THE LYMPHATIC SYSTEM

AND

THE IMMUNE RESPONSE IN THE LAMB

A THESIS

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by

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The work reported in this thesis was carried out in the Department of Experimental Pathology, John Curtin School of Medical Research, during the tenure of an Australian Wool Board Senior Post-graduate Research Scholarship. I am grateful to Professor F.C. Courtice for the opportunity to undertake this work in his department.

The electron micrographs in Chapter IV were provided by Dr. B. Morris, who also helped with some of the surgical operations. Mr. R. Hill cut and stained the histological sections. With these exceptions, the experimental work described in this thesis was done entirely by myself.

Finally, I would especially like to thank my wife, whose assistance in the preparation of this manuscript was indispensable.
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Finally, I would especially like to thank my wife, whose assistance in the preparation of this manuscript was indispensable.
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"There was a crooked man who walked a crooked mile,  
He found a crooked sixpence upon a crooked stile,  
He bought a crooked cat that caught a crooked mouse,  
And they all lived together in a little crooked house."

The capacity to discriminate and to react against "not self" material is a distinguishing feature of higher animals. This capability, while genetically determined at conception, only manifests itself after the organism has undergone considerable development and differentiation. When fully developed, the immune system of the mammal can identify and synthesize antibodies against innumerable foreign antigens, and evince a variety of cellular reactions in response to natural allergies and infections and fictitious laboratory situations such as organ and tissue transplants.

All these immune reactions depend on the reticuloendothelial and lymphoid systems and on the establishment of a responsive capability in a genealogy of lymphoid cells. Central among these cells, in the reactive sense, is the lymphocyte.

Just when and how this cell acquires immune reactivity is not known but over the last 10 years or so, there have been a vast number of papers detailing crucial experimental evidence which has lead to "an intellectually satisfying explanation" of the immune mechanism. Intellectual satisfaction is one thing, and scientific truth is another, and insofar as many aspects of immunology are concerned, the experimental results divide pretty evenly between the ayes and the noes.
Investigations into the role of the thymus in the development of the lymphoid system and immune reactivity provide an example of how a vigorously prosecuted field of research can become bog-spavined in a welter of conflicting contemporary truths and falsehoods. After more than a decade of investigation by dozens of competent scientists, the function of the thymus can fairly be said to remain unknown.

The experimental work reported in this thesis aims to examine the effect of thymectomy on the growth and development of the lymphoid system of the sheep, and to study a range of immunological reactions in thymectomized lambs at various ages after birth. An attempt has been made to study reactions to different types of antigens and to investigate a variety of cell mediated immune phenomena so that the interpretation of the results will not be too confined.

These results in the sheep have been compared with those obtained in other species to try to uncover common experimental results upon which an objective interpretation of the function of the thymus can be based.
Introduction

The Response of Newborn Animals to Antigens

It has been known since the early part of this century that foetal and newborn animals of some species are unable to produce specific antibodies in response to certain antigenic stimuli. In the domestic fowl for instance, Wywoch (1937) reported that chicken embryos and newly hatched chicks produced no antibodies in response to injections of E. coli and subsequently a wide range of antigens, such as diphtheria toxoid (Grassett, 1929), influenza virus (Burnet, 1941), yellow fever virus (Fox and Lasermart, 1947) and bacteriophage C16 and human red cells (Burnet, Stone and Sidney, 1950) were all shown to be ineffective in eliciting a humoral antibody response in these embryos. Fox and Lasermart (1947) further showed that in those chickens in which virus still persisted at the time of hatching, antibody was subsequently produced some three months later. This finding was extended by Wolfe and Drobay (1954) who found that while the response of young chickens to bovine serum was very low at birth, it increased gradually to 6 weeks of age, at which time they concluded that chickens were "sensitologically mature". However, subsequent work by Wolfe, Neuher, Nees and Tempell (1957) showed that immune mechanisms in the chicken continued to develop beyond 5 weeks of age and they found that antibody titres increased significantly as the birds grew from 6 to 96 weeks of age.

Similar results were obtained in studies on young mammals. Tschitschikine (1904) found that 6 day old rabbits produced little antibody in response to cultures of killed typhoid organisms while Moll (1908) found that 3 week old
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Similar results were obtained in studies on young mammals. Tschitschkine (1904) found that 6 day old rabbits produced little antibody in response to cultures of killed typhoid organisms while Moll (1908), found that 3 week old
rabbits had a deficient response to cholera vaccine and horse serum. Subsequent studies by Friedberger and Simmel (1913) and Valtis and Saenz (1928) on guinea pigs, Coca, Russell and Baughman (1921) on the rat, Grasset (1929) and Freund (1930) on the rabbit, seemed to establish clearly that the immunological reactivity of young mammals was inferior to that of adults.

The discovery of the inheritance of iso-agglutinins in man by von Dungern and Hirzfeld (1910), taken together with previous findings on the lack of reactivity of foetal and newborn animals to antigens, led Hirzfeld (1928) to postulate a concept of "constitutional serology" which was founded on the proposition that antibodies were biochemical entities which matured only at a specific stage in development and resulted in a state of "serological maturity". From this experimental evidence, together with the long recognized fact that young children were subject to many diseases which rarely afflicted adults (cf McKhann and Kapnick, 1938), it became a point of immunological dogma that young animals were "quantitatively and qualitatively inferior" in their ability to respond to antigenic stimuli (Baumgartner, 1934).

It is not within the scope of this thesis to detail all the different attempts to elicit specific antibody responses in young animals. With the development of sensitive techniques for measuring antibody production, it has become clear that the apparent quantitative and qualitative differences that were previously thought to distinguish antibody synthesis in young animals from that in adults may not be as great as was previously thought. The immune response elicited in any animal is subject to many variables and it is worth recording that for any comparisons based on the efficiency of antibody synthesis to be valid, these variables must be standardized.
The most important factors affecting the response of a young animal to a particular antigen are:

1. The sensitivity of the test system employed to measure antibody production,
2. The nature and dose of the antigen,
3. The influence of passively acquired maternal antibody,
4. The age at which the test is performed and the species used.

(1) **The Sensitivity of the Test System**

The sensitivity of various antibody assay systems has been reviewed by Grabar (1957). Precipitation reactions are least sensitive requiring 3-20 µg of antibody nitrogen (AbN) per ml of serum. Bacterial agglutination detects 0.5 to 1.0 µg AbN/ml, haemolytic reactions detect about 0.01 µg, while passive haemagglutination with protein or bacterial antigens detects 0.005 µg AbN/ml. These figures relate only to antibodies of high avidity which are found in hyper-immune sera and do not apply to those antibodies which are formed early in an immune response and which have a much lower avidity. Bactericidal tests are very sensitive, detecting $10^{-5}$ µg AbN/ml (Sterzl, Kostka and Lanc, 1962). Virus and phage neutralization tests are also very sensitive. Silverstein, Uhr, Kraner and Lukes (1963b) calculated that as little as 0.001 µg of antibody nitrogen could be detected in the phage neutralization assay for antibody to φX-174. Many of the early reports of failure to detect antibody production in newborn animals employed relatively insensitive assay systems, in particular toxin-antitoxin precipitation and bacterial agglutination tests. These observations are therefore of little value in discounting antibody production in young
animals, particularly in relation to antigens such as diphtheria toxoid and Salmonella organisms.

(2) The Nature and Dose of Antigen

With the use of antigens of widely different immunological characteristics, it has become clear that relatively large amounts of some antigens are required to elicit an antibody response, while with others, minute quantities are sufficient to produce a high serum titre i.e. these latter antigens are more "immunogenic" than others. Uhr, Finkelstein and Franklin (1962a) and Uhr, Dancis, Franklin, Finkelstein and Lewis (1962b) have shown that bacteriophage x-174 is one of the most potent antigens known, although an assessment such as this must be, to some extent, a reflection of the sensitivity of the antibody assay system employed with this antigen. Sterzl, Mandel, Miler and Riha (1965) found that T2 phage produces an earlier and greater antibody response than sheep red cells which in turn is better than Salmonella organisms as an antigen in newborn piglets. Smith (1960) has shown that the Salmonella 'H' antigen is, in terms of the time of detection of antibody and the peak titre, more antigenic than the 'O' antigen.

Increasing the dose rate of these particulate antigens has also been found to accelerate and enhance the antibody response. Sterzl and Trnka (1957) found that, by increasing the dose of Salmonella paratyphi B given to newborn rabbits, antibody production occurred several days earlier. Similar results have been obtained in other animals by Riha (1961) and Bellanti, Eitzman, Robbins and Smith (1963), and in children by Edsall (1955). However, Halliday (1964) could not show any earlier production of antibodies to Brucella abortus in young rats by using increasing doses of this antigen.
With soluble antigens such as serum proteins, a very different situation results. Smith (1961) found that young rabbits produce antibody in response to injections of 10 $\mu$g of bovine serum albumin per day while with doses of 200 $\mu$g no antibody production occurred. Similar results have been obtained in the guinea pig by Uhr (1960) and Humphrey and Turk (1961). They could elicit no response to a single injection of 10 mg of BSA, while a dose of 1.0 mg resulted in antibody production.

Thus the synthesis of antibody is clearly a function of the nature of the particular antigen used and the dose rate at which it is administered. It can be said that, in general, increasing the dose of a particulate antigen accelerates the immune response and leads to higher titres of antibody, while increased doses of soluble antigens result in immunological negativity (or tolerance).

(3) The Influence of Passively Acquired Maternal Antibody

In the early days of practical immunology, much effort was directed toward the development of methods of immunization against infective agents or toxic substances. As early as 1892 Von Behring and Wernicke demonstrated that antibody production against diphtheria toxin could be elicited after the injection of mixtures of toxin and antitoxin without adverse effects upon the recipient. These toxin-antitoxin mixtures subsequently became generally used for prophylactic immunization procedures. However, Smith (1909) warned that excessive antibody concentration in the mixture could reduce or completely extinguish the subsequent antibody response. It was not until much later, after the discovery of the placental and colostral transfer of maternal antibodies to the newborn (Smith and Little, 1922; Kuttner and Ratner, 1923; Ratner, Jackson and Gruehl, 1927; Mason, Dalling and
Gordon, 1930) that the inhibitory effect of antibodies in the immune response was recorded and the subsequent antigenic reactions of young animals with passively acquired antibodies was investigated.

Greengard and Bernstein (1935) found that Schick negative children reacted poorly to immunization against diphtheria toxoid, while Schick positive children reacted well. A similar finding was made in sheep by Buddle (1941), who observed that lambs born of mothers immunized prenatally against Clostridium welchii type D reacted less well at 3 months of age than did lambs born of non-immunized mothers. Subsequently there have been numerous reports of the depressive effects of colostral or placentally derived maternal antibodies on subsequent active immunization procedures. In humans these reports have mainly come from studies on the response of infants to diphtheria toxoid (Vahlquist, 1949; Barr, Glenny and Randall, 1950; Osborn, Dancis and Julia, 1952; and Butler, Barr and Glenny, 1954), polio vaccination (Perkins, Yetts and Gaisford, 1958, 1959) and Salmonella antigens (Fink, Miller, Dorward and Lo-Spalluto, 1962; and Smith and Eitzman, 1963). In domestic animals piglets have been the most commonly used subjects (Hoerlein, 1957; Riha, 1961; Aicken and Blore, 1964). These findings have been confirmed in experimental situations in which various antigens have been injected into animals previously given specific antisera (Nossal, 1957; Uhr and Baumann, 1961; and Rowley and Fitch, 1964).

There have, however, been some conflicting reports which claim that the antibody response to bovine serum albumin (BSA) is increased if the antigen is administered as an antigen-antibody complex in the presence of excess antigen (Terres and Stoner, 1962). Other observations by
Segre and Kaeberle (1962a and b), Myers and Segre (1963) and Segre and Myers (1964) have claimed that the immunological unresponsiveness of colostrum deprived piglets could be overcome by administering antigen together with small amounts of hyper-immune serum, or even serum from older colostrum deprived pigs, suggesting that, in line with Jerne's Natural Selection Theory (Jerne, 1955), preformed antibody is required to elicit a good immune response. On the other hand considerable work has been done by Sterzl and associates (Sterzl, Kostka, Mandel, Riha and Holub, 1960a; Sterzl, Kostka, Riha and Mandel, 1960b; Sterzl et al, 1962; Sterzl et al, 1965;) and by Kim, Bradley and Watson, (1966a and b), who have shown convincingly that, in their hands at least, preformed antibody is not an essential prerequisite for an immune response in newborn colostrum deprived piglets. The weight of evidence available to date would suggest that passively acquired antibody does depress rather than enhance subsequent antibody production, although the mechanism by which this is effected is, as yet, unknown (Rowley and Fitch, 1964; Uhr and Moller, 1968).

Passively acquired antibody, although in some way disadvantageous, can be of great value to the newborn in protecting them from the sudden onslaught of foreign antigens and pathogens with which they are faced at birth. Longsworth, Curtis and Pembroke (1945) noticed that human foetal blood had high levels of \( \gamma \) globulin but lower levels of \( \beta \) globulins than that of adults. This was subsequently confirmed by Franklin and Kunkel (1958) and Gitlin, Rosen and Michael (1963). These latter workers investigated the levels of immunoglobulins in the sera of newborn children to determine why they were more subject to infections with Gram negative organisms than with other bacterial species. They found that
although these infants had good levels of $\gamma_2$ (IgG) globulins, the levels of $\gamma_1$ (IgG) and $\beta_2M$ (IgM) were low and there was a lack of antibodies to S. typhosa and E. coli. This was in spite of the fact that maternal sera had relatively high levels of $\beta_2M$, and it was with this globulin fraction that the antibodies to S. typhosa and E. coli were found. Where a newborn child was shown to have antibody to these organisms in its serum, this antibody was of the 7S type and was present in quantities equal to that in the maternal serum. Thus the general neo-natal susceptibility to Gram negative bacterial infections was a direct result of a failure of the maternal antibodies to these organisms, which are in the $\beta_2M$ fraction, to cross the placenta to any significant degree. A similar pre-natal differential transfer of immunoglobulins has been shown to operate in rabbits (Hemmings, 1956) and in the rhesus monkey (Bangham, Hobbs and Terry, 1958; and Bangham, 1961).

In the rat in which most transfer of maternal antibody occurs after birth, a differential transfer also occurs but in this case there is a selective transport across the gut wall (Halliday, 1955). In the calf and pig (and probably the horse and lamb as well) where no pre-natal transfer occurs, the uptake of proteins from the gut is entirely non-selective for a short period after birth (Smith and Little, 1924; McCarthy and McDougall, 1953; Bangham, Ingram, Roy, Shillam and Terry, 1958; Payne and Marsh, 1962). In this situation however, there is a selective transfer between maternal serum and milk across the mammary epithelium (Pierce and Feinstein, 1965; Rejnek, Kostka and Travnicek, 1966).

(4) The Age at which the Test is Performed and the Species Used

For the reasons outlined above, many of the earlier
investigations on the state of immunological competence of young animals have been difficult to interpret. In recent years, however, various experimental models have been developed which have enabled greater insight to be gained into the development of immunological reactivity of young animals. The use of foetal and germ-free, colostrum deprived animals has eliminated the confounding effect of passively acquired maternal antibody on active immune responses to antigens and removed the effect of compromising exogenous non-specific stimuli.

One of the earliest attempts to study antibody production in the foetus was that of Kriedl and Mandl (1904) who found that the foetal kid could produce haemolysins against sheep red cells. In 1957, Fennestad and Borg-Petersen experimentally inoculated viable Leptospira into the uterus of pregnant cattle and found that the calves had high levels of anti-Leptospira antibody in their serum at birth before they had sucked. This experiment was later extended and it was found that the earliest foetal age at which antibody could be detected to Leptospira saxkoebing was 132 days after conception and that the production of this antibody was accompanied by the presence of plasma cells in the spleens and kidneys of the infected calves (Fennestad and Borg-Petersen, 1962). Immunization of foetal guinea pigs was carried out by Uhr (1960) who injected the foetuses with several antigens 1 to 2 weeks prior to birth and found that delayed type hypersensitivity could be produced in these animals at birth and that antibody was detectable some 6 days later.

The response of foetal guinea pigs to tuberculin was investigated by Weiss (1958) who found that foetuses could be sensitised to tuberculin with live BCG organisms given as
early as 42 days prior to birth. This finding was confirmed by Rees and Garbutt (1961) using live tubercle bacilli. However, since these foetuses were tested after birth and the sensitising organisms were alive when given, it is difficult to determine at what age they became sensitised. Hieger and Silverstein (1965) injected foetal guinea pigs with ferritin incorporated in complete Freund's adjuvant and although this resulted in an enlargement of the local draining lymph nodes, no antibody or gamma globulin could be detected in the circulating plasma. Since ferritin is known to be highly immunogenic in foetal lambs while BCG is not (Silverstein et al, 1963b), it would appear probable that the live BCG and tubercle bacilli used by Weiss (1958) and by Rees and Garbutt (1961), persisted in the foetus long enough for them to develop the competence to respond to these antigens.

The development of immunological competence in the foetal lamb has been investigated quite extensively in recent years and the results of these investigations have yielded some valuable information. Silverstein et al (1963b) challenged foetal lambs in utero at various stages of development with a variety of antigens. They found among other things, that lambs could produce antibody to bacteriophage $\phi$X-174 and ferritin as early as 65 days in utero, but not to ovalbumin until they were 120 days of gestation. At no stage of their in utero life were lambs able to produce antibodies against Salmonella typhosa or BCG organisms. These experiments indicated that immunological competence did not develop as a quantal phenomenon but occurred rather in a stepwise fashion, competence to $\phi$X-174 appearing first, then ferritin, then ovalbumin (Silverstein et al, 1963b). The ability to respond to BCG or Salmonella typhosa organisms does not
apparently appear in lambs until some time after birth.

The kinetics of the response of the foetal lamb to \(\phi X-174\) was further investigated by Silverstein, Parshall and Uhr (1966) who found that the rate of clearance from the blood and the subsequent production of 19S and 7S antibody by 100 day foetuses, was identical to that seen in adult guinea pigs exposed to this antigen for the first time (Uhr, 1964). This suggested that once an animal is able to respond to \(\phi X\) antigens the response is at once adult in character.

Similar investigations on foetal monkeys have been carried out by Cotes, Hobbs and Bangham (1966) using bovine serum albumin as an antigen. They found that foetal monkeys older than 70 days gestation could produce antibody after a dose of 1mg of antigen while doses of 100 mg (0.7 to 3.0 mg/g body weight) produced immunological tolerance. After birth a similar dose, 1.0mg/g (a total of 350mg) was required to produce tolerance, while 100mg resulted in antibody production. These figures indicate that the same dose per gram of body weight produced the same result in foetal and post-natal monkeys, thus confirming the observation of Silverstein et al (1966), that once an animal is able to respond to an antigen, the response is fully manifest and adult in character.

Information on the development of competence in the human is limited, in the main, to pathological cases. Silverstein and Lukes (1962) examined the response of human foetuses to congenital syphilis and found that infected foetuses over 6 months of age had an increased development of their lymphoid tissue and an increased production of plasma cells. In foetuses under 6 months of age, although some were positive for Treponema organisms, there was no sign of any inflammatory reaction or plasma cells. It was concluded from these findings that the human foetus attains the ability to
to respond to Treponema at around 6 months gestation (Silverstein, 1962). The response of human foetuses to another congenital disease, toxoplasmosis, was examined by Eichenwald and Schinefeld (1963). They found that the cord blood of infected foetuses contained both 7S and 19S antibodies and the 19S titre was higher than in the circulating blood of the mother. It is known that these macroglobulin antibodies are not passed from mother to foetus across the placenta in any significant amount, so these antibodies must have been produced by the foetus itself. Investigations by Uhr et al, (1962b) have shown that premature infants respond to \( \Phi X-174 \) antigens in a way that is indistinguishable from that of older, normal, full term children, again lending further support to the evidence of Silverstein et al (1966) and Cotes et al(1966) that there is no further improvement of the immune response once it has become manifest.

The newborn and young opossum have also been used as subjects for investigations into the development of the immune response, since at birth, the young of this species resembles an embryo in most respects. Kalmutz (1962) found that newborn opossums could respond fully to \( \Phi X-174 \). La Via, Rowlands and Block (1963) also found antibodies could be detected in newborn opossums 8 days after administration of S. typhi organisms. It would appear from this evidence that newborn and even foetal animals are capable of adult type immune reactions to at least some antigens.

The use of foetal animals as a model system has great advantages over the use of post-natal animals, especially if in those species used, there is no in utero transmission of maternal globulins. Since these foetal animals are normally agammaglobulinaemic and exist in a sterile environment, any
changes in the lymphoid system or appearance of immunoglobulins must be entirely the result of and specific to the antigen administered.

Qualitative Differences Between the Response of Young and Adult Animals to Antigens

It has become clear in recent years that the primary antibody response of young animals to many antigens is characterised first by the appearance of 19S antibodies and later by the appearance of 7S antibodies. This sequence of events has been reported in immune responses in young children (Smith, 1960; Uhr et al., 1962b; Gitlin et al., 1963; Eichenwald and Schinefeld, 1963; and Smith and Eitzman, 1963) and also in young animals (Bauer and Stavitsky, 1961; Bellanti et al., 1963; Silverstein, Thorbecke, Kraner and Lukes, 1963a; and Silverstein et al., 1966). These observations have led to the general acceptance of the hypothesis that the immune response in young animals is qualitatively different to that of adults. There is, however, evidence that the sequential production of 19S and 7S antibodies occurs in the primary response in adult animals to some antigens. Uhr (1964) has shown that, in adult animals injected with \( \Phi \times 174 \), 19S antibodies are produced first, followed by 7S. This observation had been reported previously by Kabat (1939) and Stelos and Taliaferro (1959).

As described earlier, there is a differential or selective transfer to the foetus or newborn of antibody of maternal origin via the placenta, yolk sac or colostrum. This results in small molecules of the fast \( \gamma \) globulin fraction passing across the placenta or being absorbed through the gut wall more readily than the larger slow \( \gamma \) or macroglobulin antibodies.
It has been conclusively shown by Smith (1960), Fink et al (1962) and Dray (1962), that passively acquired maternal antibody has a suppressive effect on subsequent autogenous production of the same antibody by newborn animals. This evidence, together with the fact that the primary immune response of foetal or premature animals is identical to that of older animals, (Sterzl et al, 1960b; Riha, 1961; Uhr et al, 1962; Silverstein et al, 1966; Cotes et al, 1966;) would indicate that the differences that have been reported by other workers are not due to any degree of incompetence, but could be entirely due to the suppressive effects of maternal antibody.

As outlined previously these problems can only be successfully overcome by using germ-free animals known to be completely free of passively derived antibody.

The Development of Delayed Hypersensitivity Reactions and Homograft Immunity

(1) Delayed Type Hypersensitivity

As was the case with the antibody response to antigens, it had been thought for a long time that hypersensitivity reactions could not be evoked in newborn animals. This is due, in part at least, to the repeated demonstrations that the skin of newborn animals is, for some reason, an unsatisfactory site in which to elicit delayed inflammatory reactions (Coca et al, 1921; Freund, 1929; Waksman and Matoltsy, 1958; Sterzl and Hrubesova, 1959; Salvin, Gregg and Smith, 1962). However, although cutaneous reactions were difficult or impossible to elicit in the skin of newborn animals, it was found that a state of hypersensitivity could,
in fact, be established. Freund (1929) found that, although the guinea pigs he infected with tubercle bacilli at birth would not respond to an intra-cutaneous test with Old Tuberculin, they were sensitive to the toxic effects of intravenously administered Old Tuberculin. This was confirmed by Sterzl and Hrubesova (1959) and Salvin et al (1962) who found that although antibody could be detected, no cutaneous reactions could be elicited. These workers also found that if leucocytes from these non-reacting neonates were transferred to adults, then a positive reaction could be elicited in the recipients.

The reverse situation, in which cells were transferred from sensitised adults to neonates, also failed to produce hypersensitivity (Waksman and Matoltsy, 1958; Warwick, Good and Smith, 1960; Warwick, Archer and Good, 1960). These experiments seemed to be fairly conclusive proof that there is some factor lacking in the neonates which will support a positive skin test. However, Weiss (1958) found that if live BCG organisms were administered to foetal guinea pigs even as early as 41 days prior to birth, then a cutaneous reaction could be elicited in the early days of their life. This was subsequently confirmed by Uhr (1960) who administered protein antigens in Freund's adjuvant to foetal guinea pigs 1 to 2 weeks before birth and found that positive cutaneous tests could be elicited at birth and that antibody could not be detected until some 6 to 11 days later. It has also been shown that human infants infected in utero with tuberculosis give positive Mantoux tests 3 weeks after birth (Kendig and Rodgers, 1958). It thus appears that, if sufficient time is allowed to elapse between infection or sensitisation, newborn animals will give positive delayed-type hypersensitivity responses.
(2) Homograft Immunity

As early as 1913, some of the most important work on transplantation immunity was done by Murphy (1913, 1914a and b, 1916). He found that rat tumour cells grafted onto chicken embryos survived until the eighteenth day of incubation when they rapidly began degenerating. Any grafts implanted into 2 day old chickens immediately underwent rejection and the rejection process in the embryo could be accelerated by the simultaneous grafting of adult spleen or bone marrow, but this procedure generally resulted in splenomegaly and damage to the embryo. Murphy's findings that chicken embryos tolerated xenogeneic grafts, recently confirmed by Lafferty and Jones (1969), led to a wave of experimental transplants. Some of these grafts were not rejected for some weeks, probably as a result of a tolerant state or a chimera being produced in the recipient. Danforth and Foster (1929) performed a large number of skin transplants on newborn chickens and found that a number of these had a prolonged survival, although the majority were rejected in a normal fashion. It was shown subsequently that the capacity of the chick embryo to reject homografts manifests itself around the eighteenth day of incubation (Trnka and Riha, 1959, and Solomon, 1963).

One of the earliest homograft studies in mammals was that of Schinkel and Ferguson (1953). They grafted foetal lambs in utero between 80 and 117 days after conception and found that these foetuses were capable of rejecting skin homografts in an adult fashion even at this early stage. Second set grafts applied at birth were rejected in a more rapid fashion, indicating that these lambs had an acquired immunity as the result of the previous in utero graft. These results have been confirmed by Silverstein, Prendergast and Kraner (1964).
who defined the time at which the foetal lamb acquires the ability to reject a skin homograft as being at about 80 days post-conception. Before this time grafts are maintained, but after this, the rejection process is as fully developed as in adult sheep.

Rawles (1955) and Medawar and Woodruff (1958) found that some newborn rats accepted skin grafts for a much longer time than adults, indicating some lack of reactivity against homografts in this species at birth. A similar situation exists in the mouse (Billingham and Brent, 1959; Howard and Michie, 1962).

In the rabbit, it has been shown by Egdahl (1958) that skin grafts are rejected actively at birth and this has been confirmed by Najarian and Dixon (1962). Ivanyi and Ivanyi (1961) have found that, although neonatal rabbits do reject homografts, the process is less rapid than that of an adult. Newborn and foetal piglets have been found capable of actively rejecting skin homografts (Sterzl et al., 1960a; Sterzl, 1963) as have premature and newborn humans (Fowler, Schubert and West, 1960). Silverstein and Kraner (1965) have found that the foetal rhesus monkey rejects skin homografts as early as 58 days post-conception.

Cell transfer experiments similar to those of Murphy (1913, 1914 a and b, and 1916) have been responsible for a considerable insight into the ability of young animals to react against grafted foreign tissues. Most of these experiments were originally designed to demonstrate that the transferred cells formed antibody. Dixon and Weigle (1957) found that lymph node cells from adult rabbits did not produce antibody when transferred with antigen to neonatal recipients. They proposed that the immunological inadequacy of young rabbits was due to an internal environment which was inimical
to the metabolic processes necessary for antibody production. However, Sterzl (1958), who repeated these experiments with similar results, interpreted these findings as evidence of a homograft reaction by the neonatal recipient against the transferred adult cells.

Spärck (1959), using neonatal rabbits and Dixon, Weigle and Deichmiller (1959), using adult X-irradiated rabbits, repeated the experiments of Sterzl (1958) with similar results. Holub (1958), Harris and Harris (1960) and Harris, Harris and Farber (1962), have all done experiments which show that the newborn rabbit is capable of mounting an immune response against adult cells, while Porter (1960) has found that homograft reactivity is present as early as the twenty-sixth day of gestation. Experiments have also been carried out in chickens (Trnka and Riha, 1959; Papermaster, Bradley, Watson and Good, 1959, 1962; and Solomon, 1963) with results which indicate that chickens similarly possess the ability to evince transplantation immunity at hatching, thus confirming Murphy's original work.

To summarise, the onset of transplantation immunity in the various species appears to occur at the following times:— in the human before birth, in the monkey at 60 days or less in utero, in the lamb at 80 days in utero, in the chicken at 18 days incubation, in the rabbit before birth, and in the rat and mouse at birth.

It thus appears that the ability to recognize foreign tissues as manifest by the homograft reaction is one of the earliest immunological activities to develop.

The Growth and Development of the Lymphoid System

The ability to mount immune reactions appears at different times after conception or birth in different
species. Since it is in the lymphoid system that these immune reactions occur, it is important to know to what stage the lymphoid system has developed at the time of the first appearance of these reactions in the various species.

In all animals which have been studied, the thymus is the first organ clearly recognizable as a lymphoid structure. In birds an additional organ, the Bursa of Fabricius, also becomes lymphoid in character at an earlier stage than the spleen or the lymph nodes. Both the thymus and the Bursa have ectodermal as well as endodermal contributions to their structure. In the very early stages of development these organs contain purely epithelial elements. It is only later that lymphoid cells begin to make their appearance.

There has been controversy between embryologists since the turn of the century as to the manner in which the embryonic epithelial thymus or Bursa comes to assume a lymphoid structure. Hammar (1905 and 1921), Maximow (1909) and others (see review by Good and Papermaster, 1964) concluded that cells of mesenchymal origin wandered into the epithelial thymus, found conditions there stimulating and proceeded to multiply. Other investigators (Maurer, 1885; Stohr, 1906 and others) claimed that the lymphoid elements are directly derived from the thymic epithelium. Auerbach (1960, 1961) found that embryonic mouse thymus developed lymphoid characteristics under the influence of a mesenchymal factor which could cross a millipore barrier. When embryonic thymuses were cultured with chicken mesenchyme or in chicken embryos, the lymphoid elements which developed were of mouse type and he concluded from this result that they had developed from the original epithelial components. Similar conclusions were reached by Ackerman and Knouff (1959, 1964)
and Ackerman (1962) in respect to the chicken bursa. However, recent studies by Moore and Owen (1967a) have shown that stem cells which initially originate in the yolk sac are capable of migrating to the thymus, and that these stem cells can subsequently be found in blood, spleen and bone marrow (Moore and Owen, 1967b). Owen and Ritter (1969) have extended these observations by using Auerbach's technique of culturing thymic rudiments in diffusion chambers in chicken embryos and they have shown convincingly that 6 day chicken thymus does not become populated with lymphoid cells in these circumstances, whereas 7 day rudiments do so to a slight extent. If the thymic rudiments are explanted directly onto the CAM, lymphoid development proceeds rapidly indicating thereby that an extrinsic source supplies the lymphoid precursors to the thymus. Using mouse thymus they found a gradual increase in the lymphoid development of 11 and 12 day rudiments, while those from 10 day embryos remained undifferentiated. It seems reasonable to assume from these results that the 12 day rudiments employed by Auerbach were already colonized by immigrant cells of yolk sac origin.

The work of Moore and Owen (1967a and b) and Owen and Ritter (1969) suggests that the same cell immigrants also colonize the secondary lymphoid organs and circulate freely in the bloodstream. There is, however, abundant evidence that these peripheral lymphoid organs do not display a lymphoid structure until some time after the thymus does. This has been observed in the human (Holyoke, 1936; Gilmour, 1941), the rat (Reinhardt, 1946), the opossum (Kalmutz, 1962), the rabbit (Archer, Sutherland and Good, 1963), the mouse (Auerbach, 1963), the dog (Kelly, 1963), the lamb (Silverstein et al, 1963b) and the pig (Pestana, Shorter and Hallenbeck, 1965). It seems unlikely that these precursor cells from
the yolk sac would travel specifically to the thymus anlage, bypassing other future sites of lymphocyte production. The reason for their apparent first appearance in the thymus is possibly that conditions there are more suitable for proliferation, or they may be subjected to an intense proliferative stimulus. If this were so, their numbers would increase more rapidly in the thymus than elsewhere, resulting in a more discernable concentration of lymphoid cells, thus giving the impression that the thymus is colonized in preference to other lymphoid organs.

It has been established that the rate of proliferation of cells within the thymus is much higher than in any other tissue (Andreasen and Christensen, 1949; Metcalf, 1964a). Provided a continuous source of stem cells is available, and these probably come from the bone marrow in adults (Dukor, Miller, House and Allman, 1965) or from the spleen, blood, yolk sac, as well as bone marrow in embryos (Moore and Owen, 1967b), lymphopoiesis proceeds at a normal rate in thymus grafts implanted into normal, thymectomized or splenectomized hosts (Pepper, 1961a; Metcalf, 1964a) and in adult or neonatal hosts (Metcalf, Sparrow, Nakamura and Ishidate, 1961). Even when up to 24 thymic grafts were implanted in the same animal (Metcalf, 1963a) lymphopoiesis proceeded at a normal rate in each of the grafts.

The rate of lymphopoiesis is highest in the thymus of foetal animals and gradually falls off with increasing age after birth (Metcalf, 1964a). However, Hess, Stoner and Cottier (1967) found a maximum rate of lymphopoiesis 3 to 4 days after birth and this finding, correlated with the reduced growth rate after birth, could mean an increased migration of cells out of the thymus (Weissman, 1967).
Observations on the development of the thymus have shown that there is a rapid growth phase in the first few weeks after birth, then a period of reduced growth, followed by a period of gradual involution (Reinhardt, 1946; Santisteban, 1960; Pepper, 1961b; Metcalf, 1964a; Adner, Sherman and Damashek, 1965). It has also been shown that the growth of the thymus, like the lymphopoietic activity going on within the organ, is controlled by some intrinsic mechanism (Metcalf et al, 1961). Extensive observations on the growth of the thymus in mice were made by Ball (1963) and Hess et al, (1967). They found that the growth rate of the thymus before birth was significantly more rapid than after birth. The relative growth rate of the thymus after birth in conventional and germ-free mice was studied by Wilson, Bealmear and Sobonya (1965) who found that although the shape of the growth curves were identical, the thymuses of the conventionally reared mice grew to a larger size than those of their germ-free counterparts.

In contrast to the thymus, the rate of lymphopoiesis in normal unstimulated lymph nodes has been found to be quite low, although many cells are constantly leaving the node (Hall and Morris, 1965). Similar comparatively low levels of lymphopoietic activity in peripheral lymphoid tissues have been found by Andreasen and Christensen (1949) and Metcalf (1964a).

Spleen grafts have been found to behave differently from thymic grafts. They grow better in splenectomized than in normal recipients and multiple spleen grafts do not grow as well as single grafts (Metcalf 1963b, 1964b).

All investigations into the development of the lymphoid system after birth have shown that the peripheral lymphoid organs (spleen and lymph nodes) reach a maximum size
some time after the thymus (Reinhardt, 1946; Santisteban, 1960; Pepper, 1961b; Metcalf, 1964a and Good, Finstad, Peterson, Kellum and Sutherland, 1965), and then remain fairly constant in size for the remainder of the animal's life. They do not display the early involutary phase which characterizes the growth and development of the thymus. In germ-free animals it has been shown that the secondary lymphoid organs remain under-developed (Thorbecke, 1959; Miyakawa, 1959; Gordon, 1959), lack plasma cells (Thorbecke, Gordon, Wostmann, Wagner and Reyniers, 1957) and exhibit a lower lymphopoietic activity than in conventional animals (Olson and Wostmann, 1966a). If however, these animals are stimulated antigenically, lymphopoiesis increases, plasma cells appear and the organs increase in weight (Olson and Wostmann, 1966b; Caster, Garner and Luckey, 1966). It thus appears that, while the peripheral lymphoid organs do develop to a certain extent after birth in the absence of antigenic stimulation, their most rapid growth occurs as a result of contact with antigens.

It has been established that there is a large pool of lymphocytes which are constantly leaving the blood stream and travelling through lymphoid tissues into lymph and thus back to the blood again (Gowans and Knight, 1964). Cells have also been shown to be continually entering the thymus and the Bursa from the general circulation (see review by Miller and Osoba, 1967). These cells originate mainly from the bone marrow in adults (Gengozian, Urso, Congdon, Conger and Makinodan, 1957) and the spleen, as well as bone marrow in embryos (Ford, Hamerton, Barnes and Loutit, 1956; Moore and Owen, 1967b). There is also evidence that cells leave the thymus and travel to the peripheral lymphoid organs.

The experiments of Weissman (1967) showed that the number of cells leaving the thymus in neonatal mice was twenty times greater than in the adult. He also showed that these
cells 'homed' to the splenic white pulp and the diffuse cortex of lymph nodes, but not to the germinal centres or to the medulla. Linna and Stillström (1966) demonstrated that in the guinea pig the cells passed from the thymus to the spleen and lymph nodes. Linna (1967) observed that labelled cells from the thymus also reached the tonsils in rabbits.

The Immunologic Reactivity of the Thymus

Even though the thymus is colonized by cells that subsequently can be found in peripheral lymphoid organs and despite the fact that cells of peripheral origin are constantly entering and leaving the thymus, it seems, paradoxically, that the thymus takes no overt part in immunological reactions, and its cells are not nearly as immunologically reactive as their counterparts of the peripheral lymphoid system.

It has been shown that antibody is not normally found within the thymus following systemic immunization (Thorbecke and Keuning, 1953; Askonas and Humphrey, 1958). Plasma cells or germinal centres are rarely found within the thymus (Askonas and White, 1956) unless antigens are injected directly into the thymic parenchyma (Sherman, Adner and Damashek, 1965) or damage has been done to the intra-thymic blood vessels (Marshall and White, 1961). Thus the thymus, as a whole organ, is unresponsive to antigens and it has also been shown that thymus lymphocytes are not nearly as capable of taking part in immune responses as their counterparts in other lymphoid organs or in the circulation. Holub (1959) has shown that thymus lymphocytes are incapable of producing a primary response to Salmonella or Brucella organisms in neonatal recipients. However, thymic cells have been shown to produce a secondary response to BSA in X-irradiated recipient rabbits (Stoner and Hale, 1955; Dixon, Weigle and Roberts, 1957; Stoner and Bond, 1963), although less well than spleen cells (Stoner and Bond, 1963).
Thymus cells have also been found to be less capable than other lymphoid cells in initiating graft versus host reactions in rats (Billingham, Defendi, Silvers and Stein-muller, 1962). However, mouse thymus cells, although still less capable than spleen or bone marrow cells, are apparently able to produce so-called "runt disease" in a high proportion of cases (Billingham and Brent, 1959; Billingham and Silvers, 1961; Cohen, Thorbecke, Hochwald and Jacobsen, 1963; Mitchell and Miller, 1968). Whereas the ability of spleen cells to produce this syndrome increases from nil at birth to be ten times as effective as thymus cells in the adult (Dal-masso, Martinez, Sjodin and Good, 1963; Sosin et al, 1966). This observation that thymus cells do not change in competency with the age of the animal has also been observed by Stoner and Hale (1955) in respect to responses to antigen, and it suggests that whatever degree of immuno-competence the thymus possesses, this competence is fully developed at birth.

The reasons for the low capacity of thymus cells to mount immune reactions are unclear, but two main explanations have been offered:-

(1) That the thymic cells do not respond to antigens at all and the responses obtained are due to contaminating competent immigrant cells,

(2) That the thymus is a source of immature cells destined to become fully immuno-competent only after they leave the thymus.

The observations that neonatal thymus cells are as capable as adult thymus cells while neonatal spleen cells are incompetent, suggest that immigrant cells in the thymus are not the source of its competence. The fact that thymectomy, at various ages after birth, apparently prevents any further maturation of the degree of competence of spleen cells supports the second alternative.
In view of the constitutional similarities between the thymus and other lymphoid organs, the fact that they are all colonized by stem cells of a common origin and that cells leave the thymus and travel to other lymphoid organs, one might expect that the thymus and the other lymphoid organs would have a similar function. The disparities in growth characteristics, rate and control of lymphopoiesis and involvement in immunological reactions indicate that the function or functions which the thymus subserves are however, different from those of other lymphoid organs.

The Effects of Thymectomy

One of the most convenient methods of studying the function of an organ is to remove it and observe the effects of this manoeuvre. The technique of thymectomy has been widely used in studies on the thymus since the middle of the nineteenth century and a variety of functions have, at some time or another, been ascribed to this organ. This review will be concerned solely with the immunological aspects of thymic function.

The early work on thymectomized animals has been reviewed by Paton and Goodall (1904) and Park (1917) who concluded that the conflicting results found up to that time were a result of incomplete thymectomy associated with infection after surgery. Whether this assertion is warranted or not the results obtained from recent work have not been any less conflicting.

The Effect of Thymectomy on Lymphoid Organs

The earliest observations of a reduction in circulating white cell counts after thymectomy was made in guinea pigs by Paton and Goodall (1904) and this was subsequently confirmed by Reinhardt and Yoffey (1956), and Clark, Williams...
and Yoffey (1965). Metcalf (1960) studied the effect of thymectomy in adult mice and found a slow but progressive fall in circulating lymphocytes and a reduction in the cell population of lymph nodes and spleen. However, the effects of neonatal thymectomy are rather more dramatic. Miller (1961) found that in normal newborn mice, the absolute lymphocyte count and the ratio of lymphocytes to polymorphonuclear cells rose during the first 8 days of life and reached values near to those found in adults. In neonatally thymectomized mice however, the lymphocyte to polymorph ratio did not alter greatly. At 6 weeks of age, the total leucocyte count of the thymectomized mice was only half that of normal mice, and was due entirely to a lymphopaenia (Miller, 1962b).

Miller, Mitchell and Weiss (1967b) have shown a great reduction in output of cells from the thoracic duct of neonatally thymectomized mice. There is also a reduction in the density of lymphocytes in the secondary lymphoid organs which appears to be confined to the diffuse cortex of lymph nodes and peri-arteriolar sheaths of the spleen (Parrot, de Sousa and East, 1966). These areas have been termed the "thymic dependant areas", and appear to bear a relation to the sites where lymphocytes have been shown to pass from the blood into the tissues. (Gowans and Knight, 1964). However, thymectomy does not appear to affect the number or density of the germinal centres, lymphoid follicles or plasma cells (Parrot et al., 1966).

In the rat, similar observations have been made on the effect of neonatal thymectomy. There is a reduction in size of the lymph nodes (Reinhardt, 1945), a reduced cellularity of the lymphoid organs (Schooley and Kelly, 1961), reduced thoracic duct lymphocyte output (Schooley and Kelly, 1964), while the number of plasma cells remains unchanged (Waksman, Arnason and Jankovic, 1962; Azar, 1966) as does the
lymphocyte status of the bone marrow (Bierring, 1963). Similar observations have been made in other species (see review by Miller and Osoba, 1967). The results of thymectomy in birds have served to emphasize that the effect is on the lymphocyte levels and not on the follicular or plasma cell development of the lymphoid organs (Isakovic and Jankovic, 1964; Jankovic and Isakovic, 1964). However, the removal of the Bursa of Fabricius in birds at an early age does result in a loss of follicular development and in the absence of plasma cells in the lymphoid tissue and spleen (Isakovic and Jankovic, 1964; Cooper, Peterson and Good, 1965; Cooper, Peterson, South and Good, 1966).

It would appear then that thymectomy results in a decreased number of lymphocytes in the blood and lymph as well as in the diffuse cortex of the lymph nodes and in the splenic white pulp i.e. in the circulating lymphocyte pool. The earlier the age at which the thymus is removed, the greater this defect and the more rapidly it occurs. On the other hand, there appears to be no coincident depression of plasma cell production or follicular development.

The Effect of Thymectomy on the Occurrence of Wasting Disease

Miller (1961) first observed that some of his thymectomized animals were afflicted by a progressive wasting disease, characterised by loss of weight, hunched posture, ruffled fur, diarrhoea and death. This condition has since been observed in many different strains of mice, rats, hamsters and guinea pigs following thymectomy (see review by Hess, 1968). The most constant histopathological changes in these animals were an even more severe depression of circulating lymphocytes than that seen in non-wasted thymectomized animals and even more atrophic lymph nodes, Peyer's Patches and
spleens (Parrot and East, 1964). No obvious evidence of specific infection could be found but Miller (1961) observed that if special precautions were taken, the incidence of wasting could be reduced. It has since been graphically demonstrated by the use of specific pathogen-free and germ-free thymectomized mice (Hess, Cottier and Stoner, 1963; Wilson, Sjodin and Bealmear, 1964; McIntire, Sell and Miller, 1964) that thymectomized animals grow as well as conventional or germ-free controls but rapidly succumb to wasting disease when exposed to a contaminated environment.

It has also been noted that if thymectomy is delayed for a few days after birth, then the incidence of wasting disease is drastically reduced (Wilson et al, 1964; Parrot and East, 1964). This could be explained on the basis of there having been sufficient delay for enough potentially immunologically competent cells to have migrated into the peripheral lymphoid organs so that, even in the absence of the thymus, the animal can resist the effects of the inevitable onslaught of foreign antigens to which it becomes exposed at birth. Parrot (1962) has investigated the variation in susceptibility to wasting disease between different strains of mice and has found considerable differences. This could be due to variations in the time at which lymphocytes migrate to peripheral lymphoid organs in different strains after birth. Although common pathogens were rarely isolated from thymectomized animals, the germ-free evidence, as well as the reported increased susceptibility to endotoxin, Candida spp. (Salvin, Peterson and Good, 1965) and various viruses (East, Parrot, Chesterman and Pomerance, 1963; Leyten, de Somer, Denys and Prinzie, 1965) strongly suggests that wasting is a product of a contaminated environment and is probably infectious in nature. This is supported by the observations of Azar,
Williams and Takatsuki (1964) who showed that the incidence of wasting disease in thymectomized mice could be reduced by the continuous feeding of tetracyclines.

The Effect of Thymectomy on the Response to Antigens

In 1961, three reports followed one another showing that thymectomy in young animals was followed by a reduced immunological capacity (Fichtelius, Laurell and Philipsson, 1961; Archer and Pierce, 1961; and Miller, 1961). Since then there have been many similar reports showing a reduced or almost total abolition of the immune response to a variety of antigens after thymectomy in several different species of animals. Inbred strains of mice have been used most frequently as experimental models and the antigens to which thymectomized mice have been shown to respond poorly include sheep red blood cells, Salmonella typhi 'H', 'O' and 'Vi' antigens, influenza virus, T2 coliphage, diphtheria toxoid, ovalbumin and BSA (see reviews by Miller and Osoba, 1967; Hess, 1968). However, the significant fact which emerges from all these investigations is that while it is incontestable that many investigators have found responses to be reduced, they are rarely completely abrogated and, in fact, some of the experimental animals react only slightly less well than normal. There also appears to be considerable strain variation in response to different antigens (as in the case of wasting) and it is extremely difficult, if not impossible, to correlate all these results due to the fact that they are confounded by the use of different strains of animals, F1 hybrids, different dose rates and modes of administration, and different time schedules in administration of antigen and time of testing.

Recently a number of reports have appeared which cast some doubt on the validity of much of this early work on the immuno-suppressive effects of thymectomy. Svet-Moldavsky,
Zinzar and Spector (1964) found that although the primary response to sheep red blood cells was reduced, they could elicit a normal response after a third injection. Brooke (1965) could detect no difference in the haemagglutinin response 3 weeks after a primary injection of sheep red blood cells at 9 weeks of age. Rogister (1965) found that the immune response at 30 days was less than that of controls, while at 60 days the secondary responses were identical to those of controls and a primary response at this age was significantly higher than that elicited at 30 days. Dukor, Dietrich and Rosenthal (1966) found a similar "recovery" of the immune response in Swiss albino mice so that the responses in the thymectomized animals were equal to that of controls at 42 days (for plaque forming cells) or 13 weeks (for serum haemolysins). However, CBA/Tif or CBAT 6T 6/Tif showed no similar "recovery". Zinzar and Svet-Moldavsky (1967) extended their previous observations using thymectomized C57Bl/6 mice and also found a gradual improvement in their ability to respond to sheep red blood cells, so that by 10 to 12 weeks of age their response was equal to that of control mice. Sinclair and Millican (1967) confirmed the observations of Dukor et al (1966) and Sinclair and Elliot (1968) extended them to show that larger doses could elicit responses at an earlier age and that although some low doses failed to produce a detectable response, these doses were sufficient to prime the thymectomized animals so that they subsequently produced a normal secondary response.

Other disturbing discrepancies have been reported in the response of thymectomized mice to other antigens, including haemocyanin, S. typhi 'O' antigen and S. typhi "H" antigen. Humphrey, Parrot and East (1964) found only a slightly reduced response of C 3H/Bi and C 57Bl x C 3H/Bi F 1 ,
mice to haemocyanin, while Fahey, Barth and Law (1965) found a more severe reduction in C3Hf/LW. Arnason, de Vaux St.Cyr and Shaffner (1964b) found a normal response to Typhoid 'O' antigen in Balb/C mice, Humphrey et al. (1964) found it considerably reduced in C3H/Bi, while Zinzar and Svet-Moldavsky (1967) obtained a depressed primary but a normal secondary response in C57Bl/6. Miller (1962b) reported a severely depressed response to Salmonella typhi 'H' antigen in neonatally thymectomized C57Bl, C3H and (Ak x T6)F1, while Brooke (1965) found a perfectly normal response to the same antigen in C57Bl/6J. Other antigens which have been tested and found to give perfectly normal responses in thymectomized mice are ferritin (Fahey et al., 1964) and pneumococcus polysaccharide (Humphrey et al., 1964; Brooke, 1965).

The neonatally thymectomized rat gives a normal immune response to sheep red blood cells and Salmonella typhi flagellar antigens (Pinnas and Fitch, 1966), pneumococcus polysaccharide and Salmonella typhi 'O' antigens (Arnason, de Vaux St.Cyr and Relyfeld, 1964a), but their response to horse serum is reduced (Azar et al., 1964) as is also the response to diphtheria toxoid (Arnason et al., 1964a) and BSA (Jankovic, Waksman and Arnason, 1962; Arnason et al., 1964a and b; and Pinnas and Fitch, 1966).

Neonatally thymectomized rabbits have been shown to have a reduced response to BSA and T2 phage (Arther, Pierce, Papermaster and Good, 1962) although the response to T2 was subsequently said by the same workers to be not significantly reduced (Good, Dalmasso, Martinez, Archer, Pierce and Papermaster, 1962). Hamsters thymectomized early in life have shown a reduced response to human gamma globulin (Sherman, Adner and Damashek, 1963).

No effect on antibody production after neonatal
thymectomy has been found in piglets (Pestana, Hallenbeck and Shorter, 1965) or dogs (Van de Water and Katzman, 1964) challenged with a variety of different antigens.

The antigens which result in an apparently consistently low response by thymectomized animals are those low molecular weight soluble proteins such as ovalbumin, heterologous gamma globulins and particularly bovine serum albumin (BSA). The response to BSA is reduced in almost every species investigated. However, as pointed out above, those antigens with a high molecular weight, such as ferritin and haemocyanin are "strong" antigens and the similarity in the response to these antigens between thymectomized animals and newborn animals is striking.

The effect of neonatal thymectomy on the humoral immune response is still incompletely understood. The wide variations in the responses to different antigens between species and even strains within a species makes it impossible to make any generalized comment about the effect of thymectomy. Many of these differences could be due to differences in experimental technique and design in relation to antigen dose, age of animal at testing and the time after thymectomy at which the animal is challenged, but perhaps the most important factor in determining the outcome of the antigenic challenge is whether the animals are healthy or suffering from wasting disease when tested. For instance, Brooke (1965) and Dukor et al (1966) observed no wasting in the mice which responded normally to sheep red cells. All reports which showed a reduced response to sheep red blood cells also showed a high incidence of wasting disease. This was also the case in the conflicting results on the response to the 'O' antigen of Salmonella typhi and haemocyanin (Arnason et al, 1964a; Humphrey et al. 1964). This suggests that animals suffering
from wasting disease may be "pre-occupied" and their lymphoid apparatus so fully committed to dealing with the extraneous antigenic influences which are gradually exhausting them that they fail to respond when a specific stimulus is applied. Investigations on germ free thymectomized animals would thus be of value in determining the specific effects of thymectomy on antigenic response. Little information is available on this aspect with the exception of the work of Hess et al (1963), Hess and Stoner (1966) and Hess et al (1967). Hess et al (1963) found that neonatally thymectomized BNL Swiss mice had only a slightly lower primary response to tetanus toxoid administered at 4 weeks of age when compared with normal animals. Surprisingly however, the secondary response 3 weeks later was, in many cases, reduced. Those animals which had a normal secondary response also produced a normal tertiary response, while those initially exhibiting a somewhat reduced primary, had a much reduced secondary and an even more reduced tertiary response (Hess and Stoner, 1966). However, the responses to pertussis vaccine and heat aggregated BSA were perfectly normal even in 26 week old, neonatally thymectomized mice (Hess and Stoner, 1967). Bealmear and Wilson (1967b) are quoted by Hess (1968) as having compared the response of conventionally raised and germ-free mice thymectomized at birth, to a primary challenge with Salmonella typhimurium and found the germ-free animals had titres equivalent to, or exceeding those, of normal, non-operated mice whereas those of the conventionally raised, thymectomized animals were severely reduced. On the other hand, Miller, Dukor, Grant, Sinclair and Sacquet (1967a) found that the response to sheep red blood cells was depressed to the same extent in both germ-free and conventionally raised C3H/Gif mice, thymectomized at birth, while non-operated control mice had much higher titres.
The Effect of Thymectomy on Transplantation Immunity

(1) Skin Homografts

Miller (1961) and Martinez, Kersey, Papermaster and Good (1962c) were the first to report that neonatally thymectomized mice did not reject skin homografts as readily as normal mice. The rejection of skin homografts in mice has since become the most widely used method of evaluating the immunological effects of neonatal thymectomy for, while the response of thymectomized animals to antigens is variable, their reaction to homografts appears to be consistently depressed (see reviews by Miller and Osoba, 1967; Hess, 1968).

Recently however, there have been several reports showing that homograft immunity is not depressed in specific pathogen free BNL Swiss mice (Hess and Stoner, 1967) and only slightly depressed in germ-free C57Bl/Ka mice (McIntire, Sell and Miller, 1964) and only moderately depressed in C3H/Gif (Miller et al, 1967a). Rogister (1965) has found also that although Swiss albino mice have a reduced capacity to reject homografts for up to 45 days after thymectomy, after this time they reject homografts perfectly well. Brooke (1965) could not produce any suppression of homograft immunity in neonatally thymectomized C57Bl/6J mice even with animals reared under conventional conditions. Brooke's mice did not display any great degree of wasting disease, whereas in all other reports in which grafts have been accepted for a prolonged time, wasting disease was present in the animals to a significant degree and in fact, many mice died of this condition with grafts still intact (Miller, 1962b,c).

Neonatal thymectomy produces variable effects on graft rejection in rats. Arnason, Jankovic, Waksman and Wennersten (1962) found that Sprague-Dawley rats thymectomized at birth accepted skin grafts for a prolonged time, while
Defendi, Roosa and Koprowski (1964) found that thymectomized Lewis rats rejected grafts normally. Fisher and Fisher (1965) found that 32 per cent of neonatally thymectomized Long-Evans rats showed prolonged rejection time at 35 days of age, 40 per cent at 56 days of age, but only 17 per cent at 92 and 13 per cent at 123 days. These results suggested that the capacity to reject homografts developed in these animals with age.

Thymectomy of rabbits under 5 days of age (Good et al, 1962a) young pups (Van de Water and Katzman, 1964), or piglets within 24 hours of birth (Pestana et al, 1965) does not result in any prolongation of the rejection time.

(2) Tumour Homografts

It appears to be easier to transplant tumours in thymectomized mice than in normal animals (Good et al, 1962; Martinez, Dalmasso and Good, 1962b; McEntegart, Ross and Best, 1963; Osoba and Auersperg, 1966) although again, in all these experiments, the prolonged acceptance of foreign tumours was associated with wasting disease. Martinez, Dalmasso and Good (1962b) found that Z(C3H) mice thymectomized at birth and grafted with a mammary adenocarcinoma of A strain origin at the same time, showed 100 per cent takes, whereas the tumour successfully took in only 62 per cent of controls. When grafted at 22 days post-thymectomy, there was an 87 per cent take in the thymectomized animals as against 5 per cent in controls, at 50 days post-thymectomy there was a 57 per cent take in the thymectomized animals as against 0 per cent in controls. A similar increase in resistance with age of thymectomized C3H/Bi mice to AKR leukaemic cells was found by Parrot and East (1965), even though these mice were coincidentally suffering from wasting.
disease. Bealmear and Wilson (1967a) found germ-free CFW and C\textsubscript{3}H mice were fully capable of rejecting reciprocally derived tumour cells, and X-irradiation abolished this resistance but only for a period of 2 months when the resistance had fully re-developed.

Neonatally thymectomized rats have also been shown to be less capable of rejecting foreign tumour grafts. Lazar (1966) found that a mammary adenocarcinoma, which is normally histo-incompatible to Sprague-Dawley rats, will grow in them after they have been thymectomized. Perri, Faulk, Shapiro, Mellors and Money (1963) obtained similar results in transplanting the Jensen sarcoma to Sprague-Dawley rats. On the other hand, Fisher and Fisher (1965) could detect no difference in the acceptance rate of Walker tumour cells between normal or neonatally thymectomized Long-Evans or Sprague-Dawley rats.

Thus the acceptance of homografts appears to be extended in mice when thymectomy is performed very early in life and when wasting disease ensues. This prolongation is greatest when donor and recipient animals are closely related (no difference at the H\textsubscript{2} locus) and least when there is a difference at the H\textsubscript{2} locus. Other species, outbred strains, and germ-free animals appear to be almost fully capable of rejecting homografts after thymectomy with the possible exceptions of some types of tumours.

(3) Graft Versus Host Reactions

(a) In Thymectomized Animals Treated with Allogeneic Lymphocytes. Thymectomized mice have been shown to be much more susceptible to the lethal effects of parenterally administered allogeneic lymphocytes than normal mice (Good et al., 1962) particularly if suffering from wasting disease.
Martinez, Dalmasso, Blaese and Good (1962a) found that neonatally thymectomized \((A \times C_3H)F_1\) were highly susceptible to parental A strain cells, but strangely, \(C_3H\) parental cells were not effective, while Parrot and East (1964) found that \(C_3H/Bi\) mice are much more susceptible to \(C_57\)Bl spleen cells after thymectomy. On the other hand, Aisenberg, Wilkes and Waksman (1962) found that thymectomy at 3 days of age had no effect on the "onset, incidence, clinical or histological picture" of runt disease in Sprague-Dawley rats caused by the injection of Long-Evans spleen cells.

(b) In Normal Animals Treated with Lymphocytes from Thymectomized Donors. As described above, thymectomized mice appear to be less capable of resisting the effects of an attack by foreign lymphocytes. It has also been shown by Good et al (1962) and Dalmasso, Martinez and Good (1962) that spleen cells obtained from thymectomized animals are less capable of causing graft versus host disease in normal \(F_1\) recipients. It was further shown by Dalmasso et al (1963) that in normal mice, the ability of spleen cells to produce a GVH reaction in \(F_1\) recipients increased gradually from birth to 35 days and beyond, but thymectomy at any time prevents any further development of this ability. Further, this lack of ability is quantitative only and provided the dose of spleen cell is large enough GVH symptoms will appear in the recipient.

A similar reduction in the ability of thoracic duct lymphocytes from thymectomized donors to produce GVH reactions has been shown in mice by Miller, et al (1967b) and in rats by Rieke (1966).

There is no information available on other species or on germ-free animals.
The Effect of Thymectomy on Hypersensitivity Reactions

Comparatively little information is available on the status of delayed type hypersensitivity (DTH) reactions in thymectomized animals. Russe and Crowle (1965) found that only 25 per cent of neonatally thymectomized CF₁ mice were capable of responding in an immediate or delayed fashion to ovalbumin or tuberculin but they gave no information on the size of the reactions obtained. Arnason et al (1962) and Jankovic et al (1962) have shown that skin reactions to BSA and tuberculin were reduced but not abolished in thymectomized rats, while Messini, Cenci and Cucchi (1964) claimed that DTH reactions to BSA were completely abolished, although this was only a small trial. Wilson, Jones and Leskowitz (1967) found that neonatally thymectomized Sprague-Dawley rats infected with Nippostrongylus brasiliensis larvae produced a much less intense passive cutaneous anaphylaxis reaction, although these rats were immune as judged by the lack of egg production following a second dose of larvae.

From this small amount of evidence it would appear that both immediate and delayed type hypersensitivity reactions are reduced, but not abolished, following neonatal thymectomy.

Reconstitution of Thymectomized Animals

A number of different methods have been tested for their capacity to restore neonatally thymectomized animals to normal in terms of circulating cell counts, cell population of peripheral lymphoid organs and immunological competence. Some of these have apparently been successful, while the results with others have been equivocal.

(1) Reconstitution by Injected Cells

(a) Thymus Cells Dissociated thymus cells have
been found by different workers to have different effects. For instance, Miller (1962c) and Parrot and East (1964) found they were incapable of protecting mice against wasting disease which follows thymectomy, even when administered in large numbers. Hilgard, Yunis, Sjodin, Martinez and Good (1964), Trainin, Law and Levey (1965) and Miller and Mitchell (1968) found injections of these cells gave partial protection to thymectomized mice, while in other studies East and Parrot (1964) and Dalmasso et al (1963) found them to be fully protective. Isakovic, Waksman and Wennersten (1965) found that the ability to produce delayed type hypersensitivity reactions in thymectomized rats was not restored following the injection of thymus cells. Similarly, efforts to restore antibody production in thymectomized mice by injection of thymus cells have been unsuccessful, even after using thymic cells from previously immunized donors (Claman, Chaperon and Triplett, 1966). On the other hand, Trainin et al (1965) and Miller and Mitchell (1968) have reported that adult thymus cells can confer immediate competence on neonatally thymectomized mice to react against sheep red blood cells.

(b) Spleen Cells Spleen cells of syngeneic origin have been found to protect thymectomized mice from wasting and to restore homograft immunity in these animals as well as the antibody response to some antigens (Dalmasso et al, 1963; Parrot and East, 1964; East and Parrot, 1964; Hilgard et al, 1964; Trainin et al, 1965; Claman et al, 1966) as have lymph node cells (Trainin et al, 1965; Isakovic et al, 1965). Thoracic duct cells also have this capacity and appear, in fact, to be much more effective in this role than thymus cells (Miller, et al, 1967b) In relation to homograft immunity, Miller (1962c) has shown that spleen or lymph node cells from C3H mice previously immunized
against A strain cells will cause rapid rejection of A strain grafts.

Thymectomized animals which have been reconstituted with syngeneic spleen cells have been shown to have cells in their spleens which are capable of eliciting GVH reactions in suitable recipients (Dalmasso et al., 1963). This also applies to recipients of thymic cells, although spleen cells from these animals are not as capable of eliciting GVH reactions as those that have been reconstituted with spleen or lymph node cells (Hilgard et al., 1964; Trainin et al., 1965). Thus it appears that cells from peripheral lymphoid organs are much more capable than thymus cells of restoring the immune capabilities of the thymectomized recipient.

(c) Bone Marrow Cells

It is well known that bone marrow cells fully restore immunological competence and prevent wasting in lethally irradiated animals. Wasting of thymectomized animals can also be prevented by bone marrow transfusions (Miller, Doak and Cross, 1963; Trainin et al., 1965). It is also claimed that the immune response can be restored in thymectomized mice by the injection of bone marrow cells. This claim however, is based entirely on observations made on the immune response to sheep red blood cells. As described in a previous section the immune response to sheep red blood cells in thymectomized animals varies between strains, laboratories and between germ-free and conventional animals. The "restoration" of the response to sheep red blood cells with various types of cell inocula will be discussed more fully in a succeeding section.

(2) Reconstitution by Thymus Grafts

Since the removal of the thymus results in a considerable impairment of various immunological reactions,
particularly in conventional animals, replacement of the thymus by way of a graft would seem a logical method for re-endowing a thymectomized animal with these lost faculties. Miller (1961) in his initial publication on the effects of neonatal thymectomy showed that these effects could be prevented by the subcutaneous grafting of a syngeneic thymus. He further showed that allogeneic grafts would restore to thymectomized animals the competence to reject third party skin grafts while conferring tolerance to skin grafts from the thymus donor (Miller, 1962c). This was confirmed by Dalmasso et al (1963) and Parrot and East (1964) who found that wasting could be prevented by thymus grafts placed under the kidney capsule but not subcutaneously. However, Hilgard et al (1964) found that wasting could not be prevented by grafting once overt symptoms were present. Neonatally thymectomized mice have also been restored to normal by the implantation of xenogeneic (rat or hamster) thymic grafts (Law, 1966) although Dalmasso et al (1963) could not restore C3H mice with rat grafts.

The regeneration of thymic tissue after grafting has been described by Dukor, Miller, House and Allman (1965). They found that syngeneic grafts implanted into normal hosts showed necrosis of the centre of the graft for the first 2 days while around the central necrotic area there was a surviving rim of epithelial and lymphoid cells. An intense proliferative phase rapidly followed in the graft so that by 5 days a full lymphoid complement had reappeared, and a normal lobulated appearance had developed by 8 days after grafting. Up until the twelfth day all the dividing cells in the graft were seen to be of donor origin (these grafts were syngeneic but carried a marker chromosome), then host cells began to appear within the thymus so that by 30 days all dividing cells
were of host type. Syngeneic grafts implanted into thymectomized recipients showed similar changes although the mitotic activity within the graft during the 6 to 10 day period was less than that in a non-thymectomized recipient. Syngeneic grafts implanted into a thymectomized and irradiated recipient showed an even slower regenerative pattern. Previously irradiated syngeneic grafts show the early necrotic phase, with no surviving lymphoid rim, and host cells enter the graft from the periphery at about 12 days.

These results clearly show that initially the donor thymus cells proliferate and subsequently seed to the periphery. This is followed by infiltration and proliferation of host lymphoid cells. The cellular component of the donor thymus can be destroyed by prior irradiation, while thymectomy of the recipient causes a slowing of host cell infiltration. This is even more noticeable if the lymphoid population of the thymectomized host is further depleted by X-irradiation. Thus there is in the thymectomized host, a population of cells which migrate into and repopulate the thymus graft.

Allogeneic grafts display a different pattern of regeneration. When implanted into normal hosts, the donor lymphoid cells rapidly disappear under the onslaught of infiltrating host cells and the graft is rejected in 7 to 11 days. In thymectomized hosts however, allogeneic grafts show a regenerative pattern identical to that of syngeneic grafts and the host subsequently becomes tolerant to grafts of other donor tissues.

These observations were extended by Miller, de Burgh, Dukor, Grant, Allman and House (1966) who showed that parental or allogeneic thymic grafts restored normal blood lymphocyte counts and prevented wasting in a proportion of the recipients even when the grafts were excised after 10 to 14 days.
Leuchars, Cross and Dukor (1965) investigated the effect of syngeneic and allogeneic thymic grafts in CBA mice thymectomized at 8 weeks of age, lethally irradiated with 850r and immediately protected with syngeneic (CBA) bone marrow. They found that syngeneic grafts partially restored the homograft response, and that an allogeneic graft (C3H) conferred tolerance to C3H skin but not third party skin such as C57Bl or AK skin. Another allogeneic thymic graft (C57Bl) also restored homograft immunity to third party skin (AK and Balb/c) but, remarkably, C57Bl (i.e. donor type) skin was rejected in a very rapid manner reminiscent of a second set response. These allogeneic thymic grafts were themselves also rejected. Thus, in this situation at least, tolerance to donor type skin can only be conferred by a thymic graft from a closely related strain. Xenogeneic grafts have also been found to restore homograft immunity to thymectomized animals and these grafts are themselves rejected (Law, 1966).

From these experimental results the following points emerge:-

(1) Cells that take part in the homograft response are derived from the bone marrow and are influenced by, or interact with some thymic component to become competent (Dukor et al, 1965; Leuchars et al, 1965).

(2) The period of interaction between the precursor cells and the thymic components need only be short (Miller et al, 1966).

(3) The evidence available to date suggests that actual traffic of these cells through the thymus is not obligatory (Dukor et al, 1965).

(4) The thymic influence is not strain or species specific (Feldman and Globerson, 1964; Leuchars et al, 1966; Law, 1966.)
Considerable effort has been directed toward the elucidation of the role of the bone marrow in restoration experiments with thymic grafts. For these experiments the adult thymectomized, lethally X-irradiated, syngeneic bone marrow protected and thymus grafted mouse has been used as the experimental model. However, it must be constantly borne in mind that this experimental situation is, to say the least, highly artificial. Therefore, extrapolation from these results to the normal situation must be considered with extreme caution.

The design of these experiments and the results obtained from them are very difficult to describe adequately in a short space. The essential findings to come from them can be summarised.

Adult thymectomy does not lead to an immediate loss of immunological competence unless it is followed by a heavy dose of X-irradiation. In these circumstances wasting and death follow. If these lethally irradiated, thymectomized animals are injected with syngeneic bone marrow cells they are protected against wasting but other immune characteristics are not restored (Davies, Leuchars, Wallis and Koller, 1966; Mitchell and Miller, 1968). Thymus grafts given to adult thymectomized, X-irradiated, bone marrow restored animals restore their immunological competence, although not completely, (Cross, Leuchars and Miller, 1964; Miller et al, 1966), whereas allogeneic thymus grafts (no $H_2$ difference) result in tolerance to donor type skin (Miller, 1962c; Leuchars et al, 1965). Allogeneic thymus grafts (with $H_2$ difference) do not induce tolerance in the recipient and lead to rejection of the donor thymus, i.e. these grafts restore immunological competence even to donor skin (Leuchars et al, 1965).
By the use of syngeneic grafts bearing a marker chromosome the identity of the cells in thymic grafts can be established. Most cells seen dividing in the donor thymus are at first of donor origin but subsequently all the dividing cells are host type (Dukor et al, 1965). The dividing cells in the peripheral lymphoid organs are mostly host type, but rather more donor-type cells can be identified in the spleen than in the lymph nodes (Leuchars et al, 1965). These donor type cells in the lymph nodes and spleen are capable of a considerable proliferative response following antigenic stimulation in the form of sheep red blood cells or a homograft (Davies et al, 1966). Although these donor (thymus) cells respond to antigen by dividing, they do not produce antibody (Davies, Leuchars, Wallis, Marchant and Elliot, 1967). The bone marrow cells are slower to undergo mitosis under antigenic stimulation than thymus cells, but they do produce antibody (Davies et al, 1967). When both thymus (donor) and bone marrow (host) cells are allowed to react to the antigenic stimulus together, the production of antibody is greater than if the bone marrow cells react on their own (Claman et al, 1966; Davies et al, 1967).

These results show that in the thymectomized, X-irradiated, bone marrow restored mouse, the cells that produce antibody are of bone marrow origin. However, with this experimental design it was not possible to determine whether thymus cells were themselves capable of antibody production in the presence of bone marrow cells.

Further complex experiments of this type designed to elucidate these inter-cellular relationships were carried out by Miller and associates (Miller and Mitchell, 1967, 1968; Mitchell and Miller, 1968; Nossal, Cunningham, Mitchell and Miller, 1968) using a combination of iso-antisera and
chromosome markers with thymectomized and irradiated recipients of various cell types and combinations. They measured the response to sheep red blood cells in terms of the number of plaque forming cells produced in the various experimental situations. Their results lead them to conclude that the thymus produces cells ("Antigen reactive cells") which either recognise or process antigen and then focus this "super antigen" on or react with "antibody forming cell precursors" of bone marrow origin. These bone marrow precursors then differentiate into antibody forming cells. Miller and his colleagues claim that the only source of "antigen reactive cells" is the thymus and thymectomized animals have an almost complete lack of this type of cell, while having a full complement of "antibody forming cell precursors" which are derived from the bone marrow. The thoracic duct lymph in normal animals contains a mixture of both these types of cell, with a predominance of the "antigen reactive cell". The thoracic duct lymph of thymectomized animals has many antibody forming cell precursors but lacks antigen reactive cells.

The implications of these results are of considerable significance. However, as the experimental designs employed by Miller et al were limited to one species and one antigen, it is essential that further confirmation of these results be obtained in the same and other species of animals using different antigens. Acceptance of the general applicability of this concept of thymus cell, bone marrow cell interactions will also depend on results being obtained in animals under more physiological conditions.

(3) Reconstitution by Thymus in Diffusion Chambers

There is considerable evidence that when neonatally thymectomized animals are implanted with Millipore diffusion chambers containing adult or neonatal thymus, some restoration
of their immune capacity results (see reviews by Miller and Osoba, 1967; Hess, 1968).

This restoration is not total, and it does not result in restoration of the peripheral lymphocyte populations to normal, as in animals given thymus grafts. These experiments with diffusion chambers suggest that thymic epithelium produces a humoral factor. There is no doubt that the thymus exerts a strong lymphopoietic effect upon cells within its confines. It is not known however, whether under normal circumstances the thymus extends this influence much beyond its boundaries. Osoba (1965) observed that thymectomized female mice regained some immunologic capacity during pregnancy and he suggested that a thymic humoral factor elaborated by the foetuses was able to cross the placenta and exert its effect in the mother.

The final proof of the thymic humoral factor (or hormone) must rest upon the isolation and characterization of a specific thymic extract. To date this has not been accomplished and thymic extracts of various types have produced results which are, to say the least, equivocal (Miller and Osoba, 1967).

SUMMARY

The literature contains dogmatic statements to the effect that immunological competence is acquired only after birth. It is clear that this does not apply in the general sense and that different species of animals acquire a capacity to mount immunological responses at different ages in their development. Apart from this, the work of Silverstein has demonstrated that the ability to respond to various antigens is acquired by the foetuses over a period of months (Silverstein et al, 1963b). For these reasons any discussion
on the state of immunological competence of young animals must be considered essentially within the framework of one species and one particular antigen. It must also be borne in mind that variations in dose rates, the use of different antibody detection tests and the presence of passively acquired antibody also effect the results obtained and this must be considered when comparing the responses of animals of different ages within the same species.

The development of immunological competence is closely correlated with the appearance and dissemination throughout the body of cells of the lymphoid series. Obviously there is some period in the development of a foetal animal when it is incapable of responding to even "strong" antigens due to a complete lack of competent cells. Once competent cells begin to appear, the capacity to produce immune reactions also appears. However, the stepwise maturation of the immune response indicates that the mere presence of lymphoid cells is not sufficient in itself, and that for different antigens, some further development is necessary before an effective immune response can be mounted. Whether this development is related to the recognition, the processing of antigen, or the synthesis of antibody is not known.

As a result of the comparatively slow rate at which antibody production to some antigens occurs following a primary stimulus at or near birth, the newborn animal is more susceptible than the adult to a range of pathogenic organisms. In the various species maternal immune globulins pass from mother to offspring before or after birth. The mode of passage varies from species to species but all have a common selective effect, allowing 7S globulins to pass more readily than 19S globulins. Although in most cases this passage of antibody is important to the health and wellbeing
of the young animal, it can in some circumstances be harmful in that the subsequent response to an antigen may be depressed while maternal antibody still persists in the circulation.

As mentioned above, the development of immunological competence is closely correlated with the development of the lymphoid system. The first organ of the foetus to develop lymphoid characteristics is the thymus. This is followed by the lymph nodes and the spleen - the so-called peripheral lymphoid organs. Recent work indicates that the lymphoid stem cells which colonize both the thymus and the peripheral organs are derived from a common source; originally this is the yolk sac and subsequently the bone marrow. The thymus is the site of intense lymphopoietic activity in the young animal and it has been shown that, in the newborn mouse, many cells leave the thymus and travel to peripheral lymphoid sites. Lymphopoietic activity in the thymus is dependant on a continuing immigration of stem cells. The thymus grows most rapidly in the young animal and although it regresses in adult life, it still retains some functional tissue.

The peripheral lymphoid organs develop more slowly than the thymus and no comparable rate of lymphopoiesis or early regression is observed in lymph nodes or spleen. It appears that the growth of these organs is, in a large measure, due to continued antigenic stimulation.

Whereas the peripheral lymphoid organs are intimately involved in responses to antigens, the thymus generally takes no direct part in these responses. The main effect of neonatal thymectomy is to reduce the number of cells in the peripheral lymphoid organs and in the circulating lymphocyte pool. This effect is probably brought about as a direct result of the removal of a site of intense lymphopoietic stimulus which normally acts on the stem cells originating in the bone marrow.
However, the number and density of primary lymphoid follicles, the development of plasma cells in the spleen or lymph nodes and the lymphoid population of the bone marrow are not affected.

The current state of knowledge on the effect of thymectomy on the subsequent development of immune reactions is confused. Almost all reports of reduced immunological capacity have been associated with some degree of wasting disease, while on the other hand, germ-free, neonatally thymectomized animals do not waste and in almost every case are immunologically fully competent. Under germ-free conditions the development of the peripheral lymphoid organs and immunological competence proceeds in the same way in thymectomized animals as in non-thymectomized, germ-free animals. There have also been some reports of continuing development of immunological competence even in non-germ-free thymectomized mice. It would seem that the absence or reduction of immune responses which follows neonatal thymectomy, is a direct result of the sudden deprivation of an important source of lymphocytes. At the same time as this source (the thymus) is removed, the newborn animal is faced with a sudden and extremely heavy antigenic burden, which in many cases, these neonatally thymectomized animals are unable to resist.

The response of thymectomized animals to some antigens is perfectly normal however, even in wasting animals. The most consistently reduced response is to serum proteins, particularly BSA. Intact newborn animals respond poorly to this antigen and this suggests that thymectomy perpetuates or prolongs the developmental process leading to a normal response to BSA.

Neonatal thymectomy in piglets and dogs has not led to any detectable reduction in the immune capacity of these animals. In these species the colonization of the peripheral
lymphoid organs has reached a much more advanced stage at birth than in rodents. Thus thymectomy in species such as the dog, pig and calf could be more analogous to adult or delayed thymectomy in rodents which does not result in wasting, and there is only a gradual reduction in immune responses over a protracted period of time.

The work described in this thesis is concerned with the development of the lymphoid apparatus in sheep before and after birth and in the events which occur in the lymphatic system around the time of birth when the young lamb is projected into an alien environment and encounters a host of antigens for the first time in its life. The importance of maternal antibodies to the newborn lamb and the significance of the thymus and the spleen in the development of immunological competence have been studied in an attempt to give some perspective to the role of these organs in cellular and humoral immunity.
Experimental Animals

**Foetal Lambs**

Intact uteri were obtained from pregnant ewes slaughtered at the local abattoir. These fetuses were examined within 12 hours of slaughter.

**Newborn Lambs**

Randomly bred pregnant, Merino or Merino × Corriedale ewes mated to Merino, Corriedale or Border Leicester rams, were purchased from local flocks on the southern Tablelands of New South Wales and the Australian Capital Territory, or from the Trangie district of the Central West of New South Wales. The ewes were run in small grassed paddocks until near to term when they were moved indoors and lambed individually in small pens. Some lambs were reared normally and others were deprived of colostrum by pinning the teats of the ewe with heavy cloth fashioned to enclose the udder and held in place by tapes tied over the backs of the ewes. These lambs were bottle fed on milk replacer in the first 7 days of life. During this time the ewes were milked twice daily and the lambs were then fostered back onto their mothers.

**Early Pregnant Ewes**

Recently mated ewes were obtained from the same sources as above. Some non-pregnant ewes were also purchased and mated under supervision to ensure that accurate conception dates were known. These ewes were used for foetal surgical operations when it was necessary to know the exact age of the foetus at the time of operation.

**Chemicals**

All chemicals used were of the Analytical Reagent grade unless otherwise stated.
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Chemicals
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FIGURE 2.1

A young lamb with a lymphatic fistula of the efferent popliteal duct.

A young lamb with a lymphatic fistula of the main intestinal duct.
Sodium Chloride Solution
9.0g of Sodium chloride were dissolved in 1 litre of distilled water to make a 0.9 per cent solution. The word saline used hereafter will refer to this solution.

Antibiotics
Penicillin - 'Crystapen'. Crystalline penicillin G (Glaxo-Allenburys (Aust.) Pty. Ltd.) was used to prevent or control bacterial growth in lymph samples or to dust on surgical wounds.

'Triplopen'. Benethamine, procaine and sodium penicillins (Glaxo-Allenburys) was used for injection purposes when required.

'Streptopen'. 250,000U. of procaine penicillin and 0.25g of dihydrostreptomycin per ml (Glaxo-Allenburys) was also used for injection purposes.

'Terramycin' Soluble. Tetracycline HCl (Pfizer Pty. Ltd.) was added to the milk fed to colostrum deprived newborn lambs.

Anticoagulants
10,000 I.U. of heparin ('Pularin', powdered heparin B.P., Evans Medical Ltd.) was dissolved in 100ml of normal saline together with 100,000 I.U. of crystalline penicillin. This solution (5ml/100ml lymph) was used to prevent lymph from coagulating during collection. For plasma samples, blood was collected into a centrifuge tube which contained a few crystals of powdered heparin.

Anaesthetics
The following anaesthetics were used.

Thiopentone sodium B.P. ('Intraval' Sodium, May & Baker Ltd.) was used as a 5.0 per cent solution in distilled water for the induction of anaesthesia.

Halothane B.P. (Fluothane, I.C.I. Ltd.) was used for maintenance of anaesthesia in a closed circuit.
Pentobarbitone sodium B.P. ('Nembutal', Abbot Laboratories Pty. Ltd.) was used for some anaesthetic procedures and for euthanasia.

Buffers

Phosphate buffer. 0.1M phosphate buffer was prepared by adding 1 volume of 0.1M \( \text{NaH}_2\text{PO}_4 \) (1.5601g/100ml) to 9 volumes of \( \text{Na}_2\text{HPO}_4 \) (1.4198g/100ml). The pH was adjusted to 8.0.

Tyrode's solution. Tyrode's solution (pH7.3) was prepared by dissolving the following chemicals in 100ml of distilled water. \( \text{NaCl} \) 0.8g, \( \text{Na}_2\text{HPO}_4 \) 0.10g, \( \text{KCl} \) 0.02g, \( \text{NaH}_2\text{PO}_4 \) 0.005g, \( \text{CaCl}_2 \) 0.01g, \( \text{MgCl}_2 \) 0.01g, Dextrose 0.10g.

Acetate buffer. 0.2M Acetate buffer (pH5.2) was prepared as follows. 10.5ml of 0.2M acetic acid solution (11.55ml per 1. of water) was added to 39.5ml of 0.2M Sodium acetate (27.2g \( \text{C}_2\text{H}_3\text{O}_2\text{Na} \ 3\text{H}_2\text{O} \) per 1. of water) and the volume made up to 100ml with distilled water.

Barbital buffer. ('Veronal' LKB Produkter AB) 24.5g of Veronal buffer was dissolved in 1500ml of distilled water to make a 0.1M solution (pH8.6).

White Cell Counting Fluid. 1.5ml of acetic acid was diluted in 98.5ml of distilled water and coloured with a small amount of methylene blue.

Saponin Solution. A stock solution of Saponin (Saponin - White, The British Drug Houses Ltd.) was prepared by dissolving 1.0g of saponin in 100ml of saline solution.

Eagle's Basal Medium. This medium was prepared in the Microbiology Department of the John Curtin School of Medical Research, and the recipe used was a slight modification of the one described by Eagle (1959). Four solutions were prepared as follows.
Solutions (a) and (b) were mixed together, then solution (c) was added, followed by solution (d) and the volume made up to 2 litres with distilled water. This solution (Solution I) was sterilized by Seitz filtering and held at 4°C until used. A second solution (Solution II) consisting of 5.84g of L-glutamine in 400ml of water was also prepared and Seitz filtered. A final product was produced by combining the 2 litres of Solution I with the 400ml of
Solution II and adding 880ml of sterile distilled water. The pH was adjusted to 7.4 by the addition of 1.4 per cent sterile sodium bicarbonate solution.

Tritiated Thymidine. Tritiated thymidine was obtained from the Radiochemical Centre, Amersham, England. It had a specific activity of 5000mc/mM.

Complement. Lyophilized guinea pig complement was obtained from the Commonwealth Serum Laboratories and was reconstituted according to the directions immediately prior to use.

Rabbit Anti-sheep Serum. Anti-sheep serum was prepared by injecting rabbits subcutaneously with 1.0ml of a mixture of equal parts of normal sheep serum and complete Freund's adjuvant (Difco Laboratories). A secondary injection was given 3 to 4 weeks later consisting of a 1.0ml dose of either serum or equal parts of serum and complete adjuvant. The rabbits were bled 10 days after the secondary stimulus and the serum obtained was pooled and held at -10°C until used.

Nutrient Broth. 8.0g of powdered nutrient broth (Difco Laboratories) was dissolved in 1 litre of distilled water and sterilized by autoclaving at 15lb. pressure for 15 minutes.

Agar for Electrophoresis. 1.0g of Special Agar Noble (Difco Laboratories) was dissolved by boiling in 25.0ml of Veronal buffer and 75.0ml of distilled water. The agar was stored at 4°C in 11.0ml portions.

Rinsing Solution for Electrophoresis. 45.0ml of methyl alcohol was added to 10.0ml of acetic acid and 45.0ml of distilled water.

Stains

Leishman. 0.15g of Leishman stain (British Drug Houses Ltd.) was dissolved in 100ml of methanol.
Amido Black. 9.0g of Amido Black (Schmid & Co., Stuttgart) was dissolved in 1500ml of rinsing solution.

Azure A. 0.1g of Azure A (Allied Chemical & Dye Corp., New York) was dissolved in 100ml of acetate buffer.

Trypan Blue. 0.5g of Trypan Blue (G.T. Gurr Ltd., London) was dissolved in 100ml of phosphate buffered saline.

Trypsin Solution. 40.0mg of Trypsin (Difco Laboratories) was dissolved in 100ml of phosphate buffer and stored frozen in 5.0ml volumes.

Periodate Solution. 0.255g of sodium periodate was dissolved in 100ml of distilled water to make a 0.11M solution.

Glycerol Solution. 3.0ml of glycerol was added to 97.0ml of saline.

Mercapto-ethanol Solution. A 0.2M solution of 2-mercapto-ethanol (Eastman Organic Chemicals) was prepared by mixing 1.5626g in 100ml of saline.

Haemagglutination Diluent. A solution was prepared by dissolving 9.0g NaCl, 0.028g CaCl₂ and 0.079g MgCl₂ in 1 litre of distilled water.

Antigens

Swine Influenza Virus. Influenza virus of swine origin, strain Shope 15, was kindly supplied by Dr. R. Webster and Dr. H. Meier-Ewart of the Department of Microbiology, John Curtin School of Medical Research. The virus was suspended in saline to give a suspension of 16-20,000 haemagglutinating units per ml.

Chicken Red Cells. Fresh chicken red cells were washed 3 times and then resuspended in saline to give a concentration of $2 \times 10^9$ red cells per ml in saline.

Salmonella Muenchen. Boiled Salmonella muenchen organisms were suspended in saline to give a concentration of $2 \times 10^9$ organisms per ml.
Ferritin. Lyophilized horse ferritin (Mann Research Laboratories) was dissolved in saline to give a 2.0 per cent solution. One volume of this solution was emulsified with one volume of incomplete Freund's adjuvant (Difco Laboratories) and 1.0ml injected subcutaneously to give sensitisation. For testing hypersensitivity reactions, a 0.5 per cent solution of the ferritin dissolved in saline was prepared.

B.C.G. 2.2mg of viable B.C.G. organisms (C.S.L.) were resuspended in 2.2ml of the diluent supplied to give a suspension of 1.0mg per ml.

Homologous Lymphocytes. Lymphocytes of sheep origin were obtained from efferent popliteal lymph. The lymph was centrifuged at 1,000 rpm and the cells were washed 3 times in Eagle's basal medium then resuspended to a concentration of 50 x 10^6 or 25 x 10^7 per ml in the same medium.

General Methods

Collection of Lymph. Efferent lymph was collected into plastic bottles containing anti-coagulant solution, which were tied to a plastic holder sutured to the lamb's skin (Fig. 1.1). Collection of lymph from lambs posed a problem, in that they had to be allowed free access to their mothers. As the lambs were continually moving about and lying down special precautions were needed to prevent the lymph from being spilt from the collection bottles. The necks of the bottles were stoppered with rubber plugs through which glass tubes had been inserted. This prevented lymph from spilling when the bottles were not upright. It was essential that the bottles were of hard plastic since the lambs commonly lay on them, and lymph was forced out by compression of soft bottles regardless of the type of stoppering arrangement employed.
Lymph collection bottles were changed every 12 to 24 hours. The volume of lymph was measured, the cells counted and the lymph flow rate and cell output calculated. A sample of lymph was then centrifuged, a portion of the cell-free lymph frozen for subsequent antibody titrations and a smear made of the cells.

**Blood Samples.** Blood samples were obtained from the jugular vein. The blood was collected into centrifuge tubes containing powdered heparin, centrifuged at 2,000 rpm for 10 minutes and the plasma drawn off and stored frozen for subsequent antibody titrations.

**Detailed Methods**

**Surgical Procedures**

All animals undergoing surgery were starved for the preceding 12 hours with the exception of very young lambs. Anaesthesia was induced by intravenously administered thiopentone sodium at a dose rate of 0.1g per 5.0Kg body weight. The sheep were then intubated with a cuffed Magill endotracheal tube and anaesthesia was maintained with a mixture of halothane and oxygen administered through the closed circuit of a Boyle's anaesthetic machine (British Oxygen Company). Where two animals were to be operated on simultaneously, as in the case of reciprocal homografts, one animal was induced with thiopentone and maintained with closed circuit halothane anaesthesia, while the other was induced and maintained with intravenously administered pentobarbitone sodium. In this case induction was obtained with a dose rate of 12.5mg per Kg and small maintenance doses were given when required.

**Cannulae.** The cannulae used for lymphatic cannulation were either polyvinyl or polyethylene (Dural Plastics,
New South Wales). Various sizes of cannulae were used depending on the size of the lymphatic duct.

Preparation of the surgical site. All wool was closely clipped from the general operative area. The skin was then scrubbed thoroughly with a 1.0 per cent solution of chlorhexidine ('Hibitane', I.C.I.), and the whole animal covered with sterile drapes leaving only the operation site exposed.

Cannulation of the efferent popliteal duct. This duct was cannulated following the method of Hall and Morris (1962). An incision is made through the skin and subcutaneous tissues beginning about 2 to 3 cm below a point midway between the trochanter major and the sciatic tuberosity, and continued down the leg for 5 to 10 cm depending on the size of the animal. The incision is continued through the aponeurosis between the posterior head of the biceps femoris and the semitendinosus muscles which are then separated with wound retractors. Muscular branches of the posterior femoral artery and vein can be seen crossing the field. These vessels are divided between two ligatures of 0 silk. Blunt dissection then reveals the posterior femoral vein and artery and the efferent popliteal duct can usually be found lying posteromedial to the artery and medially to the vein. The duct is dissected free of surrounding fat and connective tissue and a ligature of 3/0 silk placed around it and the duct tied off as far from the node as possible. A second ligature is then placed around the duct about 1 cm below the occluding tie, but this is left loose. A cannula of external diameter about the same as that of the duct is then led out through a stab incision in the skin and placed so as the cannula lies in the same direction as the duct. A small incision is then made in the duct with iridectomy scissors, taking care to
avoid any valves. The cannula is inserted into the duct, gently pushed beyond the lower ligature and then firmly tied in place. The flow of lymph is checked and provided it is running freely, the cannula is then fixed in position with further ligatures. Powdered crystalline penicillin is dusted into the wound, and the skin incision closed with Michel clips. It was not found necessary to suture the muscles together.

**Cannulation of the efferent prefemoral duct.** A vertical incision is made parallel but slightly anterior to the cranial border of the tensor fascia lata. The subcutaneous tissues are divided by sharp and blunt dissection and the duct can be found running in a proximal direction under the edge of the tensor fascia lata muscle, in association with the blood vessels supplying the prefemoral lymph node. Cannulation is effected as described for the popliteal duct.

**Cannulation of the main intestinal lymph trunk.** An incision is made on the right side of the abdomen beginning just lateral to the transverse process of the first lumbar vertebra, and continuing ventrally, parallel and just posterior to the last rib for some 10 to 15cm. The incision is continued through the abdominal muscles and peritoneum. The loops of the small intestine and the right and caudate lobes of the liver are packed off and the right kidney retracted. It is usually best to cut through the ligament which binds the caudate lobe of the liver to the vena cava to allow this lobe to be retracted. The peritoneum overlying the pancreas and the inferior vena cava is divided and blunt dissection of the connective tissue reveals the large milky main intestinal trunk. This duct runs dorsally in the mesentery and emerges through the substance of the pancreas, then turns posteriorly
and runs for a short distance parallel to the posterior vena cava before passing under this vein to join the cisterna chyli. One or more efferent ducts from the portal hepatic node and from a node adjacent to the hepatic artery can be seen joining the intestinal trunk at some part of its length now exposed. Cannulation of the trunk is done in the same manner as for other lymph ducts. The correct placement of the cannula is crucial.

Cannulation of the lumbar trunk. The lumbar trunk is located running alongside the abdominal aorta. An incision is made on the left side mid-way between the coxal tuber and the last rib extending ventrally for 10 to 15 cm from the transverse processes of the lumbar vertebrae. The intestines are packed off and the lumbar trunk isolated for a distance of about 5 to 6 cm cranially from the bifurcation of the abdominal aorta. This duct is relatively large and the cannula easy to position so as to obtain a free flow of lymph.

In Utero Foetal Surgery

Anaesthetised pregnant ewes are positioned in ventral recumbency and a mid-line incision is made extending from the umbilicus posteriorly as far as possible toward the mammary gland. The incision is continued through the linea alba and peritoneum. The pregnant uterus is brought out of the peritoneal cavity and placed in a wet polythene bag to prevent it from drying out. The foetus is palpated through the uterine wall to determine its position and the plastic bag is cut over the area where the uterine incision is to be made. This incision is sited so as to allow subsequent partial exteriorisation of the fore parts of the foetus. The uterus is incised transversely through the muscular coats taking great care to avoid any cotyledons or major blood
vessels. Bleeding is immediately stopped by electro-cautery. The incision is continued through the uterus until the foetal membranes are visible, and then widened by blunt dissection. Any vessels visible on the membranes are sealed by cautery. The foetal membranes which are now exposed consist of the allantois and amnion. If the foetus is more than 50 days of age, these two membranes are generally fused to form, in effect, one membrane. The fused membranes are picked up with forceps and the amniotic cavity opened with a small incision. The amniotic fluid is then removed by suction. 500,000 I.U. of crystalline penicillin is added to the amniotic fluid which is kept at 40°C until it is returned to the uterus. The foetus is then manoeuvered up to the now enlarged incision.

Thymectomy. The head and neck of the foetus are brought out through the incision and the head extended to reveal the ventral surfaces of jaws, neck and thorax. The foetus is packed around with gauze swabs which are kept continually moist. This is most important as the foetal tissues are extremely soft and subject to desiccation and bruising. An incision is made through the skin from the manubrium sternum to the epiglottis. The skin is gently dissected away from the underlying brachio-cephalic and sterno-thyro-hyoid muscles, which are divided along the mid-line. This procedure exposes the trachea, on each side of which can be seen the posterior portion of the cervical thymus. Further dissection in a posterior direction reveals the thoracic inlet which is gently enlarged by delicate blunt dissection. As much of the thymus as possible is removed from the thoracic inlet and dissection continued cranially on each side of the trachea. Frequently the thymus is continuous along the whole length of the neck, but occasionally it is interrupted and care must
be taken to ensure each of these small individual islands of thymic tissue are removed. This is especially important in the cranial part of the neck where the thyroid gland, which is relatively large, commonly overlies parts of the thymus. The most cranial part of the thymus consists of a mass of tissue closely apposed to the parotid salivary gland. Dissection in this region must be done extremely carefully to ensure none of the major vessels or nerves in the area are damaged and that the parathyroid glands are not removed in the process. After all the cervical thymus has been removed the skin incision is sutured with single interrupted sutures of 5/0 silk attached to an eyeless needle ('Atraloc', Ethnor Pth. Ltd.).

The foetus is then gently withdrawn further from the uterus and placed on its right side so that the left side of the thorax is exposed. The thoracic portion of the thymus is approached by making an incision behind the left shoulder. The left leg of the foetus is drawn forward and the thoracic wall incised along its whole width. The incision is continued down through the latissimus dorsi and deep pectoral muscles. The intercostal muscles and pleura between the second and third ribs is incised and the thorax opened. The thoracic thymus can be seen lying within the mediastinum just anterior to the heart. The thymus is removed by blunt dissection care being taken to avoid damage to vital structures such as the phrenic and vagus nerves and the great vessels which are immediately contiguous with the thymus. Care must be taken to ensure that no thymic tissue remains within the thoracic inlet.

The ribs are sewn together with one or two simple interrupted sutures of 5/0 silk and the skin and muscle incision is closed with one or two rows of sutures.
The foetus is then gently returned to the uterus and the amniotic fluid is returned to the sac. Closure of the foetal membranes is effected by gathering the edges of the incised membranes together and tying them off with a purse string suture. Frequently several sutures are required before the closure is complete. The uterine incision is then closed with a single row of interrupted inverting mattress sutures using size 1 plain gut. The uterus is returned to the abdomen and the abdominal incision is closed with a single row of horizontal mattress sutures using 4 silk or extra heavy Vetafil (Bengen & Co., Hannover, West Germany). The skin incision is closed with Michel clips.

**Splenectomy.** The foetus is delivered from the uterus as before and positioned to expose the left side of the abdomen. An incision is made through the skin, muscles and peritoneum just posterior to the last rib. The spleen is found lying anterior to the incision, and is gently dissected away from its attachments. The single splenic artery and vein are clamped with haemostats and removal of the intact spleen completed. Little or no bleeding occurs when the haemostats are released. The abdominal muscles and peritoneum are sutured with 5/0 silk and then the skin incision is closed in a similar manner.

**In Utero Cannulation of Lymphatic Ducts**

These operations were performed on near term foetuses. Since these foetuses are quite large, and at this stage the uterus is rather irritable, it is inadvisable to withdraw the foetus from the uterus as it then tends to contract rapidly, and return of the foetus is frequently impossible. Because of this the uterine incision is made over the general area of the foetus which is to be operated on, and the actual foetal surgery is performed through the wall of
the uterus. Under these conditions it was rarely necessary to remove any amniotic fluid. A long length of cannula was used to allow for the in utero and intra-abdominal distance to be traversed. Lymph flows were best maintained if the free end of the cannula was at a level below that of the foetus.

Main intestinal trunk. An incision is made in the uterus over the right side of the foetal abdomen. Retraction of the incision and a small degree of repositioning allows the foetal incision to be made without exteriorisation. The subsequent cannulation procedure is the same as described previously with the exception that about 1 metre of cannula is used. Location of the duct is more difficult since it is not filled with milky chyle as it is in post-natal lambs, and all foetal tissues, especially the liver, must be handled with extreme care due to their extreme fragility. After cannulation and suturing of the foetus, the cannula is led out through the uterine incision, the foetal membranes are tied off and the uterus sutured around the cannula. About 20 to 25cm of the cannula is then coiled and sutured to the uterine wall. This coiled length allows for any subsequent strain on the cannula to be taken up. The uterus is returned to the abdomen and the free end of the cannula is passed out through a stab incision high in the abdominal wall of the ewe nearest to the pregnant horn. The abdominal incision is sutured in the usual way.

Lumbar trunk. The approach to the lumbar trunk in the foetus is the same as in adult sheep. The duct is easily exposed and isolated as the foetal bowel is small and empty. This duct in the foetus is very thin walled but as the flow of lymph is quite brisk it is not difficult to cannulate. After an appropriate sized cannula has been passed into the
duct and tied in place the foetal incision is closed and the cannula led out through the foetal membranes and the uterus. Extra coils of cannula are left in the uterus and the peritoneal cavity of the ewe to allow for subsequent movements of the foetus and the uterus. The cannula is brought out through a stab incision high in the abdominal wall in front of the coxal tuber.

**Efferent popliteal duct.** The approach to and entry of the uterus and amniotic sac is accomplished as described above. One hind limb of the foetus is exteriorised and the efferent popliteal duct located and cannulated as described above for the normal post-natal lamb. The further procedures are the same as described for other foetal lymphatic cannulations.

**Caesarian Section**

The lymph flow from lambs cannulated in utero was monitored for several days and the lambs were then delivered by Caesarian section. The ewes were anaesthetised and the original abdominal and uterine incisions were re-opened. The foetus was then removed and since the ewes were under halothane anaesthesia, the lambs were also anaesthetised and frequently resuscitation procedures were necessary.

Resuscitation measures consisted of draining fluid from the trachea and bronchi by holding the lamb upside down by the hind legs and massaging the chest. If necessary an endotracheal tube was inserted and positive pressure respiration was applied using a Bird Respirator. Spontaneous respiration generally followed in a short time when all residual anaesthetic gas had been expired.

**Application of Homografts**

Homografts were applied either as full thickness fitted grafts or following the method of Schinkel and Ferguson (1953).
Full thickness grafts. Two sheep were anaesthetised and the lateral aspect of one foreleg of each sheep was clipped and scrubbed first with Hibitane and then with PhisoHex (Winthrop Laboratories Ltd.). Residual PhisoHex was washed off with saline. Two pieces of skin of identical size were then cut from the sterilized area and placed in saline to which had been added crystalline penicillin. The second sheep was subjected to the same procedure. Any bleeding was stopped by clamping the cut vessels with haemostats rather than by cautery.

Autografts were applied in each case by moving the lower piece of skin to the upper site. For homografts the skin removed from the upper site in one sheep was transferred to the lower site in the other sheep.

The grafts were sutured in place with 5/0 silk attached to an 'Atraloc' needle, using a continuous mattress suture which slightly everted the cut edges. The grafts were dusted with crystalline penicillin and covered with 'Nonad Tulle' (Allen & Hanbury Ltd.), and the leg was then bandaged with dry gauze and covered with Elastoplast (Smith & Nephew). The bandages were left intact for 5 days and then removed and the grafts were then inspected daily.

Subcutaneous grafts. These were applied using the method of Schinkel and Ferguson (1953). The skin on the thorax was scrubbed with Hibitane and PhisoHex as described previously. Small pieces of skin, approximately 0.75 x 0.5cm, were cut from the relatively wool free axillary area and placed in the penicillin saline solution. Small vertical incisions were then made over the ribs and subcutaneous pockets were produced by blunt dissection. The pieces of skin to be grafted were then inserted in the pockets, so that the subcutis of the graft was apposed to the subcutis of the
graft bed and the epidermal surface was turned in on itself. The grafts were sutured in place using 5/0 silk attached to an 'Atraloc' needle. Generally 3 autografts and 3 homografts were applied at the same time. The grafts were examined subsequently by excising the whole graft and the surrounding skin and subcutaneous tissues.

Preparation and Administration of Antigens

Chicken red blood cells. Adult hens were bled by nicking the wing vein with a scalpel blade. The blood was collected into a centrifuge tube containing powdered heparin. This was centrifuged at 2,000 rpm for 5 minutes, washed 3 times and resuspended in saline at a concentration of $2 \times 10^9$ red cells per ml. One ml was injected subcutaneously in the lower lateral aspect of the hind leg.

Swine influenza virus. Viable swine influenza virus (strain Shope 15) was grown in the allantoic cavity of 10 day old chicken embryos. The allantoic fluid was harvested 96 hours later and centrifuged at 3,000 rpm for 10 minutes at $4^\circ C$ to deposit particulate material. The supernatant was decanted and added to one tenth its volume of packed washed chicken red cells. The virus was absorbed onto the red cells at $0^\circ C$ for 30 minutes. The red cell suspension was then centrifuged at 2,000 rpm for 10 minutes and the supernatant removed. The packed red cells were then resuspended in a volume of saline to give a virus strength of 10 to 20,000 haemagglutinating units per ml. The virus was eluted off the red cells by incubation at $37^\circ C$ for 60 minutes followed by centrifugation at 3,000 rpm for 10 minutes. The supernatant containing the virus was removed and stored at $4^\circ C$.

The antigenic dose used was 10 to 20,000 haemagglutinating units suspended in 1.0ml of saline given subcutaneously into the lower hind leg.
Killed Salmonella muenchen. Salmonella muenchen cultures were grown on nutrient agar slopes at 37°C for 24 hours. The bacterial growth was then removed from the slopes and suspended in 5.0ml of saline. A 0.5ml aliquot of this suspension was poured on to the surface of a series of nutrient agar plates and incubated at 37°C for a further 24 hours, at which time the surface of the plates was covered with a confluent growth of organisms. These subcultures were then removed from the surface of the plates and suspended in a small volume of saline. The concentration of bacteria in this suspension was assayed by plating serial dilutions on to the surface of nutrient agar plates which were incubated at 37°C for a further 24 hours. The individual colonies on each plate were then counted and the titre of the original suspension thus determined. The concentration of the suspension was then adjusted to contain $2 \times 10^{10}$ organisms per ml, and then boiled for 30 minutes to kill all the bacteria then stored at 4°C. Immediately prior to use one volume of the killed suspension was diluted in 9 volumes of saline to give a concentration of $2 \times 10^9$ killed organisms per ml. The antigenic dose used was 1.0ml of this diluted suspension injected subcutaneously or intravenously.

Salmonella lipopolysaccharide. Salmonella lipopolysaccharide was prepared by the method of Halliday and Webb (1965). Stock Salmonella muenchen bacteria were subcultured on nutrient agar plates for 24 hours at 37°C and the fresh bacterial culture inoculated into 2 x 1 litre bottles of sterile nutrient broth (Difco Laboratories). The broth cultures were shaken at 37°C for 24 hours and then centrifuged at 6,000 rpm at 4°C for 30 minutes to deposit the bacteria. The supernatant was removed and the sedimented bacteria washed twice and resuspended in 400ml of saline. 16.0ml of
1N NaOH was added to the bacterial suspension and the mixture incubated at 37°C for 16 hours. The alkaline suspension was then neutralized with 1N HCl after which it was centrifuged at 6,000 rpm for 30 minutes and the supernatant decanted. The supernatant was treated with acetone (0.5 vol acetone of 1.0 vol of supernatant) and centrifuged. A further 0.5 vol of acetone was added to the supernatant and held at -40°C for 3 hours. The precipitated lipopolysaccharide was collected by centrifugation at 6,000 rpm for 30 minutes and resuspended in a small volume of acetone and again centrifuged. The final deposit was dried in a desiccator over CaCl₂ and then ground to a fine powder.

**Ferritin.** 0.2g of lyophilized ferritin (Mann Research Laboratories) was dissolved in 10.0 ml of saline to give a 2.0 per cent solution. Equal volumes of the ferritin solution and incomplete Freund's adjuvant were emulsified and 1.0ml injected subcutaneously over the right side of the thorax. A 0.5 per cent solution of ferritin was used for intradermal injections. 0.1ml was injected into the wool free area of the medial aspect of the thigh.

**B.C.G.** Viable B.C.G. (C.S.L.) was resuspended in the diluent supplied to produce a suspension containing 1.0mg of organisms per ml. The sensitising dose used was 0.5mg injected subcutaneously over the right side of the thorax. Intradermal injections of 0.1ml of tuberculin (C.S.L.) were made in the wool free area of the medial aspect of the thigh.

**Homologous lymphocytes.** Sheep lymphocytes were obtained from a 24 hour collection of efferent popliteal lymph. The lymph was centrifuged and the deposited cells were washed 3 times in Eagle's medium, then resuspended to
give a concentration of $5 \times 10^7$ or $25 \times 10^7$ cells per ml in the same medium. Viability was estimated by the trypan blue exclusion test. This test was carried out adding 0.1ml of the cell suspension to a volume of 0.5 per cent trypan blue solution in saline sufficient to give a suspension which allowed satisfactory counting of the cells in a haemocytometer. The cells were allowed to stand in the trypan blue for 3 to 4 minutes and then a haemocytometer chamber was filled and the proportion of cells which took up the stain was estimated. Usually no more than 1.0 per cent of cells were dead as judged by this test.

Two dose rates of $5 \times 10^6$ and $2.5 \times 10^7$ cells in 0.1ml were injected into the wool free skin of the medial aspect of the thigh.

**Intradermal Injection of Antigens and Measurement of Skin Reactions**

All intradermal injections were made in the wool free skin of the medial aspect of the thigh. The skin was washed with 0.5 per cent Hibitane and wiped with alcohol. The injection volume was 0.1ml given with a tuberculin syringe and 30 gauge needle.

Skin measurements were made before injection and each 12 to 24 hours subsequently, using a pair of 'Schnelltaster' skin calipers. The skin was picked up in a fold and measured so that two thicknesses of skin, one of which contained the injection or reaction site, were included in each measurement. All reactions were assessed by the increase in skin thickness and the diameter of the lesion.

**Cell Counts**

A model B Coulter Counter (Coulter Electronics, Hialeah, Florida) was calibrated with chicken red cells, human red cells, sheep red cells and latex particles of known
size (Dow Chemicals). The calibration constant for this particular machine was found to be $10.0$ at a vernier setting of 16. Sheep lymphocytes could be counted with the machine set at aperture 1, current $\frac{1}{2}$, lower threshold 10 and upper threshold disabled.

Differential size counts could be performed using the settings shown in Table 2.1.

The modal cell volume was calculated by the following formula:

$$V = KIA$$

where $V$ = volume in $\mu^3$

<table>
<thead>
<tr>
<th>Aperture Setting</th>
<th>Current Setting</th>
<th>Threshold Setting</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100</td>
<td>50 - 150</td>
</tr>
<tr>
<td>2</td>
<td>150</td>
<td>150 - 210</td>
</tr>
<tr>
<td>3</td>
<td>200</td>
<td>200 - 260</td>
</tr>
</tbody>
</table>

Thus the modal cell volume of the cells counted in window 1 is

$$V = 10 \times 1 \times \frac{1}{2} \times 14$$

$$= 70$$

Although red cells were occasionally found in lymph, these did not pose a problem in counting since the sheep red cell is smaller (mean cell volume of $35 \mu^3$) than the smallest lymphocyte. All red cells were excluded from the counts by setting the lower threshold at 10, which automatically excluded all cells with a volume of less than 50.

**Preparation of Cell Smears**

A volume of lymph containing at least $15 \times 10^6$ cells was centrifuged at 1,000 rpm for 5 minutes. The supernatant lymph was decanted and the sides of the tube wiped dry. The cell pellet was then resuspended in serum and smears prepared in the usual way. Satisfactory smears could be made if one drop of serum was used for a cell pellet containing $15 \times 10^6$ cells.
The swarms were rapidly dried in air and then stained. One ml of Leishman stain was poured onto the surface of the slide and allowed to stand for 5 minutes after which the stain was diluted in 2.5 ml of distilled water. The diluted stain was left for 15 minutes and then the slides were rinsed in water and dried in air. The swarms were examined under oil using a 54:1 objective and 10 x eyepiece. Counts were performed in a random manner beginning at the edge two thirds of the way along the smear from the origin and proceeding toward the centre until at least 500 cells were counted. All cells were measured in the portion of their morphology that permitted an accurate estimation of size. The settings used on the Coulter Counter for the arbitrary division of the cells in lymph into four classes on the basis of size.

**TABLE 2.1**

<table>
<thead>
<tr>
<th>Window</th>
<th>L.T.</th>
<th>U.T.</th>
<th>Range</th>
<th>M.C.V.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>30</td>
<td>50 - 150</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>30</td>
<td>42</td>
<td>150 - 210</td>
<td>180</td>
</tr>
<tr>
<td>3</td>
<td>42</td>
<td>62</td>
<td>210 - 310</td>
<td>260</td>
</tr>
<tr>
<td>4</td>
<td>62</td>
<td>disabled</td>
<td>310 or &gt; 500</td>
<td>405 or &gt;</td>
</tr>
</tbody>
</table>

L.T. = Lower Threshold
U.T. = Upper Threshold
Range = Size distribution in $\mu^3$
M.C.V. = Modal cell volume in $\mu^3$

The settings used on the Coulter Counter for the arbitrary division of the cells in lymph into four classes on the basis of size.
The smears were rapidly dried in air and then stained. One ml of Leishman stain was poured onto the surface of the slide and allowed to stand for 5 minutes after which the stain was diluted in 2.5ml of distilled water. The diluted stain was left for 15 minutes and then the slides were rinsed in water and dried in air. The smears were examined under oil using a 54:1 objective and 10 x eyepiece. Counts were performed in a consistent manner beginning at the edge two thirds of the way along the smear from the origin and proceeding toward the centre until at least 500 cells were counted. Cells were classified according to their morphology and staining characteristics into 5 main categories.

1. Small lymphocytes, 5-8µ diameter. These are small round cells with a dense nucleus and a thin rim of basophilic cytoplasm.

2. Medium lymphocytes, 8-11µ diameter. These are the most common cells seen in lymph. The nucleus is less dense and the cytoplasm more abundant than in the small lymphocyte. The cytoplasm is not basophilic.

3. Large lymphocytes, 11-15µ diameter. These cells are characterized by abundant non-basophilic cytoplasm and moderately dense nuclei.

4. Basophilic cells 5-15µ diameter. The nucleus of these cells is moderately dense and the cytoplasm very basophilic.

5. Blast or transitional cells. These cells are very large with abundant pale cytoplasm and pale nuclei.

**Autoradiography**

**Preparation of cells.** A volume of lymph containing 1 x 10^8 cells was centrifuged at 1,000 rpm for 5 minutes and the cells resuspended in 1.0ml of cell-free lymph. A solution of tritiated thymidine of specific activity 5000mc/mM was added to...
added to the cell suspension to give a concentration of 10µc/ml. The mixture was incubated at 37°C for 60 minutes in a water bath. The cell suspension was then centrifuged and washed 3 times in cell-free lymph and after the third wash, the pellet of cells was resuspended in 6 drops of serum. At least 6 smears of each sample were made in the usual manner on acid washed glass microscope slides.

**Preparation and development of autoradiographs.** Kodak Stripping Film AR10 was used to cover all autoradiographs. All procedures were carried out in the darkroom using a safe lamp fitted with a 'Wratten' Series 1 (Red) Safelight Filter over a 25 watt bulb. The stripping film was cut with a razor blade into pieces of 3 x 8cm and floated, emulsion side down, on distilled water at 23°C. The pieces of film were allowed to expand for at least 2 minutes and then floated onto the slide. The coated slides were then dried with the aid of a fan and then placed in a light proof box containing a small amount of CaCl₂ desiccant and kept at 4°C for 4 to 7 days after which they were developed.

Development was performed at a constant temperature of 16°C in the dark. The slides were developed for 10 minutes in Kodak D19 developer, acid fixed and dried in air. The slides were stained with 0.1 per cent Azure A in acetate buffer of pH5.2 for 15 minutes then washed in tap water, dried and examined.

**Preparation of Tissues for Histological Examination**
Tissues were removed from post-mortem specimens immediately after death and fixed in 10.0 per cent formol saline. Conventional sections of 5µ were cut and stained routinely with haematoxylin and eosin or pyronin methyl green.
Preparation of Tissues for Electron Microscopic Examination

**Tissues.** Samples of tissues were removed from anaesthetised animals and immediately cut into small pieces and placed in cold 1.0 per cent OsO$_4$ in 50 per cent Tyrode's solution. Fixation was continued for 2 hours when the samples were washed in Tyrode's solution for 1 to 2 hours then stained in 1.0 per cent aqueous uranyl acetate for a further 2 hours. Dehydration was carried out through successive changes of 50, 70 and 90 per cent and 2 changes of dry acetone, and embedded in araldite (Durcupan, Fluka AG, Switzerland).

**Cells.** A sample of lymph was centrifuged, the cell-free lymph decanted and the pellet of cells resuspended in a small amount of serum. Initial fixation was carried out by adding 3 to 5ml of 1.25 per cent glutaraldehyde (Fluka AG, Switzerland) which was allowed to stand for 10 minutes then centrifuged for 5 minutes at 1,000 rpm and the glutaraldehyde decanted. 3.0ml of 1.0 per cent osmium tetroxide in Tyrode's solution diluted 1:1 with distilled water was then added, the cell pellet broken up into small pieces and allowed to stand at room temperature for 2 hours, after which the OsO$_4$ solution was pipetted off, and replaced by 3.0ml of 1.0 per cent formaldehyde in 50 per cent calcium free Tyrode's solution. After 30 minutes the formaldehyde was removed by pipetting and replaced by 1.0ml of a 1.0 per cent aqueous solution of uranyl acetate which was allowed to stand for 2 hours. Dehydration of the samples was carried out through successive changes of 50, 70 and 90 per cent and finally two changes of dry acetone. The dehydrated samples were then embedded in araldite (Durcupan, Fluka AG, Switzerland).

Sections were cut on an LKB ultramicrotome using glass or diamond knives, mounted on 200 mesh copper grids.
(E. F. Fullam, New York), overstained with Millonig's lead stain (Millonig, 1961) for 1 hour and examined in a Philips E.M.U. electron microscope.

**Titration for Specific Antibody**

All lymph and sera samples were routinely inactivated at 56°C for 30 minutes prior to titration.

*Antibody to swine influenza virus.* Antibody to swine influenza virus was measured by the haemagglutination inhibition method of Fazekas de St. Groth and Webster (1966). This test poses two problems. Firstly, the chicken red cells which are used as the indicator in this test frequently vary in their susceptibility to the agglutinating effect of virus in the presence of serum and some fowl cells can give quite high titres even when the serum contains no specific antibody (Anderson, Burnet and Stone, 1946). Fowls were therefore selected as donors of red cells by the following method. A number of fowls were bled and their red cells washed 3 times in saline. The cells were then made up to a 5.0 per cent suspension in haemagglutinating diluent. Normal rabbit and sheep serum samples were then heated at 56°C for 30 minutes and diluted out in perspex trays in a twofold fashion from a starting dilution of 1:20. To each well was added one 0.025ml drop of virus suspension containing 4 haemagglutinating units. After standing at room temperature for 30 minutes, one 0.025ml drop of the red cell suspension was added to each well, the trays shaken, and the results read 35 minutes later. Those fowls which showed the most complete agglutination in all wells were selected as donors.

The second problem is the presence in serum of factors other than specific antibody which prevent agglutination of the red cells by virus. It was found that sheep serum regularly contained these non-specific factors, occasionally
in high titres, although lymph was relatively free. To overcome this problem the trypsin-periodate inactivation method of Jensen, Dunn and Robinson (1958) was employed. 0.2ml of plasma or lymph were mixed with 0.2ml of trypsin solution and incubated at 56°C for 30 minutes in a water bath. The samples were then cooled to room temperature and 0.2ml of 0.011M potassium periodate solution was added. The samples were allowed to stand at room temperature for 15 minutes when 1.0ml of a 3.0 per cent solution of glycerol in saline was added. This provided a 1:10 dilution of the original samples.

Measurement of IgG antibody. All samples were routinely treated with 2-mercapto-ethanol to measure the titre of IgG antibody. 0.5ml of the trypsin-periodate treated 1:10 dilution was added to 0.5ml of a 0.2M solution of 2-mercapto-ethanol in saline. The samples were incubated at 37°C for 30 minutes in a water bath. This gave a 1:20 dilution. To the 1.5ml remaining of the initial 1:10 diluted trypsin-periodate treated samples, 1.5ml of haemagglutinating diluent was added giving a dilution of 1:20.

All titrations were carried out at room temperature and done in duplicate using perspex agglutination trays. 0.25ml of haemagglutinating diluent was placed in each well with the exception of the first well in each row. 0.5ml of each sample was then placed in the first well and twofold serial dilutions were made across the tray using a 0.25ml Takatsky loop. One 0.025ml drop of virus suspension containing 4 haemagglutinating units was then added to each well using a calibrated dropper. The trays were then stood at room temperature for 1 hour when one 0.025ml drop of 5.0 per cent chicken red cells was added to each well, the tray shaken and stood for 35 minutes when the results were read.
The end point was determined by reading 7 intermediate degrees of agglutination between complete agglutination (+++) and no agglutination (-) and interpolating to the conventional partial agglutination (+) end point.

Titres were recorded in terms of $\log_2$ units, with the first well designated as 0 and subsequent wells 1 to 9. $\log_2$ units are converted to $\log_{10}$ units by multiplying by 0.3. The starting dilution of 1:20 must be allowed for by adding $\log_{10} 20 = 1.3$. For example, a titre recorded as 5.8 in $\log_2$ units is converted as follows:

$$
\begin{align*}
5.8 \\
\times 0.3 \\
1.74 \\
+ 1.3 \\
3.04
\end{align*}
$$

Thus a $\log_2$ titre of 5.8, using a starting dilution of 1:20 is 3.04 in $\log_{10}$ units. To convert this to reciprocal terms, 3.04 is the $\log_{10}$ of 1096. Thus a titre recorded as 5.8 represents a dilution of 1:1096. This is the activity of antibody per 0.25ml of lymph or serum. The accuracy of this method is ± 10 per cent (Fazekas de St. Groth and Webster, 1966).

Antibody to Salmonella muenchen lipopolysaccharide. Chicken red cells sensitised with bacterial lipopolysaccharide were used as the indicator in this test and they were prepared by the method of Halliday and Webb (1965). Equal volumes of a 5.0 per cent suspension of chicken red cells and a solution of bacterial lipopolysaccharide containing 50µg per ml in saline were incubated at 37°C for 60 minutes. The mixture was then centrifuged and the cells washed 3 times and resuspended to a 5.0 per cent concentration in saline.

The test was carried out in perspex agglutinating
trays. 0.25ml of haemagglutinating diluent was deposited in each well, apart from the first well in each row which received 0.5ml. 0.1ml of each sample was then added to the first well and serial twofold dilutions were made using a 0.25ml Takatsky loop. One 0.025ml drop of sensitised red cells was then added to each well, the trays shaken and the results read 35 minutes later. End points were read and recorded as for the swine influenza antibody titrations. Calculations were also made in a similar manner bearing in mind the starting dilution in these titrations was 1:6.

Assay for Antibody Forming Cells in Lymph

Cells producing antibody to Salmonella lipopolysaccharide were assayed using the technique of Cunningham and Szenberg (1968). The assay chambers were made by applying 2 strips of 'Scotch' brand double coated tape No. 4010 to a microscope slide 22mm apart. The backing is removed and a coverslip 22mm x 33mm is applied and pressed to seal onto the tape. This provides a chamber of approximately 0.05ml volume.

A quantity of lymph containing at least $5 \times 10^7$ cells was centrifuged at 1,000 rpm for 5 minutes. The cells were washed 3 times and resuspended in cold Eagle's medium to give a concentration of $10^5$ to $10^7$ cells per ml. To 0.5ml of this cell suspension was added 0.1ml of a 1:6 dilution in Eagle's medium of sheep erythrocytes previously sensitised to Salmonella lipopolysaccharide (as described above for chicken red blood cells) and 0.05ml of guinea pig complement from which had previously been absorbed all antibody to Salmonella lipopolysaccharide. 0.05ml of the sensitised erythrocyte-complement-lymphocyte mixture was then used to fill one counting chamber which was then sealed with heated paraffin (Vaseline) and incubated at 37°C for 30
minutes. The chambers were examined under low power and the number of plaque forming cells (pfc) counted.

The number of pfc per chamber could be converted to the number of pfc per million lymphoid cells as follows. The initial 0.5ml of lymphoid cell suspension was diluted to a volume of 0.65ml. Therefore -

Concentration of cells in the reaction mixture

\[ \text{Concentration of cells in the reaction mixture} = X \times \frac{0.5}{0.65} \times 10^6/\text{ml} \]

Since the chamber contains 0.05ml

then No. of plaques per ml of reaction mixture

\[ \text{No. of plaques per ml of reaction mixture} = 20 \times \text{No. of plaques/chamber} \]

\[ \text{pfc/million} = \frac{20 \times \text{pfc/chamber}}{X \times 0.77} \]

or more simply expressed -

\[ x = \frac{26y}{z} \]

where

- \( x \) = pfc/million
- \( y \) = pfc/chamber
- \( z \) = initial concentration of cells in the reaction mixture (\( x \times 10^6 \))

Agar Electrophoresis

Electrophoresis of lymph and sera was carried out using a LKB 6800A immuno-electrophoresis apparatus (LKB Produkter AB, Stockholm, Sweden) following the method of Scheidegger (1955) which is a modification of the original method of Grabar and Williams (1953).

Buffered agar solution was prepared by dissolving 1.0g of Special Agar Noble (Difco Laboratories) in 25.0ml of 0.1M Barbital buffer, pH 8.6 and 75.0ml of distilled water. The agar was dissolved by boiling and the solution was stored in 11.0ml volumes at 4°C until required.
Agar coated slides were prepared by pouring 10.0ml of heated agar over a row of slides placed in the special frame. It was found that prior sealing of the slides to the frame with 1.0ml of agar prevented any leakage and resulted in layers of uniform thickness. The gel layer was solidified in a humid chamber for at least 30 minutes after which the troughs and wells were cut with the gel punch. The agar plugs were then sucked out of the wells and the samples to be analysed were deposited in the wells with a 5μl micro-pipette.

The electrophoresis run was then carried out at 35 amps and 250 volts for 60 minutes. The troughs were then emptied of agar and filled with rabbit anti-sheep serum using a 0.1ml pipette. The slides were then placed in a humid chamber for at least 20 hours to allow the antigen-antibody reaction to occur and precipitation lines to develop, after which they were immersed in 1.0 per cent NaCl solution for 6 hours then in a second 1.0 per cent NaCl solution for 16 hours to remove any unprecipitated protein. The slides were then dried and allowed to stand for 10 minutes in 1.0 per cent Amido black in acetate buffer and rinsed 4 times in rinsing solution.

**Statistical Analysis**

The mean "\( \bar{x} \)" of a series of observations \( x_1, x_2, x_3, \ldots, x_n \) is given by \( \bar{x} = \frac{\sum x}{n} \) where "n" is the number of observations and \( \sum x \) is the sum of the observations.

The variance "V" is taken as the total sum of the squared deviations divided by one less than the total number of observations and is given by

\[
V = \frac{\sum (x-\bar{x})^2}{n-1}
\]
The standard error of \( n \) observations is calculated as the square root of the variance divided by the square root of the number of observations and is given by

\[
S.E. = \frac{\sqrt{\frac{V}{n}}}{n}
\]

The significance of the difference between two means is calculated using Student's 't' test and is given by

\[
t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{N_1 + N_2}{\sum (x-\bar{x}_1)^2 + \sum (x-\bar{x}_2)^2}}} \sqrt{\frac{N_1 N_2 (N_1 + N_2 - 2)}{N_1 N_2 (N_1 + N_2 - 2)}}
\]

where \( N_1 \) and \( N_2 \) represent the number of observations in each group.

The regression coefficient "b" is calculated from the formula

\[
b = \frac{\sum (x-\bar{x})}{\sum (x-\bar{x})^2}
\]

where \( \sum (x-\bar{x}) = \sum xy - \frac{\sum x - \sum y}{n} \)

Then \( y = a + bx \)

where \( a = \overline{y} - bx \)

The variance of \( y \) (\( V_y \)) is calculated as

\[
V_y = \frac{1}{n-2} \sum (y-\overline{y})^2 - \frac{\sum (y-\overline{y}) (x-\overline{x})}{\sum (x-\overline{x})^2}
\]

The variance of \( b \) (\( V_b \)) is calculated as

\[
V_b = \frac{V_y}{\sum (x-\overline{x})^2}
\]
The standard deviation of $b$ ($S_b$) is calculated as

$$S_b = \sqrt{V_b}$$

To determine the significance of the difference between two regression coefficients, the 't' test was used and is given by

$$t = \frac{b_1 - b_2}{\sqrt{V b_1 + V b_2}}$$

where the degrees of freedom are $(n_1 - 2) + (n_2 - 2)$.
CHAPTER III

THE GROWTH AND DEVELOPMENT OF THE LYMPHOID SYSTEM IN LAMBS

The development of the lymphoid system has been studied in various species, but little information is available on the overall development of the lymphoid system in the sheep. The growth of the lamb has been described by Barcroft (1946) using British breeds of sheep, and by Stephenson and Lambourne (1960) using the Australian Merino but the only data available on lymphoid development in the sheep has been provided by Wallace (1945) and Kay, Playfair, Wolfendale and Hopper (1962). These data are meagre and devoted entirely to the development of the thymus. In view of this paucity of information, and because of the relatively early development of some aspects of immunological competence in the sheep, in relation to birth (e.g. Ain et al., 1963b), the growth and development of the lymphoid organs of the lamb during foetal life and after birth were investigated. The post-natal period was also investigated to study the effect of privation of maternally derived antibody on the subsequent development of the lymphoid system.

Foetal lambs were examined at various stages of gestation. Where the date of service was not known the age of the foetus was estimated by reconciliation with data of Stephenson and Lambourne (1960). The foetuses were removed from intact pregnant uteri, weighed wet, and the thymus, spleen and a selection of peripheral lymph nodes (popliteal, prefemoral and prescapular nodes) and one abdominal node (the hepatic) were dissected out, cleaned of fat and connective tissue and weighed wet.

Agammaglobulinaemic lambs were obtained from ewes with covered udders as soon as possible after birth and hand reared without maternal contact. The development of the lymphoid system was studied in these animals.
The Growth and Development of the Lymphoid System in Lambs

The development of the lymphoid system has been studied in various species, but little information is available on the overall development of the lymphoid system in the sheep. The growth of the lamb has been described by Barcroft (1946) using British breeds of sheep, and by Stephenson and Lambourne (1960) using the Australian Merino but the only data available on lymphoid development in the sheep has been provided by Wallace (1945) and Kay, Playfair, Wolfendale and Hopper (1962). These data are meagre and devoted entirely to the development of the thymus. In view of this paucity of information, and because of the relatively early development of some aspects of immunological competence in the sheep, in relation to birth (Silverstein et al., 1963b), the growth and development of the lymphoid organs of the lamb during foetal life and after birth were investigated. The post-natal development of agammaglobulinaemic lambs was also investigated to study the effect of deprivation of maternally derived antibody on the subsequent development of the lymphoid system.

Foetal lambs were examined at various stages of gestation. Where the date of service was not known the age of the foetus was estimated by reconciliation with data of Stephenson and Lambourne (1960). The foetuses were removed from intact pregnant uteri, weighed wet, and the thymus, spleen and a selection of peripheral lymph nodes (popliteal, prefemoral and prescapular nodes) and one abdominal node (the hepatic) were dissected out, cleaned of fat and connective tissue and weighed wet.

Agammaglobulinaemic lambs were obtained from ewes with covered udders as soon as possible after birth and hand
reared on cow's milk. The same lymph nodes were examined from these post-natal lambs as from the foetuses. In addition, the mesenteric lymph nodes were weighed.

Results

Growth

Pre-natal

As has been previously reported for every other species studied, the thymus was the first organ in the lamb to show lymphoid characteristics. The earliest age at which a thymus was large enough to be dissected out macroscopically was in a foetus estimated to be 44 days post-conception. The thymus in this foetus weighed 17mg. The spleen was first visible macroscopically at around 60 days, while the particular lymph nodes that were examined could not be located before 80 days of age. Peyer's patches were not visible until relatively late in gestation (around 120 days). All these organs could be identified by microscopic examination earlier, but it would not have been possible to measure their size accurately.

The growth of the body and these individual lymphoid components is shown in Fig. 3.1. The pre-natal increase in weight in all cases occurred in a logarithmic fashion. The thymus grew rapidly from around 20mg at 45 to 50 days of age to reach a maximum weight of about 20 to 25g at around 130 days. The growth rate then slowed and the thymus did not appear to gain much more in weight until soon after birth. The total body weight followed a similar pattern and the growth rate slowed over the last 2 to 3 weeks of gestation. The spleen and lymph nodes did not show this slowing of growth just prior to birth. It must be remembered that throughout this pre-natal period, these lymphoid organs are growing in the absence of any antigenic stimulation.
FIGURE 3.1

A comparison of the body weight and weight of the lymphoid organs of sheep from 70 days gestation to 100 days after birth.
Post-natal Birth to 50 days of age. The thymus increases in weight very rapidly after birth and reaches a maximum size in both absolute terms and in relation to the whole body and other lymphoid organs at around 40 days of age. Of the other lymphoid organs the spleen grows faster than the peripheral nodes but the absolute increase in size of these organs is less rapid than that of the thymus. The mesenteric nodes which were examined in the post-natal period were found to grow at a significantly more rapid rate than the peripheral lymph nodes.

The growth rates of the lymphoid organs and the body as a whole appeared to be almost linear from soon after birth up to around 50 days of age. The growth rate of the various organs before and after birth, expressed as percentages of body weight, are given in Table 3.1. It can be seen that the thymus reaches a maximum size in relation to the body weight (0.81 per cent) at about 130 days post-conception. The slowing of the growth rate from this age to just after birth results in the relative size of the thymus to the total body weight being reduced to 0.675 per cent at 10 days of age. Subsequently the rate at which the thymus increases in weight falls slightly behind that of total body weight. The spleen continues to increase at a relatively greater rate than the total body weight, while the peripheral nodes increase at a relatively lesser rate. The mesenteric nodes, in contrast to the peripheral lymph nodes, increase at a greater rate than any other part of the lymphoid system or the body as a whole after birth. This is perhaps illustrated more simply in terms of the factorial increase in weight over this period. The total body weight increased by a factor of 2.87 times, from 10 to 50 days of age, the thymus by 2.80,
The body weight and weights of various parts of the lymphoid system expressed in absolute and relative terms (as a percentage of body weight), at various ages prior to and up to 50 days after birth. All values were calculated from regressions of weight on age.

<table>
<thead>
<tr>
<th>Age in Days</th>
<th>Body Wt. in Kg</th>
<th>Thymus in g</th>
<th>Per Cent to Body Wt.</th>
<th>Spleen in g</th>
<th>Per Cent to Body Wt.</th>
<th>Peripheral Nodes in g</th>
<th>Per Cent to Body Wt.</th>
<th>Gut Nodes in g</th>
<th>Per Cent to Body Wt.</th>
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<td>Pre-natal</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td>70</td>
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<td>0.286</td>
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<td>1.894</td>
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<td>13.425</td>
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</table>

* = Values calculated from regression coefficients which exceed observed values.
the spleen 3.0 and the peripheral nodes by 2.0, while the mesenteric nodes increased by a factor of 3.3 times. In comparison, the factorial increases over the 100 to 140 day period before birth were - body weight 4.45 times, thymus 9.36, spleen 7.82 and lymph nodes 7.62. Thus the relative rate of increase in the size of all organs is reduced after birth.

No attempt was made to measure accurately the mass of lymphoid tissue associated with the Peyer's patches, but they were seen to develop very rapidly in the post-natal period. At birth in the lamb this part of the gut associated lymphoid system consisted of a series of discrete areas of lymphoid tissue scattered along the surface of the small intestine opposite the mesenteric attachment, mainly in the ileal region and concentrated toward the terminal portion of the ileum. The patches were visible as pale, thin, oval areas which rarely measured more than 2 x 1 cm. During the first two weeks of life these areas grew very rapidly to cause an obvious thickening of the intestinal wall. The patches subsequently became confluent so that one large continuous Peyer's patch was formed extending from near the ileo-caecal junction forward for a distance of one metre or more. This single patch ranged from 1.5 to 2.0 cm in width and around 0.2 to 0.25 cm in depth thus being equivalent to a volume of around 40 ml of solid lymphoid tissue. Further forward along the small intestine additional discrete ovoid, or circular lymphoid areas developed. These were scattered along the remainder of the ileum and throughout the length of the jejunum becoming less frequent in the duodenum and absent some 0.5 to 1.0 metres distal to the abomasum. These scattered patches were assessed to contain at least half the amount of lymphoid tissue as the large single ileal patch,
so the total volume of lymphoid tissue in the gut of a lamb at about 50 days of age was something of the order of 60ml (i.e. approximately 60g).

50 days and over. The rapid growth phase exhibited by the thymus in the post-natal period slowed at around 40 days. After this time, the thymus began to regress in both relative and absolute terms, falling from a maximum weight of 90g at 40 days to a minimum post-natal weight of around 20g at about 100 to 110 days of age. After this the weight of the thymus remained fairly constant in any individual although variations occurred between individuals. The spleen, peripheral and gut associated nodes did not undergo a similar regression. Statistical analysis of the data available for lambs aged 50 days or more showed there was no correlation between the weight of the various lymphoid organs and age with the exception of the rapidly regressing thymus. The great variation in the sizes of the different lymphoid components beyond 50 days of age is probably a reflection of a number of different factors such as the plane of nutrition, the relative disease status of the flocks from which animals were obtained and other unknown variables.

As described above, the Peyer's patches, like the thymus, developed rapidly in the 0 to 50 day period and beyond. However, by 100 days or so the size of these patches in different animals was very variable and there did appear to be a definite reduction in their size with increasing age. This regression appeared to begin at around 100 days and in lambs aged 6 months or more, the patches were difficult to locate macroscopically.

Development of agammaglobulinaemic lambs. The growth and development of the lymphoid system of colostrum deprived lambs was studied in an effort to determine whether
passively acquired maternal antibody influenced this process. Eleven lambs were removed from their mothers as soon as practicable after birth and before they had sucked their mothers. Eight other lambs were allowed to suck for 24 hours and were then removed from their mothers. These served as a control group. Both groups were hand reared on cow's milk at the rate of 1 litre per day divided into 4 feeds. The lambs were housed indoors on slatted wood floors continually for the first 2 weeks after which they were allowed to run in a grassed paddock during the day and housed overnight. At 90 days of age all lambs were killed and the thymus, spleen, mesenteric nodes and both prescapular and prefemoral nodes were dissected out. The results are shown in Table 3.2.

There was no difference in the body weight, or in the weights of the thymus, spleen, gut associated or peripheral nodes between the two groups. However, when compared to lambs raised on their mothers, both hand reared groups weighed significantly less than the group reared on their mothers.

**Histological Observations**

**Pre-natal**

The thymus in the 44 day embryo has a lobulated structure and a lymphoid appearance although at this age it is not yet differentiated into cortex or medulla (Fig. 3.2a and b). By 75 days (Fig. 3.2c), the lobules have enlarged and the cortex and medulla have differentiated. There is, as yet, no sign of Hassall's corpuscles which are first seen at about 120 days gestation (Fig. 3.2d).

The spleen at 70 days shows no sign of organized white pulp and few, if any, cells which could be termed
<table>
<thead>
<tr>
<th></th>
<th>Body Weight Kg</th>
<th>Thymus g</th>
<th>Spleen g</th>
<th>Peripheral Nodes g</th>
<th>Gut Nodes g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colostrum Fed</td>
<td>15.4±0.75</td>
<td>36.8±6.3</td>
<td>32.1±3.4</td>
<td>5.2±0.7</td>
<td>42.3±3.7</td>
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<tr>
<td>Colostrum Deprived</td>
<td>15.1±0.05</td>
<td>37.5±2.8</td>
<td>37.4±3.5</td>
<td>5.3±0.3</td>
<td>43.9±2.0</td>
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<tr>
<td>Reared on Mother</td>
<td>21.4±3.06</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Hand Reared vs Reared on Mother  p = 0.05

A comparison of the body weight and the weights of various parts of the lymphoid system in colostrum fed and colostrum deprived lambs at 3 months of age. The mean observations are given as ± their standard error.
The development of the thymus in the sheep.

A 44 days gestation. There is some early lymphoid development but as yet there is no cortico-medullary differentiation.

Stain haematoxylin and eosin. Magnification 75x.

B A thymic lobule of a foetal lamb at 44 days gestation. Many lymphoid cells and large pale reticular cells are present. The lymphoid cell complement has not yet fully developed.

Stain H and E. Magnification 480x.

C 75 days gestation, showing an increased number of lymphoid cells and differentiation between cortex and medulla.

Stain H and E. Magnification 30x.

D 120 days gestation, showing the early development of a Hassall's corpuscle in the medulla.

Stain H and E. Magnification 300x.
lymphoid in appearance (Fig. 3.3a). At 85 days, some small pockets of lymphocytes can be seen and these are confined to the vicinity of small blood vessels (Fig. 3.3b). At 120 days gestation, these small pockets have enlarged to become small follicles (Fig. 3.3c) and just prior to birth the spleen has its full adult structure with lymphoid follicles and accumulations of lymphoid cells around sheathed arterioles, but no germinal centres are yet present.

The lymph nodes at 80 days are very small, without any differentiation between cortex or medulla and very few lymphoid cells (Fig. 3.4). By 120 days small follicles can be seen scattered around the periphery, but the cortex is thin and the node consists mainly of medullary type tissue. Immediately prior to birth both cortex and medulla are more cellular (Fig. 3.5).

The Peyer's patches at 120 days (Fig. 3.6a and b) are small, have a lymphoid appearance, but show little mitotic activity and a poor degree of cortico-medullary differentiation.

Post-natal

After birth, the Peyer's patches enlarge rapidly and many mitotic figures can be seen within the follicles which extend up into the intestinal villi (Fig. 3.6c and d). The peripheral lymph nodes increase in size slowly in the post-natal period and further follicles develop. However, no germinal centres become apparent. In contrast, the gut lymph nodes grow very rapidly and contain numerous follicles which lead to an increased thickness of the cortex. Many germinal centres appear in these nodes and the medulla becomes more cellular. These are the typical changes seen in a lymph node responding to antigenic stimuli.
FIGURE 3.3

The development of the spleen in the sheep.
A  70 days gestation. The spleen consists of reticulum with a few scattered mononuclear cells.
   Stain H and E. Magnification 160x.
B  85 days gestation, showing the early development of small clusters of lymphoid cells around blood vessels.
   Stain H and E. Magnification 160x.
C  120 days gestation, showing the development of sheathed arterioles surrounded by accumulations of lymphoid cells.
   Stain H and E. Magnification 160x.
FIGURE 3.4

A  A lymph node from a foetal lamb of 80 days gestation, showing an undifferentiated structure.
   Stain H and E. Magnification 40x.

B  The same lymph node showing its reticular structure, with only a few small round cells scattered throughout the node.
   Stain H and E. Magnification 160x.
FIGURE 3.5

The development of the lymph node in the sheep.
A 120 days gestation. The node is composed almost entirely of medullary tissue, but early follicular development can be seen in the outer cortex.

Stain H and E. Magnification 25x.

B A follicle in a lymph node at 120 days gestation.

Stain H and E. Magnification 100x.

C At birth. The cortex is now quite distinct but there is no evidence of germinal centres.

Stain H and E. Magnification 62.5x.
FIGURE 3.6

The development of the Peyer's patches in the sheep.
A 120 days gestation. A cross section of the ileum, showing the development of small lymphoid follicles.
   Stain H and E. Magnification 4x.
B 120 days gestation. A developing lymphoid follicle in the ileum. Little evidence of mitosis can be seen.
   Stain H and E. Magnification 160x.
C After birth (8 weeks of age). A cross section of the ileum showing extensive development of lymphoid follicles which now make up the bulk of the gut wall.
   Stain H and E. Magnification 4x.
D After birth (8 weeks of age). The follicles are very large and densely packed with lymphoid cells.
   Stain H and E. Magnification 25x.
The mononuclear changes which take place in the lymph nodes of young calves, were found to be a normal process and not due to stress or infection. In the first month of life, the lymphocyte count was lower than normal, but by the end of the first month, the count had risen to 1.3 x 10^6/ml (initial total). This rise in lymphocytes (mainly neutrophils) was most marked at one day of age. Like the lymphocytes, their numbers decreased over the first few days, but there was no subsequent increase. The ratio of lymphocytes to polymorphs was 1.13 to 1.00 at birth and at 6 months of age, the ratio was 5.54 to 1.00. The lymphocyte count showed a similar depression in these lambs as in normal lambs at birth. As with the neutrophils, a gradually rising lymphocyte count was seen in the total cell count until the third month, and this difference in lymphocyte counts between those of the lambs and those of the normal lambs continued until the experiment was terminated.
Post-natal Development of Blood Leucocyte Levels

The post-natal changes in the level of circulating white cells in the blood of young lambs are given in Table 3.3. It was found that in normal lambs the total white cell counts in the blood fell from a level of $8.0 \pm 0.93 \times 10^6/$ml at one day of age to $4.25 \pm 0.55 \times 10^6/$ml at 3 days of age. The counts then gradually rose to reach $11.05 \pm 1.69 \times 10^6/$ml at 6 months of age. This rise in total cell numbers was due entirely to a rise in the blood lymphocyte levels. At birth the lymphocyte count was $4.25 \pm 0.89 \times 10^6/$ml (52.8 per cent of the total white cells) and at 6 months of age it had reached $9.36 \pm 1.55 \times 10^6/$ml (i.e. 84.5 per cent of the total). This rise in lymphocyte numbers represented a greater than twofold increase. Other white cells (mainly neutrophils) were most numerous at one day of age. Like the lymphocytes, their numbers decreased over the first few days, but there was no subsequent increase. The ratio of lymphocytes to polymorphs was 1.13 to 1 at one day of age and at 6 months of age it was 5.54 to 1.

Blood leucocyte counts were also determined on colostrum deprived lambs from birth to 90 days of age (Table 3.3). These lambs also had an initial low L:P ratio of 1.27 to 1 at birth. As with colostrum fed lambs there was subsequently a gradual rise in lymphocyte numbers so that by 12 weeks of age the L:P ratio was 3.29 to 1. The total white cell count showed a similar depression over the first few days as seen in normal lambs. Apart from a fortuitous difference in the total cell count at 1 day of age, there was no statistically significant difference between the 2 groups until 56 days of age when lymphocyte levels of the colostrum deprived lambs exceeded those of colostrum fed lambs. This difference was maintained up to 90 days of age, when the experiment was terminated.
A comparison of the white blood cell counts of colostrum fed (CF) and colostrum deprived (CD) lambs in terms of the total leucocyte, total lymphocyte and total polymorph counts, and the ratio of lymphocytes to polymorphs (L:P) at various ages after birth. The mean results are given in cells per ml. x 10^6 ± their standard errors.
### TABLE 3.3

<table>
<thead>
<tr>
<th>Age (Days)</th>
<th>Cell Count</th>
<th>Colostrum Deprived</th>
<th>n</th>
<th>Colostrum Fed</th>
<th>n</th>
<th>p</th>
<th>L.P. Ratio</th>
</tr>
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<tbody>
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<td>1</td>
<td>T</td>
<td>5.83±0.56</td>
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<td>8.00±0.93</td>
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<td></td>
<td>L</td>
<td>3.26</td>
<td></td>
<td>4.25</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>2.57</td>
<td></td>
<td>3.75</td>
<td></td>
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<td></td>
</tr>
<tr>
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<td>4.84±0.47</td>
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<td>8.00±1.61</td>
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<td>L</td>
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<td>5.57</td>
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<td>4.01</td>
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<td>120</td>
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<tr>
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<td>2.97</td>
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<tr>
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<tr>
<td>180</td>
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<td>P</td>
<td>1.69</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

**T** = Total  
**L** = Lymphocytes  
**P** = Polymorphs  
**n** = number of observations  
**p** = probability
Discussion

In the sheep, as in all other mammals which have been studied, the thymus is the first organ to show recognizable lymphoid structure, and during foetal life it is the most rapidly growing of all lymphoid organs. This very rapid pre-natal growth period ceases at about 130 days gestation, but again accelerates almost immediately after birth. This rapid pre-natal, static or slow peri-natal and accelerated post-natal growth pattern has also been described in the mouse (Hess et al., 1967).

Of the other lymphoid organs the spleen has the second most rapid growth rate, but the significance of this is complicated by the fact that the spleen serves the two-fold function of haematopoiesis and lymphopoiesis. Much of the increase in the size of the spleen is undoubtedly due to this former function.

The lymph nodes have a slower growth rate, more in concert with the overall rate of growth of the body as a whole than with the other lymphoid organs. Despite the fact that in the lamb the lymphoid organs develop very early in relation to birth, the relative increases in their size after birth are remarkably similar to those described for the mouse.

The thymus reaches its maximum size after birth at around 40 days and then begins to regress. This observation correlates well with the data reported for the mouse (Santisteban, 1960; Pepper, 1961b; Metcalf, 1964a) and rat (Reinhardt, 1946). The age at which the maximum size of the secondary lymphoid organs is reached in the sheep could not be assessed accurately due to the large variations found between individual animals. Data available for the mouse suggest that similar difficulties are encountered in this species. Santisteban (1960) found that the spleen and lymph
nodes reached maximum size in CBA mice at around 100 days. Pepper (1961b) states that lymph nodes in Strong A strain mice reach maximum size at 25 days, while Metcalf (1964a) gives 42 days for lymph nodes of C3H mice. While these differences could reflect real strain variations it is likely also to reflect differences in the hygiene of the animal colonies of the various laboratories. The rapid post-natal development of the Peyer's patches has been reported in rabbits by Cooper et al (1966).

A comparison between species can also be made on the basis of the age at which the various lymphoid organs reach their maximum size in relation to body weight and comparing the values of these relative sizes. The available data for the thymus is summarised in Table 3.4. It can be seen that there is considerable variation between species and between strains, which makes interpretation of these data difficult. The most that can be drawn from such a comparison is that the size of the thymus in the sheep, relative to body weight, is similar to the mouse. Similar comparisons cannot be made with other lymphoid organs due to the wide variations in the reported data. The observation that the mesenteric lymph nodes of lambs increase in size at a significantly more rapid rate than peripheral lymph nodes confirms a similar observation made in rats by Reinhardt (1946). The significance of this will be more fully developed in the chapter which follows.

The post-natal development of the lymphoid system in agammaglobulinaemic lambs was no different to that in normal lambs allowed to ingest colostrum. It is well known that colostrum is the only source of passively acquired immunoglobulins in the ruminant species (Mason et al, 1930), and it is equally well known that under field conditions
A comparison of the maximum weight, relative to the body weight, of the thymus and the age at which this occurs in various species for which data is available.

<table>
<thead>
<tr>
<th>Species</th>
<th>Age at Maximum Relative Body Weight</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>At birth (270 days)</td>
<td>Kay et al. (1962)</td>
</tr>
<tr>
<td></td>
<td>Slightly before birth</td>
<td>Personal observations</td>
</tr>
<tr>
<td>Mouse</td>
<td>At birth (150 days)</td>
<td>Wallace, (1945)</td>
</tr>
<tr>
<td></td>
<td>15-16 days post birth</td>
<td>Metcalf, (1964a)</td>
</tr>
<tr>
<td></td>
<td>0.35</td>
<td>Pepper, (1961b)</td>
</tr>
<tr>
<td>Sheep</td>
<td>At birth (150 days)</td>
<td>Wilson et al. (1965)</td>
</tr>
<tr>
<td></td>
<td>0.85</td>
<td>(Strain CFW)</td>
</tr>
<tr>
<td></td>
<td>0.73</td>
<td>(Strain C3H)</td>
</tr>
<tr>
<td>Table 3.4</td>
<td>Percentage of Body Weight</td>
<td>0.89</td>
</tr>
<tr>
<td></td>
<td>0.81</td>
<td>0.35</td>
</tr>
</tbody>
</table>

TABLE 3.4
these colostrum deprived animals are difficult to rear and are particularly subject to severe enteric infections (Smith and Little, 1922; Aschaffenburg, Bartlett, Kon, Terry, Thompson, Walker, Briggs, Cotchin and Lovell, 1949a; Aschaffenburg, Bartlett, Kon, Walker, Briggs, Cotchin and Lovell, 1949b). There was no difference in body weight or in the size of any section of the lymphoid system between these colostrum deprived or colostrum fed lambs, and there were only 2 deaths out of the 17 hand reared lambs (1 colostrum fed and 1 colostrum deprived). Since no elaborate efforts were made to hand rear these lambs, it seemed that, given reasonably hygienic quarters, kept reasonably warm and well fed, the apparent disadvantages which a colostrum deprived animal normally suffers, can be almost entirely offset. The fact that the hand reared lambs, whether given colostrum or not, did not grow as well as those reared on their mothers suggests that there is some dietary factor involved. Some early colostrum deprived lambs were reared on reconstituted powdered milk and these lambs failed to do well, remained small and stunted and suffered from chronic intermittent diarrhoea. This diarrhoea could be relieved by intestinal sedatives but not by orally administered antibiotics, indicating that the condition was non-infectious and probably entirely the product of an unsatisfactory diet since diarrhoea rarely occurred in lambs fed whole cow's milk. It was also noticed that hand reared lambs were very slow to begin eating grass or hay when compared with those reared on their mothers. This would also have an effect on their subsequent growth rates.

The gradual post-natal rise in the levels of circulating blood leucocytes has been well documented in many species (Schalm, 1965) and the sudden drop in numbers soon
after birth followed by a gradual rise has also been reported previously in humans by Kato (1935). The significance of this drop is probably associated with the sudden exposure of the newborn animals to environmental antigens and will be further discussed in the next chapter.

**Summary**

Some histological features and the gross anatomical development of the lymphoid tissue in the lamb before and after birth and the post-natal changes in the blood leucocyte levels in normal and agammaglobulinaemic lambs is described. Although the lymphoid tissues have achieved a considerable degree of development prior to birth, the post-natal changes appear to closely parallel those seen in the common laboratory animals for which similar data are available.
CHAPTER IV

THE RESPONSE OF NEWBORN LAMBS TO ANTIGENS

Since the development of the foetal lamb in utero takes place in a germ-free environment and since the ovine placenta is impermeable to maternal proteins, any development of the lymphoid system up to birth takes place independently of any antigenic stimulation. It is known that the lymphoid system of germ-free animals does not develop as quickly, or to the same degree as in conventional animals. At birth however, the normal animal is abruptly thrust into an environment which compared to the ante-natal environment, is extremely contaminated with antigenic material and potential pathogens. The evidence presented in the previous chapter suggests that much of the brunt of this precipitous antigenic onslaught is borne by the gut associated lymphoid tissues such as the mesenteric lymph nodes and the Peyer's patches. In order to gain some insight into the manner in which the newborn lamb cope with the sudden change from a germ-free to a contaminated environment, two series of experiments were performed in the lymphoid system after birth, the following studies were made.

The cellular characteristics of the efferent lymph from the popliteal node, the lumbar trunk and the main intestinal lymph duct were examined in foetal lambs in utero late in gestation. The foetuses were returned to the abdomen following the establishment of the various lymphatic fistulae and pregnancy allowed to continue. Two lambs with lymphatic fistulae established in utero were subsequently delivered by Caesarian section with their cannulae intact and patent and the changes which occurred in their lymph after birth were observed. The intestinal lymph of lambs of various ages after birth was also examined. The autogenous production of immunoglobulins to non-specific antigenic stimulation was
The Response of Newborn Lambs to Antigens

Since the development of the foetal lamb in utero takes place in a germ-free environment and since the ovine placenta is impermeable to maternal proteins, any development of the lymphoid system up to birth takes place independantly of any antigenic stimulation. It is known that the lymphoid system of germ-free animals does not develop as quickly, or to the same degree as in conventional animals. At birth however, the normal animal is abruptly thrust into an environment which compared to the ante-natal environment, is extremely contaminated with antigenic material and potential pathogens. The evidence presented in the previous chapter suggests that much of the brunt of this precipitous antigenic onslaught is borne by the gut associated lymphoid tissues such as the mesenteric nodes and the Peyer's patches. In order to gain some insight into the manner in which the newborn lamb copes with the sudden change from a germ-free to a contaminated environment and the changes which occur in the lymphoid system after birth, the following studies were made.

The cellular characteristics of the efferent lymph from the popliteal node, the lumbar trunk and the main intestinal lymph duct were examined in foetal lambs in utero late in gestation. The foetuses were returned to the abdomen following the establishment of the various lymphatic fistulae and pregnancy allowed to continue. Two lambs with lymphatic fistulae established in utero were subsequently delivered by Caesarian section with their cannulae intact and patent and the changes which occurred in their lymph after birth were observed. The intestinal lymph of lambs of various ages after birth was also examined. The autogenous production of immunoglobulins to non-specific antigenic stimulation was
studied in colostrum deprived agammaglobulinaemic lambs and finally, the response of normal and colostrum deprived lambs to specific stimulation by antigens was examined in the immediate post-natal period.

Results

Observations on the Cells of Foetal Lymph

The flow rate and cell content of lymph carried in the efferent popliteal, lumbar trunk and main intestinal lymph duct of foetal lambs in utero are shown in Table 4.1.

The cells in the lymph were extremely uniform in size and morphology regardless of the duct from which they were collected and consisted of about 98 per cent small lymphocytes. Prolonged search of Leishman stained smears failed to reveal any basophilic, blast or transitional cell types, and there was no evidence of any mitotic activity. These findings reflect the "immunological innocence" of the foetal lamb. Electron microscopic examination of the foetal lymph cells showed them to be mature undifferentiated lymphocytes (Fig. 4.1a). When these cells were incubated with $^3$H thymidine, less than 0.1 per cent showed evidence of incorporation of the label into their nuclear DNA (Fig. 4.2a).

The Changes Which Occur in the Cells of Lymph Immediately After Birth

(1) Cell output

Remarkable changes were seen to take place in the intestinal lymph consequent to the abrupt transition of the lamb from its germ-free in utero environment to the highly contaminated outside world. The following events were observed in a lamb whose main intestinal duct was cannulated in utero then delivered by Caesarian section 24 hours later.
TABLE 4.1

<table>
<thead>
<tr>
<th>Source of Lymph</th>
<th>n</th>
<th>Mean Flow Rate (ml/hr)</th>
<th>Cell Count x $10^6$/ml</th>
<th>Cell Output Per hr x $10^6$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Main intestinal duct</td>
<td>4</td>
<td>5.6</td>
<td>4.7</td>
<td>26.32</td>
</tr>
<tr>
<td>Lumbar trunk</td>
<td>3</td>
<td>5.0</td>
<td>7.1</td>
<td>35.11</td>
</tr>
<tr>
<td>Efferent popliteal duct</td>
<td>1</td>
<td>0.6</td>
<td>2.3</td>
<td>1.38</td>
</tr>
</tbody>
</table>

n = number of observations

The mean flow rate and cell content of lymph from various parts of the body of foetal lambs late in gestation.
FIGURE 4.1

A  The cells in the lymph of a foetal lamb 12 hours before birth, showing their uniformity in size and morphology. There is no evidence of proliferative activity.
Magnification 5,400x.

B  A lymphocyte, typical of many which are seen in the intestinal lymph of a lamb 72 hours after birth. This cell shows signs of differentiation and contains polyribosomes and disorganized pieces of endoplasmic reticulum.
Magnification 14,850x.
FIGURE 4.2

 Autoradiographs of cells from the intestinal lymph after incubation with $^3$H-thymidine.

A The cells in the lymph of a lamb in utero, 12 hours before birth. One cell has incorporated $^3$H-thymidine into its nuclear DNA.

B The cells in the lymph of a lamb 72 hours after birth. 20 per cent of these cells (almost all of the large transitional forms) have taken up the label.

   Stain Azure A. Magnification 1600x.
output in the intestinal lymph of the lamb in utero was 4.47 x 10⁷ cells per hour. Immediately after delivery, the cell output rose to 7.3 x 10⁷ cells/hr (Fig. 4.3), but immediately following the first feed of 150ml of colostrum the cell output dropped to the pre-birth level. Over the next 3 hours the output continued to fall to reach a level of 1.2 x 10⁷ cells/hr. This level was maintained until around 24 hours after birth when the cell output progressively rose until by 72 hours, the output had increased to a peak of 13.1 x 10⁷ cells per hour. In contrast, increased cell output occurred in intestinal lymph samples from the popliteal duct showing such changes, and the cell output remained constant throughout this first 72 hour post-natal period. Such changes in the cell content of intestinal lymph were not due to changes in flow rate, since despite intermittent feeding, the flow rate remained reasonably constant throughout the observation period.

(2) Morphology
Theularity of cells in the samples which remained unchanged for the first 24 hours. However, as the cell output increased, there was a basophilic cells which make up the main precursor of lymphocytes. These cells accounted for up to 50% of the lymphocytes in intestinal lymph. Electron-microscopic examination further confirmed the character of the cells, showing characteristic ultrastructural features. In these cells, the mitochondria were numerous and prominent. In the nucleus, the chromatin was dispersed throughout the nucleoplasm, but there was some condensation of nuclear chromatin of these cells. These were described as being thin and dense at the periphery and there were clearly

A

B
The cell output in the intestinal lymph of the lamb in utero was $4.47 \times 10^7$ cells per hour. Immediately after delivery, the cell output rose to $7.3 \times 10^7$ cells/hr (Fig. 4.3), but immediately following the first feed of 150ml of colostrum the cell output dropped to the pre-birth level. Over the next 9 hours the output continued to fall to reach a level of $1.76 \times 10^7$ cells/hr. This low level was maintained until around 20 hours after birth when the cell output progressively rose until by 72 hours, the output had increased 8 fold to a peak of $13.15 \times 10^7$ cells per hour. In contrast to the increased cell output that occurred in the intestinal lymph, lymph from the popliteal duct showed no such changes, and the cell output remained constant throughout this first 72 hour post-natal period. These changes in the cell content of intestinal lymph were not due to changes in flow rate, since despite intermittent feeding, the flow rate remained reasonably constant throughout the observation period.

(2) Cell morphology

The uniformity of cell type seen in pre-natal lymph remained unchanged for the first 48 hours after birth. However, as the cell output rose, blast and immature basophilic cells began to make their appearance and by 72 hours these cells accounted for up to 20 per cent of all the cells present. Electron-microscopic examination showed that these cells had the characteristics of primitive differentiating cells Fig. 4.1b. In the cytoplasm there were many polyribosomes arranged in clusters but for the most part the ergastoplasm was represented by occasional disorganized pieces of endoplasmic reticulum. The nuclear chromatin of these cells was arranged thinly around the periphery and there were usually
FIGURE 4.3

The changes in the output of cells in the intestinal lymph, before and immediately after birth, of a lamb cannulated \textit{in utero} and delivered by Caesarian section. The cell output in efferent lymph from the popliteal node over the same time period, is shown for comparison.
Cell output per hour x 10^7

<table>
<thead>
<tr>
<th>Hours after birth</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
</tr>
<tr>
<td>24</td>
</tr>
<tr>
<td>36</td>
</tr>
<tr>
<td>48</td>
</tr>
<tr>
<td>60</td>
</tr>
<tr>
<td>72</td>
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<tr>
<td>84</td>
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<tr>
<td>96</td>
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<td>108</td>
</tr>
<tr>
<td>120</td>
</tr>
<tr>
<td>132</td>
</tr>
<tr>
<td>144</td>
</tr>
<tr>
<td>156</td>
</tr>
</tbody>
</table>

**Intestinal lymph**

**Leg lymph**

Birth
First feed

There was a rapid increase in the number and type of cells in the intestinal lymph over the same period as the onset of a significant incorporation of lipid in the lymph of lambs. The number of lambs examined at each interval was less with older lambs than with younger lambs, and the lambs were examined at shorter intervals after birth in the older lambs. The number of cells secreted in the lymph of lambs changed rapidly and was about 1.3 x 10^5 cells/hr at 3 hours after birth.
several nucleoli present. These blast and transitional forms of lymphoid cells all incorporated $^3$H thymidine into their nuclear chromatin representing a 150 fold increase over the pre-natal level of incorporation, indicating a rapidly proliferating population (Fig. 4.2b).

Examination of the number and type of cells in the efferent popliteal lymph over the same period failed to reveal any significant changes. There was no evidence of any blast cell production of increased incorporation of $^3$H thymidine.

Development of the Lymphoid System Under Continuing Antigenic Stimulation

(1) The characteristics of intestinal lymph of older lambs

The intestinal lymph of older lambs was examined at various ages from birth to 13 weeks of age. While the lymph of younger lambs almost invariably contained a high proportion of basophilic cells, these were seen less frequently in older lambs. When they were seen in these older lambs they appeared intermittently i.e. at any one time there might be relatively few to be seen, then they would suddenly appear in moderate numbers for 24 hours or so and then disappear again.

The cell output of intestinal lymph of lambs of different ages is shown in Fig. 4.4. There was a rapid increase in the cell output from about $1.3 \times 10^8$ cells/hr at 3 days of age to $1.4 \times 10^9$ cells/hr at 13 weeks of age i.e. a 10 fold increase. This large increase in the number of free floating cells in the lymph obviously reflects the increase in size of the gut associated lymphoid tissue (described in Chapter III) which, over the same time period, was seen to
FIGURE 4.4

The changes in the output of cells in the intestinal lymph of lambs of various ages from birth to 12 weeks of age shown as the regression of cell output on age. The regression of body weight on age over the same time period is shown for comparison.
increase in size by a factor of about 5. This was not related simply to the general growth of the lamb since, in the same period, the body weight of even the most well grown lambs increased by only 4.4 times, while the average increase was much less.

(2) **The development of peripheral nodes under constant non-specific stimulation**

One group of lambs aged from 120 to 160 days which were examined at post-mortem had very large prescapular and occasionally very large prefemoral lymph nodes. These lambs had been grazing in a paddock which had a strong growth of barley grass (*Hordeum leporinum*), the seeds of which are very sharp with long serrated awns. As the lambs graze, these grass seeds become matted in the wool particularly under the neck and around the shoulders and sometimes along the flanks and under the abdomen. Some of these seeds had actually penetrated the skin causing the formation of small abscesses. The result of this grass seed infestation (a common occurrence in sheep grazing long pasture) was a chronic irritation of the skin which was reflected by a great increase in the size of the regional draining nodes. The prescapular nodes in this particular group of lambs weighed 6.9 to 14.0g compared to a maximum weight of 3.65g observed in sheep of any age on short pasture with no grass seed infestation. Prefemoral nodes weighed up to 4.65g in the grass seed infested lambs, while the maximum size observed for this node in normal animals of any age was 1.35g.

**The Response of Newborn Lambs to Specific Antigenic Stimulation**

The responses of normal, colostrum fed (CF) lambs
and colostrum deprived, agammaglobulinaemic (CD) lambs to 2 specific antigens, swine influenza virus (strain Shope 15) and boiled Salmonella muenchen organisms, was investigated. Both antigens were injected subcutaneously into the lower hind limb, and the response of the popliteal node was followed in terms of the changes that occurred in the output and nature of the cells in the efferent lymph, the production of specific antibody in lymph and sera, and in the case of the response to Salmonella muenchen, the numbers of plaque forming cells appearing in the lymph.

The collection of lymph from these very young lambs posed some special problems which were outlined in Chapter II. The lambs were running free with their mothers at all times and it proved impossible to prevent lymph from spilling out of the collection bottles and despite the addition of antibiotics to the lymph, some samples became contaminated. For these and other reasons it was much more difficult to obtain continuing collections of lymph throughout the whole of an immune response. To partially overcome this difficulty a considerable number of lambs were cannulated in these experiments and from these a composite picture of the immune response has been assembled.

(1) The response to swine influenza virus

All lambs were given a primary challenge subcutaneously in both hind limbs with 10 or 20,000 haemagglutinating units of virus suspended in 1.0ml of normal saline. Some lambs were cannulated prior to stimulation and the early aspects of the response studied. Others were cannulated 48 hours after stimulation to study the later stages. The same procedures were employed to study secondary immune responses. In order to make best use of the available animals, those
lambs which had been cannulated in one leg for a primary response were given a secondary challenge in the contralateral uncannulated leg, and serum antibody titres were studied. Those not cannulated for the primary response were given a secondary challenge in both legs and then one leg was cannulated in order to study the cellular aspects of this secondary response.

The cellular response - Primary. Immediately following the administration of antigen there was a precipitous fall in the cell content of the lymph leaving the node draining the injection site. The lymph became almost free of cells within 3 to 4 hours of the injection and remained this way for almost 24 hours after which the cells suddenly began to re-enter the lymph once more. The cell content then continued to rise and reached a maximum some 84 hours after stimulation. From this time on the numbers gradually fell to return to pre-stimulation levels 6 to 7 days later. The response of CD lambs was similar in that there was an initial "shutdown" period after the injection of the antigen followed by a rise in cell output, but this rise appeared to be somewhat slower than that seen in CF lambs and the peak cell outputs were not reached until some 96 to 108 hours after stimulation. However, the peak levels of cell output were within the range found for CF lambs (Table 4.2).

Coincident with the rise in cell numbers, a change in the cell size distribution was noted. Large cells began to appear 36 to 48 hours after stimulation and reached peak numbers at 60 to 84 hours in CF lambs and at about 108 hours in CD lambs. These large cells had abundant pale staining cytoplasm and a large nucleus with fine chromatin. Soon after the first appearance of these large "transitional" type cells, basophilic cells began to appear. These cells
<table>
<thead>
<tr>
<th></th>
<th>Maximum Cell Output</th>
<th>Maximum Percentage of Large Cells</th>
<th>Maximum Percentage of Basophils</th>
<th>Maximum Antibody Titre in Lymph</th>
<th>Maximum Antibody Titre in Plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Time (hr) x 10^6 per hr</td>
<td>Time Per cent</td>
<td>Time Per cent</td>
<td>Time Log 2 Titre</td>
<td>Time Log 2 Titre</td>
</tr>
<tr>
<td><strong>Primary Response</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C.F. 72-84</td>
<td>45.0-126.4</td>
<td>60-84</td>
<td>16.3-29.9</td>
<td>108-120</td>
<td>144</td>
</tr>
<tr>
<td>C.D. 96-108</td>
<td>94.6-130.0</td>
<td>108</td>
<td>38.3-40.7</td>
<td>132</td>
<td>144</td>
</tr>
<tr>
<td><strong>Secondary Response</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C.F. 48-72</td>
<td>39.0-141.0</td>
<td>72*</td>
<td>33.8*</td>
<td>72</td>
<td>144-168</td>
</tr>
<tr>
<td>C.D. 60</td>
<td>44.8-109.1</td>
<td>84</td>
<td>30.0-47.5</td>
<td>72-84</td>
<td>120-144</td>
</tr>
</tbody>
</table>

* One determination only

A comparison of the peak responses of colostrum fed (C.F.) and colostrum deprived (C.D.) lambs injected subcutaneously in the hind limbs with 10,000 haemagglutinating units of swine influenza virus. The primary stimulus was given on the day of birth and the secondary at 14 days of age. The responses are shown in terms of the times at which the maximum output of cells and the maximum percentage of large and basophilic cells are reached in the efferent popliteal lymph, as well as the maximum antibody titres in the lymph and blood plasma and the times at which these are attained.
had less abundant dense staining cytoplasm and coarse nuclear chromatin. As the response progressed the transitional cells became fewer in number while the basophilic cells increased and became smaller. Towards the end of the response these cells were roughly the same size as the normal lymphocytes. The peak basophilic response occurred at 108 to 120 hours after stimulation in CF lambs and slightly later, at 132 hours in CD lambs, although the actual numbers of these cells were similar in both groups.

Secondary. The secondary responses followed the same pattern, with the exception that the peak cell output occurred earlier (48 to 72 hours), and the basophilic cells also appeared and reached a peak earlier than following a primary challenge. CD lambs responded to a secondary antigenic challenge in an identical way to CF lambs. The secondary responses are summarised in Table 4.2.

Antibody production was measured by means of the haemagglutination inhibition test. Trypsin-periodate inactivation of plasma samples was essential since all lambs showed considerable levels of non-specific inhibitors. Even with this treatment and adoption of a starting dilution of 1:20, some non-specific activity sometimes remained. However, as non-specific inhibition did not result in such a clear pattern as was seen with virus alone, it was generally possible to distinguish between these reactions, particularly when they were associated with rising antibody titres.

The antibody response. In general terms, the antibody response in both CF and CD lambs was the same. Following the primary injection, positive serum titres were rarely seen before 10 days after stimulation, and then only in those lambs which had been challenged in both legs and not cannulated. In those animals with popliteal fistulae low levels of antibody were found in the lymph at about 10 days after challenge.
There was no observed difference in the response of CF or CD lambs. These results are summarised in Table 4.2.

As mentioned earlier, some lambs received 20,000 haemagglutinating units of antigen as both primary and secondary stimuli. In these animals it appeared that a more vigorous cellular response resulted although the levels of antibody produced were within the range resulting from challenges with 10,000 haemagglutinating units.

All lymph and plasma samples were also treated with 0.2M 2-mercapto-ethanol. There was little reduction in titres beyond one log₂ dilution at any stage of either primary or secondary responses, indicating that the lambs formed little 19S macroglobulin antibody in response to influenza virus.

(2) The response to Salmonella muenchen organisms

One normal CF and one CD lamb were challenged on the day of birth with $10^9$ boiled S. muenchen organisms subcutaneously in both hind limbs. Neither lamb was cannulated for the first response and only serum samples were taken for antibody assay. Both the lambs were cannulated to study the secondary response in the lymph. However, only one (the CF lamb) flowed satisfactorily, and then only for the period of from 60 to 120 hours. No antibody was detected in the serum of either lamb, or in the lymph following the primary or secondary challenge. A cellular response did occur as judged by an increase in cell output. However, there was little change in the cell size distribution, very few basophilic cells were seen (a maximum of 9 per cent at 96 hours) and no plaque forming cells were detected.

One foetal lamb aged around 80 days gestation was also challenged in utero with 2 doses of $10^9$ boiled Salmonella
muenchen organisms injected subcutaneously in both hind limbs. One month later, with the lamb still in utero, a secondary challenge with the same dose was given into both legs. Ten days later the popliteal nodes were removed and examined for evidence of an immune response to the antigen, and the foetal serum was titrated for antibody against lipopolysaccharide. Although both lymph nodes were considerably larger than those of a normal foetal lamb of the same age, on examination of the nodes under the electron microscope some cells were seen to have undergone a degree of transformation (Fig. 4.5) but no plasma cells could be seen in any section of the nodes. Titration of the sera revealed absolutely no trace of antibody. There was not even any non-specific reaction. However, as will be described below, this lamb did produce sufficient \( \gamma \) globulin to be detected by immuno-electrophoresis.

The Autogenous Production of Immunoglobulins

Lambs and calves are normally born completely free of immunoglobulins in their sera (Mason et al., 1930). If sucking of the mother is prevented for the first 48 hours or so, the absorption of maternal immunoglobulins from the colostrum is prevented and any immunoglobulins that subsequently appear in the blood must have been produced by the animal itself. Since colostrum deprived animals have been reported to be highly susceptible to infections in the first week or so of life, particularly those caused by enteric organisms, it was decided to investigate the time of onset and sequence of immunoglobulin production in agammaglobulin-aemic lambs. Samples of sera were taken from lambs on
An electron micrograph of the medulla of the popliteal lymph node of a foetal lamb (120 days gestation), 10 days after a secondary challenge with $10^9$ killed S. muenchen organisms injected into the hind leg.

Magnification 7800x.
the day of birth and at weekly intervals thereafter. These samples were tested for antibody globulins by immune-electrophoresis against hyper-immune rabbit anti-sheep serum.

The normal immune-electrophoretic patterns of adult and foetal lymph and serum have been described by Barbat and Luke.{19} The pattern of immunoglobulins in adult sheep serum was shown to consist of IgG, IgM, and IgA in the order from the most negative to the most positive. This pattern was also observed in foetal serum.

Aalund has further described the patterns of antigenic lines in human and various animal sera. It was noted that the patterns were similar in the various sera, but there were differences in the intensity of the lines. The patterns were described as consisting of a series of lines, each with a characteristic intensity.

There was a degree of variation between individual samples in the intensity of precipitation in the immunoglobulin regions. 

78\% was invariably present in the 78\% region, and 

1\% was frequently weak or apparently absent. 

3\% was consistently present in lymph. Some sera showed more than one line in the 3\% region and 4 or 5 faint but distinct lines could commonly be detected.
the day of birth and at weekly intervals thereafter. These samples were tested for antibody globulins by immuno-electrophoresis against hyper-immune rabbit anti-sheep serum.

(1) **The normal immuno-electrophoretic patterns of adult and foetal lymph and serum**

The normal immuno-electrophoretic patterns of sheep sera have been described by Silverstein, Thorbecke, Kraner and Lukes (1963a), Aalund, Osebold and Murphy (1965) and Pan, Kaplan, Morter and Freeman (1968). Four main classes of immunoglobulins have been identified and designated IgG₂ (slow 7Sγ or 7Sγ₂), IgG₁ (fast 7Sγ or 7Sγ₁), IgM (β₂M or γ₁M) and IgA (β₂A or γ₁A). In this description, the terminology of Aalund et al (1965) will be adhered to since no attempt has been made to associate any antibody activity with the various fractions, and thus the IgG, IgA and IgM terminology was felt not to be appropriate in this case. An immuno-electrophoretic pattern of normal adult sheep serum and adult sheep lymph developed against rabbit anti-sheep serum is shown in Fig.4.6 (1 and 2). The major precipitation arcs are marked as 7Sγ₂, 7Sγ₁, γ₁A, β globulins, α globulins and albumin. Although there were never any major differences in the patterns of lymph and sera, lymph frequently showed a less intense staining of the arcs in the β and α regions.

There was a degree of variation between individual samples in the intensity of precipitation in the immunoglobulin regions. 7Sγ₁ was invariably present but 7Sγ₂, and γ₁M were frequently weak or apparently absent. γ₁A was constantly present in lymph. Some sera showed more than one line in the γ₁A region and 4 or 5 faint but distinct lines could commonly be detected.
The immuno-electrophoretic patterns of normal adult sheep serum (1), and lymph (2), and foetal lymph (3), and serum (4), immediately prior to birth, developed against rabbit anti-sheep serum.

5-9 show the development of serum immunoglobulins in a colostrum deprived lamb after birth. This particular lamb had low but detectable levels of $\gamma_1$M in its serum at birth.
Normal adult serum

1 Albumin α β

2 Normal adult lymph

3 Foetal intestinal lymph

4 Foetal cord serum

Serum of C.D. lamb post-birth

5 7 days

6 14 days

7 28 days

8 35 days

9 42 days

(2) The autogenous production of immunoglobulins in foetal lamb serum and the importance of specific antigenic stimulation in the production of immunoglobulins by 5 colostrum doses during a period of 6 weeks. Samples of foetal cord serum were taken at weekly intervals post-birth and subjected to immuno-electrophoresis against anti-foetal serum. Apart from the single line at birth (Fig. 1), 7S1 began to appear from 7 days, and 7S2 at 14 to 21 days after birth. Every lamb showed 2 to 3 lines in the γ1A region from birth. As can be seen, the γ1A and γ2A did not change in strength with increasing age. The γ1A occupied the region above and to the left of the γ1M line. Conversely, the γ2A occupied the region below and to the right of the γ1M line. As explained above, the γ2M is normally produced by sufficient levels of immunoglobulins.
Normal immuno-electrophoretic patterns of foetal lymph and sera immediately prior to birth are shown in Fig. 4.6 (3 and 4). Lymph showed a lack of distinct lines in the \( \beta \) region and a complete lack of any \( \gamma \) lines. Serum however, constantly showed 1 to 3 strong lines in the \( \gamma_1A \) region. This is in contrast to the results of Silverstein et al (1963a) who claimed to have invariably found small amounts of \( 7S\gamma \) and \( \gamma_1M \) in foetal serum but no \( \gamma_1A \). The justification for classifying these lines as \( \gamma_1A \) rather than \( \gamma_1M \) will be described below.

(2) The autogenous production of immunoglobulins in the absence of specific antigenic stimulation

The production of immunoglobulins by 5 colostrum deprived lambs was followed over a period of 6 weeks. Samples of serum were taken at weekly intervals and subjected to immuno-electrophoresis against rabbit anti-sheep serum. Apart from one lamb, which showed a faint line at birth (Fig. 4.6-5) \( \gamma_1M \) was not detectable before 13 days after birth (Fig. 4.7). \( 7S\gamma_1 \) began to appear between 28 to 35 days, and \( 7S\gamma_2 \) at 42 to 49 days after birth. Every lamb showed 2 to 3 lines in the \( \gamma_1A \) region from birth. As can be seen, the \( \gamma_1A \) lines did not change in strength with increasing age, and none occupy the region eventually filled by the \( \gamma_1M \) line. Conversely, only rarely was a line ever seen at birth in the area eventually occupied by the \( \gamma_1M \) line. On this basis the lines seen in foetal lymph have been classified as \( \gamma A \) rather than \( \gamma M \).

(3) The early production of immunoglobulins after specific antigenic stimulation

As described above, CD lambs did not normally produce sufficient levels of immunoglobulins to be detectable
FIGURE 4.7

The development of serum immunoglobulins in a colostrum deprived lamb which showed no detectable immunoglobulins at birth. 3 and 4 are the same sera samples as shown in 1 and 2, but electrophoresed for a longer period. The $\gamma_1 A$ lines have become more distinct since they are no longer obscured by the $7S\gamma_1$ and $\gamma_1 M$. 8 shows an extra line in the $7S\gamma_1$ region.
Serum of C.D. lamb post-birth

1. 14 Days

2. 28 Days

3. 14 Days

4. 28 Days

5. 35 Days

6. 42 Days

7. 56 Days

8. 63 Days

by immuno-electrophoresis before 14 days of age. However, when virus was administered, immunoglobulins were produced earlier. Typical results are shown in Fig. 1. A faint line can be seen in the sera at 5 days. These lines however, were not consistent and it was impossible to definitely assign this globulin to a specific class. With increasing time the serum line became more distinct and had separated into $\gamma_1 M$ and $78\gamma_1$, at about 10 days after stimulation. In contrast, the same line which was visible in lymph at 6 days, was barely detectable in the sera. As can be seen, there were differences in the $\gamma$ region by 8 days after a primary challenge. By 22 days, this line was observed as a separate $\gamma_1 M$ line and $78\gamma_1$. At the 35th day, a definite line was observed in the lymph of the secondary challenge. The $\gamma_1 M$ line was more distinct than the $78\gamma_1$ line which was strongly challenged in the serum from the lamb which was immunized against influenza A virus. There was no detectable antibody to the lipopolysaccharide, quite strong precipitation lines could be seen in the $\gamma$ region (Fig. 4.9-7). There was a faint but definite $\gamma_1 M$ line, 3 definite lines in the $\gamma A$ region, 1 of which was very strong, and 2 definite lines in the $78\gamma_1$ region which appeared to be distinct $78\gamma_1$ components rather than $78\gamma_1$ and $78\gamma_2$. 
by immuno-electrophoresis before 14 days of age. However, when a specific stimulus in the form of swine influenza virus was administered, immunoglobulins were produced earlier. Typical results are shown in Fig. 4.8. A faint line can be seen in the \( \gamma \) region in lymph and serum at 6 days. These lines however, were so diffuse that it was impossible to definitely assign this globulin to a specific class. With increasing time the serum line became more distinct and had separated into \( \gamma_1M \) and \( 7S\gamma_1 \) at about 10 days after stimulation. In contrast the same line which was visible in lymph at 6 days had disappeared by 9 days.

Following a secondary stimulus with swine influenza virus, the immunoglobulins increased very rapidly, \( 7S\gamma_2 \) was visible by 48 hours, and all the lines were reasonably distinct. In general, \( 7S\gamma_1 \) was produced in much greater quantities than \( \gamma_1M \). This correlated with the fact that there was no 2-mercapto-ethanol sensitive antibody produced in response to this antigen.

Immunoglobulins were also produced soon after stimulation by killed Salmonella muenchen. As can be seen in Fig. 4.9 faint but definite lines were seen in the \( \gamma \) region by 8 days after a primary challenge. By 9 days, this line had separated into \( \gamma_1M \) and \( 7S\gamma_1 \). At 24 hours after a secondary stimulus, the \( \gamma_1M \) line was more distinct than the \( 7S\gamma_1 \), but subsequently all 3 \( \gamma \) globulins developed strongly.

The serum samples from the foetal lamb which was challenged \textit{in utero} were also subjected to immuno-electrophoresis 10 days after the secondary challenge. Although there was no detectable antibody to the lipopolysaccharide, quite strong precipitation lines could be seen in the \( \gamma \) region (Fig. 4.9-7). There was a faint but definite \( \gamma_1M \) line, 3 definite lines in the \( \gammaA \) region, 1 of which was very strong, and 2 definite lines in the \( 7S\gamma \) region which appeared to be distinct \( 7S\gamma_1 \) components rather than \( 7S\gamma_1 \) and \( 7S\gamma_2 \).
The early development of immunoglobulins in colostrum deprived lambs following the primary subcutaneous injection of 10,000 haemagglutinating units of swine influenza virus into the hind limbs on the day of birth, followed by a secondary challenge with the same dose at 14 days of age.

1-4: the primary response in efferent popliteal lymph showing a faint $7\gamma$ line at 144 hours but which is no longer present at 192 hours.

5-8: the secondary response in efferent popliteal lymph showing $7\gamma_1$ developing earlier than $\gamma_1M$.

9-12: and 13-16: the primary response in the plasma of 2 different animals.

17-20 and 21-23: the secondary response in the plasma of 2 different animals.
1-6: The production of immunoglobulins in the plasma of a colostrum deprived lamb following a secondary challenge with $10^9$ killed S. muenchen organisms injected into the hind leg, 14 days after a primary challenge given on the day of birth. Despite the fact that there was no detectable antibody, there was a rapid production of immunoglobulins following the secondary challenge. 5 and 6 show two distinct $7S_{\gamma_2}$ lines.

7: The immuno-electrophoretic pattern of the cord plasma of a foetal lamb 10 days after a secondary challenge with $10^9$ killed S. muenchen organisms injected subcutaneously into both hind legs. Again, there was no detectable antibody but there was a faint $\gamma_1M$ line, a distinct $\gamma_1A$ and a strong "gull wing" pattern of $7S_{\gamma}$, but this is different from the normal adult $7S_{\gamma}$ pattern which is shown for comparison (8).
Primary plasma

1. The cells of foetal lymph were characterized by their uniformity and the absence of mitotic activity. Foetal lamb exists in a state of continuous lymphoid tissue of the gastro-intestinal and pulmonary tract. However, large amounts of lymphoid tissue continue to develop within the gut of the young foetal lamb. This result of the results described above is that within 72 hours of birth the tissue of the gut of the young foetal lamb has undergone extensive mitotic activity.

Secondary plasma

2. Normal adult plasma

Foetal plasma after S. muenchen

5. 75Sr

8. 75Sr

Normal adult plasma
The cells of foetal lymph were characterized by their uniformity and lack of mitotic activity. These features are indicative of the germ-free state in which the foetal lamb exists in utero. Immediately after birth however, large amounts of foreign antigens confront the lymphoid tissues of the gastro-intestinal and probably the respiratory tracts. The results described above have shown that within 72 hours of birth, the lymphoid tissue of the gut of the young lamb reacts to this antigenic stimulation. This reaction involves a significant increase in the number, mitotic activity and basophilia of the cells in the intestinal lymph, which are identical in all respects to those described by Hall and Morris (1963) in adult sheep following the deliberate specific antigenic stimulation of a lymph node. Whilst the cellular response of the newborn lamb was quite dramatic, the true response would probably be of much greater intensity, since over the course of the experiment, approximately $7 \times 10^9$ cells were drained from the lamb by way of the lymphatic fistula and this must have reduced the overall magnitude of the response.

That similar cellular changes do not occur in peripheral lymph is indicative of the localization of this initial stimulation to the central parts of the lymphoid system. The further observations that the output of cells from the gut associated lymphoid tissue increases with age more rapidly than body weight, and that there is an intermittent but frequent appearance of basophilic, mitotically active, cells in intestinal lymph (Heath, Lascelles and Morris, 1962) indicate continued antigenic stimulation. Heath (1963) found that in young rats the output of cells in the
thoracic duct lymph rose by a factor of 100 fold in the period from birth to 24 days of age. He suggested this rise may be due to an outpouring of cells from the thymus but acknowledged that it may also be due, at least in part, to the production of cells from peripheral lymphoid tissue although he did not relate this phenomenon to antigenic stimulation.

The evidence presented above that a large number of cells (some $7 \times 10^9$) enter the blood from the intestinal area in the first few days after birth suggests that, if all these cells remained in the blood, the circulating white cell count would become quite high. This does not occur and the level of white cells in the blood actually falls for a time. This initial drop in the blood white cell count has been reported in children by Kato (1935) who attributed this to "stress". Alternatively it may be due to the possibility that many lymphocytes are "recruited" to the intestinal area at this time in response to the newly encountered antigens. The large number of cells which subsequently pour into the blood from the intestinal area do not appear to remain in the blood for any length of time and since there is no general increase in the numbers of cells circulating through lymph nodes such as the popliteal, it is unlikely that many of these cells become part of the recirculating pool. They must therefore be sequestered from the circulation in some part of the lymphoid tissue.

Over the past few years Good and his co-workers have been consistently advancing the hypothesis that the gut associated lymphoid tissue of mammals subserves a function similar to that of the Bursa of Fabricius in birds. They have based this hypothesis on observations in the rabbit that the appendix, sacculus rotundus and Peyer's patches
have a similar morphological appearance to the bursa (Archer, Sutherland and Good, 1963), that there is a very high rate of proliferative activity in these organs (Meuwissen, Kaplan, Perey and Good, 1969), and that removal of these lymphoid structures early in life results in reduced antibody and immunoglobulin production, reduced circulating white cell counts and wasting disease (Sutherland, Archer and Good, 1964; Cooper, Perey, Gabrielsen, Sutherland, McKneally and Good, 1968). These reports have not as yet been confirmed in other laboratories, or in other species. If the same situation applied in the sheep, it might be expected that there would be a considerable development of the Peyer's patches before birth (since the sheep is much more immunologically mature at birth than the rabbit). This is not so however. Further, there is little evidence of proliferative activity within the lymphoid follicles of the patches prior to birth or in the cells in the lymph draining from the intestinal area. After birth the proliferative activity of the cells in the Peyer's patches increases greatly. It is known that the post-natal development of the Peyer's patches in germ-free animals is poor, whereas the thymus (and the bursa in birds) develop to almost the same degree in germ-free as in conventional animals (Miyakawa, 1959; Thorbecke, 1959; Gordon, 1959). Peyer's patches do show an involutionary phase similar to that displayed by the primary lymphoid organs (thymus and bursa), but this occurs comparatively later. This involution of the Peyer's patches has also been reported in humans by Cornes (1965a and b). It would appear then that in sheep and humans the development of the Peyer's patches resembles that of the secondary rather than the primary lymphoid organs. The post-natal development strongly suggests that it occurs under the
influence of antigen rather than under any intrinsic control mechanism. Until the results of Good and his co-workers have been confirmed in other species and in germ-free animals, the existence of a mammalian "bursa equivalent" in the form of the Peyer's patches must be considered not definitely proven.

It would seem reasonable to conclude from the evidence presented in this and the previous Chapter, that the final size of any part of the peripheral lymphoid system is closely correlated with the amount of previous antigenic stimulation to which it has been subjected. The observation that the prescapular nodes in one group of lambs with severe grass seed infestation were grossly enlarged is a graphic illustration of this point.

The results of studies on the immunoglobulin status of foetal lambs and the autogenous production of immunoglobulins by agammaglobulinaemic lambs confirmed some observations previously reported for other species. Some of the results however, differed from those of other workers.

Silverstein et al (1963a) were able to constantly detect small amounts (around 5 \( \mu \)g/ml) of \( 7S \gamma_1 \), traces of \( \gamma_1M \), but no \( \gamma_1A \) immunoglobulins by immuno-electrophoretic methods. In the present study, 2 or more lines in the \( \gamma A \) region were consistently detected but never any trace of \( 7S \gamma \) and only rarely traces of \( \gamma_1M \). This discrepancy may be nothing more than a difference in terminology, but close inspection of the patterns in Figs. 4.7, 4.8 and 4.9 reveals that the arcs termed \( \gamma A \) are definitely not \( \gamma_1M \), and correspond to the \( \gamma_1A \) arcs described by Aalund et al (1965) and Pan et al (1968).

The autogenous production of immunoglobulins by colostrum deprived lambs in the absence of specific antigenic stimulation, shows some similarities to the process as it
occurs in humans. Although one lamb had a faint, though distinct $\gamma_1 M$ line at birth which continued to increase in strength with age, no other lamb showed detectable levels until 14 days of age. $\gamma_1 S$ appeared at 28 to 35 days, followed by $\gamma_2 S$ at 42 to 49 days. In agammaglobulinaemic infants gamma globulin production is not apparent until at least 21 days of age (Zak and Good, 1959). IgM production has been reported as early as a few days after birth in normal children although IgA production does not occur until at least 3 to 4 weeks later (West, Hong and Holland, 1962), and Roth (1962) has also found early production of IgM in newborn infants, particularly in the face of infection.

The kinetics of the cellular and antibody response of newborn lambs to swine influenza virus was identical in all respects to the response of adult sheep to this antigen (Smith, 1967). Similar adult type immune responses have been described in newborn animals of various species by other authors. For example, the response of foetal monkeys to bovine serum albumin at 70 days gestation was as good as that of newborn monkeys (Cotes et al, 1966). Uhr et al (1962b) found that premature infants responded to $\phi X$-174 antigens as well as full term or older children. Kalmutz (1962) found that the foetal opossum was fully capable of responding to the same antigen, while Sterzl et al (1960b) and Riha (1961) have found that the response of young colostrum deprived piglets to Br. suis and sheep red blood cells does not improve with age.

The observation that colostrum deprived lambs were slightly slower to respond to the primary injection of antigen in terms of their cellular response, could reflect an "antigenic competition" effect since these animals had no passively acquired immunoglobulins to act as a buffer between
the lymphoid system and the wide range of antigens encountered at birth. This theory of antigenic competition has some evidence to support it (Cremer, 1963; Kim et al, 1966a). However, notwithstanding this somewhat altered primary response, the secondary response was as good, and possibly better, at least in cellular terms, than the response of the colostrum fed lambs.

The response to Salmonella muenchen organisms on the other hand was almost non-existent despite the production of levels of immunoglobulins detectable by immuno-electrophoresis. A similar complete lack of response to Salmonella antigens by foetal and newborn lambs has been reported by Silverstein et al (1963b). This failure to respond to Salmonella antigens is of importance when related to the acute susceptibility of newborn animals to the enterobacteria, particularly when they are deprived of maternal antibodies (Smith and Little, 1922; Aschaffenburg et al, 1949a and b).

It must be pointed out that the lymph and sera in the present experiments were tested for anti 'O' antibody and the test system used was the passive agglutination of lipopolysaccharide coated red cells. This is a much less sensitive test than the bactericidal test (Sterzl et al, 1962). It is also known that the 'O' antigen is less immunogenic than the 'H' antigen (Smith, 1960) although this fact is probably not of great importance.

Sterzl et al (1965) advanced evidence to show that the early antibodies produced by the young piglet were macroglobulin in type (19S) and of low avidity. If these animals are primarily challenged in utero and then given a secondary challenge after birth, 7S antibodies are produced but these are of low avidity when compared to the antibodies produced by adults. Kim, Bradley and Watson (1966b, 1967a and b, 1968)
have characterised the antibodies produced by colostrum deprived germ-free piglets in response to MSP-2 phage by various fractionation, ultracentrifugation and immuno-electrophoretic methods. They have determined that the first antibodies formed are $19S\gamma G$ followed by $7S\gamma G$. These two $\gamma G$ types are antigenically similar but distinct from $19S\gamma M$. They also identified a further class of $7S\gamma G$ (the "X" component), which is antigenically distinct from the normal $\gamma G$, $\gamma A$ or $\gamma M$, but which does not increase in concentration after antigenic stimulation. This additional $7S\gamma G$ component is frequently present at birth while the other fractions are not. $19S\gamma M$ synthesis does not appear to begin until some weeks after birth even in the face of deliberate antigenic stimulation.

Kim et al (1967a and b, 1968) have claimed that only in the colostrum deprived germ-free animal can a true primary immune response be studied. They suggest that all so-called "primary" responses in adult or non-germ-free animals are, in actual fact, "early" secondary responses.

Kim et al (1966b, 1967a and b, 1968) produced evidence that newborn, colostrum deprived, germ-free piglets produce early mercapto-ethanol sensitive $19S$ antibody in response to a viral antigen (MSP-2). In the present study, lambs challenged with swine influenza virus did not produce any $19S$ antibody early in the immune response. Inspection of the immuno-electrophoretic patterns (Figs. 4.6, 4.7, 4.8) revealed that while non-stimulated colostrum deprived lambs did not produce detectable levels of immunoglobulins until at least 14 days after birth, definite precipitation arcs could be seen in the $\beta$ or $\gamma M$ areas 6 days following challenge with influenza virus. As mentioned previously, these lines were so diffuse that it was impossible to assign a definite class to the fraction but with increasing time, the line
became more distinct and separated into $\gamma_1 M$ and presumably $7S_{\gamma_1}$. This $7S_{\gamma_1}$ may in fact be the $19S_{\gamma G}$ found by Kim and associates.

Similarly, the sera of colostrum deprived and foetal lambs also shows 2 or 3 lines in the $\gamma A$ area. One of these could be analogus to the 'X' component of Kim et al (1967). These lines are no stronger in adult sera and are frequently obscured by the strong $7S_{\gamma_1}$ line.

Although no antibody to the 'O' lipopolysaccharide antigen was detected in the sera of the foetal lamb or the post-natal colostrum deprived lamb challenged with Salmonella muenchen, definite lines in the immunoglobulin regions were visible in the electrophoretic patterns of the sera from both lambs. The pattern as seen in the foetal lamb serum 10 days after a secondary stimulus is completely different to a normal sheep serum and the $7S_{\gamma}$ line appears to be composed of 2 distinct arcs possibly analogus to $7S$ and $19S$ gamma globulins of Kim et al (1966b, 1967a and b).

However, until a much more extensive physico-chemical characterization of the sera of foetal and newborn colostrum deprived lambs before and after specific antigenic stimulation has been done, the precise identification of these protein fractions is impossible.

Summary

The changes which occur in the lymphoid system of the lamb after birth have been described. These changes are most obvious in the cellular component of the lymph draining the gut associated lymphoid tissue and are identical to the changes which occur in efferent lymph from a node deliberately challenged with antigen. It is suggested that the final size attained by the peripheral lymphoid tissue is a direct result
of the amount of antigenic challenge with which that tissue has had contact. The responses of newborn colostrum fed and colostrum deprived lambs to challenge with influenza virus and Salmonella muenchen organisms and the production of immunoglobulins after birth in stimulated and unstimulated lambs have been described.
The growth and development of thymectomized lambs

The effects of thymectomy on the growth and development of the lymphoid system and the circulating lymphocyte pool has been studied in inbred strains of mice, rats, rabbits and hamsters (see reviews by Miller and Osoba, 1967; Hess, 1968). Some of the main effects observed in these species are:

1. A reduced rate of growth and an increased number of deaths from a wasting syndrome.
2. Reduced levels of circulating lymphocytes in the blood and lymph.
3. A reduced number of lymphoid cells in the lymphoid organs.
4. Hypo-gammaglobulinaemia, although this has not been found consistently.

Neonatal thymectomy has also been performed in non-inbred larger species such as the calf (Channa, Crough and Joule, 1967; Carroll, Theiler and Broughton, 1968), the pig (Feist, Hallett and Shorter, 1965) and the dog (Van de Water and Katsman, 1964) however, in these species adverse effects have not been noted with the exception of the report by Tilney et al. (1965) that thymectomy in dogs produced the whole gamut of consequences, such as wasting, reduced blood leukocyte levels, reduced lymphoid tissues and hypo-gammaglobulinaemia. Van de Water and Katsman (1964) however, found no adverse effects in dogs following thymectomy. These larger species are known to be at a much more advanced stage of development at birth in both immunological and developmental terms than the small laboratory animals (Fennestad and Berg-Petersen, 1957, 1962; Kelly, 1963; Feist, Hallett and Shorter, 1965) and neonatal thymectomy in these large species is probably analogous to thymectomy in adult laboratory animals.
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While investigating the development of the lymphoid tissue in foetal lambs, it was felt that it would be possible to remove the thymus from foetuses early in gestation before the appearance of any great numbers of lymphocytes could be seen in the peripheral lymphoid organs and prior to the time at which these foetal lambs attain an ability to reject skin homografts and respond to BCG or Salmonella antigens.

Accordingly, pregnant sheep were obtained, their foetuses were thymectomized at various ages, returned to the uterus and allowed to develop and to be born normally at term. After birth the rate of growth, the development of the peripheral lymphoid tissue and the levels of lymphocytes in circulating blood and lymph in these thymectomized lambs were investigated. In addition, some thymectomized lambs were deprived of colostrum and the capacity of these lambs to produce immunoglobulins was followed.

Results

As it was difficult to obtain pregnant sheep with a known date of mating, flock joined ewes were operated on and the age of the foetus at the time of operation was calculated from the day of birth. Subsequently, a small number of ewes were joined by hand service and the foetuses operated on at a known time after conception. As these ewes lambed within a day or two of the expected date, the estimated ages of the group of unknown conception date were considered to be quite accurate.

The results of 45 in utero operations on foetal lambs are shown in Table 5.1. Groups I to III were ewes whose conception date was unknown, Group IV ewes were of known conception date. The ewes in Group I were the first to be operated on and the success rate was not high. As the
### TABLE 5.1

<table>
<thead>
<tr>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at</td>
<td>Age at</td>
<td>Age at</td>
<td>Age at</td>
</tr>
<tr>
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<td>Operation</td>
<td>Operation</td>
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</tr>
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<td>Result</td>
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<td>Result</td>
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<td>81</td>
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<td>76</td>
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<td></td>
<td></td>
<td>69</td>
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<td>Alive</td>
<td>88</td>
<td>65(T)(^b)</td>
</tr>
<tr>
<td>95</td>
<td>Alive</td>
<td>86</td>
<td>76(^c)</td>
</tr>
<tr>
<td>95</td>
<td>Born Dead</td>
<td>86 (Aborted)</td>
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</tr>
<tr>
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<td>80</td>
<td>85(^c)</td>
</tr>
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<td>Aborted</td>
<td>64</td>
<td>60(^T)</td>
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<td>Aborted</td>
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<td>Alive</td>
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<td></td>
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<table>
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<th>Total 13</th>
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<tr>
<td>4 Resorbed</td>
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<td>4 Aborted</td>
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\(^a\) = Twins      \(^b\) = Thymectomized and Splenectomized  \(^c\) = Splenectomized

The results of all in utero thymectomy and splenectomy operations on foetal lambs. Groups I to III include ewes whose conception date was unknown and the ewes in Group I were the first to be operated on. The foetuses in Group IV were of known gestational age.
surgical technique improved the success rate rose to close to 95 per cent (Groups II and III). The foetuses in Group IV were, in general, younger and here the success rate was lower than in Groups II and III. The reason for this was thought to be due to the fact that the foetal membranes at this age are extremely delicate and the amnion and allantois do not become fused together until around the fiftieth day post-conception. At this age the amniotic sac is quite tense and it is almost impossible to replace the amniotic fluid and reconstitute the membranes satisfactorily. These difficulties become much less of a handicap with increasing age and by 60 days the operation is relatively simple, since the allantois and amnion are now fused, the membranes are less fragile and the amniotic sac has increased in size and is much less tense. However, one lamb was successfully thymectomized at 51 days of age and subsequently born normally. This lamb fell victim of an unfortunate circumstance which led to its destruction by a colleague before it could be used for any studies.

Thus, out of a total of 45 in utero operations (41 thymectomies, 2 splenectomies and 2 combined thymectomy/splenectomies) 27 lambs (60 per cent) were born alive. If the first group is disregarded as experimental, 24 out of 32 were born alive and well, representing a 75 per cent success rate. Newborn control lambs were reared under identical conditions to the thymectomized lambs. Group II lambs were older than Group III and Group IV lambs were raised without the benefit of colostrum.

Post-natal Growth

At birth, there was no significant difference in the body weight of the thymectomized or the non-thymectomized
control lambs (Table 5.2), nor was there any significant difference between male and female lambs within or between the groups.

The post-natal growth rates of lambs in Group IV (colostrum deprived) were followed from birth to 12 weeks of age. They were found to grow at exactly the same rate over this period as the lambs in Group III (colostrum fed) which were observed up to the age of 32 weeks. The regressions of weight on age were used to compare growth rates of control lambs to thymectomized lambs (Fig. 5.1). There was no difference in the rate of growth between the two groups. It was interesting to note also that the colostrum deprived hand reared lambs grew as rapidly as the colostrum fed lambs reared by their mothers. This is in contrast to the hand reared lambs described in Chapter III which did not grow as well as those reared on their mothers. This was undoubtedly due to the fact that the thymectomized and control lambs in Group IV were better cared for and given greater amounts of milk more frequently than the early group. The mean daily milk intake of those lambs in Group IV was 1.5 litres given in 6 feeds per day. The hand reared lambs described earlier were given only one litre per day divided into 4 feeds.

The growth rate of the hand reared lambs declined when they were weaned at 3 months of age. This was almost certainly due to their reluctance to graze. They seemed to be constantly standing around waiting for the next feed, which unfortunately for them, never arrived. Those lambs reared by their mothers however, were already eating grass and hay even while they were still being fed by the ewes and when weaned they continued to do well.

The growth rates of lambs from Group II were followed over a period of 7 months from 5 to 12 months of
TABLE 5.2

<table>
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<tr>
<th>Group</th>
<th>Control</th>
<th>Thymectomized</th>
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<tbody>
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<td>Birth Wt.</td>
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<td>3.37±0.27</td>
</tr>
<tr>
<td>Sex</td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>Birth Wt.</td>
<td>3.39±0.28</td>
<td>3.04±0.22</td>
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<tr>
<td></td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>Birth Wt.</td>
<td>3.78±0.15</td>
<td>3.16±0.38</td>
</tr>
</tbody>
</table>

The birth weights of thymectomized and control lambs. The mean weights are given in Kg ± their standard errors.
FIGURE 5.1

The growth rates of control and thymectomized lambs of Group III from birth to 32 weeks of age, given as regressions of weight on age (± the standard error).
CONTROL \[ b = 1.12 \pm 0.04 \]

THYMECTOMIZED \[ b = 1.13 \pm 0.02 \]
These lambs were born in spring, 1967, and there was a severe drought over the following summer and autumn. As a result the ewes went dry earlier than normal and weaned their lambs sooner than they would normally have done. At this time the feed was poor and despite some supplementary concentrate feeding these lambs did not do well. At 5 months of age the control lambs weighed 19.35 ± 1.6Kg, while the thymectomized lambs weighed 16.19 ± 1.2Kg. Thymectomized and control lambs of Group III, on the other hand, weighed about 26Kg. at the same age indicating the more congenial environmental conditions these lambs enjoyed during the following spring of 1968 after the drought ended.

The significant difference in weights of control and thymectomized lambs of Group II at 5 months of age (19 vs 16Kg) was due to one thymectomized male lamb being particularly small (11.1Kg). From this age on, when the feeding regime was considerably improved, both groups of lambs gained weight rapidly, although the control lambs grew better than the thymectomized lambs. However, the significance of this difference was confounded by the fact that 3 out of 4 of the controls were males while 5 out of the 6 thymectomized lambs were females. As male lambs are known to grow more rapidly than female lambs, the difference in weights is almost certainly of no significance in relation to the removal of the thymus.

The Size of the Circulating Lymphocyte Pool in Thymectomized Lambs

(a) Blood leucocyte levels

Lambs thymectomized in utero were born with a reduced blood leucocyte count, a reduced blood lymphocyte count but a normal ratio of lymphocytes to polymorphs. The
blood leucocyte count of these lambs gradually increased after birth, but it always remained lower than that of control lambs over the period of observation from birth to 18 months of age, although the difference was not always statistically significant (Tables 5.3 and 5.4). The increase in blood leucocyte levels after birth involved both lymphocytes and polymorphs. The blood lymphocyte count however, always remained significantly lower than in normal lambs, whereas the polymorph count was, in fact, higher than in normal lambs. As a result, the lymphocyte:polymorph ratio was significantly lower than in normal lambs.

As the circulating blood volume of the lambs increased over this period, the production of lymphocytes was in fact, considerably greater than the cell counts indicated. Examination of Leishman stained smears revealed no obvious differences in size or morphological characteristics of the circulating blood lymphocytes of thymectomized lambs compared to control lambs.

(b) Lymphocyte levels in peripheral lymph

The thymectomized lambs were allowed to grow to at least 3 months of age before any attempt was made to investigate the lymphocyte levels in lymph. This was done for two reasons, firstly, to ensure that the lambs were big enough to give satisfactory lymph flows, and secondly, since the thymus is considered to control the maturation of lymphoid tissue, it was felt that the effects of thymectomy should be more obvious as the lambs grew older.

The efferent popliteal ducts of several thymectomized lambs were cannulated at 3 months of age and a further number at 10 months of age. The output of cells from these unstimulated popliteal nodes was examined and compared to that of normal lambs.
TABLE 5.3

<table>
<thead>
<tr>
<th>Age in Days</th>
<th>0</th>
<th>5</th>
<th>7</th>
<th>10</th>
<th>14</th>
<th>28</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Count</td>
<td>C</td>
<td>8.00±0.94</td>
<td>4.84±0.47</td>
<td>N.D.</td>
<td>5.73±0.79</td>
<td>8.00±1.61</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>3.57±0.30</td>
<td>4.10±0.06</td>
<td>5.05±1.46</td>
<td>4.84±2.06</td>
<td>4.62±0.96</td>
</tr>
<tr>
<td>Significance</td>
<td></td>
<td>0.01</td>
<td>N.S.</td>
<td>-</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>Total</td>
<td>C</td>
<td>4.25±0.89</td>
<td>3.79±0.50</td>
<td>N.D.</td>
<td>3.95±0.70</td>
<td>5.57±1.24</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>T</td>
<td>1.85±0.39</td>
<td>1.73±0.32</td>
<td>1.96±0.27</td>
<td>1.20±0.29</td>
<td>1.87±0.87</td>
</tr>
<tr>
<td>Significance</td>
<td></td>
<td>p</td>
<td>0.01</td>
<td>0.01</td>
<td>-</td>
<td>0.01</td>
</tr>
<tr>
<td>Total</td>
<td>C</td>
<td>3.75</td>
<td>1.05</td>
<td>N.D.</td>
<td>1.78</td>
<td>2.43</td>
</tr>
<tr>
<td>Polymorphs</td>
<td>T</td>
<td>1.725</td>
<td>0.37</td>
<td>3.09</td>
<td>3.64</td>
<td>2.75</td>
</tr>
<tr>
<td>L:P Ratio</td>
<td>C</td>
<td>1.13:1</td>
<td>3.61</td>
<td>-</td>
<td>2.22</td>
<td>2.29</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>1.07:1</td>
<td>4.67</td>
<td>0.63</td>
<td>0.33</td>
<td>0.68</td>
</tr>
<tr>
<td>Percentage</td>
<td>C</td>
<td>63.0</td>
<td>54.0</td>
<td>-</td>
<td>70.0</td>
<td>76.4</td>
</tr>
</tbody>
</table>

L = lymphocytes  P = polymorphs  C = control  T = thymectomized  p = significance  N.S. = not significantly different  L:P = ratio of lymphocytes to polymorphs

The white blood cell counts of thymectomized and control lambs from birth to 28 days of age. The figures are given in cells x 10^6 per ml. ± their standard error.
<table>
<thead>
<tr>
<th></th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>6</th>
<th>12</th>
<th>18</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Count</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>6.94±0.99</td>
<td>6.87±0.88</td>
<td>10.62±0.89</td>
<td>11.05±1.68</td>
<td>9.31±1.46</td>
<td>9.30±0.71</td>
</tr>
<tr>
<td>T</td>
<td>3.52±0.59</td>
<td>5.68±0.81</td>
<td>7.01±1.03</td>
<td>8.20±1.00</td>
<td>3.96±0.52</td>
<td>4.95±0.91</td>
</tr>
<tr>
<td>Significance</td>
<td>0.05</td>
<td>N.S.</td>
<td>0.05</td>
<td>N.S.</td>
<td>0.05</td>
<td>0.01</td>
</tr>
<tr>
<td>Total Lymphocytes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>5.26±0.64</td>
<td>5.58±0.52</td>
<td>7.65±0.94</td>
<td>9.36±1.55</td>
<td>7.73±1.48</td>
<td>7.66±0.66</td>
</tr>
<tr>
<td>T</td>
<td>2.01±0.51</td>
<td>3.17±0.59</td>
<td>2.36±0.31</td>
<td>4.27±0.65</td>
<td>2.00±0.45</td>
<td>2.20±0.35</td>
</tr>
<tr>
<td>Significance</td>
<td>0.01</td>
<td>0.05</td>
<td>0.01</td>
<td>0.01</td>
<td>0.05</td>
<td>0.01</td>
</tr>
<tr>
<td>Total Polymorphs</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>1.68</td>
<td>1.29</td>
<td>2.97</td>
<td>1.69</td>
<td>1.575</td>
<td>1.638</td>
</tr>
<tr>
<td>T</td>
<td>1.51</td>
<td>2.51</td>
<td>4.65</td>
<td>3.95</td>
<td>1.965</td>
<td>2.75</td>
</tr>
<tr>
<td>L:P Ratio</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>3.13</td>
<td>4.35</td>
<td>2.57</td>
<td>5.53</td>
<td>4.91</td>
<td>4.67</td>
</tr>
<tr>
<td>T</td>
<td>1.33</td>
<td>1.26</td>
<td>0.60</td>
<td>1.08</td>
<td>1.01</td>
<td>0.80</td>
</tr>
<tr>
<td>Percentage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymphocyte</td>
<td>62.0</td>
<td>43.0</td>
<td>69.0</td>
<td>54.0</td>
<td>74.1</td>
<td>71.2</td>
</tr>
<tr>
<td>Depression</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

L = lymphocytes  P = polymorphs  C = control  T = thymectomized  p = significance  
N.S. = not significantly different  L:P = ration of lymphocytes to polymorphs

The white blood cell counts of thymectomized and control lambs from 2 to 18 months of age. The figures are given in cells x 10^6 per ml. ± their standard error.
(1) **Cell output.** The output of cells from the unstimulated popliteal nodes of thymectomized lambs of 3 months of age was $4.46 \pm 0.37 \times 10^6$ cells per hour. In contrast, the output in normal lambs of the same age was $31.44 \pm 2.02 \times 10^6$ cells per hour. This difference was highly significant. The resting cell output from the popliteal nodes of two splenectomized lambs was also measured and was found to be $28.48 \pm 3.95 \times 10^6$ cells per hour. This was not significantly different from the controls.

When the cell output of 10 month old lambs was investigated, it was found that there had been a considerable rise in the circulating counts in both control and thymectomized groups, but the latter were still less than half that of the controls. These findings are summarised in Table 5.5.

(2) **The morphology of the cells in lymph.** As with the blood lymphocytes, there was no obvious difference in the morphology of the lymphocytes in the lymph of thymectomized and control lambs as seen in Leishman stained smears. In all cases about 90 per cent to 95 per cent of the cells were medium to small lymphocytes, while the remainder were very small or large lymphocytes. A more detailed examination of the size distribution of the lymphocytes with the aid of the Coulter Counter showed, however, that there were differences in the lymphocytes of the two groups of lambs at 3 months of age. Table 5.5 shows the relative size distribution of the cells in absolute and percentage terms in the two groups.

There was a relatively greater reduction in the smaller sized lymphocytes in thymectomized sheep leading to a significantly higher proportion of cells in the larger size ranges. These differences in size distribution could not be detected in stained smears. By 10 months of age there were no differences in the size distribution (Table 5.5).
<table>
<thead>
<tr>
<th></th>
<th>Age</th>
<th>Count x 10⁶/ml</th>
<th>p</th>
<th>Per Cent Depression</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cell Output</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 mths.</td>
<td>C 37.41± 5.81</td>
<td>*</td>
<td>89.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T 3.75± 0.19</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10 mths.</td>
<td>C 40.98± 4.96</td>
<td></td>
<td>59.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T 16.63± 1.74</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td><strong>Cell Differential in Percentage</strong></td>
<td></td>
<td>S</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 mths.</td>
<td>C 67.20 25.40</td>
<td>6.30</td>
<td>1.10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T 50.30 35.10</td>
<td>11.60</td>
<td>3.00</td>
</tr>
<tr>
<td></td>
<td>10 mths.</td>
<td>C 60.13 31.08</td>
<td>7.23</td>
<td>1.55</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T 62.10 25.96</td>
<td>8.66</td>
<td>2.26</td>
</tr>
<tr>
<td><strong>Cell Differential in Absolute numbers at 3 months of age</strong></td>
<td></td>
<td>C 25.13 9.50</td>
<td>2.35</td>
<td>0.41</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T 1.88 1.32</td>
<td>0.43</td>
<td>0.11</td>
</tr>
<tr>
<td><strong>Percentage Depression</strong></td>
<td></td>
<td>92.6 86.1 81.5</td>
<td></td>
<td>73.2</td>
</tr>
</tbody>
</table>

S = Small cells of modal cell volume 100 µ₃
M = Medium sized cells of modal cell volume 180 µ₃
L = Large sized cells of modal cell volume 260 µ₃
VL= Very large sized cells of modal cell volume 405 µ₃ or >

* = ± the standard error

A comparison of the output and size distribution of cells in efferent popliteal lymph of unstimulated thymectomized and control lambs at 3 and 10 months of age.
The Production of Immunoglobulins by Agammaglobulinaemic Thymectomized Lambs

Samples of serum were obtained from 3 colostrum deprived (CD) lambs (2 thymectomized and one thymectomized/splenectomized) at weekly intervals after birth and subjected to immuno-electrophoresis to determine whether the autogenous production of immunoglobulins differed in these animals from normal CD lambs. The results are shown in Figs. 5.2 and 5.3. One thymectomized lamb showed a faint $\gamma_1M$ line at 7 days of age, while the $7S\gamma_1$ line had appeared at 28 days and the $7S\gamma_2$ line at 42 days of age (Fig. 5.2; 1 to 4). The second thymectomized lamb first showed a faint $\gamma_1M$ line at 14 days, $7S\gamma_1$ at 35 days and $7S\gamma_2$ at 42 days of age (Fig. 5.3). The lamb which had neither thymus nor spleen showed a $\gamma_1M$ line at 10 days, $7S\gamma_1$ at 14 days and $7S\gamma_2$ by 35 days (Fig. 5.2; 5 to 6). All lambs showed the same 2 to 3 lines in the $\gamma_1A$ area at birth as were seen in normal CD lambs (Chapter IV). These results showed that the onset of production of immunoglobulins by colostrum deprived lambs was as rapid in thymectomized or thymectomized/splenectomized lambs as in normal control lambs.

The sera of several 9 month old thymectomized lambs were examined for the presence of immunoglobulins and the immuno-electrophoretic patterns of 3 of these lambs are compared against control lamb sera in Fig. 5.4. It can be seen that one thymectomized lamb had a strong $7S\gamma_1$ line but no $7S\gamma_2$ and only a faint $\gamma_1M$ line. The second lamb had strong $7S\gamma_1$ and $\gamma_1M$ lines, but only a very faint $7S\gamma_2$ line. The third lamb had distinct lines in all three areas.
FIGURE 5.2

1-4: The immuno-electrophoretic patterns of serum from a thymectomized, colostrum deprived lamb, from birth to 28 days of age, showing the gradual development of immunoglobulins.

5-6: The immuno-electrophoretic patterns of serum from a thymectomized/splenectomized lamb at 14 and 28 days after birth, showing production of $\gamma_1M$ and $7\gamma_1$ immunoglobulins.
Thymectomized C.D.

Birth

1

7 days

2

14 days

3

28 days

4

Thymectomized—splenectomized

14 days

5

28 days

6
Another example of the immuno-electrophoretic patterns of serum from a colostrum deprived, thymectomized lamb from birth to 63 days of age, showing the gradual development of immunoglobulins.
Thymectomized C.D.

1

14 days

2

21 days

3

28 days

4

35 days

5

42 days

6

56 days

7

63 days
Three representative examples of the immuno-electrophoretic patterns of sera from 10 months old thymectomized lambs (2, 4 and 6), compared to sera of 3 different control lambs of the same age (1, 3 and 5).
The Growth and development of Lymphoid Tissue in Thymectomized Lambs

Since relatively few of these experimental animals were produced and since it was necessary to preserve as many of them as possible for a systematic study on the effect of thymectomy on the development of the lymphoid tissue was attempted on the material of lymphoid tissue obtained from lambs of both sexes. The spleens, mesenteric, popliteal, prefemoral and prescapular lymph nodes were dissected out and the ileum was examined for the presence of Peyer's patches. Sections of these tissues were fixed and examined histologically.

The lambs had significantly smaller lymphoid tissue than the controls; however, were in all cases, within the normal range. Histologically there was a general reduction in the cellular population of the spleen and lymph nodes. The findings are summarised in Table 5.6.

In the case of two lambs (No. 63 and 64) the lambs were incompletely thymectomized (No. 63), while the other (No. 62) had been thymectomized relatively late in gestation (at 89 days). The lymph nodes and spleens of all other lambs showed some degree of reduced cellularity which varied from partial to severe reduction, with gradations in between. In most cases there was a general reduction in the numbers of lymphoid cells in the cortex of the lymph nodes (Fig. 5.5), and sometimes the follicular areas of...
The Growth and Development of Lymphoid Tissue in Thymectomized Lambs

Since relatively few of these experimental animals were produced and since it was necessary to preserve as many of them as possible, no systematic study on the effect of thymectomy on the development of the lymphoid tissue was attempted. However, the lymphoid organs of 11 thymectomized and 2 thymectomized/splenectomized lambs of various ages from birth to 56 weeks of age were examined. The spleen, mesenteric, popliteal, prefemoral and prescapular lymph nodes were dissected out and weighed and the ileum was examined for the presence of Peyer's patches. Sections of these tissues were fixed and examined histologically.

All but two of these lambs had significantly smaller lymph nodes (both peripheral and mesenteric) than normal lambs of the same ages. The spleens however, were in all cases, within the normal range. Histologically there was a general reduction in the cellular population of the spleen and lymph nodes. These findings are summarised in Table 5.6.

The lymph nodes and spleens of two lambs of 16 and 17 weeks of age appeared to have a normal lymphoid structure despite reduced lymphocyte levels in their blood and lymph. One of these lambs was found at post-mortem to have been incompletely thymectomized (No.69), while the other (No.62) had been thymectomized comparatively late in gestation (at 89 days). The lymph nodes and spleens of all other lambs showed some degree of reduced cellularity which varied from partial to severe reduction, with gradations in between. In most cases there was a general reduction in the numbers of lymphoid cells in the cortex of the lymph nodes (Fig.5.5), the peri-arteriolar and sometimes the follicular areas of...
Details of the size and histological appearance of the lymphoid organs of thymectomized and splenectomized lambs of various ages at post-mortem. The figures given in brackets are the size ranges seen in normal lambs at the same age.

TABLE 5.6
<table>
<thead>
<tr>
<th>Lamb No.</th>
<th>Age at P.M. in weeks</th>
<th>Age at op.</th>
<th>Sex</th>
<th>Weight (kg)</th>
<th>Spleen (gm)</th>
<th>Lymph nodes (gm)</th>
<th>Gut nodes (gm)</th>
<th>Histological Findings</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>120</td>
<td>0</td>
<td>M</td>
<td>2.65</td>
<td>N.D.</td>
<td>2.22 (4.95)</td>
<td>N.D.</td>
<td>Reasonably normal</td>
<td>Died at birth</td>
</tr>
<tr>
<td></td>
<td>61</td>
<td>0</td>
<td>M</td>
<td>2.50</td>
<td>3.75 (13.0)*</td>
<td>1.75 (4.95)</td>
<td>N.D.</td>
<td>Depleted</td>
<td>Died at birth</td>
</tr>
<tr>
<td>37</td>
<td>62</td>
<td>4</td>
<td>M</td>
<td>4.0</td>
<td>N.D.</td>
<td>2.0 (9.7-12.3)</td>
<td>0.75 (4.95)</td>
<td>Grossly depleted</td>
<td>Died of unknown cause</td>
</tr>
<tr>
<td>69</td>
<td>97</td>
<td>16</td>
<td>F</td>
<td>14.0</td>
<td>40.0 (20.0-87.0)</td>
<td>6.1 (7.4-9.7)</td>
<td>18.4 (21.1-28.7)</td>
<td>Normal</td>
<td>Normal growth</td>
</tr>
<tr>
<td>62</td>
<td>89</td>
<td>17</td>
<td>M</td>
<td>13.6</td>
<td>63.8 (78.4-92.7)</td>
<td>5.6 (10.0 approx)</td>
<td>13.0 (30.6-43.0)</td>
<td>Normal Trace thymus remaining</td>
<td>Normal growth</td>
</tr>
<tr>
<td>55</td>
<td>99</td>
<td>18</td>
<td>F</td>
<td>N.D.</td>
<td>35.0 (32.2-48.3)</td>
<td>6.2 (10.0 approx)</td>
<td>10.5 (27.6-47.1)</td>
<td>Normal</td>
<td>Normal growth</td>
</tr>
<tr>
<td>34</td>
<td>69</td>
<td>22</td>
<td>M</td>
<td>28.2</td>
<td>30.2 (42.1-62.3)</td>
<td>11.0 (10.0 approx)</td>
<td>43.0 (29.1-41.9)</td>
<td>Partially depleted</td>
<td>Normal growth</td>
</tr>
<tr>
<td>14</td>
<td>62</td>
<td>24</td>
<td>F</td>
<td>20.5</td>
<td>51.4 (29.8-78.0)</td>
<td>5.55 (11.1-15.3)</td>
<td>26.7 (36.9-37.2)</td>
<td>Depleted</td>
<td>Normal growth</td>
</tr>
<tr>
<td>26</td>
<td>87</td>
<td>24</td>
<td>M</td>
<td>14.5</td>
<td>53.0 (29.8-78.0)</td>
<td>5.4 (11.1-15.3)</td>
<td>15.0 (36.9-37.2)</td>
<td>Partially depleted</td>
<td>Normal growth</td>
</tr>
<tr>
<td>83</td>
<td>71</td>
<td>40</td>
<td>F</td>
<td>40.0</td>
<td>56.2</td>
<td>12.75</td>
<td>47.5</td>
<td>Partially depleted</td>
<td>&quot;Wasting&quot;</td>
</tr>
<tr>
<td>31</td>
<td>88</td>
<td>56</td>
<td>F</td>
<td>15.0</td>
<td>53.8 (90.5-120.0)</td>
<td>8.1 (19.9-20.2)</td>
<td>25.6 (29.4-57.6)</td>
<td>Grossly depleted</td>
<td>Poor growth</td>
</tr>
<tr>
<td>16</td>
<td>60</td>
<td>1</td>
<td>M</td>
<td>4.6</td>
<td>-</td>
<td>5.3 (8.1)</td>
<td>4.0 (6.2)</td>
<td>Normal</td>
<td>Normal growth</td>
</tr>
<tr>
<td>22</td>
<td>60</td>
<td>17</td>
<td>F</td>
<td>15.0</td>
<td>- (2.5)**</td>
<td>3.45 (10.0 approx)</td>
<td>19.2 (30.6-43.0)</td>
<td>Partially depleted</td>
<td>Normal growth</td>
</tr>
</tbody>
</table>

N.D. = Not done  
* = Normal observed values  
** = Partial splenectomy
FIGURE 5.5

Popliteal lymph nodes from a normal lamb (A) and a thymectomized lamb (B) of 22 weeks of age. The cortex of the node from the thymectomized lamb contains relatively few cells. Germinal centres, although still present, appear relatively empty of cells.

Stain H and E. Magnification 100x.
the spleen (Fig. 5, 6; a, b and c). The lymphocyte content of the Peyer’s patches was reduced to varying degree (Fig. 1, 7).
the spleen (Fig. 5.6; a, b and c). The lymphocyte content of the Peyer's patches was reduced to a varying degree (Fig. 5.7). In all cases however, plasma cells were present in abundance.

In the severe cases there was a gross depletion of lymphocytes from the cortical areas of lymph nodes (Fig. 5.8; a) and the follicular areas of the spleen and the Peyer's patches. Some lymph nodes still had germinal centres but these presented a relatively empty appearance (Fig. 5.8; b). Despite this extensive depletion, plasma cells were present in abundance in the medullary cords of the lymph nodes (Fig. 5.8; c and d), the peri-arteriolar regions of the spleen (Fig. 5.6; d) and in the sub-mucosal areas of the intestine just outside the borders of the remaining atrophic follicles of the Peyer's patches (Fig. 5.7; f).

The Occurrence of Wasting Disease

Out of 27 lambs which were born normally following in utero thymectomy, only 2 showed signs of a condition which might have been considered analogous to the syndrome described in thymectomized mice (Miller, 1961). Both of these lambs were poorly grown and suffered from chronic intermittent diarrhoea which was not apparently due to any parasitic infestation. These 2 lambs were members of Group II which suffered the effects of drought and all animals in this group were comparatively poorly grown. However, after the drought ended, all these lambs began to gain weight again. The 2 "runted" lambs were examined at post-mortem. One (No.31, at 56 weeks of age) had grossly depleted lymphoid tissues, while in the other (No.26, which was 24 weeks of age) these tissues were only partially depleted of cells, even though the lymph nodes were much smaller than normal.
FIGURE 5.6

A The spleen of a normal lamb of 24 weeks of age. The white pulp is well developed with numerous follicles and germinal centres.

Stain H and E. Magnification 25x.

B The spleen of a thymectomized lamb of 24 weeks of age showing a depletion of the cells of the white pulp, small follicles and a reduced number of germinal centres.

Stain H and E. Magnification 62.5x.

C A peri-arteriolar area in the spleen of a thymectomized lamb showing a reduction in the density of the lymphoid follicles which normally surround the arterioles.

Stain H and E. Magnification 400x.

D A peri-arteriolar area in the spleen of a thymectomized lamb. Despite a general lack of lymphoid cells, many pyroninophilic cells are present.

Stain pyronin-methyl-green. Magnification 250x.
FIGURE 5.7

Lymphoid follicles in a Peyer's patch of a normal lamb of 8 weeks of age (A) and in thymectomized lambs of different ages (B to E) showing varying degrees of cellular deplection.

Stain H and E. Magnification A 25x.

B 62.5x.
C 62.5x.
D 100x.
E 100x.

Despite the almost complete disappearance of the great bulk of the lymphoid follicles, many plasma cells are present in the submucosal areas of the ileum. (Thymectomized lamb aged 24 weeks of age).

Stain pyronin-methyl-green. Magnification 250x.
FIGURE 5.8

Poopileal lymph nodes from thymectomized lambs of various ages.

A Cortex of a lymph node showing a considerable reduction in cellularity.

Stain H and E. Magnification 400x.

B A germinal centre containing only a few lymphocytes and other cells.

Stain H and E. Magnification 400x.

C Cortex and medulla showing an overall reduction in the cells of the cortex; the germinal centres appear reasonably normal in this case. Many pyroninophilic cells are present, particularly in the medullary cords.

Stain pyronin-methyl-green. Magnification 400x.
Although these 2 thymectomized lambs were the only experimental lambs to suffer this condition, several control lambs showed a similar stunted growth and intermittent diarrhoea. Cases of so-called "runting" have recently been observed in a large group of normal lambs which failed to sustain a normal growth rate after 6 weeks of age and remain poorly grown at 9 months of age. This "wasting disease" was uncommon in thymectomized lambs and the phenomenon of "runted" growth was found to occur with equal frequency in normal lambs. This condition as seen in sheep is probably associated with dietary rather than infectious factors. As a general statement then, it can be said that the growth rates and physical condition of the thymectomized lambs were identical to those of the control lambs.

Only 2 thymectomized lambs died of natural causes, one at one week of age (No. 16), the other at one month of age (No. 37) and both succumbed to an apparent acute toxaemic condition of unknown etiology. The lymphoid tissues of lamb 37 were grossly reduced, while those of lamb 16 (which was thymectomized and splenectomized) were reasonably normal in histological appearance, though reduced in size. One control lamb died at 3 months of age from an acute attack of enterotoxaemia (Clostridium perfringens Type D toxaemia). This lamb was extremely well grown with normal lymphoid tissues. Thus there appeared to be no correlation between the incidence of disease and thymectomy under the conditions of this experiment.

Discussion

It was found that lambs thymectomized in utero between 60 and 100 days gestation have a much reduced level of lymphocytes in their lymph at three months of age and a
consistently reduced number of lymphocytes circulating in
the blood at all ages, at least up to 18 months after birth.
The growth and development of the secondary lymphoid organs
was also found to be reduced below that in normal sheep. In
these respects the effect of thymectomy in lambs is similar
to that seen in all other species (Miller, 1961, 1962b;
Arnason et al, 1962, 1964b; Parrot, 1962; Parrot and East,
1962).

**In utero** splenectomy had no effect on lymphocyte levels in lambs, as has also been reported for mice
(Moody and Reed, 1968), although McBride, Dacie and Shapley
(1968) have found raised lymphocyte and total leucocyte
levels in splenectomized humans.

Although the lymph nodes of thymectomized
lambs were smaller than normal, the size of the spleen in all
cases fell within normal limits. This has also been observed
in rats (Waksman, Arnason and Jankovic, 1962).

Histologically the lymphoid organs of some
thymectomized lambs had a near normal complement of lymphocytes, while in others the numbers of lymphocytes were severely reduced. The most severe reduction was seen in the most poorly grown animals, particularly in one lamb which suffered from chronic diarrhoea, and in another which died at an early age from unknown causes. In these severe cases the lymphoid organs and their germinal centres were almost completely empty of lymphocytes. However, even in both these cases there appeared to be an abundance of plasma cells in the spleen and lymph nodes. In less severely affected cases, the extent to which the lymph nodes were depleted of lymphocytes was not so great, while plasma cells were always prominent.

These observations confirm those of Parrot, de
and Azar (1966) in the rat. They found that the depletion of lymphocytes which followed thymectomy occurred mainly in the diffuse cortex of the lymph nodes and in the peri-arteriolar and follicular areas of the spleen. There was little change except in severe cases in the germinal centres, while in all cases there appeared to be an abundance of plasma cells. In one of the lambs the depletion of lymphocytes in the Peyer's patches was so great that they only appeared as small accumulations in the submucosal tissues. Plasma cells, however, could still be seen scattered throughout the submucosal tissue of the gut.

As a result of observations in other species the thymus has been credited with influencing the production of lymphoid cells in a variety of ways. It is thought

(1) to produce lymphocytes in situ, which are then seeded to the peripheral lymphoid organs,
(2) to exert an influence on immigrant stem cells, inducing them to divide rapidly to produce descendants, which emigrate to the peripheral lymphoid organs,
(3) to produce a humoral factor, which influences lymphopoiesis at some distant site.

The recent evidence of Moore and Owen (1967a and b) and Owen and Ritter (1969) has indicated that thymic lymphocytes are derived from immigrant stem cells rather than directly from the thymic epithelium as suggested by Auerbach (1960, 1961). There is considerable evidence, both direct and circumstantial, that the thymus seeds cells to the periphery (Fichtelius, 1958; Murray and Woods, 1964; Nossal, 1964; Ernstrom, Gyllensten and Larsson, 1965; Linna and Stillstrom, 1966; Linna, 1967; Weissman, 1967). There is little evidence to support the hypothesis that the thymus produces a humoral factor (Metcalf, 1956) insofar as lymphopoiesis is concerned.
Regardless of whether peripheral lymphocytes are produced in the thymus either from epithelial cells or from immigrant stem cells, or whether stem cells are stimulated to divide in the periphery by a humoral factor, it would be expected that if lymphoid development was entirely dependant on an intact, functional thymus, no further development should take place following its removal. However, this is not the case. As described above, although lambs which had been thymectomized in utero some 2 to 3 months previously had reduced numbers of lymphocytes in their blood and probably lymph and smaller lymphoid organs at birth, there was a considerable though reduced development in size and cellularity of these lymphoid organs between the time of thymectomy and birth (Table 5.7).

This continued development of the lymphoid organs in the absence of the thymus could be explained on the basis of one of the following hypotheses. Firstly, lymphoid development may continue under the influence of a humoral factor produced by the thymus of the mother. To date there has been one report that the thymus produces a lymphocytosis stimulating factor (Metcalf, 1956). There have, however, been several reports detailing the restoration of immune faculties of thymectomized mice by using thymus grafts enclosed in millipore chambers (Osoba and Miller, 1963, 1964; Law, Trainin, Levey and Barth, 1964a). However, only Law et al (1964a) found any significant degree of restoration of circulating lymphocyte counts. There has also been one report of the restoration of these faculties in adult female mice following pregnancy (Osoba, 1965). This result was advanced as evidence for a humoral factor produced by the foetal thymuses.

The above hypothesis could be tested by mating adult ewes which had been thymectomized in utero as early
A comparison of the weights of the lymph nodes and spleens of normal foetal lambs at 80 days gestation and of normal and thymectomized lambs at birth showing that some development of the lymphoid system does occur in the absence of the thymus.

<table>
<thead>
<tr>
<th>Age</th>
<th>Total Lymph Node Weight (g)</th>
<th>Spleen Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>80 days in utero</td>
<td>0.1096</td>
<td>0.285</td>
</tr>
<tr>
<td>Birth (normal)</td>
<td>4.95</td>
<td>13.3</td>
</tr>
<tr>
<td>Birth (Thymectomized in utero at 80 days)</td>
<td>1.98</td>
<td>3.75</td>
</tr>
</tbody>
</table>

A comparison of the weights of the lymph nodes and spleens of normal foetal lambs at 80 days gestation and of normal and thymectomized lambs at birth showing that some development of the lymphoid system does occur in the absence of the thymus.
as possible. If no subsequent development of the lymphoid tissues of the thymectomized foetuses occurred, this would be evidence in favour of a humoral factor. On the other hand, if the same degree of development occurred as is seen in thymectomized lambs from non-thymectomized mothers, this would be evidence against a humoral factor. Sufficient thymectomized female lambs have now reached the age at which it will be possible to do this experiment.

However, even now it seems doubtful whether a humoral factor is of importance in the present situation, since not only does the lymphoid tissue develop in utero after thymectomy, but it continues to develop after birth. It is difficult to imagine any factor exerting this effect on the lymphoid tissue unless it was being produced continually.

Neonatally thymectomized laboratory animals also show a considerable development of their lymphoid tissues, although this has not previously been stressed (Law, Dunn, Trainin and Levey, 1964b; Register, 1965; Parrot et al, 1966).

A second possible explanation for the continuing growth of lymphoid tissue after thymectomy could be that sufficient stem cells had escaped from the thymus prior to thymectomy to act as progenitors of the lymphoid cells which subsequently appear.

It has been reported that if mice are not thymectomized until several days after birth, the incidence of wasting is significantly reduced (Parrot and East, 1964). It has been suggested that this delay enables sufficient cells to migrate to the peripheral lymphoid organs, so that any infectious agents to which the newborn animal is exposed can be dealt with adequately (Hess, 1968). This protective effect of delayed thymectomy seen in mice could be analogous to the
present situation in lambs, since many of the lambs were thymectomized between 70 and 90 days post-conception, when the thymus was quite definitely lymphoid in structure.

However, as mentioned above there is also a considerable development of the lymphoid tissues after neonatal thymectomy in laboratory animals (Law et al., 1964b; Parrot et al., 1966). In any event, whether cells that have left the thymus prior to its removal are influenced by a humoral factor or not, it would be necessary for them either to have a very long life span or for few of them to be required to give rise to huge numbers of daughter cells. If either of these explanations are valid and the thymus has performed a major part of its function at a very early age, it is difficult to understand why, in the normal animal, it subsequently grows to such a large size increasing in weight by at least 250 times between 80 days before and 50 days after birth.

A third possible explanation could be that the thymus is a source of, or influences the production of, one particular class of lymphocyte while having no effect on the production of others. In thymectomized rats and mice, most of the depletion of the lymphoid organs is due to a reduction in the cells in the diffuse cortex of lymph nodes, in the peri-arteriolar sheaths and follicular areas of the spleen, and not in the germinal centres or in the number of plasma cells (Waksman et al., 1962; Parrot et al., 1966; Azar, 1966). The present results show that the same situation holds in thymectomized lambs.

It seems reasonable to assume that, while the thymus is responsible in some way for the generation of a high proportion of the cells in the circulating pool of
lymphocytes (probably by a direct seeding effect), it is not directly responsible for the production or differentiation of those cells which form the germinal centres, nor does it appear to be essential to the production of plasma cells.

The observation that young thymectomized lambs had a higher proportion of large cells in their lymph than control lambs of a similar age is in line with a report by Rieke (1966) for cells in the thoracic duct lymph of neonatally thymectomized rats. In this respect, the size distribution of the cells in the lymph of thymectomized animals resembles that of animals with chronic lymphatic fistulae (Glenn, Bauer and Cresson, 1949; Gesner and Gowans, 1962) and reflects the lack of the large bulk of circulating small lymphocytes which normally dilute out this large cell component. This situation was not observed in the older thymectomized lambs which had significantly higher total lymphocyte levels in their lymph.

The observation that thymectomized lambs had consistently raised levels of polymorphs in their blood has also been reported in mice and rats (Metcalf, 1960; Parrot and East, 1962; Law et al., 1964b). The significance of this observation is not known.

Despite the fact that the thymectomized lambs had a reduced level of circulating lymphocytes in both the blood and the lymph, their production of immunoglobulins (studied in colostrum deprived thymectomized lambs) was not delayed, nor did there appear to be any defect in the range of the different immunoglobulins they produced. Experiments with other species on the effects of thymectomy on the production of immunoglobulins have shown that thymectomized mice and rats are capable of producing all types of immunoglobulins (Humphrey, Parrot and East, 1964; Fahey, Barth and Law, 1965).
but there are wide differences between individuals, as was the case in thymectomized lambs.

Wasting disease has not been a feature of thymectomy in lambs. Although some thymectomized lambs grew rather slowly and intermittent diarrhoea also occurred in one or two cases, there were no cases of severe wasting. Several control lambs also grew poorly, but in all cases it was felt that dietary rather than infectious factors were largely responsible. The growth rate of splenectomized lambs was perfectly normal. These results in lambs taken together with those of many other workers (Parrot, 1962; Hess, Cottier and Stoner, 1963; Wilson, Sjodin and Bealmear, 1964; McIntire, Sell and Miller, 1964; Isakovic et al, 1965; Fisher and Fisher, 1965) lend further emphasis to the fact that wasting disease is not an obligatory consequence of thymectomy.

Summary

The effects of in utero thymectomy on the subsequent growth rate, the levels of circulating white blood cells, the levels of lymphocytes in lymph and the size and development of the peripheral lymphoid organs have been investigated in lambs. Consistent and severe depressions were observed in the circulating lymphocyte counts in both blood and lymph of thymectomized lambs of 3 months of age, while at 10 months of age, although these levels were still significantly less than normal, considerable development had occurred. Histological observations showed that the lymphoid tissues were depleted of cells to varying degrees. Lambs thymectomized at 80 to 90 days post-conception generally showed less depletion than those lambs which were thymectomized at 60 to 70 days post-conception. Well grown
thymectomized lambs and those examined soon after birth, showed less depletion than older or poorly grown animals. The classical "wasting syndrome" described for thymectomized mice was not seen.

These results in the lamb suggest that while the thymus is a source of lymphocytes, a considerable proportion of the lymphocytes present in normal animals is not derived directly from the thymus.
The response of thymectomized lambs to antigenic stimulation

The sheep has been used experimentally as a model for the study of the response of individual lymph nodes to specific antigenic stimulation. Cannulation of the lymphatic duct efferent to a node draining the site of injection has enabled much information to be gained about the cellular events which occur in lymph in response to various antigens (Hall and Morris, 1963; Hall, Morris, Moreno and Bessis, 1967).

In the past 8 years many reports have appeared in the literature purporting to show that thymectomized animals fail to respond, or have greatly reduced responses, to a variety of antigens, in terms of the production of specific antibody (Miller and Osoba, 1967). Little information however is available on the cellular aspects of immune responses in thymectomized animals.

The results described in the foregoing chapter indicated that in utero thymectomy in the lamb resulted in a reduction in the number of circulating lymphocytes and in a reduction in the cellularity of various secondary lymphoid organs. These findings were similar to those that have been found in rodents following neonatal thymectomy. Since the circulating pool of small lymphocytes has been shown to be intimately involved in the immune response to antigen (Cowans, McGregor, Cowen and Ford, 1962) and since thymectomized lambs had a greatly reduced number of these cells, it was thought that if any specific cellular lesion is produced by thymectomy, it may be revealed by studying the cellular reactions and kinetics of antibody formation in the lymph coming from a single lymph node after antigenic challenge.
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Three different antigens have been employed in these studies; swine influenza virus (strain Shope 15), boiled Salmonella muenchen organisms and chicken red cells. The immune response to swine influenza virus in normal adult sheep has been described in detail by Smith (1967). A dose rate of 20,000 haemagglutinating units was used in the present experiments. Boiled Salmonella muenchen organisms were used as an antigen to study the production of antibody forming cells, thus providing a further parameter of the immune response. The challenging dose used was $10^9$ organisms. Chicken red cells were used to parallel the studies on thymectomized mice where sheep red cells have been used as an antigen. The dose of chicken red cells in the present experiments was $2 \times 10^9$ cells.

All antigens were injected in 0.5 to 1.0ml of saline, subcutaneously into the lower lateral aspect of the hind leg, after the efferent popliteal lymph duct had been cannulated. The subsequent immune response was measured in terms of changes in the numbers and types of cell in the lymph, the titres of specific antibody in the lymph and where possible, the numbers of antibody forming cells.

A second group of lambs (including 2 in utero splenectomized lambs) was challenged with $10^9$ killed S. muenchen organisms injected intravenously, in order to determine whether the antibody response was any different from that which resulted from a local challenge.

**Results**

**General Observations on the Cellular Response in Lymph to Antigens Injected Locally**

The subcutaneous injection of an antigen into the leg resulted in consistent changes in the number and character
of the cells leaving the popliteal node in the efferent lymph. These changes occurred in both primary and secondary responses no matter what antigen was used, the only difference in the responses to different antigens was in the quantitative aspects of these changes.

**The Primary Response**

**Changes in cell output**

The typical changes in the output of cells in the lymph from the popliteal nodes of a control and a thymectomized lamb challenged with swine influenza virus are shown in Fig. 6.1.

Within 1 hour of injection of the antigen there was a precipitous fall in the cell output so that by 3 hours, only a very few cells were present in the lymph (in some cases as few as $1 \times 10^5$ cells per ml have been observed). This very low cell output was sustained over the next 12 to 24 hours. This "shutdown" period varies in duration and severity with the different antigens used. Chicken red blood cells caused only a slight fall in cell output which lasted for 7 to 10 hours, while S. muenchen organisms caused an intermediate depression, which lasted for 12 to 18 hours. Of the three antigens used, influenza virus caused the most severe and sustained depression in cell output and the "shutdown" period lasted for almost 24 hours.

At the end of this time there was a sudden outpouring of cells from the node into the lymph which lasted only for a half to one hour. Then followed a gradual rise in the cell output to reach a peak at 72 to 96 hours. From this time on there was a gradual fall in the total cell output until resting levels were reached some 10 to 12 days after the initial antigenic challenge.
FIGURE 6.1

The typical changes which occur in the total cell output in the efferent popliteal lymph of a control and a thymectomized lamb, following a primary challenge with 20,000 haemagglutinating units of swine influenza virus injected subcutaneously into the hind legs.
Changes in Cell Morphology

There were little change in the morphology or in the size range of the lymphocytes were leaving the node after the primary antigenic stimulation. However, there were often considerable numbers of polymorphonuclear neutrophils in the lymph during this early period, particularly after the injection of murine. This was most noticeable in thymectomized lambs where 80 to 90 per cent of all cells in the lymph during this period were polymorphs. This undoubtedly reflected the high rate of polymorphs to lymphocytes in the blood of thymectomized lambs.

The production of large numbers of lymphocytes entering the lymph increased to reach a peak between 84 and 108 hours after injection and gradually increased to reach a peak between 84 and 108 hours. These cells were extremely large, with abundant slightly basophilic cytoplasm and a large nucleus with thin clumped chromosomes. They were classified as "blasts" or "transitional" cells. These cells gradually fell off in number and returned to a resting level some 6 to 7 days after an initial antigenic challenge.

The production of basophilic cells was not of any basophilic cells with a combination of basophilic and mast. Subsequently these cells appeared in the lymph in great numbers, reaching a peak 2 to 3 days after an initial antigenic challenge. The basophilic cells ranged in size from very large (25 μm diameter in smears) to very small (5-8 μm); all had the common feature of an intensely basophilic cytoplasm. As the response progressed, the large basophilic cells became

---

**CELL OUTPUT PER HOUR x 10^6**

**HOURS AFTER ANTIGEN**

---

**CONTROL**

**THYMECTOMIZED**
Changes in Cell Morphology

(1) Immediate effects. There was little change in the morphology or in the size range of the lymphocytes seen leaving the node in the first 36 hours after a primary antigenic stimulation. However, there were often considerable numbers of polymorphonuclear neutrophils in the lymph during this early period, particularly after the injection of S. muenchen, and to a lesser extent, after influenza virus. This was most noticeable in the thymectomized lambs where 80 to 90 per cent of all cells in the lymph during this period were polymorphs. This undoubtedly reflected the high ratio of polymorphs to lymphocytes in the blood of thymectomized lambs. (See Chapter V).

(2) The production of large cells. The number of large cells entering the lymph increased at around 36 hours after injection and gradually increased to reach a peak between 84 and 108 hours. On examination of Leishman stained smears, these cells were seen to be extremely large, with abundant slightly basophilic cytoplasm and a large nucleus with fine unclumped chromatin; they were classified as "blast" or "transitional" cells. These cells gradually fell off in numbers and returned to a resting level some 6 to 7 days after the initial antigenic challenge.

(3) The production of basophilic cells. Few if any basophilic cells were seen in the lymph before 48 hours. Subsequently these cells appeared in the lymph in great numbers, reaching peak proportions at 84 to 108 hours after challenge. After this time their numbers gradually decreased. The basophilic cells showed great variations in size from very large (25 µ diameter in smears) to very small (5-8 µ); all had the common feature of an intensely basophilic cytoplasm. As the response progressed, the large basophilic cells became
less common and the medium to small sized forms predominated. For the purposes of comparison, all basophilic and transitional cell types were grouped together under the classification of "basophilic" cells.

The Secondary Response

All the changes described above for the primary response were also seen in response to a secondary challenge, the only difference being in the numbers of cells involved and in the time at which they appeared.

In general it can be said that the "shutdown" period was similar in degree and duration to that following a primary challenge. The subsequent outpouring of cells into the lymph however was more rapid and the total cell output and numbers of large and basophilic cells involved were greater in the secondary response. After the peak period of the response was reached, the cell picture in the lymph returned to resting levels more rapidly.

Specific Observations on the Response of Thymectomized Lambs to Antigen

(1) The Response to Swine Influenza Virus

The primary response of the popliteal lymph node to a challenge of 20,000 haemagglutinating units of swine influenza virus (strain Shope 15) was studied in 4 thymectomized lambs and the secondary response was studied in 3 thymectomized lambs.

(a) The cellular response. The total cell output and the output of large cells and basophilic cells were determined for each 12 hour period after challenge, and the values for each determination at each period, were compared against similar determinations made in control lambs.
Fig. 6.2 shows the changes in total cell output. Apart from the "shutdown" period, the lymph of control lambs always contained significantly greater numbers of cells than the lymph of thymectomized lambs. However, it can be seen that the thymectomized lambs responded in the same manner as the controls insofar as the cellular kinetics of the response were concerned.

Figure 6.3 shows the production of large cells. During the primary response, the control lambs produced a significantly greater number of large cells at each time period (with the exception of one collection period between 144 to 156 hours) than did the thymectomized lambs. During the secondary response however, there was no significant difference in the number of large cells in the lymph of thymectomized and control lambs between the period 48 to 96 hours.

Figure 6.4 shows the production of basophilic cells. In general, there were fewer of these cells in the lymph of the thymectomized lambs in both primary and secondary responses than in the controls but this difference was not statistically significant. Again the kinetics of the response, insofar as these cells were concerned, was similar in both groups.

Inspection of the figures 6.2, 6.3 and 6.4 will show that although the total cell output was always less in thymectomized lambs, there was a relatively much higher proportion of both large and basophilic cells to total cells than in the lymph of control lambs (Table 6.1). Figure 6.5 shows the size distribution range of the cells in the lymph of a thymectomized and a control lamb at the peak of a secondary response to influenza virus.

(b) The antibody response. The antibody production following a primary challenge with influenza virus is quite low and appears very late in the course of the response after
FIGURE 6.2

The changes in the total output of cells in efferent popliteal lymph of control and thymectomized lambs following primary and secondary challenges with 20,000 haemagglutinating units of swine influenza virus injected subcutaneously into the hind legs, 14 days apart. The primary responses represent the mean of 3 control and 4 thymectomized lambs, while the secondary responses represent the mean of 2 control and 3 thymectomized lambs.
CONTROL

THYMECTOMIZED

PRIMARY

SECONDARY

CELL OUTPUT PER 12 HOURS x 10^8

DAYS AFTER ANTIGEN
The changes in the number of large cells, as determined by the Coulter Counter, in efferent popliteal lymph of control and thymectomized lambs following primary and secondary challenges with 20,000 haemagglutinating units of swine influenza virus injected subcutaneously into the hind legs, 14 days apart. The primary responses represent the mean of 3 control and 4 thymectomized lambs, while the secondary responses represent the mean of 2 control and 3 thymectomized lambs.
CONTROL

THYMECTOMIZED

PRIMARY

SECONDARY

CELL OUTPUT PER 12 HOURS $\times 10^6$

DAYS AFTER ANTIGEN

0 2 4 6 8 0 2 4 6 8

5 10 15 20 25
FIGURE 6.4

The changes in the number of basophilic cells in efferent popliteal lymph of control and thymectomized lambs following primary and secondary challenges with 20,000 haemagglutinating units of swine influenza virus injected subcutaneously into the hind legs, 14 days apart. The primary responses represent the mean of 3 control and 4 thymectomized lambs, while the secondary responses represent the mean of 2 control and 3 thymectomized lambs.
### TABLE 6.1

<table>
<thead>
<tr>
<th>Control</th>
<th>Thymectomized</th>
<th>Control</th>
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</tr>
</thead>
<tbody>
<tr>
<td><strong>Per Cent Large Cells</strong></td>
<td></td>
<td><strong>Per Cent Small Philip Cells</strong></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>Range</td>
<td>Hours after Antigen</td>
<td>Mean</td>
</tr>
<tr>
<td>P 18.70</td>
<td>12.7-22.2</td>
<td>96</td>
<td>12.0</td>
</tr>
<tr>
<td>S 17.05</td>
<td>16.0-19.9</td>
<td>60</td>
<td>13.7</td>
</tr>
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<td><strong>Thymectomized</strong></td>
<td></td>
<td><strong>Thymectomized</strong></td>
<td></td>
</tr>
<tr>
<td>P 31.50</td>
<td>25.8-41.6</td>
<td>96</td>
<td>30.8</td>
</tr>
<tr>
<td>S 47.45</td>
<td>30.8-54.2</td>
<td>60</td>
<td>61.4</td>
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</table>

Primary response: P = Primary peak cellular responses in terms of the output of large and basophilic cells in efferent popliteal lymph of control and thymectomized lambs follow antigenic stimulation of the first doses of 20,000 haemagglutinating units of swine influenza A virus, injected simultaneously into the hind legs 14 days apart.

---

**DIAGRAM:**

- **CONTROL**
- **THYMECTOMIZED**

**Cell Output per 12 Hours x 10^6**

**Days after Antigen**

- **Primary**
- **Secondary**

Legend:
- 25
- 20
- 15
- 10
- 5
- 0
<table>
<thead>
<tr>
<th></th>
<th>Per Cent Large Cells</th>
<th>Per Cent Basophilic Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
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<td></td>
<td>S</td>
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</tr>
<tr>
<td></td>
<td>S</td>
<td>47.45</td>
</tr>
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</table>

P = Primary response  
S = Secondary response

The mean peak cellular responses in terms of the output of large and basophilic cells in efferent popliteal lymph of control and thymectomized lambs following antigenic stimulation in the form of 2 doses of 20,000 haemagglutinating units of swine influenza virus, injected subcutaneously into the hind leg 14 days apart.
The typical size distribution, as determined by the Coulter Counter, of the cells in efferent popliteal lymph of a control and a thymectomized lamb, at the height of the response to a secondary subcutaneous injection into the hind legs of 20,000 haemagglutinating units of swine influenza virus. The cell volume is expressed in µ³.
most of the cell changes in the lymph have died away. Only 2 out of 3 thymectomized lambs and one out of 3 control lambs had detectable titres in their lymph at any stage. The secondary response, however, was vigorous and high titres could be detected in the efferent lymph and plasma of both control and thymectomized lambs (Fig. 6.6). Although mean titres for the thymectomized lambs in this figure were 35% lower than for the controls, this difference was not statistically significant.

Lymph and plasma were routinely checked for 19S macroglobulin antibody by incubating the samples with 0.3M 2-mercaptoethanol. At no stage was there any significant reduction in the antibody titre after treatment, indicating that none of the antibody produced in response to swine influenza virus was 19S in type.

(2) The Response to Swine Organisms

The local response to this antigen was studied in 16 lambs, grouped as follows:

(a) Two young (6 months of age) thymectomized lambs,
(b) Two old (12 months of age) thymectomized lambs,
(c) Two young (6 months of age) control lambs,
(d) Four old (12 months of age) control lambs.

All these lambs were challenged with the afferent popliteal ducts had been transected. Of these, 3 "old" (12 months of age) control lambs and 3 "old" (10 months of age) thymectomized lambs were also challenged locally but their popliteal ducts were not cannulated, and only the production of antibody in the circulating blood was studied in these animals.

(a) The cellular response. In addition to studying the changes in total cell output, the production of large and
most of the cell changes in the lymph have died away. Only 2 out of 3 thymectomized lambs and one out of 3 control lambs had detectable titres in their lymph at any stage. The secondary response, however, was vigorous and high titres could be detected in the efferent lymph and plasma of both control and thymectomized lambs (Fig. 6.6). Although the mean titres for the thymectomized lambs in this figure are less than for the controls, this difference was not statistically significant.

Lymph and plasma were routinely checked for 19S macroglobulin antibody by incubating the samples with 0.2M 2-mercapto-ethanol. At no stage was there any significant reduction in the antibody titre after treatment, indicating that most of the antibody produced in response to swine influenza virus was probably 7S in type.

(2) The Response to Salmonella muenchen Organisms

The local response to this antigen was studied in 16 animals grouped as follows:-

(a) Two "young" (3 months of age) thymectomized lambs,
(b) Two "old" (10 months of age) thymectomized lambs,
(c) Two "young" (3 months of age) control lambs,
(d) Four "old" (10 months of age) control lambs.

All these lambs were challenged after their efferent popliteal ducts had been cannulated. In addition to these, 3 "old" (10 months of age) control lambs and 3 "old" (10 months of age) thymectomized lambs were also challenged locally but their popliteal ducts were not cannulated, and only the production of antibody in the circulating blood was studied in these animals.

(a) The cellular response. In addition to studying the changes in total cell output, the production of large and
FIGURE 6.6

The $\log_2$ antibody titres in efferent popliteal lymph and blood plasma of control and thymectomized lambs following primary and secondary subcutaneous injections of 20,000 haemagglutinating units of swine influenza virus into the hind legs, 14 days apart. Each point is the mean of 2 - 8 determinations.
basophilic cells and the antibody titres, the number of antibody forming cells in the lymph was also measured using the haemolytic plaque technique.

There were considerable differences in the magnitude of the cellular response between the "old" and "young" groups. It was unlikely that these differences were fortuitous since greater cell output was recorded with thymectomized and control groups of "young" lambs. In the case of the thymectomized lambs this was despite the fact that the "old" lambs had higher antibody titres than the young animals. The responses in the "old" and "young" animals in the two groups are shown in figures 6 and 7. The cellular response to influenza virus were repeated in the present situation. Again it was apparent that both age groups responded in a similar fashion as lambs, although in each case there was greater reduction in cell output. The time of appearance and numbers of large and basophilic cells in the efferent lymph were also within the normal range, as was the case for the response to influenza virus.

(b) The Antibody Titres. The numbers of antibody forming cells in the lymph was measured in terms of cell producing antibody titres. These cells began to appear after primary challenge and peak numbers occurred after 24 hours later. After this time they slowly fell until few were present in the lymph by 160 hours. The appearance of these cells following a secondary challenge paralleled the overall cellular response, in that they were present in the lymph as early as 60 hours and had disappeared almost completely by 120 hours. There was considerable variation in
basophilic cells and the antibody titres, the number of anti-body forming cells in the lymph was also measured using the haemolytic plaque technique.

There were considerable differences in the magnitude of the cellular response between the "old" and "young" groups. It was unlikely that these differences were fortuitous since greater cell responses were recorded in both thymectomized and control groups of "young" lambs. In the case of the thymectomized lambs this was despite the fact that the "old" animals had higher resting cell outputs than the young animals.

The responses in the "old" and "young" animals in both groups are shown in Figures 6.7 and 6.8. It can be seen that the cellular changes in the lymph described for the response to influenza virus were repeated in the present situation. Again it was apparent that thymectomized lambs of both age groups responded in a similar fashion to control lambs, although in each case there was greatly reduced total cell output. The time of appearance and numbers of large and baso-philic cells in the efferent lymph were also within the normal range as was the case for the response to influenza virus.

(b) The Antibody forming cell response. The numbers of antibody forming cells in the lymph was measured in terms of cells producing haemolytic plaques. These cells began appearing in the efferent lymph about 72 hours following a primary challenge and peak numbers occurred about 24 hours later. After this time their numbers gradually fell until few were present in the lymph by 168 hours. The appearance of these cells following a secondary challenge paralleled the overall cellular response, in that they were present in the lymph as early as 60 hours and had disappeared almost completely by 120 hours. There was considerable variation in
FIGURE 6.7

The cellular response seen in efferent popliteal lymph of old, (10 months of age), control and thymectomized lambs following primary and secondary challenges with $10^9$ killed S. muenchen organisms injected subcutaneously into the hind legs, 14 days apart. The solid lines represent the total cell output in a 12 hour collection period, the interrupted lines - the output of large cells, the stippled lines - the output of basophilic cells, and the open circles joined by solid lines represent the $\log_2$ antibody titres in the lymph. The left half of each of the figures for the responses in both control and thymectomized lambs represents the primary response, while the right side in each represents the secondary response.
The cellular response seen in efferent popliteal lymph of young, (3 months of age), control and thymectomized lambs following primary and secondary challenges with $10^9$ killed S. muenchen organisms injected subcutaneously into the hind legs, 14 days apart. The solid lines represent the total cell output in a 12 hour collection period, the broken lines - the output of large cells, the stippled lines - the output of basophilic cells, and the open circles joined by solid lines represent the $\log_2$ antibody titres in the lymph. The left half of each of the figures for the responses in both control and thymectomized lambs represents the primary response, while the right side in each represents the secondary response.
the numbers of these cells in the lymph of different lambs, particularly in the secondary response. The thymectomized lambs produced as many, and frequently, more of these antibody forming cells than did the control lambs. These data are given in Tables 6.2 and 6.3.

(c) The antibody response. Despite the obvious differences in the cellular response of young and old animals, there were no differences in the antibody titres in either the lymph or the plasma of these two groups. Thus all the data were combined and the titres of control lambs were compared to the titres of thymectomized lambs in each age group.

The mean antibody titres found in the lymph and plasma of thymectomized and control lambs at different times during the primary and secondary responses to S.muenchen are shown in Figure 6.9. There was no significant difference in the titres at any stage of the primary or secondary responses in either lymph or plasma of thymectomized and control lambs.

All samples were subjected to treatment with 0.2M 2-mercapto-ethanol and it was found that almost all the antibody produced was sensitive to mercapto-ethanol, and was therefore presumed to be 19S macroglobulin-type antibody. The relative titres of 7S antibody to total antibody are shown in Figure 6.9.

(3) The Response to Chicken Red Blood Cells

The responses of 6 thymectomized lambs and 2 splenectomized lambs to chicken red cells was compared to that of 6 control lambs. As mentioned previously, the "shutdown" period which followed the subcutaneous injection of this antigen was not as severe or as prolonged as with influenza virus or S.muenchen organisms. Nevertheless, there were cellular responses in the lymph of all the lambs and these
<table>
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<th>Total</th>
<th></th>
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<th>Total</th>
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<td>96 - 108 hr</td>
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The number of plaque forming cells (pfc) per million total cells and the total number of pfc \( \times 10^6 \) in efferent popliteal lymph of control and thymectomized lambs at various times following the primary subcutaneous injection of \( 10^9 \) killed S. muenchen organisms into the hind leg.
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<td>84 - 96 hr</td>
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<td>pfc</td>
<td>Total</td>
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<td>Total</td>
<td>pfc</td>
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<tr>
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<td></td>
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The number of plaque forming cells (pfc) per million total cells and the total number of pfc \((x \times 10^6)\) in efferent popliteal lymph of control and thymectomized lambs at various times following the secondary subcutaneous injection of \(10^9\) killed S. muenchen organisms into the hind leg.
A comparison of the mean antibody titres in efferent popliteal lymph and blood plasma of control and thymectomized lambs following the local subcutaneous injection of two doses of $10^9$ killed S. muenchen organisms in the hind leg, 14 days apart. The upper part of the graph shows the total antibody titres, while the lower part shows the titres after treatment with 2-mercapto-ethanol.

- Solid lines represent control lambs.
- Interrupted lines represent thymectomized lambs
- Closed circles represent the antibody titre in serum.
- Open circles with a central dot represent the antibody titre in lymph.
occurred at the bronchi, and over the lungs, as were observed after challenge with influenza virus and 
organisms. In general however, these cellular changes were not as large as were seen with the other antigens. The 
production of plasmacytoid cells was investigated and 
again there was considerable variation between animals, the numbers of these cells did not reach the same proportions as with influenza antigens.

Antibody titres provided by each particular agglu-
tination and haemagglutination test were comparatively low in both 
primary and secondary responses and the serum levels were the same for some of the animals. The 
details of the two types of plasmacytoid cells were summarized in Table 1.

DAYS AFTER ANTIGEN

LOG 2

PRIMARY SECONDARY

ANTIBODY TITRE (S.D. 1.6)

9
8
7
6
5
4
3
2
1

2 4 6 8 10

2 4 6 8 10

284 30, 31)

Within 72 hours of antigen injection, the animals 
noticed to be listless and were found to have severe symptoms of anaesthesia, dyspnoea, diarrhoea, and both finally collapsed and died, the control
occurred at the same times and over the same periods, as were observed after challenge with influenza virus and S. muenchen organisms. In general however, these cellular changes were not as large as were seen with the other two antigens. The production of plaque forming cells was also investigated and again there was great variation between lambs, and the numbers of these cells did not reach the same proportions in lymph as with S. muenchen organisms.

Antibody titres determined by both passive haemagglutination and haemolysin tests were comparatively low in both lymph and plasma and both measurements gave the same titres. The details of the responses to chicken red cells are summarized in Table 6.4.

The Antibody Response to Intravenously Administered Salmonella muenchen Organisms.

Eight animals were challenged by the intravenous injection of $10^9$ boiled S. muenchen organisms suspended in 2.0ml of saline. These animals were divided into 4 groups as follows:

(a) 2 splenectomized,
(b) 2 thymectomized,
(c) 2 thymectomized/splenectomized,
(d) 2 normal controls.

Within 2 hours of the injection, all 8 animals were noticed to be listless and had ceased feeding. By 8 hours after injection the 2 splenectomized lambs had returned to normal. One thymectomized and one control lamb remained listless for 24 hours before recovering. The other control lamb and one thymectomized/splenectomized lamb developed severe symptoms of ataxia, extremely rapid respiration, diarrhoea, and both finally collapsed and died, the control
The peak primary and secondary responses of control and thymectomized lambs following the subcutaneous injection of $2 \times 10^9$ chicken red blood cells into the hind legs. The results are given as the number of cells $\times 10^6$ put out in the efferent popliteal lymph over 12 hour periods at the height of the responses. The mean results are given $\pm$ their standard errors.
lamb at 10 hours, the thymectomized/splenectomized lamb at 13 hours after the injection. Post mortem examination revealed no significant lesions. This syndrome is identical to that described for endotoxin shock (Thomas, 1954) with the exception that pyrexia was not a prominent feature. In order to prevent any further losses, the surviving lambs were injected intravenously with 20 units of Corticotrophin B.P. (C.S.L.) 60 minutes before injecting the secondary challenge. This treatment effectively prevented the appearance of symptoms of endotoxin shock, and did not appear to affect the subsequent antibody response.

The titres produced by the surviving animals are shown in Figure 6.10. There was no difference between any of the groups in either the time course of the response or in the amount of antibody produced. As was the case with the local response, most of this antibody was mercapto-ethanol sensitive, presumably 19S macroglobulin.

Discussion

The results described above have shown that despite the removal of the thymus at an early age in utero, the immune response of thymectomized lambs to 3 different antigens, tested at different ages after birth, was no different from that of normal, un-operated, control lambs. Despite the fact that the thymectomized lambs had significantly smaller numbers of circulating lymphocytes and a reduced cellularity of their secondary lymphoid organs, the response of the popliteal node as judged by cellular changes in the efferent lymph was as good as that of control lambs. The kinetics of the cellular response and the numbers of large and basophilic cells appearing in the efferent lymph of thymectomized lambs were
A comparison of the mean antibody titres in the plasma of control, thymectomized, splenectomized and thymectomized/splenectomized lambs given two intravenous doses of $10^9$ killed S. muenchen organisms, 14 days apart. The upper part of the graph shows total antibody titres, while the lower shows the antibody titres after treatment with 2-mercapto-ethanol.
LOG 2 ANTIBODY TITRE (S.D 1:6)
no different from the controls and the number of plaque forming cells produced in response to S. muenchen organisms and chicken red blood cells also fell within the normal range.

Neonatal thymectomy in mice has been shown to reduce the response to several antigens similar to those used in the present study. For example, Good et al (1962a) found that the antibody response to $T_2$ phage was severely impaired, Basch (1966) found a reduced secondary response to MS-2 phage and Defendi et al (1964) a reduced response to polyoma virus. The response to Salmonella antigens has also been reported to be reduced in thymectomized mice (Miller, 1962b; Humphrey, et al, 1964) and many workers have reported a reduced or absent response to heterologous red cells in mice (Hess, 1968).

In direct contrast to these findings are a number of reports which show the other side of the coin where thymectomy has been found to cause no reduction in response to these antigens. For example, in mice Ting and Law (1965) found a normal response to polyoma virus, Arnason et al (1964b) found a normal response to Salmonella S Typhi 'O' antigen, while Brooke (1965) found the responses to Salmonella Typhi 'H' antigen and sheep red cells were normal. Dukor et al (1966) reported that the impairment in antibody production and plaque forming cell production in response to sheep red blood cells lasted only for 3 months in neonatally thymectomized Swiss mice but that there was no comparable restoration of reactivity in CBA strains of mice. A similar improvement in the ability of Swiss mice to respond to sheep red blood cells with increasing age has been shown by Rogister (1965) and Sinclair and Millican (1967).

If there is, in fact, some strain variation in the ability to mount an antibody response to some antigens, this surely could not explain all the anomalous results described
above. It is significant that in many cases where reduced responses to any antigen have been observed, there was a high incidence of wasting among the thymectomized animals; in those instances where normal responses were obtained, wasting did not occur to any significant degree. This is even more striking when one investigates a specific example such as that of Dukor et al (1966). They found that Swiss Albino mice, which did not waste, gradually improved in their ability to respond to sheep red blood cells, while $(CBA \times CBAT_6T_6)^F_1$ mice which suffered a high incidence of wasting disease showed no such recovery. Similarly, the results of Basch (1966) with mice, indicated that they gave a normal primary response to MS-2 phage when injected on the day of birth, while the secondary response some 25 days later was reduced. At this age the mice were also challenged with BSA and sheep red blood cell stromata with negative results. However, wasting was already in evidence at this stage with 50 per cent deaths by 7 weeks of age. The existence of wasting disease at the time of antigenic challenge could also be a factor in the reduced response of thymectomized animals to the 'O' antigen of Salmonella typhi reported by Humphrey et al (1964), since thymectomized mice with no wasting syndrome show no reduction in response (Arnason et al, 1964a).

The evidence derived from studies on germ-free mice lends further support to the proposition that concurrent infections result in a reduced response to an antigenic challenge. Hess and Stoner (1966, 1967) and Bealmear and Wilson (1967b) have shown perfectly normal responses to a variety of antigens in germ-free thymectomized Swiss mice, with the exception of some cases which showed a reduced secondary response to tetanus toxoid (Hess et al, 1963; Hess and Stoner, 1966). On the other hand however, Miller et al (1967a) claimed
that germ-free thymectomized C₃H mice did not show any improvement in response over conventional thymectomized mice.

Since there are such great differences reported between strains of mice in response to antigens after neonatal thymectomy and since wasting disease appears to have a strong influence on these responses, the true position of the thymus and its involvement in the immune response will not be clarified until many more inbred and outbred strains have been tested against those antigens for which a reduced response has been reported following thymectomy. Such experiments will need to be done under reproducible conditions. Until such time as this is done, the role of the thymus in the development of the capacity of an animal to produce antibody must remain inconclusive.

There is little information in the literature on the kinetics of the cellular response to antigen by thymectomized mice, apart from information on the production of plaque forming cells in the spleen in response to intravenously injected sheep red blood cells. In all cases, the numbers of these cells have been reduced when the antibody response was reduced (Miller, de Burgh and Grant, 1965; Dukor et al, 1966; Miller et al, 1967a; Miller et al, 1967b) and normal when the antibody response was normal (Dukor et al, 1966) Much work has been done, however, on the interaction between thymus and bone marrow derived cells in the response to antigenic challenge with sheep red blood cells. However, this information has been derived in the artificial experimental model of the "adult thymectomized, lethally whole-body X-irradiated, syngeneically bone marrow restored, thymus grafted or thymus cell and bone marrow cell inoculated, highly inbred mouse."

The conclusions drawn from this work are that, while thymus derived cells respond to sheep red blood cell antigens by a
mitotic response (Davies et al., 1966), it is the cells derived from the bone marrow and not the thymus which elaborate the antibody (Davies et al., 1967). In some way the thymus derived cells and the bone marrow derived cells act synergistically (Claman et al., 1966; Davies et al., 1967). However well thought out and executed this work may be and however valid the results may be for the inbred mouse-red cell antigen model, it is essential that these conclusions be substantiated by experiments in other species challenged with other antigens. As has been pointed out, there is much evidence to show that the immune response to some antigens is not affected at all by thymectomy, that the response of germ-free animals is on the whole normal, and that there is considerable continued development of the ability of thymectomized animals to respond to some antigens despite absence of the thymus (Rogister, 1965; Dukor et al., 1966; Sinclair and Millican, 1967). The results described here for the sheep are similar to those reported for germ-free and non-wasted thymectomized mice. Thus, although thymectomy results in a loss of a large proportion of the lymphocyte complement in the lamb, it has no effect on the numbers of cells reacting to antigen, on the numbers of antibody forming cell precursors or the actual cells which make antibody.

Perhaps the most significant fact which emerges from the present study is that although the thymus was removed from the lambs in utero some 120 days prior to the age at which sheep normally attain the capacity to respond to Salmonella antigens (Silverstein et al., 1963b and see Chapter IV), this capacity developed subsequently to a completely normal degree, despite the absence of the thymus. It is highly unlikely that sufficient cells had escaped from the thymus prior to its removal and survived long enough to be responsible for the
considerable subsequent development and differentiation of Salmonella reactive cells; it is also highly unlikely that a thymic hormone could have been responsible for this development.

The antibody response to intravenously administered Salmonella muenchen organisms by either thymectomized or splenectomized lambs and by lambs which had both thymus and spleen removed in utero, was normal. This was a surprising find, in view of the generally accepted proposition that the spleen is essential for a normal response to intravenously administered antigens. This concept has been accepted since the report by Rowley (1950) showed that removal of the spleen from rats at or near to the time of injection of an antigen (sheep red blood cells and Salmonella typhi) resulted in a greatly depressed antibody response. Similar results have been obtained in studies on the response of splenectomized rabbits to sheep red blood cells (Taliaferro and Taliaferro, 1950). These experiments confirmed some of the earliest investigations into the cellular basis of immunity by Pfeiffer and Marx (1898) and Deutsch (1899) who found that splenectomy resulted in a reduced antibody response. However, a search of the literature revealed, as is so often the case in matters relating to immunology, that an apparently simple situation is not, in fact, as simple as it first appears. Fitch and Winebright (1962) and Winebright and Fitch (1962) found that the response of splenectomized animals to antigenic challenge depended on the type of antigen used. Particulate flagella, for instance, resulted in a more delayed and reduced response in splenectomized animals than in controls, while the response to soluble flagellin was not significantly depressed. The situation became more confused when Campbell and La Via (1967) confirmed the observations of Rowley (1950), while Pierce (1967),
working in the same laboratory as Rowley and Fitch, reported that while there was no primary antibody response to bovine gamma globulin (BGG) in control rats, splenectomized rats did produce antibody.

Splenectomy is recognized as a procedure liable to render children much more susceptible to infections (Erickson, Burgert and Lynn, 1968), but this is generally thought to be due to the fact that the efficiency of phagocytosis is depressed following the removal of a large part of the reticulo-endothelial system (Ellis and Smith, 1966). This is also considered to be the case in mice (Shinefield, Steinberg and Kaye, 1966) and cattle (Klauss and Jones, 1968).

Recently it was reported that neonatal splenectomy in C3H/Bi mice resulted in a high incidence of wasting with many deaths (Kalpatsoglou, Yunis and Good, 1967). However, this work has since been repeated by two groups of workers with several strains of mice - C57/BL, C3H/Bi, C3H/He, Balb/c and (Balb/c x C57/BL)F1, with absolutely negative results (Moody and Reed, 1967; Kubai and Auerbach, 1968). This is just another example of the blatant inconsistencies that exist in immunological literature and which make a rational appraisal of the current state of knowledge on many issues impossible. One of the differences between the present experiments and others on the effect of splenectomy, is that whereas these lambs were splenectomized in utero and challenged at least 5 months subsequently, most other experiments have been done on animals challenged near to or at the same time as splenectomy. This time lag could be a factor in explaining the difference in responses, in that a compensatory increase in the reticulo-endothelial system may have occurred during the period between splenectomy and challenge. In this regard, Taliaferro and Taliaferro (1950) found that splenectomy in
rabbits, when done some 14 weeks prior to challenge, had no effect on the antibody response to sheep red blood cells. Thus while the spleen is an important site of antibody production in the normal animal, for intravenously injected particulate antigens it is not essential for antibody production, provided sufficient time is allowed to elapse between splenectomy and antigenic challenge.

The antibody produced by sheep in response to Salmonella muenchen lipopolysaccharide was mainly mercaptoethanol sensitive (presumably 19S). This result confirms previous findings by LoSpalluto, Miller, Dorward and Fink (1962) and Fink et al (1962) that the response to Typhoid 'O' antigen was always 19S in type in children. Some 7S antibody production to Salmonella antigens has been reported in rabbits by Weidanz, Jackson and Landy (1964), and Pike and Schulz (1964) found that the subcutaneous injection of S. typhosa organisms resulted in higher 7S titres than did intravenous injections. This is in contrast to the present result where the titres of 7S antibody were higher in the efferent lymph leaving the node draining the site of injection than were present in the sera of the same animals.

Summary

The responses of thymectomized lambs to 3 different antigens, swine influenza virus (strain Shope 15), boiled Salmonella muenchen organisms, and chicken red blood cells have been studied in terms of the cellular and antibody responses in lymph coming from the popliteal node.

It was found that, although thymectomized lambs had a greatly reduced total cell output from the popliteal node, the significant features of the immune response in these animals was unaffected. There were no significant differences
between thymectomized and control lambs in terms of the production of large cells or basophilic cells, neither were there any differences in the number of plaque forming cells leaving the node, in total antibody production in lymph or sera, nor any qualitative differences in the type of antibody produced.

The response of thymectomized or splenectomized lambs or lambs with both thymus and spleen removed, to the intravenous injection of S. muenchen organisms was also studied. The type and the quantity of antibody produced in the circulating blood of these animals was no different from normal control lambs. It was concluded from these results that in the lamb neither the spleen nor the thymus is essential for the development of an adequate cellular or humoral response to swine influenza virus, to Salmonella muenchen organisms or chicken red cells.
Hypersensitivity Reactions and Homograft Rejection

in Thymectomized Lambs

The rejection of skin homografts and delayed type hypersensitivity reactions (DTH) are processes which are thought to be effected by cellular rather than humoral mechanisms (Cell, 1959; Häsek, Longerova and Arha, 1961). In consequence, these immunological phenomena have been termed "cell mediated reactions".

These cell mediated immunological reactions are considered to be severely depressed in thymectomized animals. In this regard, neonatally thymectomized mice and rats have been shown by many workers to have a greatly reduced capacity to reject homografts of skin and transplants of various types of tumours (Arnason et al. 1967; 1967; Hess, 1968). The effect of neonatal thymectomy on delayed type hypersensitivity reactions is not so well documented, but the immediate type hypersensitivity, or Arthus (IIT) reactions, (Arnason et al. 1962; Jankovic et al. 1962; Russ and Crowley, 1963).

Since thymectomy in lambs, as in other species, leads to a greatly reduced number of circulating lymphocytes, and as "cell mediated" responses are considered to depend on these cells, the ability of thymectomized lambs to reject homografts and mount hypersensitivity reactions of the delayed and immediate types was investigated.

Homograft Rejection

Three types of grafting techniques were used to transfer autografts and homografts from donor to recipient animals; split thickness grafts, subcutaneous full thickness grafts and fitted full thickness grafts.
Hypersensitivity Reactions and Homograft Rejection in Thymectomized Lambs

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Homograft Rejection

Three types of grafting techniques were used to transfer autografts and homografts from donor to recipient animals; split thickness grafts, subcutaneous full thickness grafts and fitted full thickness grafts.
Split thickness grafts were found to be unsatisfactory as both homografts and autografts hardened, even though protected from the air, and became escharotic and usually sloughed off within 7 days of grafting. It was felt that this occurred because of the demands made by the dense population of wool follicles these very thin grafts carried. In addition these grafts were placed on the side of the chest where it was difficult to keep them adequately covered.

In view of these results, the subcutaneous grafting method of Schinkel and Ferguson (1953) was employed. This method did not allow visual examination of the grafts but did provide good histological specimens. A total of 30 homografts and 30 autografts on 10 animals were examined by this method.

Fitted full thickness grafts were applied to the lateral aspect of the foreleg in 6 thymectomized and 4 control lambs. These were uniformly satisfactory and their progress could be followed by visual inspection.

**Results**

Thymectomized lambs from 4 to 18 months of age were found to be as capable of rejecting skin homografts as normal lambs, as judged grossly by visual observations and histologically by biopsy.

**Fitted Grafts**

The appearances of full thickness homografts and autografts 5, 7 and 10 days after grafting on thymectomized lambs are shown in Figure 7.1.

Up until the fifth day there was little obvious difference between the homografts and the autografts. By the fifth day the homografts showed the first signs of oedema and
Fitted full-thickness skin grafts on the foreleg of a thymectomized lamb. The graft on the left is a homograft, that on the right is an autograft.

A 7 days after grafting. The homograft is swollen and darker in colour than the surrounding host skin. The autograft appears normal.

B 10 days after grafting. The homograft is dark and hard to the touch, while the autograft, from which the sutures have been removed, has healed into place.

C 14 days after grafting. The homograft is escharotic, while the autograft is normal apart from a loss of wool cover.
were darker in colour than the autografts. At 7 days, the autografts were pink, soft and vital in appearance, while the homografts which had continued to grow darker in colour, had lost their flexibility, and were now raised above the surrounding skin. At 10 days, the autografts continued to present a perfectly normal appearance except for a loss of wool cover. The homografts on the other hand were now very dark and quite hard to the touch. In many cases they could be peeled off the graft bed exposing a raw granulating surface of host tissue beneath.

There was never more than a 1 to 2 day difference between control and thymectomized lambs in the time that the signs of rejection - swelling, darkening and hardening of the graft, appeared. The only gross difference observed was a tendency for the grafts on thymectomized lambs to show rather less swelling than those on normal lambs. The homografts were rejected at about the same time, in both thymectomized and control lambs while, in all cases, the autografts were accepted. Figure 7.2 shows homografts 10 days after application in a control and thymectomized lamb and one autograft in a thymectomized lamb at the same stage. Splenectomized lambs also rejected homografts in a manner similar to normal control lambs.

Subcutaneous Grafts

Histological examination of the subcutaneous grafts confirmed the results obtained by visual observations on fitted grafts, and revealed no obvious difference in the rejection process in thymectomized lambs. Histologically, at 7 days there was a strong vascular reaction with stasis of blood vessels and extravasation of red cells in the base of the
The histological appearance of fitted skin homografts in a control lamb (A), and a thymectomized lamb (B), 10 days after grafting. There is a loss of histological detail, most of the epithelium has been shed, the wool follicles have degenerated and the grafts have begun to be undermined by host epithelium.

By comparison, an autograft at the same stage (C) shows a thickened epithelium, but otherwise an essentially normal appearance.

Stain H and E. Magnification 62.5x.
These progressive changes were seen in homografts in both thymectomized animals and those that were not, and there was no observable difference in terms of the severity or time of rejection that occurred.

Subcutaneous tissue thinned and the sebaceous glands were reduced. Subsequent hair growth and wool growth proceeded. At some stage a local circular reaction occurred. These findings are consistent with those reported in the foetal sheep (Green, 1951).

Immediate Type Reactions

Six months after splenectomy, splenectomized lambs were sensitized with consecutive intranasal injection of 10 mg of ferritin incorporated into incomplete Freund's adjuvant. Eight weeks later these sheep were tested for
graft, and a diffuse cellular infiltration by mononuclear cells and polymorphs throughout the whole graft. The graft epithelium was thin and in parts lifted off the underlying sub-epithelial tissues. At the base of the graft there was a dense accumulation of mainly large, pale cells. (Fig. 7.3, A & B). At 10 days the florid cellular reaction had abated and the number of cells in the graft was reduced. Throughout the graft there were collections of necrotic material and the whole tissue appeared amorphous and stained poorly. The beginnings of under-running of the graft by host epithelium was apparent at this time. The reaction at the base of the graft had also subsided and a few pyroninophilic cells were visible. (Fig. 7.3 C & D). By 14 days, this under-running had progressed further and by 21 days the graft had "shelled" out and lay in a "cyst-like" cavity. (Fig. 7.4).

These progressive changes were seen in homografts in both thymectomized and in normal animals, and there was no observable difference between the two groups, in terms of the severity or the time at which these changes occurred.

Subcutaneous autografts underwent a thinning of their epithelium, a loss of follicles and some of the sebaceous glands. Subsequently new follicles developed and wool growth proceeded. At no stage did any violent cellular reaction occur. These findings were essentially similar to those reported in the foetal sheep by Schinkel and Ferguson (1953).

Immediate Type Hypersensitivity Reactions

Six normal, 6 thymectomized and 2 splenectomized lambs were sensitised by the subcutaneous injection of 10.0 mg of ferritin incorporated into 1.0 ml incomplete Freund's adjuvant. Eight weeks later these sheep were tested for
Subcutaneous skin homografts in control and thymectomized lambs.

A 7 days post grafting - control lamb.
B 7 days post grafting - thymectomized lamb.
In both cases, there is a loss of structure in the graft, a violent cellular reaction and stagnation and rupture of blood vessels in the dermis.

C 10 days post grafting - control lamb.
D 10 days post grafting - thymectomized lamb.
The cellular reaction in the dermis has subsided, but has increased in the deeper tissues. The grafts are now clearly non-viable.

Stain H and E. Magnification 100x.
Subcutaneous skin homografts and autografts in a control lamb and a thymectomized lamb, 21 days after grafting. The homografts in each case have been rejected, while the autografts are essentially normal in appearance.

A Homograft - control lamb
B Homograft - thymectomized lamb
C Autograft - control lamb
D Autograft - thymectomized lamb

Stain H and E. Magnification 100x.
injection of saline into the skin overlying the site of injection for signs of swelling at 4, 24 and 48 hours. The same degree of swelling was noticeable in the control group of each animal. At 84 hours, the degree of swelling was increased in the order of magnitude of the injection of the test substance, compared on the same animals with the same site which was not injected. The swelling increased significantly after injection of the test substance compared to the control group. In circumflexion, the reaction was not observed.
hypersensitivity to ferritin by the intradermal injection of 0.1 ml of a 0.5 per cent solution of ferritin in saline into the wool free area of the medial aspect of the thigh. The site was inspected at intervals after the injection for signs of erythema and swelling, and the thickness of the skin overlying the injection site was measured at 3, 8, 12, 24 and 48 hours. Subsequently, some lambs were injected with a test dose of ferritin and biopsies of the lesions taken at 4, 24 and 48 hours after injection.

**Results**

The changes that occurred following the injection of the test dose of ferritin were essentially the same in all groups of animals. Within 2 hours there was a slight degree of erythema in the immediate area of the injection site which persisted for up to 48 hours. At no time, however, did it become intense. At the same time there was a noticeable swelling which rapidly increased to reach a maximum at 3 hours and then gradually subsided to return to normal between 72 and 84 hours after injection (Fig. 7.5). The degree of swelling was quite severe, and in one case the skin thickness increased by 1.01 cm. The general increase however, was of the order of 0.4 to 0.5 cm.

As can be seen from Figure 7.5, the thymectomized lambs reacted as well as, if not better than the control lambs. These reactions to ferritin did not result in circumscribed lesions as the affected areas tended to spread, in some cases more than others, and for this reason comparison on the basis of the increase in skin thickness was not strictly valid.

Histologically these lesions at 4 hours after injection were grossly oedematous with the tissue diffusely
A comparison of the increases in skin thickness at the site of the intradermal injection of 0.1ml of a 0.5 per cent solution of ferritin into previously sensitised lambs. Each point represents the mean of 6 measurements in control and thymectomized lambs and 2 measurements in splenectomized lambs.
infiltrated with polymorphonuclear leucocytes (Fig. 7.6, A and B). Many of the blood vessels contained granulocytes and in many cases these appeared as though they were adherent to the wall. Only a few mononuclear cells could be seen. At 24 hours the oedema had subsided, many of the polymorphs showed pyknotic changes, and many of the small vessels contained thrombi in their lumina (Fig. 7.6, C and D). By 48 hours most of the polymorphs had disappeared, and the cellular infiltrate was now predominantly composed of mononuclear cells, a few of which still stained with haematin (Fig. 7.6, E and F). These cells resembled those described by Gell and Coombs (1955) for certain type hypersensitivity reactions.

The study of delayed hypersensitivity was carried out by the subcutaneous injection of 20 mg of live streptococci organisms. Two 18 month old normal lambs, two 12 month old thymectomized lambs, and two 12 month old thymectomized lambs were used for this experiment. Two weeks after injection of the DHC, the lambs were tested for sensitivity by injecting 0.2 ml of tuberculin intradermally into the wool-free skin of the medial aspect of the thigh. The injection was given at 12 hourly intervals and the changes in the skin thickness measured. Since growth in the skin as erythema were not noticeable, and since the reactions were quite localized, it was considered that comparisons based on the increases in skin thickness were valid in this case.
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Delayed Type Hypersensitivity Reactions

To study DTH reactions the sheep were sensitised to tuberculin by the subcutaneous injection of 0.5 mg of live BCG organisms. Two 18 month old normal lambs, two 18 month old thymectomized lambs, two 12 month old normal lambs, and two 12 month old thymectomized lambs were used for this experiment.

Eight weeks after injection of the BCG, the lambs were tested for sensitivity by injecting 0.1 ml of tuberculin intradermally into the wool-free skin of the medial aspect of the thigh. The injection site was inspected at 12 hourly intervals and the changes in the skin thickness measured. Since gross changes such as erythema were not noticeable, and since, unlike the immediate type hypersensitivity response to ferritin, these tuberculin reactions were quite localized, it was considered that comparisons based on the increases in skin thickness were valid in this case.
FIGURE 7.6

The histological appearance of an immediate type hypersensitivity reaction produced in the skin of a previously sensitised thymectomized lamb by the injection of ferritin.

A 4 hours. The skin is grossly oedematous.

B 4 hours, showing some early cellular infiltration.

C 24 hours. The oedema has subsided and a cellular reaction has become evident.

D 24 hours. The cellular reaction consists mainly of polymorphs, many of which show pyknotic changes.

E 48 hours. The cellular reaction has become more localized.

F 48 hours. The polymorphs have disappeared and have been replaced by mononuclear cells.

Stain H and E.
Results

There was a gradual increase in the skin thickness at the site of injection over a period of 48 to 60 hours, at which time the lesions had reached their maximum size. Statistical analysis of the results failed to show any significant difference in the intensity of the reactions between the two age groups of control and thymectomized lambs. Thus the responses of all the control lambs were taken together and compared to that of all thymectomized lambs. Control (normal) lambs of both age groups reacted strongly to the intradermal injection of tuberculin, while the reaction by thymectomized lambs of both age groups was consistently depressed. Based on skin thickness measurements, these differences were significantly lower at all times in thymectomized lambs (Table 7.1 and Fig. 7.7).

When these DTH lesions were examined histologically at 24 hours in normal animals, there was a diffuse infiltration of the dermis by mononuclear cells similar to the histological appearance of the skin lesions 48 hours after the injection of ferritin, but much more intense. A characteristic feature of the DTH lesions was the presence of 'peri-vascular' accumulations of mononuclear cells. This picture was relatively unchanged at 96 hours. Over the 48 to 96 hour interval the lesions contained large numbers of pyroninophilic cells particularly in the peri-vascular accumulations (Fig. 7.8, A, C and E).

The histology of the lesions in thymectomized lambs at 48 and 96 hours showed a much reduced mononuclear cell response although pyroninophilic cells were present in abundance as in the control lambs (Fig. 7.8, B, D and F).
The increase in skin thickness following the intradermal injection of tuberculin into previously sensitised control and thymectomized lambs. The mean results (in mm) are given together with their standard errors.

<table>
<thead>
<tr>
<th>Hours After Injection</th>
<th>12</th>
<th>24</th>
<th>36</th>
<th>48</th>
<th>60</th>
<th>72</th>
<th>84</th>
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<tr>
<td>Control Lambs</td>
<td>54.5±6.2</td>
<td>71.0±10.3</td>
<td>131.0±24.6</td>
<td>153.0±24.3</td>
<td>119.7±19.9</td>
<td>116.0±20.8</td>
<td>106.0±17.0</td>
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<td>n</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
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<td>4</td>
</tr>
<tr>
<td>Thymectomized Lambs</td>
<td>27.5±7.35</td>
<td>31.25±5.67</td>
<td>35.0±8.11</td>
<td>39.5±6.1</td>
<td>40.75±3.77</td>
<td>39.0±2.7</td>
<td>37.6±2.84</td>
</tr>
<tr>
<td>n</td>
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n = No. of observations
p = probability

TABLE 7.1
FIGURE 7.7

A comparison of the increases in skin thickness at the site of the intradermal injection of 0.1 ml of tuberculin into control and thymectomized lambs, previously sensitised with 0.5mg of BCG. Each point represents the mean of 4 determinations and the vertical bars represent the standard error.
INCREASE IN SKIN THICKNESS IN mm

CONTROL

THYMECTOMIZED

HOURS AFTER INJECTION

12 24 36 48 60 72 84 96 108 120
FIGURE 7.8

The histological appearance of delayed type hypersensitivity reactions 96 hours after the intradermal injection of tuberculin into the skin of sheep previously sensitised with BCG.

A Control
B Thymectomized. The overall cellular reactions is less than in the control.

Stain H and E. Magnification 25x.

C Control. The cellular infiltration is composed mainly of mononuclear cells.

Stain H and E. Magnification 400x.

D Thymectomized. Infiltrating mononuclear cells can be seen forming peri-vascular islands in the base of the dermis.

Stain H and E. Magnification 400x.

E Pyroninophilic cells in the peri-vascular accumulations in the dermis of a control lamb.

Stain pyronin-methyl-green. Magnification 750x.

F Similar accumulations of pyroninophilic cells in the 'peri-vascular islands' in the skin of a thymectomized lamb.

Stain pyronin-methyl-green. Magnification 750x.
long-term rats showed prolonged acceptance of homografts was surprising from previous studies (Brooke, 1965). In burn-free thymectomized rats, Millson and Millson (1967) showed that skin homografts had a prolonged survival time. Good et al. (1964) reported that thymectomized mice having homografts up to 125 days showed a significantly different pattern than thymectomized mice. It was reported to immunosuppress the rat, which is not greatly preoccupied.
Discussion

Neonatal thymectomy has the effect of greatly prolonging the rejection time of skin homografts in mice and rats. This observation has been confirmed many times since it was first reported by Miller (1961) (see review by Hess, 1968). However, in almost every instance the thymectomized animals did reject these grafts indicating that homograft immunity was not completely abrogated by thymectomy. Further, in almost every instance, the thymectomized animals that showed prolonged acceptance of homografts were suffering from wasting disease. There is only one report of a normal rejection time in conventionally reared thymectomized mice (Brooke, 1965). There are, however, several reports that germ-free thymectomized mice are as fully capable (Hess and Stoner, 1967) or only slightly less capable (McIntire et al, 1964; Miller et al, 1967a) than normal mice of rejecting skin homografts. Thymectomy early in life does not alter the rejection time of homografts in rats, rabbits, piglets or puppies (Good et al, 1962a; Defendi et al, 1964; Van de Water and Katzman, 1964; Pestana, Hallenbeck and Shorter, 1965). Fisher and Fisher (1965) found that although 32 per cent of neonatally thymectomized rats showed prolonged acceptance of homografts up to 35 days of age, the rejection time shortened as the rats grew older so that at 120 days of age only 13 per cent showed any prolonged acceptance. The ability of thymectomized mice to reject tumour homografts has also been reported to improve with age (Martinez et al, 1962b; Parrot and East, 1965; Bealmear and Wilson, 1967a).

It appears then that neonatal thymectomy does not remove an animal's capacity to recognize and reject foreign tissue grafts, and the survival of homografts is not greatly prolonged unless the recipients are simultaneously preoccupied with severe wasting disease.
with severe wasting disease. Given sanitary conditions the capacity to reject homografts can develop considerably after removal of the thymus and even after X-irradiation (Bealmear and Wilson, 1967a).

It was shown originally by Schinkel and Ferguson (1953) that foetal lambs of between 80 and 117 days gestation were capable of rejecting skin homografts. This has been confirmed by Silverstein et al., (1964). These workers found that while foetal lambs of 60 to 64 days gestation are incapable of rejecting skin homografts, those of 77 days or more can do so in the same way as adult sheep. Silverstein et al., (1964) postulated that the ability of foetal lambs to reject homografts was attained at around 80 days post-conception and that the transition from a state of acceptance to a state of rejection was an abrupt rather than a gradual process. If the thymus is essential for the normal maturation of this specific cell mediated immune response, then thymectomy performed prior to 77 days should prevent any further maturation or subsequent development of the ability of the lamb to mount a homograft response.

All lambs used in the present experiment were thymectomized prior to 80 days gestation, some as early as 60 days. As a development of the foregoing argument, these lambs should have been unable to reject homografts or at least, the rejection process should have been greatly prolonged, especially in the older lambs. Some of these older lambs had been thymectomized 21 months previously and it is difficult to accept that any residual thymic influence could still be operative. All the 16 lambs used in this study rejected skin homografts in normal fashion, showing that neither the final development nor the maintenance of the cellular mechanisms involved in this response is dependant upon the presence of an intact thymus.
There are comparatively little data available on the effect of thymectomy on hypersensitivity reactions. Russe and Crowle (1965) found that 75 per cent of neonatally thymectomized CF₁ mice were incapable of giving either an immediate or a delayed type reaction to ovalbumin or tuberculin. Jankovic et al. (1962) found that rats thymectomized in the first week of life, when tested at 8 weeks of age, had significantly reduced Arthus reactions to BSA 9 to 12 days after sensitisation. More than 50 per cent however, responded as well as controls at 19 to 21 days after sensitisation i.e. the development of sensitivity was delayed but not abolished. Similarly only 20 per cent showed a delayed reaction to BSA 9 to 12 days after sensitisation, while 50 per cent reacted strongly at 19 to 21 days. Arnason et al. (1962) found that the delayed response of thymectomized rats to tuberculin was almost equal to that of normal rats at 24 hours after testing, but that the reaction decreased much more quickly than in controls.

The present results showed that thymectomized lambs gave vigorous immediate type (Arthus) reactions, while the delayed response to tuberculin was significantly depressed. If Arthus reactions are caused by an antigen-antibody interaction on the walls of blood vessels (Gell, 1959), it was not surprising that these lambs were capable of producing this type of reaction, as it was shown previously (Chapter VI) that thymectomy did not cause any reduction in the antibody producing capacity of these animals. However, although the DTH response was greatly reduced, it was not completely abolished, since even in those animals which showed only a comparatively slight reaction in terms of an increase in skin thickness, histologically a cellular reaction was seen in the tissues. It has been shown by Silverstein et al. (1963b) that
foetal lambs are unable to produce antibody to BCG at any time of their intra-uterine life. Although it is doubtful whether antibody is involved in DTH responses, it is probable that the ability to mount a DTH response to the same antigen also develops rather late. Thus these lambs were probably thymectomized well before the time at which the capacity to respond to BCG in a delayed fashion is normally attained. It is likely then that the thymus does provide cells which take part in DTH responses. However, although the DTH response was reduced in these lambs, it was not completely abolished, indicating that the thymus is not the sole source of these cells.

The histological appearance of the immediate type hypersensitivity lesions in normal and thymectomized lambs was identical to that described for the rabbit by Gell and Hinde (1954). The fact that the initial polymorph invasion gives way to a mononuclear infiltration indicates that the immediate type reaction is superseded by a delayed type reaction. Gell and Hinde (1954) claimed that plasma cells were produced from the mononuclear cell infiltrate about 96 hours after administration of the test dose, but also claimed that these cells are never produced in the classical delayed type response elicited by tuberculin (Gell and Hinde, 1951). However, as was described above the DTH lesions elicited in both control and thymectomized sheep showed many pyroninophilic plasma cells, particularly in the centre of the perivascular islands and deep in the dermis. The presence of plasma cells in DTH reactions has also previously been reported in guinea pigs by Waksman (1960).
Summary

Lambs thymectomized in utero prior to the eightieth day of gestation have been shown to be capable of rejecting grafts of allogeneic skin as vigorously as normal lambs even when grafted as long as 21 months after thymectomy. Histologically, the cellular reactions involved were the same in thymectomized lambs as in control lambs. Since all these lambs were thymectomized prior to the time at which they normally develop the capacity to reject allogeneic skin grafts, it was concluded that the thymus plays no essential part in the final development or maintenance of this particular immune reaction.

Thymectomized lambs were also found to be fully capable of mounting an immediate type hypersensitivity response to ferritin, although the delayed type hypersensitivity response to tuberculin was severely reduced. Since it has been shown that foetal lambs are incapable of responding to BCG at any time before birth, it is possible that the thymus is a source, although not the sole source, of cells which take part in delayed type hypersensitivity responses.
CHAPTER VIII

THE NORMAL LYMPHOCYTE TRANSFER REACTION IN THYMECTOMIZED LAMBS

Neonatally thymectomized mice have been shown to be less capable than normal mice of resisting a graft-versus-host reaction caused by the parenteral administration of parental or allogeneic lymphoid cells (Good et al., 1962; Martinez et al., 1962a; Parrot and East, 1964). The reverse situation also applies, in that lymphoid cells from thymectomized mice are less capable than cells from normal mice of causing a graft-versus-host reaction in normal F1 recipients (Good et al., 1962; Delmasco et al., 1962; Delmasco et al., 1963; Miller et al., 1967b). This is also the case in rats (Biske, 1966), but thymectomized rats, in distinction to mice, do not appear to be more susceptible to the graft-versus-host reaction (Aisenberg et al., 1964).

The normal lymphocyte transfer reaction, i.e., the reaction seen in the skin following the intradermal inoculation of lymphoid cells, has been described as a local graft-versus-host reaction (Brent and Medawar, 1966). For the reaction to occur, the donor cells must be alive (Brent, Brown and Medawar, 1962; Jones and Lafferty, 1969). However, there is also a significant host component to this response, since the recipient must be immunologically competent and genetically dissimilar to the donor (Brent and Medawar, 1964, 1966; Ramsay andBillingham, 1966). It has been further shown that this reaction is a result of an interaction which occurs between donor and host lymphocytes and not a reaction of the donor cells to host antigens (Ramsay and Streilein, 1965; Ramsay and Billingham, 1966; Elkins and Gutmann, 1968) Jones and Lafferty, 1969).

This NLT reaction has been shown to occur in guinea
The Normal Lymphocyte Transfer Reaction in Thymectomized Lambs

Neonatally thymectomized mice have been shown to be less capable than normal mice of resisting a graft versus host reaction caused by the parenteral administration of parental or allogeneic lymphoid cells (Good et al., 1962; Martinez et al., 1962a; Parrot and East, 1964). The reverse situation also applies, in that lymphoid cells from thymectomized mice are less capable than cells from normal mice of causing a graft versus host reaction in normal F1 recipients (Good et al., 1962; Dalmasso et al., 1962; Dalmasso et al., 1963; Miller et al., 1967b). This is also the case in rats (Rieke, 1966), but thymectomized rats, in distinction to mice, do not appear to be more susceptible to the graft versus host reaction (Aisenberg et al., 1962).

The normal lymphocyte transfer reaction, i.e. the reaction seen in the skin following the intradermal inoculation of 5 million allogeneic lymphocytes, has been described as a local graft versus host (GVH) reaction (Brent and Medawar, 1966). For the reaction to occur, the donor cells must be alive (Brent, Brown and Medawar, 1962; Jones and Lafferty, 1969). However, there is also a significant host component to this response, since the recipient must be immunologically competent and genetically dissimilar to the donor (Brent and Medawar, 1964, 1966; Ramseier and Billingham, 1966). It has been further shown that this reaction is a result of an interaction which occurs between donor and host lymphocytes and not a reaction of the donor cells to host antigens (Ramseier and Streilein, 1965; Ramseier and Billingham, 1966; Elkins and Guttmann, 1968; Jones and Lafferty, 1969).

This NLT reaction has been shown to occur in guinea pigs (Brent et al., 1964), hamsters (Ramseier and Billingham, 1967) and sheep (Ford, 1967).
pigs (Brent and Medawar, 1963), chickens (Warner, 1964), hamsters (Ramseier and Billingham, 1966), rats (Ford, 1967) and sheep (Jones and Lafferty, 1969). Since both host and donor lymphocytes are involved, it was thought that the normal lymphocyte transfer reaction could be employed as a means of assessing the capacity of thymectomized lambs to react in a graft versus host situation. By injecting lymphocytes from thymectomized lambs into normal sheep, it would be possible to assess whether these lymphocytes were defective in their ability to cause a graft versus host reaction. Conversely, by injecting lymphocytes from normal sheep into thymectomized lambs, it would be possible to determine whether there was any defect in the ability of these lambs to resist a graft versus host reaction.

Experimental Design

Only a small number of thymectomized lambs was available for this experiment and to minimize the chance of donor lymphocytes being obtained from genetically similar sheep, each recipient (thymectomized and normal), was injected with lymphocytes from 3 different normal donors. Donor cells were obtained from the efferent lymph draining from an unstimulated popliteal lymph node. In all cases, 5 or 25 x 10^6 lymphocytes suspended in 0.1 ml of Eagle's medium were injected intradermally into the wool free skin of the medial aspect of the thigh. Lymphocytes from normal adult sheep, normal (control) lambs and thymectomized lambs, were injected at separate sites into adult, control and thymectomized recipients. In all, 9 different donors (3 adult, 3 control and 3 thymectomized) and 26 recipients (15 adult, 6 control and 5 thymectomized) were used. A total of 120 lymphocyte transfer reactions were observed in 9 different donor-recipient combinations.
The development of the skin reactions after the injection of the lymphocytes was followed by measuring the thickness of the skin at the injection site every 24 hours and the results were recorded as an increase in skin thickness. Reactions were considered to be positive if there was an increase in thickness of 1.0 mm or more. Skin sections from some sheep were taken at 5 and 7 days after injection for histological examination.

Results

Immediately following injection of the lymphocytes there was a slight degree of erythema which soon faded, only to reappear 12 to 24 hours later. This was followed by a gradual increase in the skin thickness which reached a maximum at 5 to 6 days and then regressed. These reactions varied in size. In some cases there was no noticeable change, while in others, large lesions with central necrotic areas developed. In these cases, the lesions regressed slowly probably due to superimposed infection of the necrotic skin.

The incidence of positive reactions in the recipients of each group (adult, control and thymectomized) to the injection of lymphocytes from normal animals is shown in Table 8.1. It can be seen that fewer thymectomized recipients (32 per cent) reacted than did control recipient lambs (63.3 per cent), while almost all adult sheep produced positive reactions (96.6 per cent). The overall positive reactors in the normal (adult and control lambs) group was 80 per cent which approximated the expected incidence of 85 per cent in normal adult sheep found by Lafferty and Jones (1969).

The incidence of positive reactions in each group to cells from thymectomized lambs is shown in Table 8.2. Again fewer thymectomized lambs reacted than did control
<table>
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<th>Combination</th>
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<th>Number Positive</th>
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</tr>
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<td>3</td>
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<tr>
<td>Total Thymectomized Recipients</td>
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</tr>
<tr>
<td>Control</td>
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<td>9</td>
<td>60.0</td>
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<tr>
<td>Adult</td>
<td>15</td>
<td>10</td>
<td>66.6</td>
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<tr>
<td>Total Control Recipients</td>
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<td>63.3</td>
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<tr>
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<td>24</td>
<td>96.0</td>
</tr>
<tr>
<td>Adult</td>
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<td>100.0</td>
</tr>
<tr>
<td>Total Adult Recipients</td>
<td>30</td>
<td>29</td>
<td>96.6</td>
</tr>
<tr>
<td>Total Normal Recipients</td>
<td>60</td>
<td>48</td>
<td>80.0</td>
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</tbody>
</table>

The incidence of positive reactions in the skin of normal adult sheep, control lambs and thymectomized lambs, following the intradermal injection of $5 \times 10^6$ lymphocytes from normal adult sheep or control lambs.
The incidence of positive reactions in the skin of normal adult sheep, control lambs and thymectomized lambs, following the intradermal injection of $5 \times 10^6$ lymphocytes from thymectomized donors.

<table>
<thead>
<tr>
<th>Combination</th>
<th>n</th>
<th>Number Positive</th>
<th>Per Cent Reactors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor Cells</td>
<td>Recipient</td>
<td></td>
<td></td>
</tr>
<tr>
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<td>Thymectomized</td>
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<td>1</td>
</tr>
<tr>
<td>Thymectomized</td>
<td>Control</td>
<td>15</td>
<td>7</td>
</tr>
<tr>
<td>Thymectomized</td>
<td>Adult</td>
<td>17</td>
<td>6</td>
</tr>
</tbody>
</table>
lambs or adult sheep (25 per cent, 46.7 per cent and 35.3 per cent respectively), while the incidence of reactions in both normal groups of sheep was significantly less than that following the injection of lymphocytes from normal sheep.

The relative size of the reactions elicited in each group are shown in Figs. 8.2, 8.3 and 8.4 (an explanatory legend is shown in Fig. 8.1). These curves were constructed from the daily measurements of the increase in skin thickness following the injection of lymphocytes. All reactions less than 1.0 mm were disregarded in calculating the incidence of positive reactors. Statistical analysis of the data revealed the following points:

(1) There was no statistically significant difference at any time in the overall size of the reactions produced in normal adult sheep, or normal control lambs, by normal cells (i.e. cells from adult sheep or control lambs) (Figs. 8.2 and 8.3).

(2) The size of the reactions produced in normal adult sheep, or normal control lambs, produced by the injection of normal cells, was significantly greater (p = 0.001) at each time interval than the size of the reactions produced following the injection of cells from thymectomized animals (Figs. 8.2 and 8.3).

(3) There was no difference in the size of the reactions produced in thymectomized lambs by the injection of cells from any of the 3 donor types (adult, control or thymectomized). (Fig. 8.4).

These same reactions, plotted on a 'donor' basis are shown in Figs. 8.5, 8.6 and 8.7, and illustrate the following points:

(1) Adult sheep gave larger reactions in response to normal cells than did normal control lambs, although this
An explanatory legend for the symbols used in Figs. 8.2 to 8.12. The lines represent the recipients in each case and the square and circles represent the type of cells injected.

For example: A solid line joining solid circles represents the reaction in the skin of adult sheep following the intradermal injection of lymphocytes from an adult sheep.

A broken line joining open circles represents the reaction in control lambs following the injection of lymphocytes from a thymectomized lamb.

A finely stippled line joining solid squares represents the reaction in thymectomized lambs following the injection of lymphocytes from a control lamb.
ADULT SHEEP RECIPIENTS

CONTROL LAMB RECIPIENTS

THYMECTOMIZED LAMB RECIPIENTS

• CELLS FROM ADULT SHEEP

■ CELLS FROM CONTROL LAMBS

○ CELLS FROM THYMECTOMIZED LAMBS
Comparison of the increases in skin thickness in sheep following the intradermal injection of $5 \times 10^6$ allogeneic lymphocytes.

8.2. The reactions in adult sheep following the injection of cells from normal adults (each point is the mean of 5 measurements), cells from control lambs (17 measurements) and cells from thymectomized lambs (17 measurements).

8.3. The reactions in control lambs following the injection of cells from normal adult sheep, control lambs and thymectomized lambs. Each point is the mean of 15 measurements.

8.4. The reactions in thymectomized lambs following the injection of cells from normal adult sheep (each point is the mean of 15 measurements), cells from control lambs (13 measurements) and cells from thymectomized lambs (4 measurements).
Comparison of the increases in skin thickness in sheep following the intradermal injection of $5 \times 10^6$ allogeneic lymphocytes.

8.5 The reactions following the injection of cells from normal adult sheep into normal adult sheep recipients (each point is the mean of 5 measurements), control lamb recipients (15 measurements) and thymectomized lamb recipients (15 measurements).

8.6 The reactions following the injection of cells from control lambs into normal adult sheep recipients (each point is the mean of 25 measurements), control lamb recipients (15 measurements and thymectomized lamb recipients (13 measurements).

8.7 The reactions following the injection of cells from thymectomized lambs into normal adult sheep recipients (each point is the mean of 17 measurements), control lamb recipients (15 measurements) and thymectomized lamb recipients (4 measurements).
was statistically significant ($p = 0.01$) only in the case of the adult recipients given normal cells (Figs. 8.5 and 8.6).

(2) The injection of normal cells into thymectomized lambs resulted in reactions which were significantly smaller ($p = 0.001$) than those produced by normal cells injected into normal adult recipients (Figs. 8.5 and 8.6).

(3) Cells from thymectomized lambs produced only very small reactions (Fig. 8.7).

Increasing the dose of injected lymphocytes from $5 \times 10^6$ to $25 \times 10^6$ increased the incidence and size of the reactions in each recipient group. There was one exception to this rule, that the number of thymectomized recipients which showed positive reactions to injection of cells from control lambs did not change with an increase in dose (2 out of 5 reacted with $5 \times 10^6$ and 2 out of 5 reacted to $25 \times 10^6$). The reactions were more intense. These results are given in Table 8.1 and the increase in size of the reactions is shown in Figs. 8.10, 8.11, and 8.12.

The NLT reactions which occur in the skin of normal sheep following the intradermal injection of lymphocytes from thymectomized lambs are much smaller than those which occur in the same recipients injected with cells from normal sheep.

(2) The NLT reactions which occur in the skin of thymectomized sheep following the injection of cells from normal donors are much smaller in size than the reactions produced by these same cells when injected into normal recipients.

(3) Virtually no NLT reactions are produced in the skin of thymectomized sheep following injection of cells from thymectomized donors.

DAYS AFTER INJECTION
was statistically significant (p = 0.01) only in the case of the adult recipients given normal lamb cells (Figs. 8.5 and 8.6).

(2) The injection of normal cells into thymectomized lambs resulted in reactions which were significantly smaller (p = 0.001) than those produced by normal cells injected into normal adult sheep and lambs (Figs. 8.5 and 8.6).

(3) Cells from thymectomized lambs produced only very small reactions in any of the recipient groups (Fig. 8.7).

Increasing the dose of injected lymphocytes from $5 \times 10^6$ to $25 \times 10^6$ increased the incidence and size of the reactions in each recipient group. There was one exception to this rule in that the number of thymectomized recipients which showed positive reactions to the injection of cells from control lambs did not change with an increase in dose (2 out of 5 reacted with $5 \times 10^6$ cells and 2 out of 5 reacted to $25 \times 10^6$ cells) but the reactions were more intense. These results are given in Table 8.3, and the increase in size of the reactions is shown in Figs. 8.8, 8.9, 8.10, 8.11 and 8.12.

These results can be summarised as follows:-

(1) The NLT reactions which occur in the skin of normal sheep following the intradermal injection of lymphocytes from thymectomized lambs are much smaller than those which occur in the same recipients injected with cells from normal sheep.

(2) The NLT reactions which occur in the skin of thymectomized sheep following the injection of cells from normal donors are much smaller in size than the reactions produced by these same cells when injected into normal recipients.

(3) Virtually no NLT reactions are produced in the skin of thymectomized lambs following the injection of cells from thymectomized donors.
TABLE 8.3

<table>
<thead>
<tr>
<th>Combination</th>
<th>n</th>
<th>No. of Reactors at 5 x 10^6</th>
<th>No. of Reactors at 25 x 10^6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Thymectomized</td>
<td>5</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Adult Thymectomized</td>
<td>2</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Thymectomized Thymectomized</td>
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<td>3</td>
</tr>
<tr>
<td>Thymectomized Control</td>
<td>5</td>
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<td>5</td>
</tr>
<tr>
<td>Thymectomized Adult</td>
<td>5</td>
<td>2</td>
<td>5</td>
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</table>

The incidence of positive reactions in the skin of normal adult sheep, control lambs and thymectomized lambs following the intradermal injection of 25 x 10^6 lymphocytes from adult, control or thymectomized donors.
Comparison of the increases in skin thickness in the skin of sheep following the intradermal injection of 5 or $25 \times 10^6$ allogeneic lymphocytes. The lower curve in each case is the response to $5 \times 10^6$ cells, while the upper curve is the response in the same recipients to $25 \times 10^6$ cells from the same donors.

8.8 The reactions in the skin of thymectomized lambs to the injection of lymphocytes from control lambs. Each point is the mean of 5 determinations.

8.9 The reactions in the skin of control lambs injected with lymphocytes from thymectomized lambs. Each point is the mean of 5 determinations.

8.10 The reactions in the skin of thymectomized lambs injected with lymphocytes from thymectomized lambs. Each point is the mean of 4 determinations.

8.11 The reactions in the skin of thymectomized lambs injected with lymphocytes from normal adult sheep. Each point is the mean of 2 determinations.

8.12 The reactions in the skin of normal adult sheep injected with lymphocytes from thymectomized lambs. Each point is the mean of 5 determinations.
Histological Observations

The histological appearances 120 hours after the injection of allogeneic lymphocytes into the skin of normal sheep were of a dense cellular infiltration of the skin around the site of injection. Dense collections of cells were scattered throughout the tissues, while at the borders of the lesions there were small islands of cells and a large nucleus containing one or more nucleoli. In addition, mononuclear cells with darker staining nuclei were seen scattered sparsely through the tissue. The large blast cells were faintly pyroninophilic, but there were some strongly pyroninophilic, medium sized cells scattered around the periphery of the cellular accumulations. Other groups of medium sized pyroninophilic cells were present in the small blood vessels in the deeper layers of the dermis beneath the site of the main cellular reaction.

7 days after injection the picture was similar, the only difference being that there was a slight reduction in the number of cells present. Large blast cells were still the most prominent feature. Gradations of severity in the cellular reaction could be seen, from minimal change in those animals which had no significant increase in skin thickness following the injection of allogeneic lymphocytes, to those which reacted very strongly (particularly at the site of the lesion), in which the central area of the lesions became histologically indistinguishable from normal skin. In contrast, injection of cells from normal sheep did not produce any of these effects (Figs. 8.13 and 8.14).
Histological Observations

The histological appearance 120 hours after the injection of allogeneic lymphocytes into the skin of normal sheep was of a dense cellular infiltration of the skin around the site of injection. Diffuse collections of pale staining cells were scattered throughout the tissues, while at the base of the dermis there were smaller islands of cells separated by bands of connective tissue. At higher magnification, most of these cells were seen to be large blast cells with a large nucleus containing one or more nucleoli. In addition mononuclear cells with darker staining nuclei were present, scattered sparsely through the tissue. The large blast cells were only faintly pyroninophilic, but there were some intensely pyroninophilic, medium sized cells scattered around the periphery of the cellular accumulations. Other groups of deeply staining, medium sized pyroninophilic cells were present adjacent to small blood vessels in the deeper layers of the dermis beneath the site of the main cellular reaction.

At 7 days the picture was similar, the only difference being that there was a slight reduction in the number of cells present. Large blast cells were still the most prominent feature. All gradations of severity of the cellular reaction could be seen, from minimal changes in those animals which showed no significant increase in skin thickness following the injection of allogeneic lymphocytes, to those which reacted very strongly (particularly at a high dose), in which the centre of the lesions became frankly necrotic. The histological features of typical NLT reactions in the skin of control and thymectomized lambs following the injection of cells from normal animals are shown in Figs. 8.13 and 8.14.
FIGURE 8.13

A comparison of the intensity of the cellular reactions which occur in the skin of control and thymectomized lambs following the intradermal injection of $5 \times 10^6$ allogeneic lymphocytes.

A  Control lamb at 5 days
B  Thymectomized lamb at 5 days
C  Control lamb at 7 days
D  Thymectomized lamb at 7 days

Stain H and E. Magnification 25x.
FIGURE 8.14

The histological appearance of NLT reactions produced in the skin of thymectomized and control lambs 5 days after the intradermal injection of $5 \times 10^6$ allogeneic lymphocytes.

A Control. The "blast" cell response. These cells are large, with a pale staining nucleus and abundant cytoplasm.

B Thymectomized. Despite a reduced overall cellular response, clusters of "blast" cells can be seen scattered along the base of the dermis. These blast cells are usually found in the immediate peri-vascular areas.

Stain H and E. Magnification 750x.

C Control. Pyroninophilic cells in the NLT reaction. Most of the "blast" cells are faintly pyroninophilic but more intensely staining cells can be seen, particularly in the accumulations of cells in the peri-vascular areas.

D Thymectomized. A similar view of the pyroninophilic cells in the peri-vascular regions.

Stain pyronin-methyl-green. Magnification 400x.
Discussion

The normal lymphocyte transfer reaction has been shown to be the result of a complex cellular interaction involving genetically dissimilar lymphocytes (Jones and Lafferty, 1969). The reaction consists of graft versus host and host versus graft components, in which the donor and recipient lymphocytes participate. These reactions probably occur simultaneously, since the injection of dead lymphocytes evokes no response (Brent et al., 1962; Jones and Lafferty, 1969), nor does a reaction ensue if live lymphocytes are injected into a recipient which has been depleted of lymphoid cells by X-irradiation (Ramseier and Billingham, 1966; Elkins, 1966).

The results described above show that while lymphocytes from normal donors react strongly in normal recipients, these same cells when injected into thymectomized recipients elicit much smaller responses and there is a much lower incidence of positive reactions. Since the thymectomized recipients have a greatly reduced circulating lymphocyte pool, this reduction in the ability of normal cells to produce a reaction in these thymectomized recipients could be explained on the basis of a lack of opportunity for contact between the donor and recipient lymphocytes. However, when $5 \times 10^6$ lymphocytes from a thymectomized donor were injected into normal recipients, fewer and smaller reactions occurred than when normal cells were injected into the same normal recipients. The lack of reaction in this situation cannot be explained on the basis of lack of opportunity for contact. It must therefore be due, either to the fact that in thymectomized animals there is a deficiency in a particular type of lymphocyte which is involved in the NLT reaction, or, that there is a deficiency in the ability of these cells from
thymectomized lambs to recognize and react with foreign lymphocytes.

Thus thymectomy in the lamb results in a reduction in the ability of lymphocytes from these animals to produce a graft versus host reaction, and a reduction in the ability to mount a host versus graft reaction, i.e. a reduced ability to resist a graft versus host reaction. Although NLT reactions have not been studied following thymectomy in any other species, it has been found that lymphocytes from thymectomized mice are less capable of causing graft versus host reactions in susceptible recipients. Thymectomized mice are also less capable of resisting the graft versus host reaction which results from the parenteral injection of allogeneic lymphocytes (Good et al., 1962; Martinez et al., 1962b; Dalmasso et al., 1962, 1963; Parrot and East, 1964). Lymphocytes from thymectomized rats have also been found to be less efficient in causing graft versus host reactions (Rieke, 1966).

Although larger reactions and an increased number of positive reactors were obtained when the numbers of injected cells from thymectomized animals was increased 5 fold, it was not possible to deduce from the results whether this lack of graft versus host activity was due to a reduction in the number of specifically reactive cells, or whether these reactive cells, although present in normal numbers had a diminished capacity to recognize or react with genetically dissimilar lymphocytes.

**Summary**

In utero thymectomy of lambs results in a reduced ability of the lymphocytes of these lambs to cause a normal lymphocyte transfer reaction when injected into the skin of
normal sheep. The reaction in the skin of thymectomized lambs following the intradermal injection of lymphocytes from normal donors was also greatly reduced. These results indicate that an analogous situation exists following thymectomy in the sheep to that which occurs following neonatal thymectomy in mice and rats, in that there is a reduction in the ability of thymectomized animals to produce a graft versus host reaction as well as a reduction in the ability of these animals to resist a graft versus host reaction. It is not known whether this deficiency is due to a reduction in the number of competent cells, or a reduction in the competence of individual cells.
CHAPTER IX

GENERAL SUMMARY AND CONCLUSIONS

In general terms the growth and development of the lymphoid system of the lamb has been found to be similar to that described for other species such as the mouse (Satiristahan, 1960; Pepper, 1961b; Metcalf, 1964a), the rabbit (Good et al., 1965) and the rat (Bainhardt, 1946). In all these species the thymus is the first organ to have recognizable lymphoid characteristics, followed by the lymph nodes and the spleen. The growth curves of the thymus and other lymphoid organs after birth are similar to those described for the mouse by Metcalf (1964a). This is despite the fact that in the sheep, the thymus is recognizable as early as 110 days prior to birth, as against only 7 to 8 days in the mouse. This suggests that the post-natal development of the thymus is correlated with birth and the events which occur about this time.

Observations of Wilson et al. (1965) that the growth of the thymus in germ-free mice follows a similar course to that of conventionally raised mice, suggest that the post-natal growth of the thymus is not dependent on antigenic stimulation. On the other hand, Wilson et al. (1965) observed further that the thymus of germ-free mice does not grow to as large a size as in conventionally reared mice, indicating that the final size of this organ is in some way, related to antigenic stimulation.

The size reached by the lymph nodes is undoubtedly dependent, to a very large extent, on the degree of antigenic stimulation to which each node is subjected. It has been well documented that lymph nodes are poorly developed in germ-free animals (Thorbecke, 1959; Gordon, 1959; Miyakawa, 1959) and when germ-free animals are exposed to a contaminated environment, there is an increase in lymphoid cells and an increase in weight of the lymph nodes (Thorbecke, 1959; Olm
General Summary and Conclusions

In general terms the growth and development of the lymphoid system of the lamb has been found to be similar to that described for other species such as the mouse (Santisteban, 1960; Pepper, 1961b; Metcalf, 1964a), the rabbit (Good et al, 1965) and the rat (Reinhardt, 1946). In all these species the thymus is the first organ to have recognizable lymphoid characteristics, followed by the lymph nodes and the spleen. The growth curves of the thymus and other lymphoid organs after birth are similar to those described for the mouse by Metcalf (1964a). This is despite the fact that in the sheep, the thymus is recognizable as early as 110 days prior to birth, as against only 7 to 8 days in the mouse. This suggests that the post-natal development of the thymus is correlated with birth and the events which occur about this time. The observations of Wilson et al (1965) that the growth of the thymus in germ-free mice follows a similar course to that of conventionally raised mice, suggest that the post-natal growth of this organ is not dependent on antigenic stimulation. On the other hand, Wilson et al (1965) observed further that the thymus of germ-free mice does not grow to as large a size as in conventionally reared mice, indicating that the final size of this organ is in some way, related to antigenic stimulation.

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The events which occur in the lymphoid system following the transition from a germ-free to non-germ-free state, have been investigated in this present study by examining the intestinal lymph of young lambs immediately prior to and after birth. There are remarkable changes in the number and character of the cells seen leaving the gut in the intestinal lymph soon after birth, and these changes are identical to those seen following deliberate antigenic stimulation. No such changes occur in lymph from other lymph nodes at this time. As a consequence of the antigenic stimulation to which the intestinal lymphoid tissue is subjected, the mesenteric nodes and Peyer's patches rapidly increase in size during the first few weeks of life. Other lymph nodes do not display the same rapid increase in size unless they are similarly subjected to a constant antigenic barrage, such as occurred in a group of lambs suffering from chronic grass seed infestation. The late pre-natal and rapid post-natal development of the Peyer's patches and the evidence of Thorbecke (1957), Gordon (1957) and Miyakawa (1957), that the Bursa of Fabricius develops normally in germ-free chickens, while other lymphoid organs such as Peyer's patches remain under-developed in germ-free animals, casts considerable doubt on the concept that the gut associated lymphoid tissue, particularly the Peyer's patches, are the "bursal equivalent" in mammals, as suggested by Archer et al (1963), Sutherland et al (1964), Cooper et al (1968) and Meuwissen et al (1969).

It has become clear that the degree of immunological immaturity displayed by young animals varies between species, and that, insofar as many antigens are concerned, newborn animals are quite capable of mounting effective immune responses. Failure to detect a satisfactory response
in the newborn may be due to the presence of specific antibody of maternal origin, as these passively acquired antibodies are known to depress the immune response (Smith, 1960; Fink et al, 1962; Dray, 1962). There have been some suggestions that pre-formed antibody is essential for an adequate immune response to occur (Segre and Kaeberle, 1962a and b; Myers and Segre, 1963; Segre and Myers, 1964). It was found however, that colostrum deprived lambs are as capable of responding to swine influenza virus as colostrum fed lambs, and this finding supports the evidence of Sterzl and associates (Sterzl et al, 1960a and b, 1962, 1965) and Kim et al (1966a and b) who found that pre-formed antibody is not essential for the expression of an adequate immune response. Passively acquired immunoglobulins have been considered extremely important in protecting newborn animals against attacks by pathogenic organisms, particularly the enterobacteria. The present study has shown however, that provided colostrum deprived (agammaglobulinaemic) lambs are kept warm, dry and well fed, the apparent disadvantages which these animals normally suffer, can be almost entirely offset. The autogenous production of immunoglobulins by colostrum deprived (agammaglobulinaemic) lambs is similar to that described in humans (Zak and Good, 1959; Good et al, 1962) in that $\gamma_1 M$ is produced first (at about 14 days after birth) then $7S\gamma_1$ (at 28 to 35 days) followed by $7S\gamma_2$ (at 42 to 49 days).

While there must be some period in the early developmental life of an animal before the lymphoid system is formed, when it is completely unresponsive to antigenic stimulation, the failure of very immature animals to mount an immune response may be only relative, rather than absolute. For instance, the foetal mouse has been shown to have cells
capable of producing immunoglobulins in the yolk sac and liver at 9 days gestation, 3 days before the thymus contains any lymphoid cells (Tyan and Herzenberg, 1968a and b). On the other hand, a fully developed lymphoid system does not necessarily imply fully developed immunological competence. The work of Silverstein et al (1963b) has graphically demonstrated that foetal lambs acquire competence to different antigens at different times in their development. In particular, and this was confirmed in the present study, foetal or newborn lambs are incapable of responding to Salmonella organisms as judged by the local cellular response or by the production of antibody, whereas at the same age they are fully capable of responding to other antigens such as bacteriophage ϕX-174, ferritin, ovalbumin and swine influenza virus. Further, although the foetal lamb does not produce specific antibody to Salmonella lipopolysaccharide, nor can any plasma cells be found in the lymph nodes draining the site of injection, immunoglobulins can be found in the serum. Thus the presence of a developed lymphoid system does not necessarily imply full immunological competence, nor does the lack of an organized lymphoid system imply a lack of immunologically competent cells.

In utero splenectomy of foetal lambs does not depress the ability of these animals to form antibodies to S. muenchen organisms when challenged either locally or intravenously some 5 to 9 months later. It has generally been considered that splenectomy reduces the antibody response, particularly in relation to an intravenous challenge with antigen (Pfeiffer and Marx, 1898; Deutsch, 1899; Rowley, 1950; Taliaferro and Taliaferro, 1950; Campbell and La Via, 1967; De Carvalho, Borel and Miescher, 1967). However, most studies have been done on animals splenectomized either
immediately before, at the time of, or soon after antigenic challenge. In the present study lambs were splenectomized several months prior to challenge and in these animals the immune response was normal. It would appear then that, while the spleen is a site of antibody production in the normal animal, it is not essential, and normal immune responses can be obtained following the intravenous administration of antigen, provided sufficient time has elapsed between splenectomy and challenge for some compensatory mechanism to be developed. This probably involves some hypertrophy of the reticulo-endothelial system, since a large part of this normally resides in the spleen (Ellis and Smith, 1966; Shinefield et al, 1966; Klauss and Jones, 1968).

In utero splenectomy of the foetal lamb had no apparent effect on growth rate, or on the levels of circulating lymphocytes in the blood or lymph, nor did splenectomy result in wasting disease.

In utero thymectomy in the foetal lamb, at an early stage of gestation, resulted in a great reduction in the levels of lymphocytes in the lymph. Although the numbers of all classes of lymphocytes (based on their size distribution) were reduced, the most severe reduction occurred in the population of small lymphocytes. Neonatal thymectomy has been reported to have a similar effect on the size distribution of cells carried in the thoracic duct lymph of rats (Rieke, 1966).

The blood lymphocyte levels were also reduced in thymectomized lambs, but at the same time, there was an increase in the number of polymorphonuclear neutrophils. This result has also been reported previously in thymectomized mice and rats (Metcalf, 1960; Parrot and East, 1962: Law, Dunn, Trainin and Levey, 1964b).
There was also a reduction in the cellularity of other lymphoid organs, such as the lymph nodes, spleen and Peyer's patches. There was not, however, any comparable reduction in the number of germinal centres or plasma cells in these tissues. In these respects, the thymectomized lamb resembles other species of conventionally raised, neonatally thymectomized animals (see review by Miller and Osoba, 1967). In contrast to conventionally reared, neonatally thymectomized mice however, thymectomized lambs kept under reasonably hygienic conditions did not suffer from "wasting disease", even when they were deprived of antibodies from the maternal colostrum.

The time when autogenous production of immunoglobulins occurred in in utero thymectomized lambs, and the sequence in which the γ globulin fractions were synthesized, was no different to that seen in normal, colostrum deprived lambs. This confirmed the results found for other species, that thymectomy does not affect the amount or type of immunoglobulins produced (Humphrey et al, 1964; Fahey et al, 1965).

Although thymectomized lambs showed a greatly reduced lymphocyte population in their circulating blood and lymph, and greatly reduced numbers of lymphocytes in their lymphoid organs, they responded in the same way as normal lambs to antigenic challenge with swine influenza virus, killed S. muenchen organisms, or chicken red blood cells. These immune responses were assessed by measuring the production of basophilic cells, large or transitional type cells, and where possible, plaque forming cells, in the lymph. In addition, the amount and type of antibody that was produced after local or intravenous antigenic challenge was also measured. In terms of these parameters, in utero thymectomy at an early age did not affect the recognition of antigens
or the subsequent events which lead ultimately to the production of specific antibodies of both 7S and 19S type. If there is in fact, some cellular interaction in the sheep between "antigen reactive cells" and "antibody forming cell precursors" as postulated for the mouse by Miller and co-workers (Miller and Mitchell, 1967, 1968; Mitchell and Miller, 1968; Nossal et al, 1968), then thymectomy in the lamb has none of the effects of reducing the number of "antigen reactive cells" as it has been claimed to have in conventionally reared mice. Lambs thymectomized in utero before 80 days gestation, were found to be fully capable of rejecting skin homografts, even as long as 18 months after thymectomy despite, as mentioned before, a paucity of circulating lymphocytes. This was an unexpected finding, since the homograft reaction is generally considered to be a cell mediated phenomenon. If this is indeed so, then early thymectomy does not effectively reduce the cell type involved in this response.

The immediate type hypersensitivity reaction to ferritin was also found to be unimpaired in thymectomized lambs. It has been shown by Silverstein et al (1963b) that foetal lambs challenged with ferritin as early as 65 days post-conception, subsequently produce antibody to this antigen. Antibody production appeared to be normal in thymectomized lambs, and since ITH responses are thought to be due largely to a reaction between antigen and antibody the normal ITH response to this antigen was not unexpected.

Two immunological reactions were found to be depressed in thymectomized lambs; the delayed type hypersensitivity reaction to tuberculin and the normal lymphocyte transfer reaction. The DTH reaction to tuberculin following sensitisation with BCG, was significantly depressed in
comparison to the same reaction elicited in normal lambs. Nevertheless, this response was not completely abrogated and could always be detected in thymectomized lambs, even though greatly reduced in size and intensity.

The normal lymphocyte transfer (NLT) reaction which followed the intradermal injection of homologous lymphocytes from normal adult sheep or normal lambs of the same age into thymectomized lambs, resulted in much smaller skin reactions than were seen when the same cells were injected into normal adult sheep or lambs. Conversely, the size of the reactions produced in the skin of normal recipient sheep or lambs following the injection of lymphocytes from thymectomized lambs, was also significantly reduced. Virtually no responses occurred in the skin of thymectomized lambs injected with lymphocytes from thymectomized donors.

The NLT reaction is thought to be the combination of a graft versus host reaction mounted by the injected cells, and a host versus graft reaction mounted by cells of the recipient animal. The results obtained in this present study indicate that not only are cells from thymectomized animals less capable than cells from normal animals of producing a graft versus host reaction in any recipient (thymectomized or normal), but these cells from thymectomized lambs are also less capable of mounting a host versus graft reaction in response to the injection of cells from competent or thymectomized donors. At this stage it is not known whether these results are due to a quantitative deficiency in a particular cell line or a qualitative deficiency in the ability of particular types of cells to engage in these reactions.

These results can be interpreted as follows. Since the homograft response is considered to be "cell mediated", as is also the DTH response, and since thymectomy reduces
the DTH reaction but not the homograft response, it follows that either different cell types are involved in these two responses or the one cell type effects these responses in two different ways. It has been shown by Lafferty and Jones (1969), Jones and Lafferty (1969) and Jones, Yamashita and Lafferty (1969), that the NLT reaction is not mediated by any hypersensitivity mechanisms, neither do the lesions of DTH and NLT reactions resemble one another histologically. If this evidence is taken together with the present results, then DTH and NLT reactions must be considered to be distinct, both from each other and from the homograft reaction. This would conflict with the evidence of Brent and Medawar (1963) who claimed that the severity of the NLT reaction was correlated with the survival of homografts.

The effects of thymectomy have served to illustrate that, in the sheep, at least three different types of immunological reactions can be differentiated—delayed type hypersensitivity, the normal lymphocyte transfer reaction and the homograft reaction. These reactions are distinct from each other and appear to be mediated either by different types of cells or by the same cell in different ways. If the homograft reaction is also purely a "cell mediated" reaction (and this is as yet not certain) then these, taken together with the immune response leading to classical humoral immunity, imply that there are probably four modes of expression of immune reactivity, mediated by different cell lines or by different cellular interactions.

The discrepancies between the effects of thymectomy obtained in the sheep in this present study and the effects of thymectomy in the mouse could be due to one or a combination of the two following possibilities. Firstly, in utero thymectomy may have been performed too late or secondly,
development of the immune system may have proceeded normally in the foetus in utero under the influence of a thymic hormone of maternal origin. The thymus of a lamb at 60 to 70 days in utero appears to contain more lymphoid cells than does the thymus of the mouse at birth. However, the evidence presented in Chapter III has shown that while the thymus has lymphoid characteristics at this stage of gestation, its subsequent post-natal growth and development is identical to that seen in the mouse. Furthermore, at 70 days gestation, the thymus weighs about 350 mg and constitutes 0.262 per cent of the body weight, while the thymus of the newborn mouse weighs about 5 mg and constitutes about 0.22 per cent of the body weight. In these terms then, the thymus was removed from the lambs when it was at a size comparable to that in newborn mice. The thymus in the sheep subsequently grows to a maximum size of about 90g i.e. a 250 fold increase, while the thymus of the mouse grows to reach a maximum size (at the same time after birth as in the lamb) of about 60 mg, only a 12 fold increase. In view of these facts it would seem that, if the thymus is essential for the development of immunological competence, its removal from the foetal lamb at 70 days gestation should lead to even more severe immunological deficiencies than are seen following neonatal thymectomy in the mouse. This did not occur.

Since the thymus of the foetal lamb at 60 to 70 days gestation contains lymphoid cells, it is probable that some migration of lymphocytes out of the thymus had occurred prior to its removal. However, the lymph nodes and spleen of foetal lambs at this age have virtually no lymphoid characteristics at all. The situation in the neonatally thymectomized mouse is probably not very different, for it is certain that migration of lymphoid cells out of the thymus has already
occurred before birth. At birth there are circulating lymphocytes in the blood and tissues of the mouse. It has also been shown that potentially immunologically competent cells can be found in the thymus, liver, lung, spleen, gut, bone marrow and blood of the foetal mouse prior to birth (Tyan, 1968; Tyan and Herzenberg, 1968a and b). Thus even if there is more "peripheralisation" of thymus derived cells in the foetal sheep at say 70 days gestation than in the mouse at birth, the difference can only be one of degree. Further, it is unlikely that sufficient cells had escaped from the thymus of the foetal lamb at this early stage to be responsible for the considerable development of the peripheral lymphoid tissues and the vigorous immune responses which occur months after thymectomy. It has also been shown that these peripheral lymphoid tissues develop in the mouse to a certain degree after thymectomy (Law et al, 1964b; Parrot et al, 1966) as also does their ability to respond to antigens (Rogister, 1965; Sinclair and Millican, 1966) and to reject homografts (Martinez et al, 1962b; Brooke, 1965; Parrot and East, 1965). It may be that the better development and performance of the immunological capacities of lambs following thymectomy are due to a continued development under the influence of a humoral factor or hormone of thymic origin produced by the mother. However, the existence of such a factor has yet to be proven.

The immune capabilities of thymectomized lambs did not deteriorate as they grew older. Thymectomized lambs of 18 months of age gave just as effective immune responses as did young lambs. Further, all lambs used for homograft studies were thymectomized at least 10 to 20 days prior to the time when they would normally attain the ability to reject skin homografts (Silverstein et al, 1964) so in these
circumstances the subsequent development of an immunological capacity to recognize and reject foreign tissues occurred in the absence of the thymus. Similarly, the development of the ability to respond to S. muenchen organisms was unaffected in lambs which were thymectomized several months before they would normally be able to react to this antigen. These results demonstrate convincingly that the development of immunological competence, at least to S. muenchen organisms and to foreign skin grafts, is not dependent on the thymus in any way and casts great doubt on any theory that a thymic humoral factor of maternal origin has any influence whatsoever on the development of these reactions.

There were some striking differences between the present results in the lamb and those that have been obtained in other species, in particular in the mouse. Firstly, wasting did not occur in lambs thymectomized in utero, secondly, the response of thymectomized lambs to challenge with several antigens was not affected and thirdly, their response to homografts was similarly unaffected. In these respects, the results obtained with lambs were similar to those obtained in germ-free, thymectomized mice which do not waste, have little or no reduction in their ability to respond to antigens and can reject homografts (Hess et al., 1963; Wilson et al., 1964; McIntire et al., 1964; Hess and Stoner, 1966; Miller et al., 1967a and b; Hess and Stoner, 1967b; Bealmear and Wilson, 1967b).

Despite the fact that the lambs were not germ-free and were given no special treatment, they did not suffer from wasting disease even when deprived of maternal colostral antibodies. The reason for the different results of thymectomy in lambs and mice could be explained by the fact that the procedure of thymectomy and the events that follow in the
lamb and mouse are entirely different. In the foetal lamb thymectomy is performed under strict aseptic conditions and the nourishment of the foetus via the placenta is never disturbed. Upon completion of the operation, the foetus is returned to the uterus where, under perfect conditions over the next 3 months or so, it recovers from the effects of the surgical procedure. During this long recovery period, the remaining lymphoid organs continue to develop without being subjected to any antigenic stimulation from the environment. The thymectomized lamb is then born, in a state of robust health, without any interruption to its normal functions or to its supply of food. The residual lymphoid system, which has had a considerable time to develop, can now apparently withstand the initial antigenic barrage to which it is subjected after birth and post-natal growth continues in the same fashion as in normal lambs.

When one considers the neonatally thymectomized mouse, a completely different situation obtains. These mice are removed from their mother i.e. from their source of food and warmth, within 24 hours of birth. They are cooled on ice or in a refrigerator and are subjected to a crude operation which results in rupture of blood vessels, loss of blood, possible damage to nerves and important lymphatics such as the thoracic duct and other tissues which are situated near the thymus. At the time of operation, these naked, immature animals encounter foreign antigens for the first time in their lives, many of these undoubtedly entering through the wounds resulting from the thymectomy. Those that survive these initial insults, obtain sufficient antibodies from their mother via the milk to survive and even grow normally up until weaning. Following the withdrawal and disappearance of these maternal antibodies, wasting disease sets in,
particularly in unhygienic conditions, and it seems likely
that this is a consequence of an overloading of the under-
developed lymphoid system with foreign antigens. These anti-
gens need not be pathogenic in order to pre-occupy the avail-
able immunologically competent cells. Since the thymus,
which is the source of the bulk of these cells in the normal
animal, is lacking, these animals have insufficient non-
committed cells to deal with pathogenic organisms. It is
likely that endotoxins may also be involved in this
'streasing' process as thymectomized animals are more sus-
ceptible to endotoxin than are normal animals (Salvin et al.,
1962). The poor response to specific antigens, poor homo-
graft reactions, and frequent death of thymectomized mice,
may well be the consequence of this "overloading" of the
immune system. None of these effects are seen if these neo-
natally thymectomized mice are kept germ-free and certainly
none of these effects are seen in the in utero thymectomized
lamb.

This interpretation places the thymus in no special
position in the lymphoid system and certainly refutes the
proposition that it is the only source of a particular type
of immunologically competent cell such as the "antigen re-
active cell" of Mitchell and Miller (1968). However, these
results can also be interpreted as showing that the thymus
supplies or conditions cells which take part in delayed type
hypersensitivity and normal lymphocyte transfer reactions,
and in this context the thymus may be important in establish-
ing the discriminatory techniques which enable an animal to
distinguish "self" from "not self", although not in precisely
the same way as was originally postulated by Burnet (1962).
However, it must be borne in mind that none of the several
types of immunological reactions tested in thymectomized
lambs were completely defective and this fact strongly suggests that although the thymus may be the main source of the cells concerned in these reactions, it cannot be considered unique.
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