the level seen before stimulation. The spleen at day 12 showed very little reaction.

On no section was a germinal centre or the beginnings of a germinal centre seen. No change in the number of lymphocytes was observed in the spleen and there was no infiltration of polymorphonuclear leucocytes.

Plate 10a. Spleen of germ-free chicken before stimulation (day 0). In the centre of the field a group of ellipsoids is seen surrounded by immature lymphoid cells.

Methyl-green-pyronin x 300.

Plate 10b. Spleen of germ-free chicken 4 days after stimulation with HSA (11 days of age). The immature lymphoid cells have disappeared and immunocytoblasts are seen round the ellipsoid in the centre of the field.

Methyl-green-pyronin x 300.

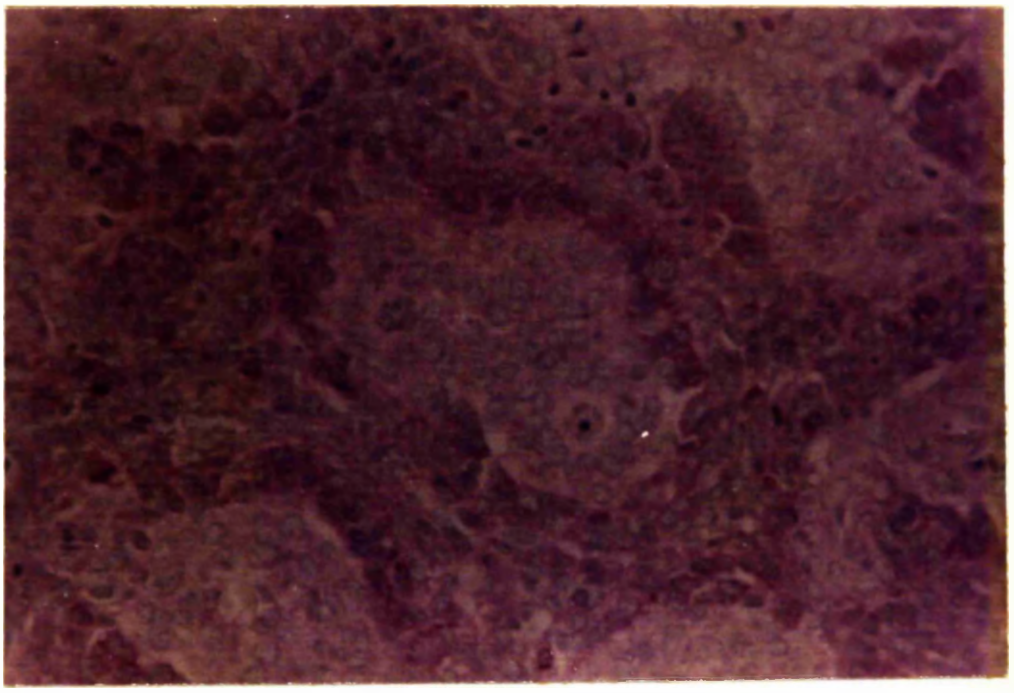


Plate 10a

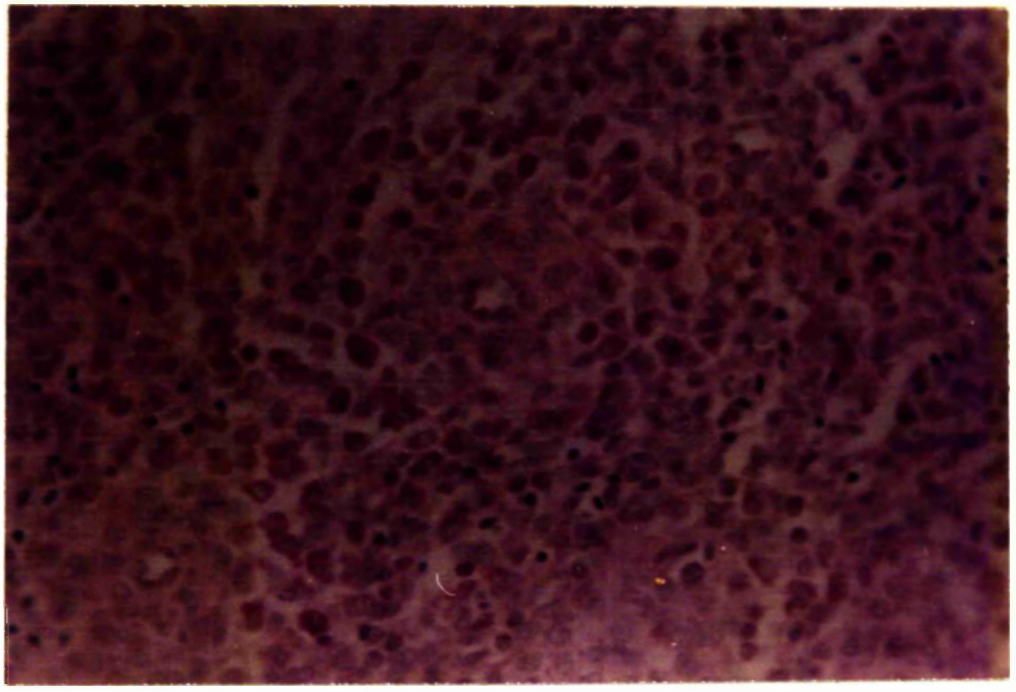


Plate 10b.

Germinal centre counts

A section from 7 different levels of each spleen was examined. No germinal centres were seen on any section.

The caecum showed no cellular reaction to the antigen. Germinal centres were first seen at day 2 after antigen administration and were present in the same numbers in all the chickens removed thereafter. The second day after administration of antigen was the seventh day after addition of feed.

At the cellular level the bursa of Fabricius and the thymus showed no definite change in architecture during the period of examination; the organs merely increased in size. Histologically the bursal and caeca were indistinguishable from the adult organs or from the 4 week old germ-free organs.

Staining with fluorescent anti-HSA

Several sections from each spleen were stained by the direct method for the detection of antigen and by the sandwich method for the detection of antibody. Neither antigen nor antibody was found in section from the HSA stimulated germ-free birds. At each staining session the conjugate was used to stain by the direct and sandwich methods spleen sections from adult birds known to contain antigen and antibody. On all occasions both antigen and antibody were detected.

Staining with fluorescent anti-chicken gamma globulin

Spleen sections were stained with fluorescent anti-chicken gamma globulin by the direct method. At day 0 no gamma globulin containing cells were seen. At day 2 after injection of HSA a few isolated gamma globulin containing cells were seen in the red pulp. At day 4 the number of gamma globulin containing cells had

increased. There appeared to be fewer gamma globulin containing cells at day 6 but at day 8 there were numerous gamma globulin containing cells. On days 10, 12 and 14 many gamma globulin containing cells were seen though there did not seem to be as many as at day 8. The staining was considerably reduced though not eliminated by mixing the fluorescent rabbit anti-chicken gamma globulin with an equal volume of chicken gamma globulin, spinning down after 1 hour at room temperature and staining with the supernatant. The fact that the rabbit anti-HSA failed to stain also acted as a control.

Antibody to HSA. Serum from the chickens was assayed for antibody content by a modification of the Farr Test. The results are presented in Plate 9.

The amount of antibody was extremely low but was present and it is seen that the amount of antibody rose sharply from 8-10 days, day 12 was poor but the amount of antibody was about the same at day 14 and day 10.

Summary

Although no germinal centres were seen after intraperitoneal injection of HSA in germ-free chickens a pyroninophilic cell reaction was seen in the spleen. No antibody containing cells were detected by the fluorescent antibody technique but very small quantities of antibody were detected by a modification of the Farr test.

3. Induction of germinal centres after intraperitoneal injection of a soluble protein antigen (HSA) at day 7 in conventional chickens

Experimental design

The experiment conducted in germ-free chickens was repeated in conventional

chickens. These chickens were the same breed and age but were kept under normal conditions of husbandry. The experimental design was otherwise as in experiment 2.

Results

Weights

	Day 0	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12	Day 14
Wt. of bd. (gms)	35	43	50	50	75	90	85	75
Wt. of sp.	0.086	0.074	0.102	0.084	0.091	0.13	0.092	0.13

Histology. The spleen from the conventional chicken at 7 days of age (day 0), before stimulation, contained more plasmacytic cells than the comparable germ-free spleen. At day 4 after injection of HSA there was an increase in the number of plasmacytic cells though this was not as obvious as in the comparable germ-free spleen because of the relative plasmacellular activity in the conventional spleen. On the following days the conventional spleens differed from the comparable germ-free spleens in that the plasma cellular reaction did not subside but continued at a higher level.

Germinal centre count. From the Leishman stained frozen sections taken from five different levels of the spleen the following values were obtained:-

	Day 0	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12	Day 14
Average no. of GC/ 25 sq. mm.	0	0	0	0.7	2.6	0.68	0.62	1.35

Germinal centres were first seen in the spleen at day 6 and were present at all subsequent days on which the spleen was examined. Germinal centres appeared in the caecum from day 0 onwards and more cells of the plasmacytic series were seen than in the comparable germ-free caecum.

The bursa and thymus did not differ histologically from the comparable germ-free bursa and thymus.

Fluorescent anti-HSA. Using the direct method of staining on spleen sections no antigen was seen at day 2 but at day 4 a few very faintly staining antigen containing cells were seen. These cells seemed to be arranged round the ellipsoids but they were few in number and extremely faint. No antigen was found at any subsequent day. Using the sandwich technique antibody containing cells were first seen at day 6. These cells occurred in the red pulp though not many were seen (Plate IIb). Faintly staining antibody containing cells were seen at day 8 but none was seen in the spleen from any subsequent day. Neither antigen nor antibody was seen in a germinal centre at any stage.

Fluorescent anti-chicken gamma globulin. A few gamma globulin containing cells were seen at day 0. The number of these cells increased at days 2, 4, and 6 to reach a peak at day 8. At this stage (Plate IIa) the red pulp contained many gamma globulin staining cells. On the following days the sections seemed to contain as many gamma globulin containing cells as at day 8. The number of stained cells on every day in the conventional chickens was greater than the number on the comparable day in the germ-free chickens.

Antibody to HSA. Serum antibody to HSA was estimated by a modification of the Farr Test. As in the germ-free birds the titre rose sharply between days 8-10 but whereas the germ-free animals rose to 3.125% antigen bound at day 10 the conventional animals rose to 14% antigen bound at day 10 (Plate 12).

Plate 11a. Gamma globulin containing cells in the spleen of a conventional chicken 8 days after stimulation with HSA (15 days old).

Fluorescent rabbit anti-chicken gamma globulin, direct staining x 300.

Plate 11b. Two cells containing antibody to HSA in the white pulp of the spleen of a conventional chicken 6 days after injection of HSA (13 days old).

Fluorescent rabbit anti-HSA, sandwich staining x 300.

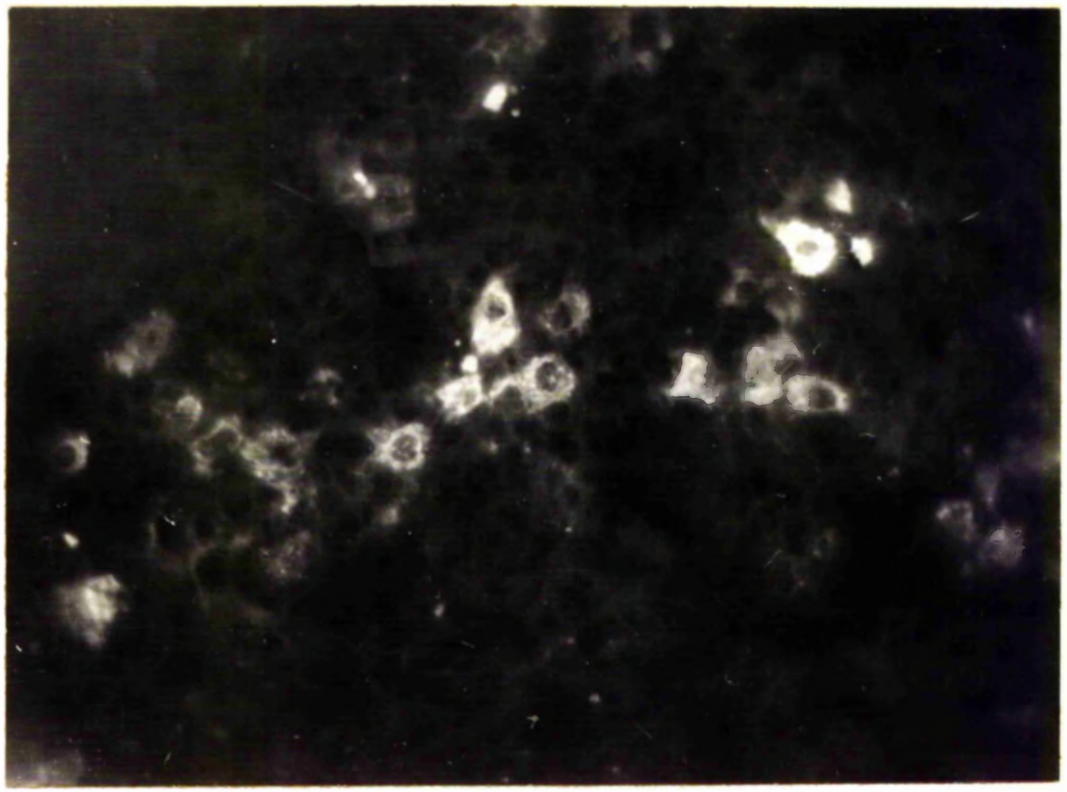


Plate 11a.

Plate 11b.

Plate 12. Comparison of antibody production to HSA expressed as % antigen bound by 0.1 ml undiluted serum in conventional and germ-free chickens. The antigen was administered at 7 days old (day 0).

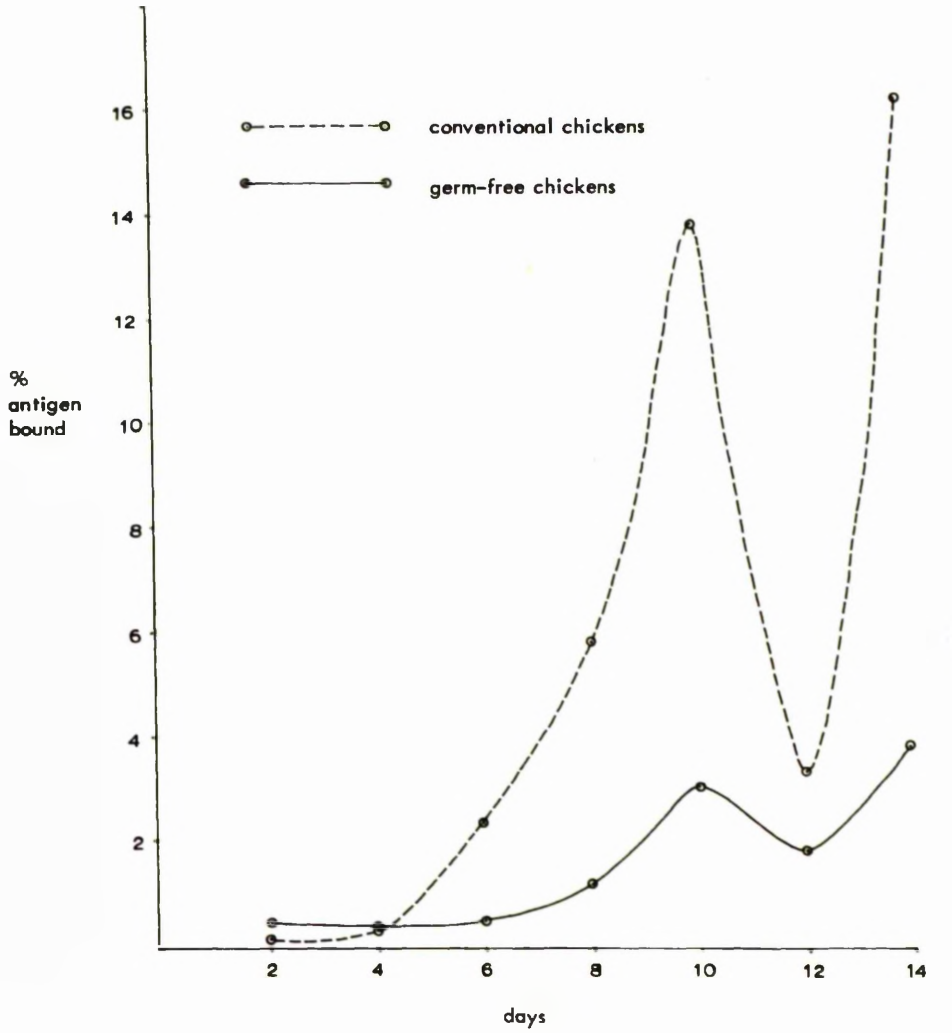

conventional chickens
germ-free chickens
% antigen bound
16
14
12
10
8
6
4
2
2
4
6
8
10
12
14
days

Plate 12.

Summary

Greminal centres and a pyroninophilic cell reaction were seen in the spleen of conventional chickens given HSA by the intraperitoneal route. A few specific antibody containing cells were detected in the red pulp and antibody to HSA was found in the serum by a modification of the Farr test.

4. Induction of germinal centres after intravenous injection of a particulate antigen (staphylococcus) in germ-free chickens at 7 days old

Experimental design

Germ-free chickens were injected intracardia with 0.25 ml of the Standard Staphylococcus Suspension at 7 days old. One chicken was removed without having been injected and one of the remaining chickens was removed at days 2, 4 and 6 after injection.

Results

Body and spleen weights

	Day 0	Day 2	Day 4	Day 6
Wt. of bd (gms)	50	51	65	77
Wt. of sp (for 100 gm bdy wt)	0.06	0.075	0.12	0.10

Histology. Spleen - In these sections no differential cell frequency counts were made since the reaction was not uniform throughout the spleen.

At day 0 (7 days old, before injection of antigen) the ellipsoids were prominent in the spleen because they were surrounded by immature lymphoid cells. These cells occurred at the junction of the ellipsoids and the red pulp. In the pulp cords a few haemocytoblasts were seen and occasionally an immature plasma cell was seen but a

mature plasma cell was rare. Blood cells were present in the sinusoids. The periarteriolar lymphocyte sheaths (PALS[8]) were not an obvious feature of the sections. The PALS[8] were small in diameter and were only seen around some arterioles.

At day 2 after injection of antigen the ellipsoids were not prominent because they were not ringed by immature lymphoid cells. This cell type was reduced in number. However the prominent feature at this stage was the hyperplasia of the periarteriolar lymphocyte sheaths (Plate 13a). The sheaths were increased in number and in size. The lymphocyte was the predominent cell in the sheath but scattered between the lymphocytes were haemocytoblasts (Plate 13b). These haemocytoblasts tended to occur in greatest number towards the periphery of the PALS. Only occasionally was an immature plasma cell seen in the PALS and no mature plasma cells were seen. In the red pulp cords all the stages between the haemocytoblast and the mature plasma cell were seen.

At day 4 after injection of antigen immature lymphoid cells were again seen round the ellipsoids. Immature plasma cells and mature plasma cells were prominent in the pulp cords. The PALS[8] were fewer in number and less reactive but the striking feature was that in a few PALS[8] the haemocytoblasts had become aggregated (Plate 15a). This clump of cells, almost entirely haemocytoblasts, was clearly delineated though not circumscribed by fibrous tissue as in a mature germinal centre. Yet the aggregation was quite clearly a germinal centre. One such centre contained only haemocytoblasts except for three or four reticuloendothelial cells. Another centre contained haemocytoblasts but as well as these and reticuloendothelial cells small lymphocytes were seen.

Plate 13a. Spleen of germ-free chicken 2 days after injection of staphylococcus into a 7 day old bird. A number of hyperplastic periarterialar lymphocyte sheaths are seen one of which is arrowed.

Giemsa x 60.

Plate 13b. High power (x 300) of a periarterialar lymphocyte sheath showing haemocytoblasts at the periphery; several are arrowed.

Methyl-green-pyronin x 300.

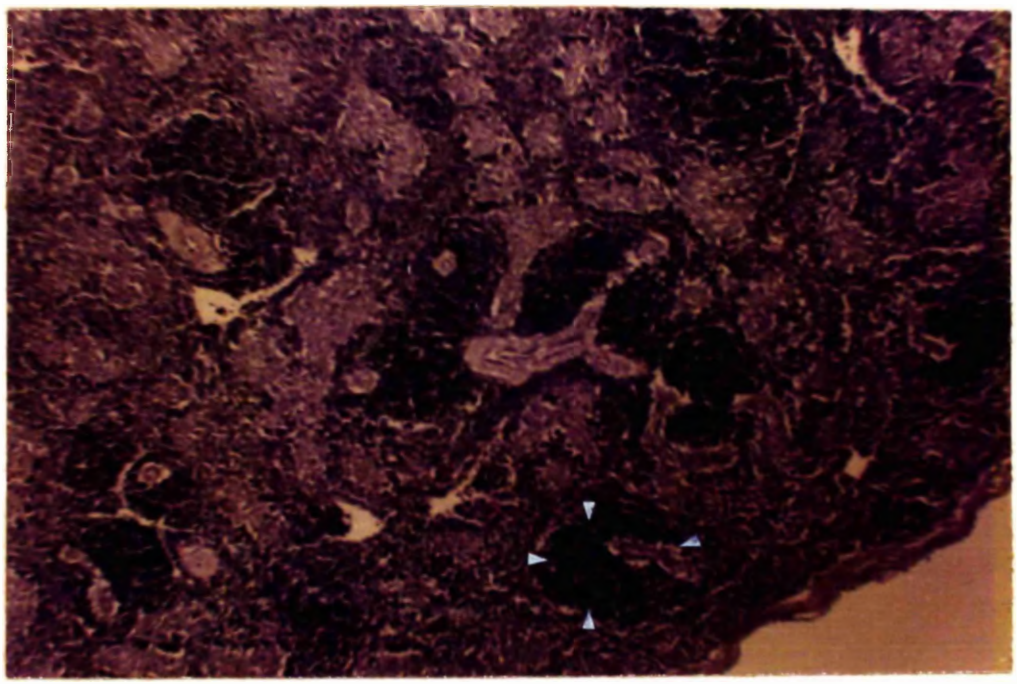


Plate 13a.

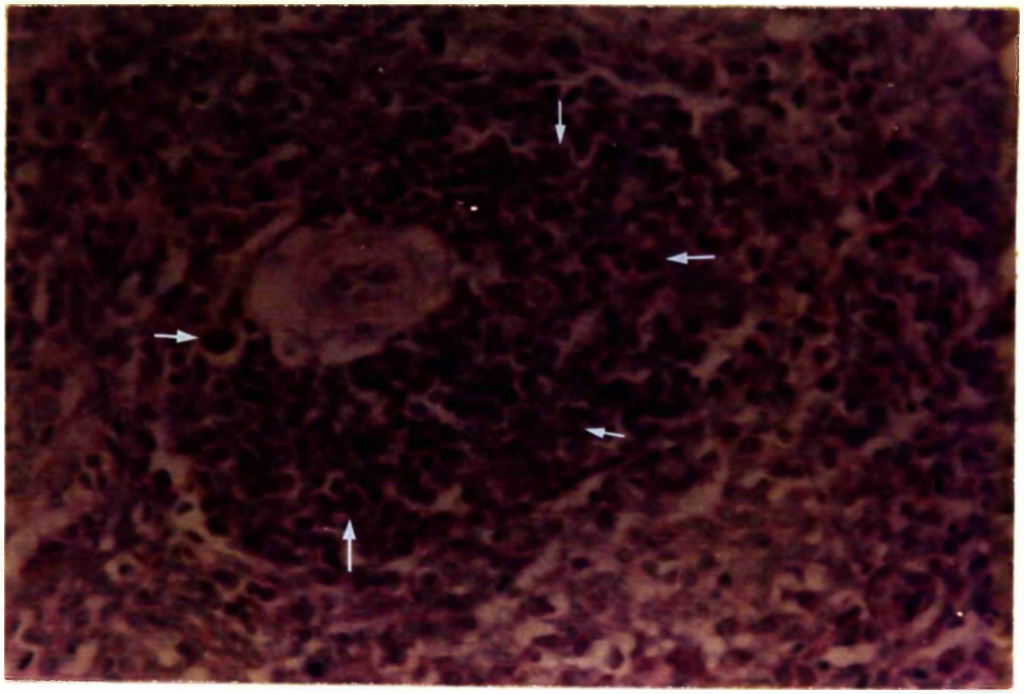


Plate 13b.

Plate 14. Spleen of germ-free chicken 3 days after injection of staphylococcus into a 21 day old bird. Haemocytoblasts have accumulated in the periarteriolar lymphocyte sheath. Methyl-green-pyronin x 300.

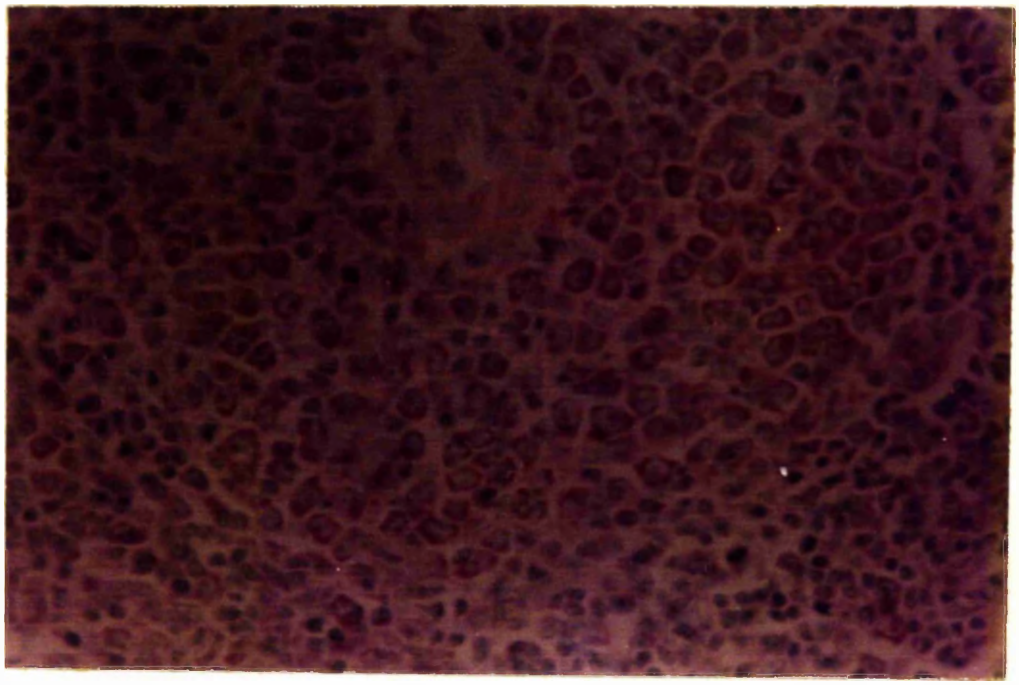


Plate 14.

Plate 15a. Spleen of germ-free chicken 4 days after injection of staphylococcus into a 7 day old bird. The haemocytoblasts in the periarterioler lymphocyte sheath have aggregated into a recognisable germinal centre.

Methyl-green-pyronin x 300.

Plate 15b. Spleen of germ-free chicken 5 days after injection of staphylococcus into a 21 day old bird. The definitive circumscribed germinal centre is seen containing both haemocytoblasts and lymphocytes.

Methyl-green pyronin x 300.

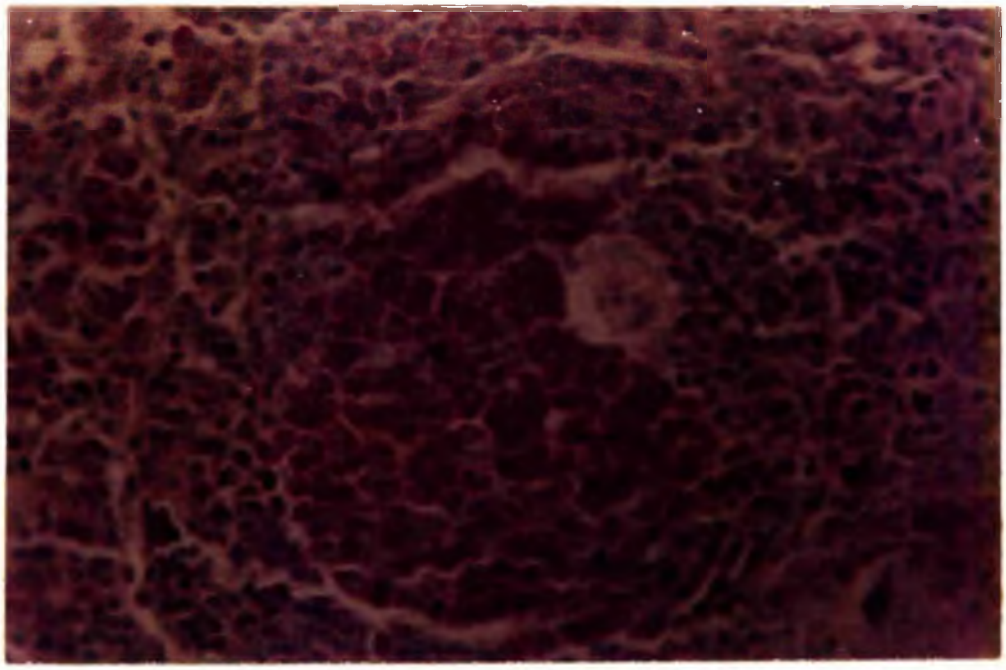


Plate 15a.

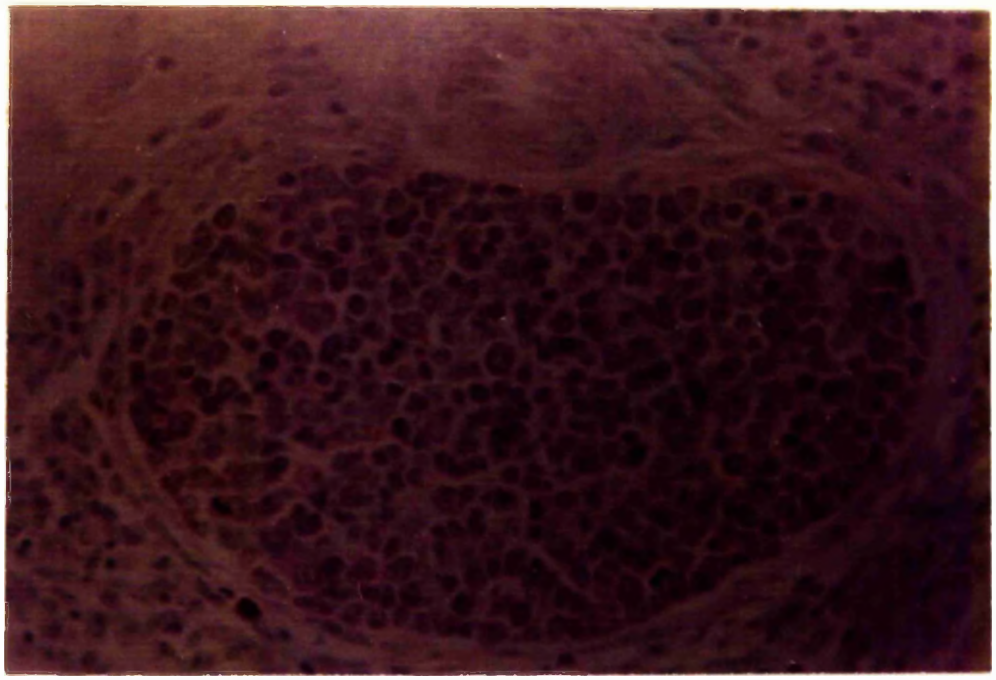


Plate 15b.

Plate 16. Spleen of germ-free chicken 7 days after a secondary injection of staphylococcus into a 21 day old bird. Five circumscribed germinal centres are seen.

Giemsa x 60.

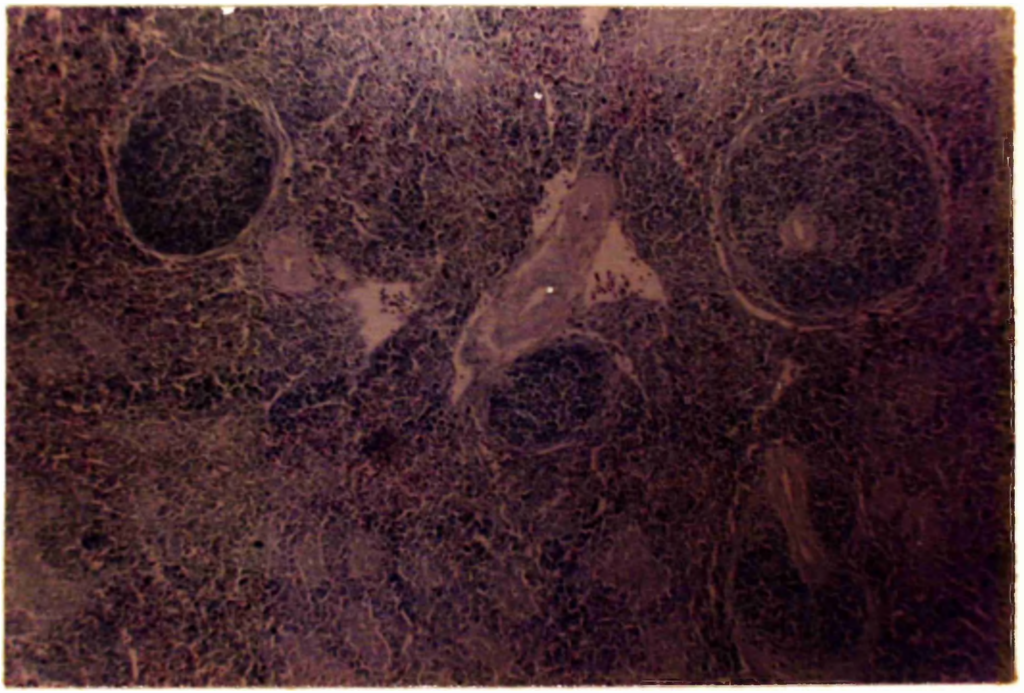


Plate 16.

At day 6 after injection of antigen the spleen appeared very like the spleen at day 0. The ellipsoids were surrounded by immature lymphoid cells, very few PALS[5] were seen and no germinal centres were seen in any of the paraffin sections (9 sections). The spleen at day 6 differed from that at day 0 in that immature and mature plasma cells were seen in the pulp cords in greater numbers.

Caecum - No reaction to antigenic stimulation was seen in the caecum. Germinal centres were present in the caecum at day 2 (7 days after addition of diet) and remained at a constant level throughout.

Thymus and bursa - No histological changes were seen in these organs.

Germinal centre count. From the Leishman stained frozen sections taken from 5 different levels of the spleen the following values were obtained.

	Day 0	Day 2	Day 4	Day 6
Average no. of GC[5] per 25 sq. mm.	0	0	3.3	1.23

Staining with fluorescent anti-chicken gamma globulin. At day 0 no gamma globulin containing cells were seen but at days 2, 4 and 6 after injection of antigen a few such cells were seen in the red pulp. There seemed to be about the same number of gamma globulin containing cells on each of these days. No germinal centres were seen in any of the sections.

Staphylococcus agglutination test. An agglutination test was performed to detect antibodies to staphylococcus using sera from days 0, 2, 4 and 6. No antibodies were found in any of these sera.

Summary

Following intravenous injection of staphylococci into germ-free chickens (7 days old) germinal centres were found in the spleen. The cellular reaction was characterized by a hyperplasia of the periarterioiar lymphocyte sheath and a proliferation of pyroninophilic cells. Germinal centres were formed in the periarterioiar lymphocyte sheath and in the early stages consisted almost entirely of haemocytoblasts. No antibody to staphylococcus was found in the serum.

5. Induction of germinal centres after intravenous injection of a particulate antigen (staphylococcus) in germ-free chickens at 21 days old

Experimental design

Three germ-free chickens were given 0.25 ml of Standard Staphylococcus Suspension intracardia at 21 days of age. One chicken was removed at days 3, 5 and 7 after injection.

Results

Body and spleen weights

	Day 3	Day 5	Day 7
Wt of bd	120	128	140
Wt. of sp (for 100 gm bdy wt)	0.24	0.16	0.15

Histology. Spleen - The reaction in the spleen following the primary injection of staphylococcus at day 21 followed the same pattern as that seen following the primary injection of particulate antigen at 7 days old. The reaction initiated at 21 days was, however, more intense. At day 3 after the injection of antigen

PALS[5] were hyperplastic and haemocytoblasts were seen between the lymphocytes. Haemocytoblasts had accumulated in the PALS without having formed a clearly circumscribed centre (Plate 14). There was a paucity of immature lymphoid cells round the ellipsoids but immature and mature plasma cells were present in the red pulp.

At day 5 after injection of antigen encapsulated germinal centres were seen (Plate 15b). These centres contained many haemocytoblasts but small lymphocytes and reticulo-endothelial cells were also present. The PALS[5] were less prominent but immature and mature plasma cells were seen in the red pulp and immature lymphoid cells surrounded the ellipsoids.

At day 7 after injection of antigen clearly circumscribed germinal centres were present. In these germinal centres there were fewer haemocytoblasts and more small lymphocytes than in germinal centres at day 5. The PALS[5] were not hyperplastic and the red pulp contained plasmacytic cells as well as immature lymphoid cells.

Caecum, thymus and bursa. These tissues showed no signs of lymphoid reaction.

Germinal centre count. From Leishman stained frozen sections of the spleen taken at 5 different levels the following values were obtained.

	Day 3	Day 5	Day 7
Av no of GC[5] per 25 sq. mm.	3.3	4.25	13.8

Staining with fluoresent anti-chicken gamma globulin. At day 3 gamma globulin containing cells were seen in the red pulp. No germinal centres staining was seen. At days 5 and 7 as well as gamma globulin containing cells in the red pulp germinal centres were seen which, when stained, gave a dendritic pattern.

Agglutination test. The sera were tested for the presence of agglutinins to staphylococcus. None was found.

Summary

Following intravenous injection of staphylococci into germ-free chickens at 21 days of age germinal centres were found in the spleen. The cellular reaction in the spleen followed the same pattern as in the previous experiment but with greater intensity and the formation of more germinal centres. No antibody to staphylococcus was found in the serum.

6. Induction of germinal centres after a secondary intravenous injection of a particulate antigen (staphylococcus) in germ-free chickens at 21 days old

Experimental design

Five germ-free chickens were injected intracardiac with 0.25 ml of Standard Staphylococcus Suspension at day 7 (7 days old).

Fourteen days later these birds received a further 0.25 ml of Standard Staphylococcus Suspension intracardia.

One bird was removed from the unit at days 2, 3, 4, 5 and 7 after the last injection.

Results

Body and spleen weights

	Day 2	Day 3	Day 4	Day 5	Day 7
Wt of bd	140	165	93	104	155
Wt of sp (for 100 gm bdy wt)	0.16	0.19	0.17	0.22	0.14

Histology. Spleen - Again, no differential cell frequency count could be made since the reaction was not uniform throughout the spleen.

At day 2 after injection of antigen the prominent feature was the periarteriolar lymphocyte sheaths. Haemocytoblasts were seen between the lymphocytes of the sheath and they were evenly scattered; they did not appear to be more numerous towards the periphery. The ellipsoids were surrounded by immature lymphoid cells but only a few haemocytoblasts and immature plasma cells were seen in the red pulp. Mature plasma cells were rare in this area. No germinal centres were seen.

At day 3 following the injection of antigen the reaction in the PALS[6] was greater than at day 2 and haemocytoblasts were more numerous. Some PALS[6] consisted almost entirely of haemocytoblasts with only a few lymphocytes round the periphery. Immature lymphoid cells were seen round the ellipsoids and haemocytoblasts, immature plasma cells and mature plasma cells were present in the red pulp.

At days 4 and 5 after injection of antigen the haemocytoblasts in the PALS[6] were grouped into recognisable centres. Some of these centres were circumscribed by fibrous tissue, others were not. The haemocytoblast was the predominant cell in most centres but some centres contained many small lymphocytes. The lymphocyte sheath was less prominent round the arterioles. Haemocytoblasts, immature and mature plasma cells were seen in the red pulp.

At day 7 after injection of antigen clearly circumscribed germinal centres were present (Plate 16). These centres contained haemocytoblasts but the predominant cell was the small lymphocyte. The PALS was barely detectable round most arterioles. Immature lymphoid cells surrounded the ellipsoids and haemocytoblasts,

immature plasma cells and mature plasma cells were present in the red pulp though these latter cells were not as numerous as at day 5.

Caecum, thymus and bursa. No signs of lymphoid reaction were seen in the caecum and the thymus and bursa did not differ histologically from the conventional tissues.

Germinal centre count. From Leishman stained frozen sections taken from 5 different levels of the spleen the following values were found.

	Day 2	Day 3	Day 4	Day 5	Day 7
Av no of GCs per 25 sq. mm.	0	7.24	12.8	25.65	26.0

Staining with fluorescent anti-chicken gamma globulin. Frozen sections of spleen were stained directly with fluorescent rabbit anti-chicken gamma globulin. At days 2 and 3 after injection of antigen a few gamma globulin containing cells were seen in the red pulp. At days 4, 5 and 7 more gamma globulin containing cells were seen (Plate 17). In these sections germinal centres were seen and the staining showed a dendritic pattern (Plate 18). The cells in the germinal centre stained lighter than the cells in the red pulp, and seemed to have processes which joined up to form a network within the centre. No plasma cells were seen within a centre.

Agglutination test. The sera were tested for the presence of antibodies to staphylococcus by an agglutination test incorporating appropriate controls. No antibody was detected in any of the sera.

Immunoelectrophoresis. Although immunoelectrophoresis is not a quantitative technique if a constant volume of serum and antiserum is used and the immunoelectrophoresis is carried out under standard conditions the results give some indication of the amount of the different globulins present. Serum from a chicken 6 days after

Plate 17. Numerous gamma globulin containing cells in the white pulp of the spleen 7 days after a secondary injection of staphylococcus into a 21 day old germ-free chicken.

Fluorescent rabbit anti-chicken gamma globulin, direct staining x 300.

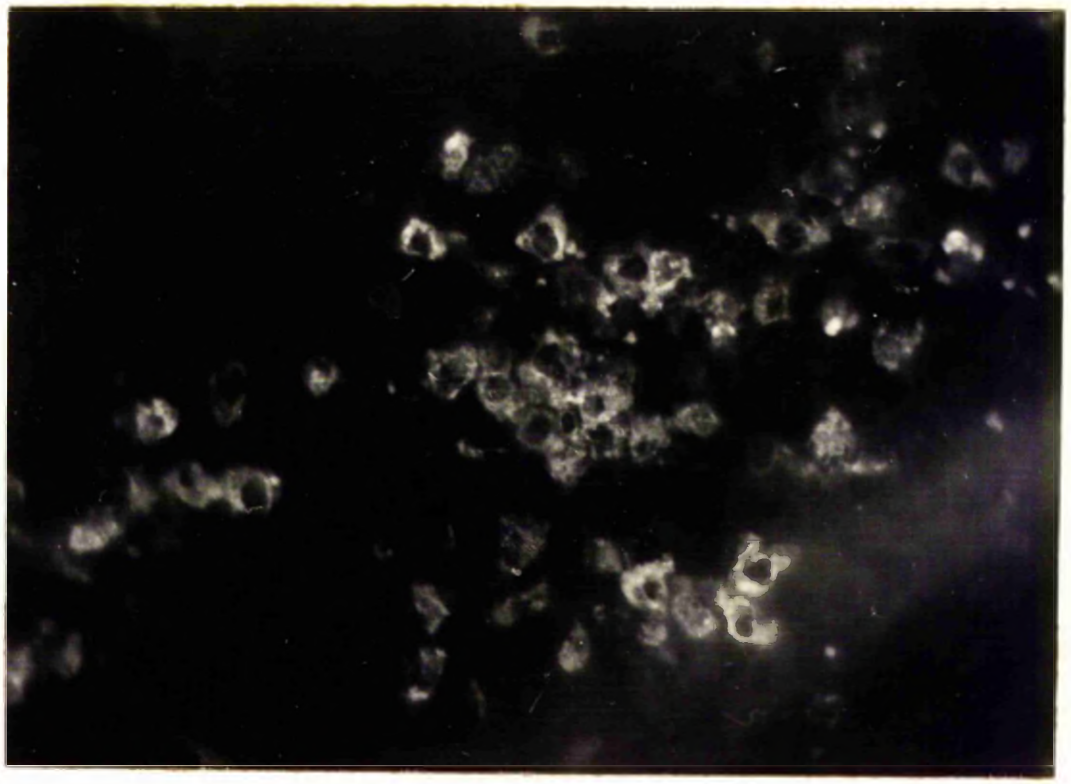


Plate 17.

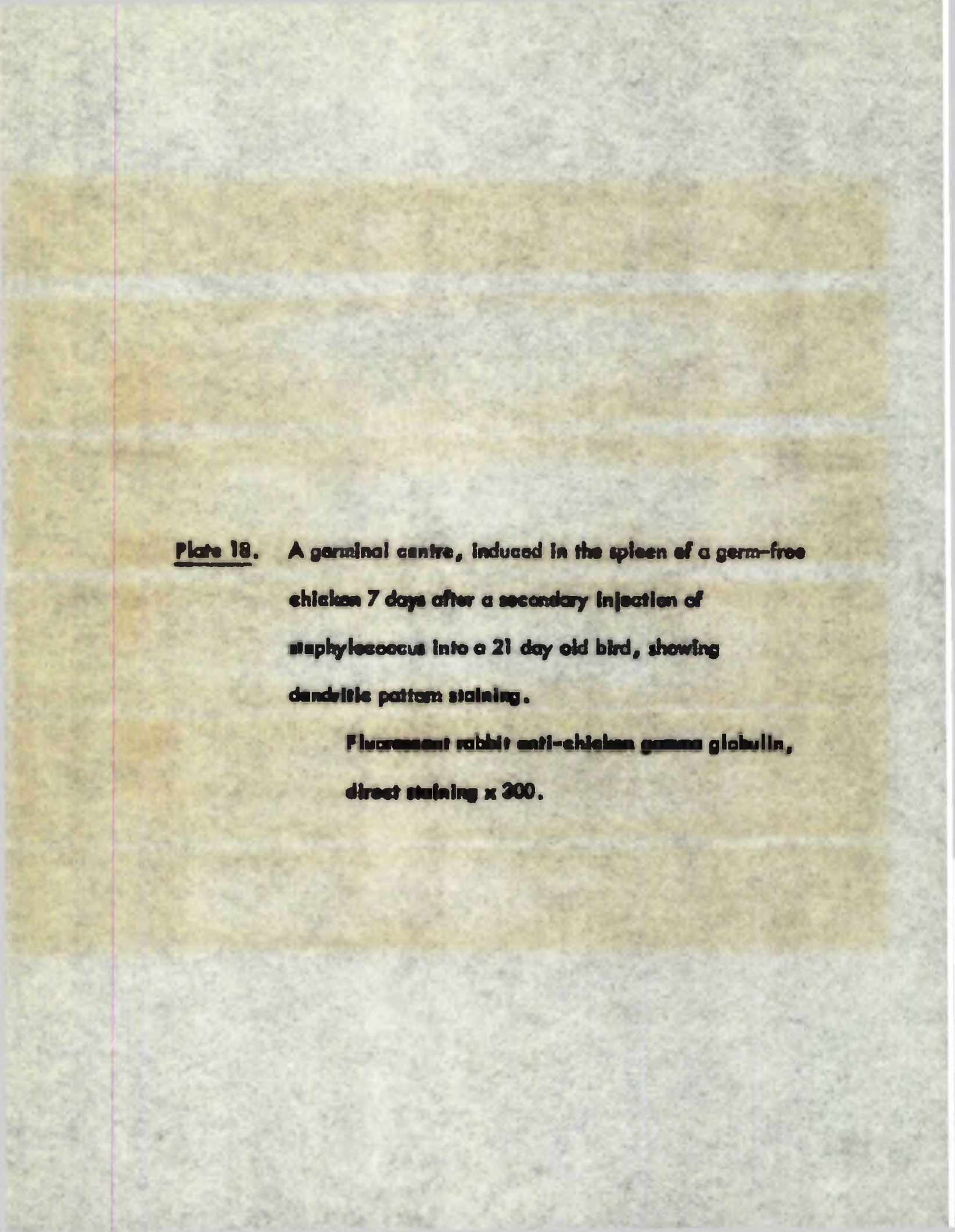


Plate 18. A germinal centre, induced in the spleen of a germ-free chicken 7 days after a secondary injection of staphylococcus into a 21 day old bird, showing dendritic pattern staining.

Fluorescent rabbit anti-chicken gamma globulin, direct staining x 300.

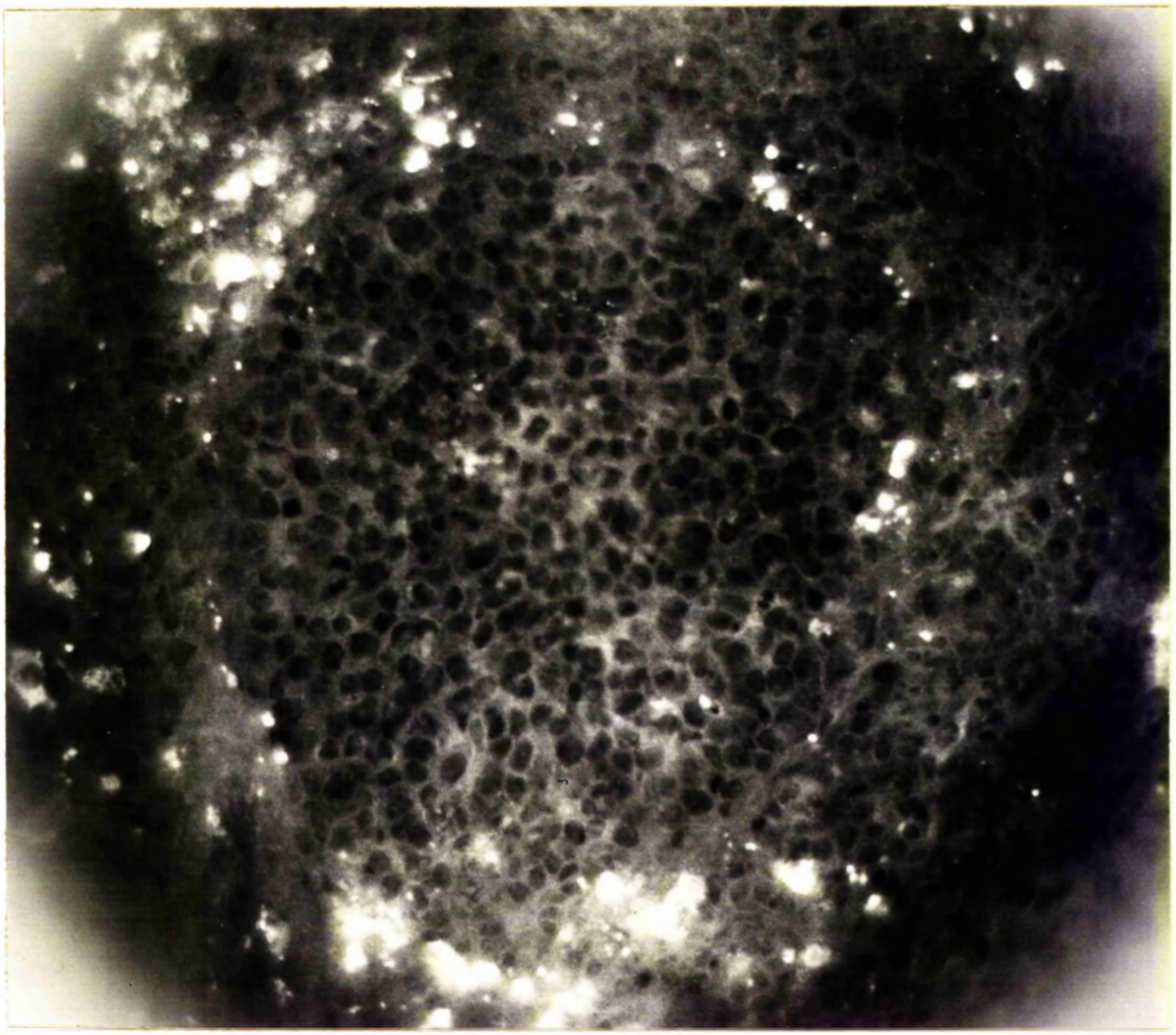


Plate 18.

Plate 19. Immunoelectrophoresis. All the troughs contain rabbit anti-chicken gamma globulin. Well 1 contains serum from a conventional chicken. Well 2 contains serum from a germ-free unstimulated chicken. Well 3 contains serum from a germ-free chicken 6 days after injection of staphylococcus (13 days old). Well 4 contains serum from a germ-free chicken 6 days after a secondary injection of staphylococcus (27 days old; primary at 7 days old). The IgG line in the serum from the germ-free bird has been arrowed. The corresponding line can be seen in the other sera.

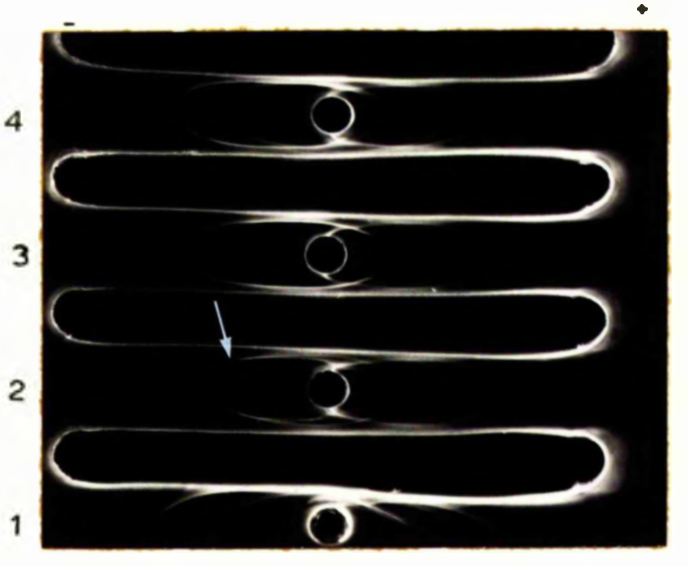


Plate 19.

a primary challenge with staphylococcus at day 7, and a secondary at day 21 following a primary at day 7 were electrophoresed along with a serum from a 4 week old germ-free bird and a conventional chicken serum. Rabbit anti-chicken gamma globulin was put in the troughs and precipitin lines formed (Plate 19). From the plate it was seen that the chicken given a primary injection of staphylococcus at 7 days had more IgG than the germ-free animal but less than the chicken given a secondary injection of staphylococcus at 21 days. The chicken given a secondary injection at 21 days seemed to have more IgG than the chicken given a primary at 7 days but less than the conventional chicken.

Summary

Following a second intravenous injection of staphylococci at 21 days of age (primary at day 7) germinal centres and a pyroninophilic cell reaction were seen in the spleen. The cellular reaction seemed to begin not round the ellipsoids as in the primary response but in the periarteriolar lymphocyte sheath. More germinal centres were formed in this area than in the primary response at 21 days of age. No antibody to staphylococcus was detected in the serum.

Discussion

In the introduction to the thesis it was suggested that the formation of germinal centres could be induced by immunogenic stimuli. The object of this work was to test that hypothesis. As explained in the introduction it was decided to reduce the immunogenic effect of the environment to as low a level as possible by rearing the chickens under germ-free conditions. The value of this method is well illustrated in experiment I (Section III) in which a total of 80 µg of Shigella

flexneri was injected intraperitoneally into 3-5 week old conventional chickens. The control birds received saline. The number of germinal centres per unit area in the spleen of the birds in each group was counted and it was found that there was no significant difference in the number of germinal centres per unit area between the groups. Indeed, on average, there were more germinal centres per unit area in the control group than in the stimulated group. From that experiment it might be concluded that antigen did not result in an increase in germinal centre numbers or even that antigen actually depressed germinal centre formation (considering the average values), but on the other hand it cannot be concluded that the antigen did not induce the formation of some new germinal centres since nothing was known of the commitment of the germinal centres and nothing was known of the number of germinal centres per unit area before stimulation.

In the same way in experiment 3 germinal centres were first seen at day 6 after intraperitoneal injection of HSA into 7 day old conventional chickens and germinal centres were seen on each subsequent day in which the spleen was examined. Now these germinal centres may have been induced by the HSA but they may equally well have been induced by an organism which had entered the circulation from the intestine. Further, in that experiment the proliferation of cells of the plasma cellular series was already underway at day 0, before the antigen was injected, and towards the end of the period of observation the reaction did not subside but continued at a higher level of activity. The difficulty, then, when working with conventional animals, lies in being able to sort out what reaction is due to the test antigen. The time and nature of the origin is complicated

by the existence of proliferative reactions that are already under way; the formation of new germinal centres is in doubt because of germinal centres which may have been induced by other antigens; and the end of the reaction is obscured by proliferative reactions which may just be beginning at that stage. The study of immune reactions in conventional chickens is complicated by the reactions to other antigens which are taking place simultaneously. The great value of the germ-free animal as a research tool in immunology lies in this - that it reduces to a minimum the lymphoid reaction to antigens other than the test antigen by eliminating as far as possible these other antigens.

In the first experiment using germ-free chickens (experiment 2) HSA was injected intraperitoneally at 7 days of age. No germinal centres were found in the spleen of chickens killed at days 2, 4, 6, 8, 10, 12 or 14 after injection of antigen. While it would be wrong to conclude that no germinal centres were induced - germinal centres may have been induced in a part of the spleen not examined - it is fair to conclude that HSA did not induce the formation of an appreciable number of germinal centres.

If the antigen (HSA) had resulted in the formation of an appreciable number of germinal centres then the research would have taken the following course. First, an attempt would have been made to find a dose-response relationship; in other words, a relationship between the number of germinal centres induced and the dose of antigen used. Secondly, the effect of a second challenge with HSA on germinal centre formation would have been investigated. Thirdly, a non-antigenic substance such as indian ink would have been injected into the

germ-free chickens in the anticipation that no germinal centres would be formed in response to this material. Fourthly, the chickens would have been rendered tolerant to the antigen (HSA) at hatching and at a later date challenged with a test dose of the antigen in the expectation that germinal centres would not be formed.

However, no germinal centres were found when the 7 day old germ-free chickens were stimulated with HSA therefore the direction of research had to change. It would have been possible to vary the dose of antigen (HSA) in the hope that a higher dose would induce germinal centre formation, and the antigen could have been administered intravenously instead of intraperitoneally. It was, however, decided to change from HSA which is a relatively simple antigen to staphylococcus, a more complex antigen. The purified protein antigen (HSA) was replaced by a particulate antigen (staphylococcus). The staphylococcus contains carbohydrates, proteins, nucleoproteins, enzymes and toxins all of which are antigenic and it was expected that this more complex immunogenic stimulus would result in the formation of germinal centres. The results show that a primary injection of staphylococcus at 7 days old or at 21 days old induced the formation of germinal centres and a secondary challenge at 21 days following a primary at 7 days induced the formation of an even greater number of germinal centres.

This immediately raises the problem of why HSA did not appear to induce the formation of germinal centres. It may have been that the immunogenic stimulus of the purified protein was simply not strong enough; or it may have been that the particular clone of cells responsive to HSA had not appeared; or that the number of

immunologically competent cells available was insufficient to bring about a complete response; or that a very small number of germinal centres was induced none of which was detected. However, it must be remembered that despite the fact that no germinal centres were found antibody to HSA was found. Now this antibody was present in very small quantities. The greatest amount of antibody produced following the injection of HSA into 7 day old germ-free chickens was found at day 14 and corresponds to 0.00625 μg of bound antigen. When this was estimated using the conventional Farr test the antigen binding capacity at 30% binding (ABC_{30}) at 1 μg HSA was less than 1 μg/ml of undiluted serum. Thus there is evidence of an immunologic response to the HSA in terms of antibody production although the amount of antibody formed was very small. . This very low level of antibody production would seem to indicate the involvement of only a small number of cells either because not many immunologically competent cells were available or because the antigenic stimulus was insufficient to stimulate many immunologically competent cells. The formation of a very small number of germinal centres would be consistent with the involvement of a small number of immunologically competent cells. However, irrespective of the explanation, the observation that the neonatal germ-free chickens did not respond to the two different antigens in the same way is valid although it is not clear whether the response was qualitatively different (i.e. no germinal centres) in HSA stimulated chickens or merely quantitatively different (fewer germinal centres) from staphylococcus stimulated chickens.

When the response of the 7 day old germ-free chicken to staphylococcus is compared to the response of the 21 day old chicken to the same antigen two points

are noticed. Firstly, although both reactions involved the same cells, the response was more intense in the older birds. Secondly, a greater number of germinal centres was induced in the older birds. It would seem from these observations that the cellular response during the neonatal period was not entirely dependent upon the immunogenicity of the injected material - since the same dose elicited a quantitatively different response at 7 and 21 days old - but partly dependent upon the ability of the chicken to respond to the stimulus. This ability to respond may depend on the number of immunologically competent cells; the older animal presumably having more such cells. Thus it seems that not only do neonatal germ-free chickens respond to different antigens in different ways depending upon their antigenicity, but they respond to a constant dose of the same antigen in a quantitatively different way depending upon the age at stimulation.

Despite the fact that serum antibody was found by the modified Farr test in the 7 day old HSA stimulated germ-free chickens no antibody was detected in the spleen using the fluorescent antibody technique. It is suggested that this was because the level of antibody present was below the sensitivity of the fluorescent antibody test. Support for this comes from the experiment in which HSA was injected into 7 day old conventional chickens (expt. 3). In these birds where the level of serum antibody was higher than in the germ-free chickens a few antibody containing cells were seen in the spleen. In the adult bird where the ABC_{30} may be as high as 50 µg/ml many antibody containing cells are seen in the spleen (White et al. 1967).

In the experiments in which germinal centres were induced by staphylococcus no antibody was detected to the antigen. When HSA was used as antigen no germinal centres were found but antibody to HSA was found. The failure to detect antibody to staphylococcus can perhaps be attributed to the limitations of the test. In the first place only agglutinating antibody was measured; other antibodies may have been present. Secondly, agglutination was looked for at a magnification of only x 5; agglutination may have been present at a higher magnification. Thirdly, the lower limit of sensitivity of the agglutination test is considered to be 0.0625 µg antibody protein (Humphrey and White, 1964); antibody may have been present below this value. On the other hand, the Farr test is considered to be a highly sensitive test (Minden et al. 1966). Thus while it is very difficult to compare these two very different tests it seems possible that antibody to staphylococcus which could not be detected by the test used, may have been present.

A valid comparison can be made between antibody production in 7 day old germ-free (expt 2) and 7 day old conventional (expt 3) chickens, since antibody was measured by the same method. The antibody response to HSA, as expressed as % antigen bound, was greater in the conventional chickens injected at day 7 than in the germ-free chickens injected at the same age (Plate 12). Westmann and Olson (1964), using 13 week old conventional and germ-free chickens, also found that germ-free chickens made a quantitatively poorer response than the conventional chickens. Now the difference between these two groups was that the conventional chickens had an intestinal flora and were kept in a contaminated environment. It may be that the immunogenic stimulus provided by the living

micro-flora primes the lymphatic tissue so that a greater response is made to an antigen. The value of such a priming would be considerable if the antigen were pathogenic.

A common reaction in all the experiments was the proliferation of cells of the plasma cellular series. This reaction was identified on tissue sections stained with methyl-green-pyronin. The basis of this technique is the competitive action of the two basic dyes, methyl-green and pyronin, one of which can bind through a single charged group while the other can combine with two charged groups. Both dyes will stain DNA and RNA, pyronin will stain more actively than methyl-green, and will drive the methyl-green out of all dye-substrate combinations where the dye is held by a single charged group. However, where methyl-green can bind to a substrate by two bonds, as it can when it links with highly polymerized and correctly orientated DNA, it is not displaced by the highly competitive pyronin. Thus pyronin stains all the nucleic acid containing structures except the DNA of the chromatin which when polymerized and correctly orientated stains with methyl-green. The red cytoplasmic staining by pyronin indicates a high content of RNA and this pyroninophilia is taken to mean that protein is being synthesized. Ehrich et al. (1949) related the increase in RNA which followed antigenic challenge with antibody production and Leduc et al. (1955) using adjacent sections of lymph node, one stained by the fluorescent antibody technique and the other stained by Giemsa, showed that antibody was contained in cells of the plasma cellular series. Thus, it seems true that all antibody synthesizing cells are stained by methyl-green-pyronin but the converse, that all cells stained by methyl-green-pyronin

are antibody synthesizing cells, is not necessarily true.

There is some evidence for the production of gamma globulin which is not antibody. Askonas and White (1956) and Askonas and Humphrey (1958) found that antibody production in the guinea-pig and in the rabbit was accompanied by the production of gamma globulin which was not antibody to the injected antigen. In the present experiments the number of gamma globulin containing cells was found to exceed the number of specific antibody containing cells (experiments 2 and 3); and the birds challenged with staphylococcus were shown by fluorescent antibody staining (Plates 11a & 17) and by Immunoelectrophoresis (Plate 19) to produce gamma globulin though no antibody was detected. Since these animals were germ-free and the germ-free unstimulated animals showed very low levels of gamma globulin by Immunoelectrophoresis (Plate 8) and by fluorescent antibody staining (experiment 4 day 0; experiment 2 day 0) the gamma globulin formed must have been induced by the injected antigen.

This gamma globulin may be agglutinating antibody below the level of the sensitivity of the agglutination test used, or it may be non-agglutinating antibody. That is, true antibody which could be detected if the correct antibody/antigen test were known, sensitive enough and applied. On the other hand, this may be gamma globulin which will not combine with any antigenic determinent in the injected mixture but is induced in some non-specific way by that mixture.

The results indicated that neither the conventional nor the germ-free environment altered the histological structure of the bursa of Fabricius or the thymus and the addition of an antigen to germ-free chickens did not alter the cellular

structure of the bursa or the thymus. This constant appearance of the bursa and the thymus is consistent with the idea of these organs as central lymphoid organs insensitive to antigens (Miller, 1968).

Three topics - the origin of antibody producing cells; the formation of germinal centres; and the secondary response, germinal centres and immunological memory - will be discussed speculatively in the light of the experimental findings.

Origin of antibody producing cells. There seems to be no doubt about the sequence haemocytoblast - immature plasma cell - mature plasma cell, though the haemocytoblast is known by different names, e.g. activated reticulum cell, transitional cell, plasmablast. However it is not yet agreed as to which cell gives rise to the haemocytoblast.

In an autoradiographic study of the rat lymph node Nossal and Makela (1962) showed that the plasma cell proliferation during the secondary response seemed to be derived from primative lymphocytes. Further studies (Nossal et al. 1963) using a combination of autoradiography and single cell micro-manipulation confirmed that the primative lymphocyte was the plasma cell precursor in the primary response. However Reike et al. (1963) could find no evidence which supported this finding. They said that the plasmablast was the most primitive cell found. Langevoort (1963) working on the rabbit spleen also described the plasmablast as the earliest cell in the primary response. Plasmablasts were first seen between the lymphocytes of the periarteriolar lymphocyte sheath from which they moved to the border of the red pulp. Here they entered the sinuses and were lost to the blood stream so that the spleen was depleted of plasma cells after the 6th day.

Germinal centre activity began on the 4th day and increased to the 10th day after which it subsided. This activity was associated with the appearance of lymphocytes in the spleen. Langeveart (1963) concluded that the plasmablasts were derived from the lymphocytes of the periarteriolar lymphocyte sheath. Combining sublethal X-irradiation with these studies Keuning et al. (1963) presented further evidence that the small lymphocyte was the cell from which the plasma cell was derived.

Most authors have, however, found that the precursor of the plasma cell is a primitive reticular cell. Fagraeus (1948) found this in the rabbit and Marshall and White (1950) in a detailed study of the rabbit spleen reached the same conclusion. They described the primitive reticular cell as a non-phagocytic cell with an oval or rod-shaped nucleus and a colourless nucleoplasm with very fine and scanty strands of chromatin which formed condensed dots at their intersection. No nucleolus was visible. The cytoplasm showed no basophilia and the cell outline was often ill-defined. Ringertz and Adamson (1950) also described the reticulum cell as the precursor cell and more recently Thiery (1960) reached the same conclusion.

In an autoradiographic study of germinal centres in mice during the early intervals of the immune response to sheep red blood cells Hanna (1964) postulated that the antigen disrupted the steady state of the germinal centres. There was an increased number of dividing cells in the germinal centres following stimulation and cells migrated into the lymphocyte mass and red pulp. This process resulted in the complete dissociation of germinal centres. Hanna (1965) suggested that the primitive lymphoid cells which migrated from the germinal centres were the precursors of the plasma cells.

Thus the nature of the precursor cell of the plasma cell remains unsettled. A lymphocyte (Nossal et al. 1963; Langevoort, 1963) a primitive reticular cell (Fagraeus, 1948; Marshall and White, 1950) and a germinal centre cell (Hanna, 1965) have all been suggested as the precursor cell.

In this present study the primary response described in experiments 2, 3, 4 and 5 all indicate that the cell called an immature lymphoid cell is the precursor cell. This cell was present in the periellipsoid area before stimulation, haemocytoblasts first appeared in the location of the immature lymphoid cells, the immature lymphoid cells fell in number after the haemocytoblasts appeared, and the immature lymphoid cells then reappeared in their periellipsoid site. Morphologically the immature lymphoid cell is very similar to the primitive reticular cell described by Marshall and White (1950). Thus, of the three suggested precursor cells the results agree most favourably with those of Fagraeus (1948) and Marshall and White (1950), though a close analogy would be dangerous since these authors did not study the chicken.

From the germ-free experiments it is clear that in the primary response the reaction begins in the red pulp round the ellipsoids and not in the peri-arteriolar lymphocyte sheath. It therefore seems unlikely that the small lymphocyte is the precursor cell. Finally, in experiment 2 a plasma cellular reaction was seen in the absence of germinal centres. This would strongly indicate that in the primary immune response the germinal centre did not produce the precursor of the plasma cell.

Theoretically these immature lymphoid cells could be "bursacytes" - lymphoid cells of bursal origin - which have come to rest in the spleen. They would then be immunologically competent and only waiting for antigen to differentiate to haemocytoblasts. The immature lymphoid cells would reach their perielliposid site by passing between the reticulo-endothelial cells lining the venous sinusoids and settling in the pulp cords adjacent to the ellipsoid. The immature lymphoid cell would then be well placed to respond to an antigen if it were to come through the ellipsoid or directly into the pulp cords.

The suggestion that the immature lymphoid cell is the "bursacyte" in the peripheral lymphoid tissue is consistent with the idea of seeding of cells from the bursa (Woods and Linna, 1965) and the failure of cells of the plasmacytic series to develop after early bursectomy (Cooper et al. 1969). This is also consistent with and extends the work in Section II in which immature lymphoid cells were seen in the unstimulated germ-free spleen in the absence of haemocytoblasts, immature plasma cells and mature plasma cells.

Germinal centre formation. Several entities seem to be involved in the formation of germinal centres in the chicken spleen. Using the fluorescent antibody technique White (1963) studied the fate of intravenously injected HSA. The antigen could not be detected at 24 or 36 hours but at 48 hours after injection antigen was detected in or on elongated spindle-shaped cells which were distributed in the white pulp near the origin with the red pulp. At 6 days antigen appeared in germinal centres as a pattern of surface fluorescence outlining the body and dendritic processes of scattered germinal centre cells. No antigen was seen

outside the germinal centres at this stage. The cells bearing the antigen were thought to be reticulo-endothelial in origin and were called dendritic cells.

In an autoradiographic study of the chicken spleen White et al. (1969) showed the same pattern of antigen movement. Forty hours after intravenous injection of ^{125}I labelled HSA the antigen could be seen associated with cells dispersed along the branches of the central arterioles of the white pulp. At 88 hours the number of antigen laden cells seemed to migrate down the arteriole to the bifurcation of two arterioles where a germinal centre was formed. At 6 days all labelled antigen-bearing cells were clearly seen to be within germinal centres.

When chicken spleen sections were stained with fluorescent rabbit anti-chicken gamma globulin the same kind of dendritic or reticular pattern was seen as when stained directly with fluorescent rabbit anti-HSA at 6 days after intravenous injection of antigen (White et al. 1967). These authors argued that there were antigen/antibody complexes on the surface of the dendritic cells in the germinal centres. This idea was supported by the fact that chickens rendered tolerant to HSA failed to localize the antigen in the germinal centres. The chickens were able to localize antigens to which they were not tolerant.

White (1969) suggested that in the presence of newly made antibody following an intravenous injection of protein antigen, antigen/antibody complexes are formed which are taken up by the dendritic macrophages at the periphery of the ellipsoids. These dendritic cells then migrate with their antigen/antibody load along the penicillary arterioles. In the course of this migration they encounter small lymphocytes which they net and hold by an in vivo agglutination reaction

to form the beginning of a germinal centre. By the sixth day this process of agglutinative growth has reached the proportion of a recognisable germinal centre.

When the chicken spleen is stained by the sandwich method with fluorescent rabbit anti-HSA it has been shown (White, 1963; White et al. 1967) that antibody containing cells are already present in the red pulp cords by the 3rd day after injection of antigen. These cells have the appearance of haemocytoblasts and immature plasma cells. The antibody containing cells reach a peak at 6-8 days but by 10-12 days very few antibody containing cells are seen in the pulp cords. However from 13 days onward antibody containing cells appeared within a proportion of the germinal centres of the white pulp and remained there for up to 42 days (White et al. 1969).

Thus antigen and antibody in the form of antigen/antibody complexes, dendritic cells, lymphocytes and cells of the plasmacytic series are all implicated in the formation of germinal centres. Further support for the association between plasmacytic cells and germinal centres comes from the work of Cooper et al. (1969) in which chickens were bursectomized as embryos. Both germinal centres and plasmacytic cells were eliminated.

This present study was directed towards the plasmacytic cells and their role in germinal centre formation. From the experiments in which chickens were given a primary injection of antigen (particulate or soluble protein) the stages involved in the formation of germinal centres may be proposed. The immature lymphoid cells (Plate 10a) which are present in the unstimulated germ-free spleen seem to differentiate to haemocytoblasts (Plate 10b) when stimulated by antigen. This

stimulation takes place round the ellipsoids but the cells rapidly move into the red pulp adjacent to the ellipsoids. Some of the haemocytoblasts remain in this site and develop through the immature plasma cell to the mature plasma cell (day 2 onwards). Other haemocytoblasts migrate from the red pulp to the periarteriolar lymphocyte sheath. The PALS[8] become hyperplastic (Plate 13a), probably in response to the antigen, and the haemocytoblasts invade the sheath (Plates 13b and 14). Next (day 4) the haemocytoblasts within the sheath aggregate to form a clearly limited clump of cells (Plate 15a). A fibrous ring forms round these cells and the haemocytoblasts seem to give rise to small lymphocytes, giving the characteristic appearance of the germinal centre (day 6) (Plate 15b).

The problem arises of blending all the entities involved in germinal centre formation. The results presented are consistent with the view that antigen/antibody complexes are formed and taken up by the peripheral macrophages of the ellipsoid. These cells would then migrate with their load of antigen/antibody complex to the periarteriolar lymphocyte sheath. As they migrate they would encounter not small lymphocytes (White, 1969) but haemocytoblasts which would be carried into the PALS[8]. The dendritic cells with their antigen/antibody complexes would act as the binding cells in the germinal centre formation in the same kind of agglutination reaction proposed by White et al. (1969). The finding of dendritic pattern staining (Plate 18) in germinal centres in experiments 5 and 6 lends support to this theory. The plasma cells which develop from the haemocytoblasts in the red pulp as well as producing circulating antibody would produce the early antibody to form the complexes on the macrophages.

It is difficult to see what part the periarterioler hyperplasia plays at this stage since no lymphocytes appear to be incorporated into the germinal centre. However lymphocytes appear to be produced within the germinal centre from the haemocytoblasts.

The significance of this aggregation theory of germinal centre formation is that it is quite different from the idea of germinal centre formation implicit in the literature for many years (Conway, 1937; Cottier et al. 1967) that germinal centres developed by rapid multiplication of a small focus of cells.

The secondary response, germinal centres and immunological memory. In 1960 White (1960) suggested that "the lymphocytopoietic (germinal) centres could provide an important part of the morphological basis of the primary response, which does not necessarily result in much production of antibody but which lays the basis for a vigorous secondary response".

This idea of the connection between germinal centres and immunological memory was taken up by Thorbecke et al. (1962) who suggested the following sequence of events. During the primary response lymphocytes of the white pulp would transform to large blast cells by exposure to antigen. These large blast cells would then lose their antigen and follow one of two pathways. Some would migrate towards the periphery of the periarterioler lymphocyte sheath where more antigen was present. They would then proliferate and differentiate into antibody producing cells. The blast cells which remained in the white pulp would also start to proliferate but because of the relative lack of antigen they would not form antibody; however they would retain the information for antibody production.

This information would be retained in the germinal centre and the cells containing this information would be long-lived lymphocytes (Thorbecke et al. 1965).

Thorbecke and her co-workers, in a series of papers (Cohen et al. 1966; Wakefield et al. 1967; Wakefield and Thorbecke, 1968a and b; Jacobsen and Thorbecke, 1968) studied the relationship between immunological memory and germinal centres by preparing cell suspensions of white pulp from primarily immunized animals and producing a secondary response which was measured in different ways. Thus a body of evidence exists which suggests that the germinal centre is involved in immunological memory. However it must be appreciated that in these experiments the germinal centre cells were only indirectly implicated because of the technical difficulty of obtaining a cell suspension containing only germinal centre small lymphocytes. In most cases the suspension used must have been a rather heteregeneous population of white pulp cells which also included germinal centre small lymphocytes.

Yet, this indirect evidence linking germinal centres with immunological memory cannot be ignored and the small lymphocyte seems to be the cell involved. Evidence to support this comes from an experiment in which X-irradiated rats gave a secondary type response to a first injection of bacteriophage after they had been given thoracic duct cells from primarily immunized donors (Gowans and Uhr, 1966). The cells responsible for initiating the secondary response were found to be small lymphocytes.

In the present experimental work the secondary response to a particulate antigen was characterized by the formation of many germinal centres: more centres than formed after a primary challenge at the same age. While this implies some

immunological memory of the antigen it does not prove that germinal centres house or manufacture memory cells.

However several observations may be made. In the secondary response it was noticed (expt. 6 day 2) that the haemocytoblasts first appeared in the PALS, not in the red pulp. This would suggest that in the secondary response the precursor cell was in the PALS and not in the periellipsoid area as in the primary response; and that the haemocytoblasts migrate from the PALS to the red pulp where they differentiate through immature plasma cells to mature plasma cells. Now an obvious candidate for the role of precursor cells in the PALS is the small lymphocyte of the germinal centre.

Further, no germinal centres were seen in the spleen at day 0 (expt. 4) after the secondary injection of staphylococcus despite the fact that germinal centres were produced following a primary injection at day 7 (expt. 4). It may have been that by chance a germinal centre was not cut but, on the other hand it may have been that all the germinal centres formed after the primary injection "dissociated" quickly after the secondary injection. Hanna (1964) described such a dissociation and earlier workers (Ehrlich, 1929b, c; Ehrlich and Harris, 1942) described this obliteration of germinal centres which occurred very soon after injection of antigen. A dissociation would result in dissemination of germinal centre cells first of all throughout the PALS[a] then into the red pulp. Now, if these germinal centre cells were memory cells which, when re-exposed to antigen, became haemocytoblasts this would account for the observation, in the present experiment (expt. 6), of haemocytoblasts first in the PALS[a] then in the red pulp.

Again, marked periarteriolar hyperplasia was seen in the secondary response. Since existent germinal centres are located in this area such a hyperplasia might well result in their dissolution. But this hyperplasia was observed in the primary response as well as in the secondary response and Ehrich and Harris (1942) observed that all the centres were obliterated, not just some of the centres. This seems to indicate a non-specific mechanism which brings about germinal centre dissociation. Now there are two theories about the fate of germinal centres, assuming that a limited number of germinal centres are induced by an antigen. Either they are permanent structures or they are temporary structures. If they are permanent it follows that if an animal is injected with a great number of different antigens then a great number of germinal centres would be produced and if this process were continued the spleen would become full, if not distended, with germinal centres. However, there is no evidence that the number of germinal centres can be dramatically increased in this way. The number of germinal centres in the stimulated birds in expt. 1, though stimulated with only one antigen, did not even exceed the number of germinal centres in the control group. It seems more likely that the germinal centre is temporary in nature. That being so, a non-specific periarteriolar hyperplasia resulting in germinal centre dissociation would clean the slate, as it were, for new germinal centre formation. At the same time it would disseminate memory cells, assuming that these cells are located in the germinal centre.

The events linking secondary response, germinal centres and immunological memory could be constructed thus - an antigenic challenge (whether primary or

secondary) causes periarterielar hyperplasia which dissociates all germinal centres. However the germinal centre cells (small lymphocytes) which have been induced by the antigen in the primary response respond to it on re-encounter by developing to haemocytoblasts. Some of these haemocytoblasts migrate to the red pulp where they differentiate to antibody producing cells. Some of the haemocytoblasts stay in the PALS[5] and form aggregates which are held together by antigen/antibody loaded dendritic cells as in de novo formation of germinal centres. So a new population of germinal centres is formed.

There is, however, only suggestive evidence of such a process. Work still has to be done on the effect of multiple antigen injections in germ-free chickens and on the suggested germinal centre clearing mechanism.

Conclusion. It is clear from the experimental work that the cellular architecture of the lymphoid tissue and in particular the spleen, is dependent upon immunogenic stimulus. However, the response to different antigens administered at the same age is not the same. In the germ-free chickens both the soluble protein antigen (HSA) and the particulate antigen (staphylococcus) induced proliferation of cells of the plasma cellular series but germinal centres were only found in the spleen following stimulation with staphylococcus. Further, the response to the same dose of the same antigen varies with the age of the chicken during the neonatal period. A greater number of germinal centres and a greater proliferation of cells of the plasma cellular series was induced by injection of staphylococcus into 21 day old germ-free chickens than into 7 day old germ-free chickens.

The 7 day old conventional chicken is able to produce a greater amount of antibody to human serum albumin (HSA) than the 7 day old germ-free chicken.

A secondary challenge with staphylococcus in germ-free chickens at 21 days old, following a primary at 7 days old, induces the formation of a greater number of germinal centres than a primary injection of staphylococcus at 21 days old.

The germinal centre appears to be formed in the spleen not by rapid multiplication of a small focus of cells but by aggregation of haemocytoblasts in the periarteriolar lymphocyte sheath. Lymphocytes do not appear to be incorporated in germinal centre formation but seem to be formed from haemocytoblasts within the germinal centre.

Antigenic stimulation of germ-free chickens does not appear to induce any cellular changes in the thymus or the bursa of Fabricius however it is quite clear that the cellular architecture of the spleen is dependent upon immunogenic stimulation.

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