HUMORAL IMMUNE RESPONSES IN FOETAL SHEEP

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IN FOETAL SHEEP

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by

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STATEMENT

The estimations of the concentration of immunoglobulin in foetal sera were performed in collaboration with Dr. M.R. Brandon, Department of Experimental Pathology, JCSMR. Mr. R. Hill and Mrs. Kath Brown cut and stained the histological sections. With these exceptions, the experiments reported in this thesis were carried out by myself.

K. Foley

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SUMMARY

The kinetics of immune responses in foetal sheep were studied by injecting antigens separately or collectively into foetuses of different ages and subsequently taking blood samples from them over periods of several weeks. Primary antibody responses to seven of the eight antigens used were first detected between 64 and 82 days gestation; the foetuses did not synthesize antibody to a primary injection of *Salmonella typhimurium* organisms.

The character of the antibody responses matured throughout the last half of gestation. Antigens injected into foetal sheep prior to 80 days gestation induced only IgM; after this time both IgM and IgG_1 were synthesized. IgG_2 did not appear until very late in the response of older foetuses, while IgA was never detected. The magnitude and duration of the antibody responses to several antigens also increased with gestational age. The onset of competence to synthesize antibody to most antigens occurred at a relatively discrete stage of development, although the ability to respond to ovalbumin and chicken gamma-globulin was quite variable until late in gestation.

The magnitude of the antibody response to polymerized flagellin (POL) increased with gestational age. IgM was the predominant immunoglobulin synthesized during the primary immune response to POL, and appeared to be mostly "non-specific". During the secondary response, IgG_1 was the major class of immunoglobulin synthesized and was mostly "specific" anti-POL antibody. The correlations between immunoglobulin concentration and antibody titre were not absolute and probably reflected the different affinities of the antibodies synthesized by different foetuses.

The ability to respond to antigens did not seem to depend on

lymphoid development which was quite sparse at the time most foetuses could mount antibody responses. Antigenic stimulation had a marked effect on the cellularity of lymph nodes draining the site of injection and the spleen. Germinal centres, pyroninophilic cells and plasma cells, absent from normal foetuses, appeared following the injection of antigen.

Thymectomizing foetal sheep prior to 60 days gestation resulted in most foetuses either failing to respond or mounting weak, 2-mercaptoethanol sensitive, antibody responses to chicken red blood cells (CRBC) and POL, when challenged between 100 and 120 days gestation. Although these latter foetuses synthesized IgM, few synthesized any IgG_1. The plaque forming cell responses to CRBC also appeared to be absent or delayed and reduced in thymectomized foetuses compared with responses detected in normal foetal sheep of the same age. A second injection of CRBC restored the competence of thymectomized foetal sheep to synthesize 2-mercaptoethanol resistant antibody and IgG_1.

Antigenic stimulation also altered the ability of young foetal sheep to synthesize IgG_1 in response to a second injection of antigen. The synthesis of IgG_1 was first detected, during primary immune responses, at 87 days gestation. In most primed foetuses, rechallenged around 70 days gestation, the synthesis of IgG_1 was detected between 74 and 80 days gestation.

It seems therefore that foetal sheep respond to most of the antigens used in this study around mid-gestation and that the magnitude and character of the responses detected, mature throughout the remainder of the gestation period in parallel with lymphoid development. Thymectomy reduced the ability of foetal sheep to respond to CRBC and POL and to synthesize IgG_1. Antigenic stimulation promoted lymphopoiesis, renovated the responsiveness of thymectomized foetal sheep and induced the earlier onset of IgG_1 synthesis in normal foetal sheep.

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The development of immunological competence in the mammalian foetus does not seem to depend on the histological maturation of the lymphoid apparatus (Silverstein and Prendergast, 1970; 1971; Fahey and Morris, 1974) although immune responses probably do not occur before lymphocytes are present. The mammalian foetus was thought to be immunologically inert *in utero*, because weak or undetectable responses to antigenic challenge were observed in neonates (Osborn, Dancis and Julia, 1952; Dixon and Weigle, 1957; Bridges, Condie, Zak and Good, 1959) and because tolerance could be induced easily in young animals (Billingham, Brent and Medawar, 1953; Smith and Bridges, 1958; Chase, 1959). Burnet and Fenner (1949) put forward the hypothesis that during foetal development antigenic stimulation would not only fail to elicit an immune response but would actually induce a state of unresponsiveness to the antigen. This hypothesis was developed to explain why immunologically mature animals fail to respond to "self" antigens.

The first demonstration that foetal sheep were able to reject allogeneic skin grafts was made by Schinckel and Ferguson (1953) and this observation effectively rejected the basis of the "null" state hypothesis (Burnet and Fenner, 1949; Bridges *et al*, 1959). Subsequent experiments in foetal sheep (Silverstein, Uhr, Kraner and Lukes, 1963a; Silverstein, Thorbecke, Kraner and Lukes, 1963b; Silverstein, Prendergast and Kraner, 1964) and foetal cattle (Fennestad and Borg-Petersen, 1962) confirmed that at least in these species the foetus could manifest immune reactivites *in utero*. Rodents on the other hand, certainly did seem to fit the "null" hypothesis for they only produced

antibody or rejected skin grafts at or after birth (Uhr, 1960; Steinmuller, 1961a, 1961b; Adler, Curry and Smith, 1969).

The lymphatic tissues of foetal animals show few of the morphological characteristics that have come to be associated with immune competence. Differentiation between the cortex and medulla in lymph nodes is indistinct and germinal centres and plasma cells are absent (Bridges *et al*, 1959; Silverstein and Lukes, 1962). Thorbecke (1959) also found that germ-free animals had fewer germinal centres and plasma cells compared to conventionally reared animals, while Caster, Garner and Luckey (1966) showed that the development of the lymphoid tissues in germ-free mice could be stimulated by antigen. The appearance of lymphoid tissues in the foetus therefore, reflects the relatively antigen-free environment in which the foetus develops rather than an absence of immunologically competent cells.

THE APPEARANCE OF IMMUNE CAPABILITIES IN THE MAMMALIAN FOETUS AND NEONATE

(a) Sheep (gestation period 149 days)

There have been various reports on the immune reactivity of foetal sheep over the past 20 years. The observations of Schinckel and Ferguson (1953) were followed a decade later by the more extensive studies on foetal immune responsiveness by Silverstein and his colleagues. Silverstein *et al* (1963a) exposed foetal sheep surgically at different stages of gestation and injected them with a mixture of antigens, usually in Freund's complete adjuvant (FCA). The foetuses were delivered by hysterotomy at various times after the antigens were injected and blood was obtained from their umbilical cords. When the sera were analysed for their antibody content it was found that the foetuses had not produced antibodies to all of the antigens and there appeared to be a relationship between the age of the foetus and its capacity to respond to each antigen. Foetal sheep produced antibody to the bacteriophage

ϕX174 at 66 days gestation, to ferritin at 80 days gestation and to ovalbumin at 123 days gestation. They failed to respond to diphtheria toxoid, *Salmonella typhosa* or BCG. Silverstein *et al* (1964) further demonstrated that foetal sheep of 77 to 88 days gestation rejected skin allografts, while animals younger than 67 days gestation did not. These data on antibody synthesis have been reviewed in several reports (Silverstein and Kraner, 1965; Silverstein and Prendergast, 1970) which defined in an even more precise chronology the time at which foetal sheep react to a variety of antigens. The details of these experiments and the data however have not been published. These reports indicated that specific antibody could be detected 6 days after injecting ϕX174 into a 35 day old foetus, while a response was found to ferritin at 56 days gestation and to haemocyanin at 80 days gestation. On the basis of these results Silverstein and Prendergast (1970) concluded that the foetus does not develop the capacity to produce antibody to all antigens simultaneously, but that there is a "stepwise maturation of immunological competence to different antigens at different stages of development". They stated further that there was "remarkable precision with which the foetus develops competence to a given antigen at a given stage of gestation" and that "the first immunological activity of the foetus was in no way immature and that it had at its command all the essential components of the adult response" (Silverstein and Prendergast, 1970).

Niederhuber, Shermeta, Turcotte and Gikas (1971) showed that foetal sheep were capable of rejecting renal allografts from other foetuses during the last half of gestation with the same rapidity and intensity as adult animals. Foetal sheep have also been shown to produce IgM complement-fixing antibody by 91 days gestation in response to viable *Brucella ovis* injected into the uterine cavity (Osburn and Hoskins, 1969), while animals injected 25 to 35 days before birth with

killed *Brucella abortus* had good antibody levels when tested at birth (Richardson, Beck and Clark, 1968). One foetus injected 70 days before birth had a low level of anti-brucella antibody when tested after birth. Richardson, Conner, Beck and Clark (1971) showed that foetal sheep injected at 50 to 65 days gestation with *B. abortus* organisms gave a secondary response when rechallenged with these organisms 50 days later. Foetal sheep were able to produce neutralizing antibody against blue tongue virus vaccine by 122 days gestation (Osburn, Johnson, Silverstein, Prendergast, Jochim and Levy, 1971), while antibodies were produced against *Escherichia coli* antigens when they were injected into the amniotic sac of foetal sheep 19 to 22 days before birth (Conner, Richardson and Carter, 1973).

Studies on foetuses in other species, although not as complete as those reported in sheep, have extended the spectrum of immune responses which have been demonstrated to occur *in utero*.

(b) Pigs (gestation period 120 days)

Foetal pigs injected with allogeneic lymphocytes at 80 days gestation subsequently reject allogeneic skin grafts, from the same strain, at birth in a second-set fashion. Animals injected with allogeneic cells at 60 days gestation did not become sensitized (Binns, 1967).

A recent report has shown that antibody to porcine parvovirus can be detected in foetal pigs born to sows infected with the virus after 55 days gestation (Bourne, Curtis, Johnson and Collings, 1974). Direct inoculation of porcine parvovirus into the amniotic sac on or before 55 days gestation caused the death of the foetal piglets. Foetuses survived infection after 72 days gestation and developed high titres of antibody (Bachmann, Sheffy and Vaughan, 1975). Foetal pigs injected with *Leptospira grippotyphosa* at 60 days gestation, had agglutinating antibodies in their serum at 101 days gestation (Fennestad,

Borg-Petersen and Brummerstedt, 1968). Foetal pigs of 70 days gestation produced neutralizing antibody, susceptible to 2-mercapto-ethanol, 4 days after ϕX174 was injected into them (Hajek, Kovaru and Kruml, 1969); the titre of antibody was found to depend on the dose of virus injected. Antibody responses to sheep red blood cells (SRBC) have been demonstrated at 70 days (Tlaskalova, Sterzl, Hajek, Pospisil, Riha, Marvanova, Kamarytova, Mandel, Kruml and Kovaru, 1970) and 74 days gestation (Schultz, Wang and Dunne, 1971a). Tlaskalova *et al* (1970) showed that the 70 day foetus was capable of producing haemagglutinins and haemolysins in response to SRBC and neutralizing antibodies in response to ϕX174. They also found that the response to ϕX174 was dose dependent and that a much larger dose of antigen (100 to 1,000 times) was required to induce a detectable immune response in foetal and neonatal animals. Schultz *et al* (1971) found that a 74 day old foetus produced IgM antibodies in response to SRBC and they showed that the number of specific plaque-forming cells (PFC) increased in foetuses challenged at older ages. The flagellar antigens of *Salmonella oranienberg* injected after 80 days gestation induced the production of 2-mercaptoethanol sensitive antibody; animals first injected with these antigens as foetuses gave a secondary response when they were rechallenged after birth (Binns and Symons, 1974a).

Foetal pigs, like foetal lambs, seem unable to respond to somatic antigens, in particular heat-inactivated *Salmonella paratyphi B* and *Brucella suis* (Sterzl, Mandel, Miler and Riha, 1965).

(c) Cattle (gestation period 285 days)

The bovine foetus has been found to respond to *Anaplasma marginale* at 141 days gestation (Trueblood, Swift and Bear, 1971) and to ferritin at 150 days gestation (Gibson and Zemjanis, 1973). Antibody to *Leptospira saxkoebing* (Fennestad and Borg-Petersen, 1962) and the bovine

parainfluenza-3 virus (Swift and Kennedy, 1972) could be detected in foetuses injected at about 165 days gestation. Antibody responses to *B. abortus* were found in foetuses of 180 days gestation and to ovalbumin at 210 days gestation (Gibson and Zemjanis, 1973). Kendrick and Osburn (1973) found antibody to the infectious bovine rhinotracheitis virus (IBR) 56 days after injecting a 3 month old foetus, while foetuses between 190 and 200 days gestation produced antibodies in response to the virus which causes bovine diarrhoea (Casaro, Kendrick and Kennedy, 1971; Braun, Osburn and Kendrick, 1973). *In utero* infection with *Vibrio foetus* is fatal in foetal calves younger than 212 days gestation, whereas foetuses older than 226 days gestation produce IgM antibody against the organism and survive (Osburn and Hoskins, 1971). With few exceptions the immune responses listed above cannot be taken as indicating the onset of competence to the particular antigen, as often the youngest animal tested gave a response or the observations were made on only a few animals (rev. Schultz, 1973).

(d) Dogs (gestation period 63 days)

Results obtained on the immune responsiveness of dogs may be confounded by the presence of maternal antibody as unlike pigs, sheep and cattle, the placenta of the dog is permeable to these molecules (Brambell, 1970). Foetal dogs 40 to 48 days old have been shown to reject skin allografts, although the mean survival time of grafts performed at this age was four times longer than in newborn or adult animals (Dennis, Jacoby and Griesemer, 1969). Leukaemic cells infused into foetal dogs survive in animals younger than 43 days gestation; the percentage of "takes" falls off after this time (Bryant, Shifrine and McNeil, 1973). Antibody responses to ϕX174 have been elicited at 40 days gestation and to SRBC at 48 days gestation. The strength of these responses as measured by serum antibody levels and numbers of PFC

increased with the age at which the foetus was first challenged. Bovine serum albumin (BSA) did not induce a response in foetal dogs unless it was injected in FCA (Jacoby, Dennis and Griesemer, 1969). Again, as with other species there appeared to be antigens to which the foetal dog could not respond. Shifrine, Smith, Bulgin, Bryant, Zee and Osburn, (1971) found that although foetal dogs were capable of responding to *Brucella canis* organisms at 50 days gestation, they were incapable of responding to keyhole limpet haemocyanin, *Mycoplasma gallisepticum*, IBR virus and chicken red blood cells (CRBC) until near or after term.

(e) Rodents (gestation periods - rats and mice 21 days, rabbits 30 days and guinea pigs 65 days)

Analysis of the development of the immune system in these common laboratory species is complicated by the fact that they do not respond to most antigens at birth or for some days or weeks thereafter. These species receive maternal antibodies from the yolk sac during gestation and newborn rats and mice receive additional maternal antibody from the colostrum (Brambell, 1970) for several days after birth. Immunological competence is acquired in these species over a relatively short period of time in the presence of environmental antigens and maternal antibodies.

Several reports relating to rodents are of relevance to the results already discussed. Steinmuller (1961a) demonstrated that rats gradually developed the ability to reject allografts in "adult" fashion during the first few weeks of life and that there was a difference in the time this response occurred in the two strains of rats he examined. The ability of these rats to reject hamster xenografts developed in a similar fashion to their ability to reject allografts. Mice on the other hand appear to reject allogeneic skin grafts in "adult" fashion at birth (Steinmuller, 1961b), although tumour transplants are generally accepted

more readily by newborn mice than adult mice (Attia, de Ome and Weiss, 1965). Thymus cells from neonatal mice are capable of mounting graft versus host (GVH) reactions, although possibly not as efficiently as cells from adults (Cohen, Thorbecke, Hochwald and Jacobson, 1963).

In general mice cannot respond to an injection of SRBC before 3 to 4 days after birth (rev. Auerbach, 1972). The onset of antibody responses to SRBC differs in various strains of mice (Hetchel, Dischon and Braun, 1965b; Playfair, 1968), although the actual time at which a particular strain can produce antibody was not altered by either the route of administration of the antigen or by the dose of antigen (Hetchel *et al*, 1965b). Shalaby and Auerbach (1973) found that the time at which mice can respond to erythrocyte antigens could be specifically altered by previous injections of the antigen.

Adjuvants can affect the ability of the developing animal to respond to an antigen. Newborn rabbits injected with BSA did not produce anti-BSA antibody until 28 days after birth, while rabbits injected with BSA in FCA produced antibody 19 days after birth (Good, Condie and Bridges, 1962). Similarly, rabbits were found to produce antibody to diphtheria toxoid injected with adjuvant 7 days after birth, while without adjuvant the response could not be detected until 19 days of age (Pernis, Cohen and Thorbecke, 1963). Not all adjuvants however, influence the time at which immune responses to antigen first appear. Hetchel, Dischon and Braun (1965a) found that the time of onset of the PFC response to SRBC in the spleens of newborn mice was not affected by administration of oligodeoxyribonucleotides, although the response in the pancreatic and mesenteric lymph nodes did appear 1 to 3 days earlier in mice injected with adjuvant than in the controls.

(f) Humans (gestation period 280 days)

There are few reports in the literature concerning the immune capabilities of the human foetus and most observations were as a result of natural infection of the foetus. Eichenwald and Shinefield (1963) demonstrated macroglobulin antibody to Toxoplasma in the cord blood of infants as early as the 28th week of gestation, while Silverstein and Lukes (1962) reported the presence of plasma cells in the lymphoid tissues of human foetuses congenitally infected with syphilis or Toxoplasma after the 5th or 6th month of gestation. Premature infants have been found to produce neutralizing antibody to ϕX174 nearly as rapidly as 2 to 10 year old children (Uhr, Dancis, Franklin, Finkelstein and Lewis, 1962a), although delayed-type hypersensitivity to 2, 4, dinitroflurobenzene (DNFB) could be induced in only about 20% of neonates; the intensity of the reaction was less than that observed in 2 to 12 month old infants (Uhr, Dancis and Neumann, 1960). Fowler, Schubert and West (1960) found that a premature infant of approximately 32 weeks gestation was capable of rejecting a paternal skin graft within 12 days; in contrast, exchange transfusions in newborn infants have induced some degree of tolerance to skin grafts from the corresponding donor, provided the transfused blood was freshly drawn.

Although the data on the immune capabilities of the human foetus are limited, there is no reason to believe that it exists in an immunological "null" state throughout gestation; in fact it seems that the repetoire of immune responses of the human foetus is quite extensive at least during the last trimester.

THE DEVELOPMENT OF THE CELLULAR AND MOLECULAR COMPONENTS OF IMMUNE RESPONSES

The ability to mount an immune response to an antigenic challenge is obviously the end result of a complex interaction of molecules and cells. If the effect of the dose of antigen or its route

of administration is ignored for the moment, the failure to respond may reflect the immature or incomplete development of any one of the components involved in the final production of antibody.

The cell populations which participate in immune responses are principally mononuclear phagocytes (Van Furth, Cohn, Hirsch, Humphrey, Spector and Longevoort, 1972) and lymphocytes. The former are commonly called macrophages, while the latter can be divided functionally into thymus derived or T-lymphocytes and cells derived from the mammalian equivalent of the avian bursa of Fabricius or B-lymphocytes. T-lymphocytes are thought to be active in "cell-mediated" immune functions such as graft rejection, delayed hypersensitivity and GVH reactivity, while B-lymphocytes are ultimately responsible for the production of antibody and give rise to the specialized antibody-producing plasma cells. These cell types may interact in a variety of ways depending on the antigen and the nature of the immune response. It follows therefore that the ability to respond to a particular antigen cannot be present until each essential component of these interacting systems has developed.

(a) Maturation of Mononuclear Phagocytes and Opsonins

In any discussion on the development of phagocytic activity it is essential to consider the level of opsonins in the system as well as the ability of mononuclear phagocytes (macrophages) to engulf and digest fully opsonized particles or cells.

Dlabac, Miler, Kruml, Kovaru and Leon (1970) showed that 61 day old foetal piglets were able to eliminate rough forms of *Salmonella typhimurium* organisms from the circulation and that this process became more rapid as the gestation period advanced. Foetal piglets, like newborn piglets, were unable to clear smooth forms of the organisms unless the bacteria had first been opsonized with rabbit antiserum. Furthermore, the ability to kill ingested rough forms of *S. typhimurium*

organisms was not manifest until 100 days gestation, as the organisms continued to multiply in the tissues of foetal piglets up to 80 days old. The rate at which organisms were killed in foetuses older than 100 days gestation was slow and did not increase significantly after birth.

Opsonins for *S. typhimurium* organisms in the blood of neonatal rats remained at a fairly constant level for the first 2 weeks after birth, but at the end of the first month their levels increased rapidly (Reade, Turner and Jenkin, 1965). These opsonins were low molecular weight proteins in the foetus, probably IgG, while they appeared to be IgM in adult rats. Whether the IgG opsonins were synthesized by the foetus or were derived from the mother was not resolved. Reade and Jenkin (1965) found that *S. typhimurium* organisms were cleared from the blood of foetal rats at an increasing rate between 16 and 22 days gestation. The rate of clearance of *E. coli*, carbon particles and different strains of Salmonella was also found to increase throughout gestation. Karthigasu, Reade and Jenkin (1965) showed that the bactericidal activity in the liver of foetal and newborn rats increased as the animals became older.

Sera from infants with low birth weights acted as poor opsonins for *Staphlococcus aureus* and *Serratia marcescans* when tested *in vitro* with either autologous or normal adult leucocytes, whereas opsonins in sera from adults enabled cells from normal term infants and premature infants to phagocytose and kill these organisms effectively (Forman and Stiehm, 1969). They concluded that the low level of IgG in the sera of premature infants was mainly responsible for the poor phagocytic and bactericidal activity displayed against bacteria.

Studies on the phagocytic activity of the Kupffer cells of newborn piglets have shown that these cells were able to phagocytose carbon, whether or not the piglets were deprived of colostrum. The

rate of clearance was the same as that observed in adult animals (Mouton, Biozzi, Bouthillier and Stiffel, 1963). When piglets were deprived of colostrum the rate of clearance of *S. typhimurium* organisms was reduced and most of the organisms were taken up by the spleen and not the liver (Mouton *et al*, 1963). Rough strains of *E. coli* organisms were removed rapidly from the circulation of germ-free, colostrum-deprived piglets, whereas smooth strains were eliminated slowly (Sterzl *et al*, 1965; Miler, Tlaskalova, Mandel and Travnicek, 1968). Smooth strains were removed much more quickly after opsonization *in vitro* and *in vivo* by specific immune sera. These results demonstrated that the phagocytic capabilities of cells in newborn piglets were well developed provided specific or non-specific opsonins were available. Newborn piglets also seemed to have natural opsonins for rough forms of *E. coli*.

Ultrastructural studies on macrophages by Reade (1968) indicated that cells from foetal rats, a few days before birth, possessed fewer mitochondria and fewer osmiophilic and acid phosphatase positive granules than did macrophages from adult rats. The number of these organelles in the macrophages increased up until 10 days after birth when the cells appeared similar to those from adults. Macrophages from newborn rats were reported to have rough endoplasmic reticulum and large numbers of free ribosomes, characteristic of active protein synthesis, while a large proportion of macrophages from adult rats showed smooth endoplasmic reticulum and few free ribosomes.

The antibody response to Shigella organisms in irradiated adult mice cannot be restored by the transfer of cells from peritoneal exudates of neonatal mice, although cells in peritoneal exudates from adult mice do this effectively (Hardy, Globerson and Danon, 1973). Since equivalent numbers of peritoneal exudate cells were transferred in these experiments the results were taken to indicate that the immunological

incompetence of newborn mice was a qualitative defect in neonatal macrophages rather than a simple lack of macrophages in the neonate.

Macrophages from adults, when transferred to immunologically incompetent neonatal animals appear to confer on the recipients the ability to respond to antigens to which they would otherwise be unresponsive. Martin (1966) found that 40% of newborn rabbits injected with peritoneal cells from exudates from adult rabbits together with alum-precipitated BSA, were able to produce antibody to a second injection of BSA given 14 days later; only 15% of the animals which did not receive exudate cells responded to the second injection of BSA. A deficiency in macrophage activity was postulated as the reason for the majority of newborn rabbits being rendered tolerant following the administration of alum-precipitated BSA. When peritoneal exudate cells from adult mice were transferred to newborn mice the response to SRBC was found to mature earlier (Argyris, 1968). In these transfer experiments syngeneic peritoneal exudate cells were more effective than allogeneic cells, which in turn were more effective than xenogeneic cells. Cells in exudates from very young or very old syngeneic mice were less effective than cells from 3 to 9 month old mice (Argyris, 1969). Experiments also showed that the antibody response to SRBC and burro red blood cells (BRBC) could be induced in rats at birth by injecting syngeneic peritoneal exudate cells from adults into them. Rats normally do not respond to SRBC until 3 to 5 days after birth and to BRBC until 10 to 14 days after birth (Blaese, Henrichon and Waldman, 1970). Brucella organisms or *E. coli* endotoxin, but not FCA, given simultaneously with erythrocytes promoted haemolysin antibody responses to erythrocyte antigens in newborn rats. Blaese *et al* (1970) concluded that it was the state of maturation or "activation" of macrophages which determined the ability of neonatal rats to

produce antibodies to certain antigens.

These transfer experiments have used cells from peritoneal exudates as a source of phagocytic cells and the results are confounded because these cell populations contain many immunologically competent lymphocytes. If one accepts the data of Auerbach (1972) that the minimum number of mouse spleen cells needed to produce an effective response to SRBC *in vitro* is no fewer than 10^5 spleen cells, then as little as a 1% contamination of lymphocytes in the 10 to 20 million peritoneal exudate cells commonly used in transfer experiments (Martin, 1966; Argyris, 1968) could account for the observed responses. Only Blaese *et al* (1970) used lymphocyte-free cultivated peritoneal exudate cells and found these cells still able to confer competence on the recipient animals. They claimed however, that a similar number of spleen cells could not transfer this reactivity, although Argyris (1968) found spleen cells to be as active as peritoneal exudate cells when transferred to newborn mice. The finding that Brucella organisms and *E. coli* endotoxin could evoke antibody responses to erythrocyte antigens may in part be explained by the finding of Rank, Di Pauli and Flugge-Rank (1972) that all enterobacterial lipopolysaccharides containing lipid A induce antibodies which cross react with erythrocyte antigens. "Activation" of macrophage activity may therefore not be the only explanation for the antibody that occurs to SRBC and BRBC in these rats (Blaese *et al*, 1970).

Fidler, Chiscon and Golub (1972) found that the inability of spleen cells from young mice to respond to SRBC *in vitro* was due to a defect in the lymphocyte-rich non-adherent cell fractions. When the macrophage-rich, adherent cell population from newborns was mixed with the non-adherent spleen cells from adult mice, a normal PFC response was detected.

From the available data it would seem that macrophages from neonatal and foetal animals are quite capable of phagocytosis, even in the absence of specific antibody, provided suitable opsonins are available. The ability of these cells to kill ingested organisms subsequently or to participate in an immune response to an antigenic challenge is still in doubt. This question particularly concerns newborn rodents, since we know that foetal sheep, pigs and cattle can mount immune responses to a wide variety of antigens. If the participation of macrophages is obligatory for immune responses to occur we can assume that these cells are fully functional well before birth in these species.

(b) Maturation of B-lymphocytes

(i) Chickens

Most data relating to the development of antibody forming lymphocytes and plasma cells (Howard and Gowans, 1972) come from studies in the chicken where a discrete lymphoid organ, the bursa of Fabricius, has been identified as the site of differentiation of cells involved in antibody responses (Glick, Chang and Jaap, 1956; Glick, 1961).

In the chicken, the thymus acquires its first lymphocytes at about 11 to 12 days of incubation (Venzke, 1952; Ackerman, 1966); the bursa at 12 to 13 days of incubation (Ackerman, 1966) and the spleen on day 14 (Sandreuter, 1951). Soon after this date lymphocytes can be found in the walls of the gastrointestinal tract and in lymphoid nodules throughout the body.

Moore and Owen (1967) made a systematic study of the migration of lymphoid cells in the chick embryo, identifying migrant cells by a chromosome marker peculiar to chickens, (Moore and Owen, 1965). Haemopoietic stem cells, originating in the yolk sac were found to enter the epithelial thymus and bursa via the blood circulation and there

they underwent extensive and rapid proliferation (Rubin, Cooper and Kraus, 1971). In the bursa these dividing cells formed the lymphoid follicles. The cortex and medulla were established in the follicles just before birth. Lymphocytes migrate from the bursal follicles to the spleen (Durkin, Theis and Thorbecke, 1971) and to peripheral lymphoid organs (Cooper, Lawton and Kincade, 1972b) and from the thymus to the spleen (Durkin *et al*, 1971) to the tonsilla caecalis and to the bone marrow (Linna, Back and Hemmingsson, 1971).

Cells in the bursal follicles of 14 day old chick embryos were found to synthesize IgM, which could be detected by direct immunofluorescence (Kincade, Lawton, Bockman and Cooper, 1970). Cells synthesizing immunoglobulin were not detected in other lymphoid tissues in embryos of this age (Kincade and Cooper, 1971). Cells containing IgG appeared in the bursa 7 days later, at about the time of hatching. These IgG-containing cells were found in the follicles which already contained IgM-producing cells. IgA-containing cells appeared only after birth and were distributed in follicles in a similar fashion to the IgG-containing cells. The appearance of cells containing immunoglobulin in peripheral lymphoid tissues closely paralleled that in the bursa, but was delayed by several days (Kincade and Cooper, 1971; 1973).

The synthesis of immunoglobulin by bursal cells was not thought to be influenced by antigen. Thorbecke, Warner, Hochwald and Ohanian (1968) found no difference *in vitro* in IgM synthesis by bursal lymphocytes from 8 day old chicks raised in either germ-free or conventional environments, while Kincade and Cooper (1971) found that intentional intravenous antigenic stimulation of embryos did not alter the appearance or proliferation of IgM and IgG-containing cells in the bursa. Stimulation by environmental antigens however did promote the development of both IgM and IgG-containing cells in peripheral lymphoid

tissues of conventionally raised chickens, compared to that observed in chicks raised in a germ-free environment. Conversely, antigenic stimulation has been shown to induce specific antibody synthesis in the bursa. Although the intravenous injection of SRBC failed to induce a PFC response in the bursa (Dent and Good, 1965), introducing SRBC into the cloaca induced a moderate PFC response (Van Alten and Meuwissen, 1972).

About half of those bursal cells which contained IgG determinants were also stained by anti-IgM, while cells producing both IgG and IgM were rare in control suspensions of spleen cells (Kincade and Cooper, 1971). This suggested that the development of IgG synthesizing cells in the bursa may involve a "switch" in the expression of those genes which direct the formation of the constant region of immunoglobulin heavy chains.

When 13 to 14 day old embryos were given an injection of purified goat anti-chicken IgM sera and then surgically bursectomized at birth, it was found that the animals lacked circulating immunoglobulin, plasma cells and germinal centres, while "cell-mediated" immune functions remained intact (Kincade *et al*, 1970; Kincade and Cooper, 1973; Leslie and Martin, 1973). It was also found that those animals treated with the anti-IgM sera not only failed to synthesize IgM but also IgG and IgA (Kincade *et al*, 1970; Kincade and Cooper, 1973; Leslie and Martin, 1973). The important control in these experiments was that IgM synthesis was suppressed in birds bursectomized at birth and then treated with anti-IgM, whereas IgG synthesis was unaffected. This indicated that suppression of IgM synthesis by anti-IgM occurred only in the bursal environment; the precursor cells involved in IgG synthesis were not inhibited by antisera once they had left the bursa (Kincade *et al*, 1970). Chickens which were injected with anti-IgM as embryos but not

bursectomized failed to produce IgM and IgG for only a short period of time; production in animals given serial injections of anti-IgM antisera was suppressed for a longer period (Leslie and Martin, 1973).

Observations on the ontogeny of B-lymphocytes in the chicken led to the proposal (Cooper, Lawton and Kincade, 1972a; Lawton, Kincade and Cooper, 1975) that "clonal development" starts when stem cells migrate to the bursal environment where they differentiate and begin to synthesize IgM. Most of this IgM becomes incorporated into the membranes of those cells synthesizing it. Under the influence of the bursal micro-environment the lymphocytes undergo division and some of the daughter cells "switch" from the expression of the Cμ gene to the Cγ gene. The specificity of the clone need not change as the C_L and V_H and V_L genes remain unaltered. The model then proposed a similar switch from Cγ to Cα to commit precursor cells to IgA synthesis. These cells migrate to the peripheral lymphoid tissues where clones of B cells composed of cells committed to IgM, IgG and IgA synthesis, become established. This maturation process in the bursa was independent of contact with exogenous antigen and once the B-lymphocytes left the bursa they were committed to the production of a single species of antibody. The thymus may also play a role in the "switch" from IgM to IgG and IgA, as chickens thymectomized at hatching have depressed IgG and IgA levels, while chickens thymectomized and bursectomized at hatching have no IgA whatsoever (Perey and Bienenstock, 1973).

(ii) Mammals

How B cells mature in mammals is not as well established as in chickens and this is due to the lack of knowledge about where these events take place. There has been an extensive search for an organ in mammals with functions equivalent to those of the bursa of Fabricius,

with the bone marrow and the gut-associated lymphoid tissues being early favourites. Suffice it to say that neither of these now seems likely to qualify (Lawton *et al*, 1975). Recently the foetal liver has been implicated as the place where a suitable inductive environment exists for B-lymphocyte differentiation (Owen, Cooper and Raff, 1974) although not all data support this concept (Nossal and Pike, 1972).

The presence of surface immunoglobulin has been generally accepted as the marker which identifies B-lymphocytes (Coombs, Fienstein and Wilson, 1969; Pernis, Forni and Amante, 1970; Raff, 1970), although it has been stressed recently that no surface marker exists which is common to *all* B cells and that the presence of immunoglobulin receptors demonstrably synthesized by the cell is simply the best marker presently available (Moller, 1974). Evidence suggests that an antigen-binding B cell has the same antigen-binding specificity and the same heavy and light chains in its surface immunoglobulin molecules as are present in the antibody secreted by the cell. Walters and Wigzell (1970) treated lymphocytes with anti-L chain or monospecific anti-Ig sera and specifically prevented antigen-binding cells from being removed during their passage through antigen-coated columns. They found that treating the cells with anti-lymphocyte sera did not prevent the specific removal of antigen-binding cells, while passing cells through an unrelated antigen-coated column did not interfere with the ability of the filtered cells to produce an *in vitro* PFC response to the test antigen (Wigzell, 1970). Surface immunoglobulin is therefore thought to be a component of the triggering mechanism which initiates proliferation of B cells to form memory cells and plasma cells (Davie and Paul, 1974; Wigzell, 1974). The presence of surface immunoglobulin will be taken to define B cells in much of the following discussion.

(a) Sheep

Surface immunoglobulin has been detected on peripheral blood leucocytes in foetal sheep at 56 days gestation (0.3% positive cells); by 78 to 87 days gestation up to 15% of the cells are positive and this percentage falls to 2% at 117 days gestation. A slight rise occurs again towards term (Binns and Symons, 1974b; Symons and Binns, 1975). Decker and Sercarz (1974) found cells binding β-galactosidase and ferritin in the thymus (0.05 to 0.09%), spleen (0.08 to 0.14%) and bone marrow (0.6 to 1.0%) of a 58 day old foetus. The percentage of spleen cells binding β-galactosidase, ferritin and haemocyanin did not increase significantly between 58 and 140 days gestation and remained only a fraction of the percentage found in adult animals; an exception being ovalbumin-binding cells, where levels similar to those found in adult sheep (0.25%) occurred in foetuses near term. More interestingly, ovalbumin-binding cells were present long before foetal sheep have been shown experimentally to be able to produce antibodies against this antigen (Silverstein *et al*, 1963a). The percentage of antigen-binding cells found in lymphoid tissues of foetal sheep by Decker and Sercarz (1974) however, did not reflect the increase in immunoglobulin-positive cells in the peripheral blood between 70 and 80 days gestation reported by Binns and Symons (1974b).

(b) Pigs

Binns and Symons (1974a) found that in foetal pigs the percentage of surface immunoglobulin positive cells in the peripheral blood increased rapidly between 70 and 80 days gestation from approximately 0.7% to 8.4%. This increase, detected by an anti-immunoglobulin rosette technique, was claimed to coincide with the earliest detectable

antibody responses (Hajek *et al*, 1969; Tlaskalova *et al*, 1970; Schultz *et al*, 1971a). However, this interpretation is incorrect as Bourne *et al*, (1974) have shown that foetal pigs can respond to parvovirus as early as 58 days gestation, well before the number of immunoglobulin-bearing cells rises. Binns and Symons (1974a) also found that the injection of several antigens into foetal pigs at 59 days gestation increased the percentage of immunoglobulin-positive cells estimated 10 days later (8.8% positive cells in untreated controls as against 17.0% to 19.1% in animals which had received antigen). The high control values could possibly have been due to the surgical manipulation or cross-stimulation from antigenically challenged litter mates.

IgM containing cells have been found by immuno-fluorescence techniques in the spleen of foetal pigs at 55 days gestation and in the thymus and mesenteric lymph nodes at 65 to 70 days. IgG containing cells first appear in the thymus at 60 to 65 days gestation and in the spleen and lymph nodes at 70 days gestation. IgM and IgG containing cells were not found in intestinal lymphoid follicles until 80 to 90 days gestation (Chapman, Johnston and Cooper, 1974). Since the number of positive cells was not recorded, it is not known whether the percentage of cells which contain immunoglobulin varies in the tissues throughout gestation as reported for cells with surface immunoglobulin in the peripheral blood (Binns and Symons, 1974a).

A report by Jaroskova, Trebichavsky, Riha, Kovaru and Holub (1973) indicated that immunoglobulin could not be detected by immuno-fluorescence on the surface of spleen cells from foetal pigs until near term, although surface IgM determinants could be detected on 1% of splenic lymphocytes from an 80 day old foetus, using an electron-microscopic technique. The antisera in these studies were conjugated with horseradish peroxidase. Only IgM determinants were found on

unprimed lymphocytes in the spleen and bone-marrow from foetuses at term (112 - 114 days) and from germ-free piglets before they were fed colostrum. Surface IgG was found on lymphocytes of conventionally reared piglets or germ-free piglets after immunization with SRBC, human serum albumin and bacterial antigens. Jaroskova *et al*, (1973) concluded that the antigenic recognition unit or "primary" receptor on uncommitted lymphocytes was IgM, while IgG could only be detected on cells after antigenic stimulation (c f. Kincade and Cooper, 1971).

(c) Rabbits

Elfenbien, Harrison and Mage (1975) examined spleen cells from newborn to one month old rabbits to see if surface immunoglobulin and complement receptors were present and if *in vitro* proliferative responses to anti-immunoglobulin sera and mitogens occurred. They reached the conclusion that lymphocyte surface markers and functional responses appeared "asynchronously" during the development of the rabbit. In the newborn rabbit 45% of cells from the spleen had receptors for C_3 while only 15% were stained by anti-immunoglobulin sera. Although the percentage of complement-binding cells in the spleen did not increase in adult rabbits, the anti-immunoglobulin positive cells increased to 77% of the total cells.

(d) Guinea pigs

A great majority of DNP-binding cells in foetal guinea pigs bear surface IgM. Moreover in the earliest foetus examined (54 days gestation) the ratio of cells which bound both anti-IgM and DNP to total anti-IgM binding cells was similar to that found in term foetuses (68 days) and in unimmunized adults. This suggested that the ability to recognize a wide range of antigens is established very soon after immunoglobulins appear on the surface of lymphocytes (Davie, Paul, Asofsky and Warren, 1974). Conversely, the ratio of cells which bound

both anti-IgG_2 and DNP to total anti-IgG_2 binding cells increased from late foetal life to adulthood. This indicated that antigenic stimulation plays a role in the appearance or expression of IgG on the surface of lymphocytes. The finding that cultures of spleen cells taken from foetal guinea pigs could synthesize IgA, while IgG synthesis could only be found in cultures of spleen cells taken from animals after birth, makes it unlikely that the ontogenic sequence IgM→IgG→IgA holds true for the guinea pig (c f. Cooper *et al*, 1972a).

(e) Mice

In the mouse, treatment of neonatal animals with anti-IgM will suppress IgM, IgG and IgA production, while anti-IgG at best only depresses IgG levels (Lawton, Asofsky, Hylton and Cooper, 1972a; Manning and Jutila, 1972). Anti-IgM suppressed the antibody response to ferritin (Lawton *et al*, 1972a) and the direct and indirect PFC response to SRBC (Manning and Jutila, 1972), while anti-IgG only suppressed the indirect PFC response. Neonatal mice treated with anti-IgM had few germinal centres in their spleens and lymph nodes, smaller numbers of mature plasma cells, smaller numbers of immunoglobulin-bearing cells of all classes and no IgA synthesizing plasma cells in the gut (Lawton *et al*, 1972a).

Anti-IgM serum suppressed the synthesis of all immunoglobulin classes in *in vitro* primary immune responses to SRBC, whereas anti-IgG only suppressed the IgG response and not the IgM or IgA response (Pierce, Solliday and Asofsky, 1972a). In *in vitro* secondary responses, anti-IgM only depressed IgM and IgA PFC responses and had little or no effect on IgG PFC responses; the IgG PFC responses could, however, still be suppressed by anti-IgG. These changes in the susceptibility of precursor cells to anti-IgM were antigen specific (Pierce, Solliday and Asofsky, 1972b). The results suggested that surface immunoglobulin

classes differ between normal and immune cells and that the membrane receptors for antigen can change during an immune response. Furthermore, it appears that the ontogenic sequence IgM→IgG→IgA does not hold for mice and that a direct switch from IgM→IgA could be postulated from these results.

Greaves and Hogg (1971) have also demonstrated that antigen influences surface receptors. They found that the majority of cells forming rosettes with SRBC from non-immune mice had surface IgM, while the percentage of rosette-forming cells having surface IgG increased in mice primed with SRBC.

Using a haemolytic plaque technique and micromanipulation of single plaque-forming cells, Nossal, Warner and Lewis (1971) found a small percentage (1.5%) of cells which produced both IgG and IgM antibodies to SRBC. It was suggested that these cells may have been "caught" switching from IgM to IgG synthesis. Similarly, Greaves (1971) found that many rosette-forming cells simultaneously express IgM and IgG determinants on their surface. Some rosette-forming cells may also express IgA. They found cells with several classes of immunoglobulin on their surface in the early part of the primary response to SRBC; 15 and 30 days after the injection of antigen, only a single class of immunoglobulin could be detected on the surface of any particular rosette-forming cell. Single antibody-forming cells have also been reported which have IgM on their surface and IgG in their cytoplasm (Pernis, Forni and Amante, 1971).

Recently an IgD-like molecule has been reported to appear on mouse lymphoid cells during ontogeny (Vitetta, Melcher, McWilliams, Lamm, Phillips-Quagliata and Uhr, 1975). In young mice IgD appears on spleen cells after the appearance of IgM, while in adult mice it occurs on lymph node cells but not bone-marrow or thymus cells. Vitetta *et al* (1975)

concluded that the first receptor on virgin B-lymphocytes was IgM and that IgD represented a "switch" of cell surface receptors during differentiation; this is contrary to the scheme proposed for humans (c f. Rowe, Hug, Forni and Pernis, 1973b). Lymphocytes from athymic mice or germ-free mice have both IgM and IgD on their surface, suggesting that the "switch" can occur in the absence of T-lymphocytes and possibly in the absence of antigen.

Lymphocytes from the spleen of newborn mice do not have complement receptors although they do have surface immunoglobulin (Gelfand, Elfenbien, Frank and Paul, 1974). Complement receptors do not appear until 1 to 2 weeks after birth and do not reach adult levels until 6 to 12 weeks after birth, while the proportion of immunoglobulin positive cells reach adult levels 1 week after birth (c f. rabbits - Elfenbien *et al*, 1975).

(f) Humans

Lawton, Self, Royal and Cooper, (1972b) found both surface IgM and IgG on liver cells from human foetuses at 9.5 weeks gestation and by 11.5 weeks gestation IgM, IgG and IgA were found on liver and spleen cells. They found that one of three preparations of thymus cells from foetuses of 11.5 weeks gestation was positive for all immunoglobulin classes, while one of three bone marrow preparations was positive for IgM and IgG, but negative for IgA. Results obtained from older foetuses also showed great variability in regard to the presence or absence of the various immunoglobulin classes on lymphoid cells from different tissues. The number of peripheral blood leucocytes that was positive for immunoglobulin was similar in some foetuses to the number found in neonates, children and adults, but again this finding was quite variable. These experiments, however, failed to demonstrate that the IgG on the surface of the cells was synthesized by them; it may have been adsorbed

maternal IgG. Similarly, the binding of heterologous anti-immunoglobulin sera by liver cells from foetuses may in fact be non-specific binding of antigen, possibly by non-lymphoid cells, following the finding of Dwyer and Mackay (1972) that the binding of antigen by liver cells from foetuses could not be inhibited by anti-immunoglobulin sera.

Rowe, Hug, Page Faulk, McCormick and Gerber (1973a) found that approximately 14.5% of lymphocytes in cord blood from humans had surface IgD, while 8.5% were positive for IgM, 2.9% for IgG and 0.8% for IgA; IgD was thus by far the most common surface immunoglobulin on lymphocytes from newborns, even though IgD could not be detected in the sera (less than 100 ng/ml). Although the biological functions of IgD are not known, its absence from the sera of human foetuses would suggest that IgD does not cross the placenta (Rowe, Crabbe and Turner, 1968). IgD has been detected on 3 to 4% of lymphocytes in blood from adult humans. Many cells in normal individuals have both IgM and IgD on their surface, but cells with both IgG and IgD on their surface have not been found. Based on these observations Rowe, Hug, Forni and Pernis (1973b) have proposed that IgD was the first class of immunoglobulin to appear on the surface of lymphocytes.

The production of immunoglobulins by lymphoid tissues from human foetuses has been studied *in vitro*. Van Furth, Schuit and Hijman (1965) found that spleen cells from foetuses of about 20 weeks gestation produced both IgG and IgM when cultivated *in vitro* in the presence of ovalbumin. In similar experiments by Gitlin and Biasucci (1969) IgM was produced by spleen cells from a 10.5 week old foetus, while IgG was produced by cultivated spleen cells from a foetus of 12 weeks gestation. The physiological significance of these findings may be difficult to interpret following the finding of Silverstein *et al* (1963b) that

cultures of spleen and lymph node cells from antigenically stimulated foetal sheep produced β2M (IgM), β2A (IgA), fast γ (IgG_1) and slow γ (IgG_2) *in vitro*, even though β2A could not be found in the sera of these animals. These *in vitro* results which occur outside the physiological constraints of the foetus, may demonstrate an ability of cells to produce immunoglobulin which is not normally expressed *in vivo* by foetuses in response to antigenic stimulation.

To sum up, we know very little of the way in which B cells mature in the foetal animal. The factors which regulate the maturation of B cells from the time they can first be detected until the time they differentiate into mature plasma cells producing antibody of one immunoglobulin class and one antigenic specificity remains a mystery. Some observations can be made from the available data. The first B-lymphocytes that can be recognized seem to bear surface IgM in all species so far examined and they appear frequently before any encounter with antigen occurs. The IgM present on the surface of these lymphocytes may be monomeric, rather than the pentameric form found in the serum (Vitetta, Baur and Uhr, 1971; Parkhouse, 1973). Later in development cells with surface IgG and IgA can be detected and these may arise following antigenic stimulation. That there is a "switch" in the class of surface immunoglobulin expressed by B-lymphocytes during development has been strongly suggested although the initiator of the switch and the direction of the switch, have not been resolved (Manning, 1975). In the chicken it has been suggested that some cells switch from the production of IgM to IgG and then from the production of IgG to IgA (Cooper *et al*, 1972a). The available evidence in mammals tends to suggest that the switch of IgG to IgA may not occur and in fact the switch may be from IgM to IgG, IgM to IgA or even IgM to IgD. Whether the switch from the production of surface IgM to the production of other

surface immunoglobulin classes is antigen independent or occurs during immune responses remains a controversial point (Jones, Craig, Cebra and Hertzenberg, 1974). The evidence that serum IgM and IgG antibodies formed during an immune response have the same antigen binding site is better established (Oudin and Michel, 1969). In chickens it appears that the switch in immunoglobulin synthesis is antigen independent, since exogenous antigen did not stimulate the maturation of lymphocytes containing IgG or IgA in the bursa (Kincade and Cooper, 1971). The antigen used in this experiment, however, was administered intravenously and may have had little or no effect on the bursa of Fabricius which seems to have a rather special relationship with antigens located in the gut (Van Alten and Meuwissen, 1972; Schaffner, Hess and Cottier, 1974). In mammals it does seem that B cell maturation can be greatly accelerated by antigenic stimulation (Binns and Symons, 1974a; Davie *et al*, 1974).

(c) Maturation of T-lymphocytes

Since it was demonstrated that the thymus plays a fundamental role in the differentiation of cells active in "cell-mediated" immune responses (Miller, 1961) and that thymus-derived (T) lymphocytes are involved in certain humoral immune responses (Miller and Osoba, 1967) the role of the thymus and T-lymphocytes in immune reactions has been studied extensively.

The surface antigens described for thymocytes and T-lymphocytes have been used as tools to understand the differentiation and maturation of thymus-derived lymphocytes. The importance of these surface markers would be enhanced if they can be shown to reflect functional differences between cells. This may prove difficult as it must be assumed that thymus-derived lymphocytes are immutably locked into various differentiative pathways which lead to T cells with different functions.

The various cell markers which have been described may simply indicate the antigenic determinants on thymus-derived lymphocytes which are expressed at different stages of their differentiation, rather than indicate their functional competence; it still remains to be shown that these surface antigens remain as stable constituents of the cell membrane throughout the life of the cell or that the life histories of lymphocytes are determined once and for all by events which occur in the organs and tissues from which they are derived.

Studies on the function of the thymus, like those on the bursa of Fabricius, have been largely based on the effects that follow its surgical removal (Miller, 1961). Thymus cells are known to be a heterogenous population in respect to their size and electrical charge (Droege, Zuker and Jauker, 1974) their density (Schlesinger, Gottesfeld and Korzash, 1973) and the properties of their surface antigens (Raff and Cantor, 1971; Schlesinger, 1972). In the mouse, thymus cells have been divided empirically into two sub-populations which differ in these various characteristics. The majority of the cells in the thymus of the mouse are of relatively small size, have a high density (Konda, Stockert and Smith, 1973) and a low electrophoretic mobility (Droege *et al*, 1974). This population of cells is characterized by a distinctive thymus antigen, TL in TL-positive strains of mice (Leckband, 1970; Raff, 1971). They have a high density of theta (θ) antigen (Raff and Owen, 1971) and a low density of histocompatibility (H-2) antigen on their surface (Raff, 1971). The second population of thymus cells in the mouse (approximately 5% of the total) is composed of relatively large cells which have a low density (Konda *et al*, 1973) and a high electrophoretic mobility (Droege *et al*, 1974). These cells have a high density of H-2 antigen but a low density of θ antigen and they lack the TL antigen (rev. Dyminski, Forbes, Gebhardt, Nakao, Konda and Smith, 1974).

As mentioned previously, in chicken embryos multipotential stem cells, derived from the yolk sac (Moore and Owen, 1967) enter the thymus and undergo extensive proliferation. They emerge later, via the circulation, to populate peripheral lymphoid tissues. In the mammalian foetus stem cells from the yolk sac and liver migrate to the epithelial thymic anlage. The maturation of T cells in the thymus may be seen simplistically as stem cells→thymocytes→"mature" thymic lymphocytes→ peripheral T-lymphocytes (Raff, 1973).

In embryonic mice it has been shown that immigrant stem cells from the yolk sac and liver do not express θ or TL antigens on their surfaces (Owen and Ritter, 1969). These markers are thought to be acquired during differentiation from stem cells to thymocytes (Owen and Raff, 1970). The smaller population of thymus cells, which lack the TL antigen, are relatively resistant to cortisone (Blomgren and Andersson, 1969; 1971; Andersson and Blomgren, 1970) and have been shown to migrate preferentially to lymph nodes (Raff, 1971; Levey and Burleson, 1972) and to participate in GVH immune reactions (Blomgren and Andersson, 1969; 1971; Leckband, 1970). This population of thymocytes closely resembles peripheral T-lymphocytes. Whether the smaller population of TL negative thymocytes develop from precursor cells in the larger TL positive population (Boyse and Old, 1969; Raff and Cantor, 1971) has recently been questioned (Schlesinger, 1974). The alternative suggestion is that the two cell types develop in parallel from different stem cells in the thymus.

The fact that neonatal thymectomy in rodents impairs immune functions ascribed to T-lymphocytes (Good and Gabrielsen, 1964; Miller and Osoba, 1967; Hess, 1968) and that a functional thymus is necessary to restore these activities to animals given doses of anti-lymphocyte serum (Monaco, Wood and Russell, 1965) or irradiation (Cross, Leuchars

and Miller, 1964) have been taken to indicate that the thymus provides the environment critical for T cell maturation (Blomgren and Andersson, 1969; Raff and Cantor, 1971; Owen, 1972). Exactly how the thymus influences lymphocyte differentiation has not been determined, but humoral factors or hormones produced by the thymus may be involved (Goldstein and White, 1973; Stutman and Good, 1973; Trainin and Small, 1973).

Not all evidence, however, indicates that the thymus provides an essential environment for T cell maturation. Cole and Morris (1971a; 1971b; 1971c; 1971d) found that lambs thymectomized *in utero* at 60 to 80 days gestation, showed little or no immunological deficiencies after birth. The only abnormal findings were lymphopenia and a reduced ability of the thymectomized lambs to mount a normal lymphocyte transfer reaction. The ability to produce antibodies and reject skin allografts had matured in the absence of the thymus. The usual criticism of these findings is that thymectomy was performed after the thymus had played its "critical" role and that T cells seeded to the peripheral tissues prior to thymectomy account for the immunological competence observed. Silverstein and Prendergast (1973) performed similar experiments to Cole and Morris but after thymectomy they treated the foetus with anti-lymphocyte serum. This resulted in an almost complete ablation of lymphoid tissues and of circulating lymphocytes in the foetus. Despite this drastic treatment, lymphocytes began to appear in these animals after birth and although a marked lymphopenia persisted, they produced antibodies to ferritin, ovalbumin and bovine gamma-globulin by 2 to 3 months of age and rejected skin allografts with almost the same proficiency as normal control animals. The "essential" role of the thymus at least in so far as it determines the specific immune functions of certain classes of lymphocytes must therefore remain unproven.

A further subdivision of lymphocytes derived from the mouse thymus has been proposed for cells found in the peripheral blood and lymph (Raff and Cantor, 1971). One population was found to have a higher density of θ antigen (T_1) than the other (T_2), although both had a lower density of θ antigen than cells isolated from the thymus, (Aoki, Hammerling, de Harven, Boyse and Old, 1969). T_1 cells were found predominantly in the spleen and preferentially homed to the spleen. They were short-lived cells, did not recirculate and were relatively resistant to anti-lymphocyte serum. T_2 cells were found mainly in the blood, thoracic duct lymph and lymph nodes. They were long-lived, homed preferentially to lymph nodes, recirculated and were susceptible to anti-lymphocyte serum. Raff and Cantor (1971) suggested that T_1 cells were immature T_2 cells, whose maturation was conditioned by antigen. T_2 cells alone were claimed to be responsible for the T-lymphocyte component of the immune response, while T_1 cells became T_2 cells in response to antigenic stimulation.

T-lymphocytes have been found to specifically bind certain antigens to their surface and the search for the antigen receptor on T cells has resulted in controversy. For obvious reasons it was considered that the receptor on T cells was most probably immunoglobulin, but the initial experiments of Raff (1970) and most others have failed to find immunoglobulin on the surface of thymocytes or T cells. Nossal, Warner, Lewis and Sprent (1972) did find immunoglobulin on T cells and some investigators have also shown immunoglobulin on the surface of thymocytes (Grey, Colon, Campbell and Rabellino, 1972; Nossal *et al*, 1972), which after solubilization seems to be 7S IgM (Cone, Sprent and Marchalonis, 1972; Marchalonis, Cone and Atwell, 1972a; Marchalonis, Atwell and Cone, 1972b). The controversy has recently been analysed by Burckhardt, Guggisberg and von Fellenberg (1974) and may be resolved

by the fact that those investigators who showed that immunoglobulins were present on the surface of thymocytes used antisera against normal IgM, while those who failed (Rabellino, Colon, Grey and Unanue, 1971; Vitetta *et al*, 1971; Lamelin, Lisowska-Bernstein, Matter, Ryser and Vassalli, 1972; Vitetta, Bianco, Nussenzweig and Uhr, 1972) used antisera against the IgM myeloma protein - MOPC 104E, which may lack antigenic determinants present on the surface immunoglobulins on thymocytes.

There is some indirect evidence from inhibition studies that surface immunoglobulin occurs on T-lymphocytes and acts as the antigen receptor. Lymphocytes from mice which are sensitive to anti-θ serum and complement (T-lymphocytes), have been shown to form rosettes with SRBC (Greaves and Moller, 1970; Schlesinger, 1970) although characteristically these lymphocytes bind fewer red cells than do B-lymphocytes (Elliot and Haskill, 1973). T cells can be inhibited from forming rosettes with SRBC by treating them with anti-Fab antisera. This result indicates that most rosette-forming cells in the normal mouse have L-chain determinants on their surface. After immunization with SRBC both L and μ chain determinants can be detected on rosette-forming T-lymphocytes. Neither γ nor α determinants could be detected by these inhibition studies (Greaves and Hogg, 1971). Wybran, Carr and Fudenberg (1972) proposed that rosette-forming cells in humans were lymphocytes derived from the thymus. They found that up to 65% of cells obtained from the thymuses of two 15 to 16 week human foetuses formed rosettes with SRBC. Rosette-forming cells were first found in the thymus and blood of foetuses of 11 weeks gestation, in the spleen of foetuses at 13 weeks gestation and in the liver of foetuses at 17 weeks gestation. The extent to which rosette-forming cells could be inhibited by anti-μ chain antiserum increased from 11 weeks gestation to 19 weeks

gestation, when rosette formation could be completely inhibited by the antiserum. From 12 to 19 weeks gestation rosette-forming cells from the thymus could be inhibited by anti-γ chain antiserum. On the contrary, Whittingham and Mackay (1973) have published results which showed that thymus cells from human foetuses which form rosettes could not be inhibited by anti-immunoglobulin sera. The percentage of thymus cells from human foetuses which formed rosettes in their experiments ranged from 0.8 to 22% of cells.

Thymus cells from human foetuses have been shown to bind antigens and it has been presumed that they do this through antigen receptors on their surface. Dwyer and Mackay (1972) found that thymic cells from a 12 week old human foetus specifically bound flagellin. The proportion of cells binding flagellin reached a maximum of about 3% in foetuses between 16 and 17 weeks gestation, decreasing to 0.7% in foetuses at 30 weeks gestation. Ninety per cent or more of these antigen-binding thymus cells were inhibited by polyvalent anti-human immunoglobulin serum. Hayward and Soothill (1972) similarly found β-galactosidase-binding cells in the thymuses of 13 week old foetuses, although the percentage decreased from about 1% in foetuses at 13 weeks gestation to 0.05% in foetuses at 20 weeks gestation. These binding cells could be inhibited by anti-L chain serum but not anti-μ serum. Cells capable of binding flagellin did not appear in the spleens of foetuses until 16 weeks gestation (Dwyer and Mackay, 1972) and the proportion of these cells increased to 2% at 30 weeks gestation. Only about 75% of these binding cells could be inhibited by anti-immunoglobulin serum.

The function of surface immunoglobulin on T cells, if it does exist, cannot be inferred from treatment of neonates with anti-IgM or anti-IgG sera, since neither affects the subsequent ability of treated

mice to reject skin grafts (Manning and Jutila, 1972), confirming the earlier observation made in chickens (Kincade *et al*, 1970). Anti-L chain sera however, have been shown to inhibit the GVH activity of T cells from mice (Mason and Warner, 1970) and chickens (Rouse and Warner, 1972).

There is no doubt that in certain mammalian species, T cell function as manifest by graft rejection develops *in utero* (Silverstein *et al*, 1964; Binns, 1967; Dennis *et al*, 1969; Niederhuber *et al*, 1971) although the rejection process may be slower in foetuses than in adults (Dennis *et al*, 1969). Delayed type hypersensitivity has also been induced in premature infants by DNFB (Uhr *et al*, 1960). One infant which gave a positive reaction was estimated to be of only 30 weeks gestation when sensitized.

The maturation of T cell function has also been examined *in vitro* using various assays and tests which purport to reflect the immunological competence of T cells. One such *in vitro* test is the ability of lymphocytes from foetuses and neonates to respond to phytohaemagglutinin (PHA) by undergoing blast transformation and incorporating radioactively labelled nucleotides. In humans this phenomenon can be demonstrated to occur in thymocytes from foetuses between 12 and 16 weeks gestation (Pegrum, Ready and Thompson, 1968; Kay, Doe and Hockley, 1970; Papiernik, 1970; Carr, Stites and Fundenberg, 1973). PHA responsiveness appeared in thymus cells before it could be demonstrated in cells from the spleen or the blood, while bone marrow cells did not respond at all (Carr *et al*, 1973).

Thymocytes from foetal pigs obtained between 72 and 90 days gestation gave a greater response to PHA *in vitro* (as measured by ^{3}H-thymidine incorporation) than did spleen cells from foetuses of the same age. After 90 days gestation spleen cells responded as well as

thymocytes (Rodey, Day, Holmes-Gray, Good, 1972). The intensity of the response of thymocytes to PHA did not change much as the foetal pig developed and persisted at about the same level into adult life (Weber, 1966; 1967). Thymocytes from mature pigs differ from thymocytes from adult humans for these are unresponsive to PHA (Winkelstein and Craddock, 1967; Claman and Brunstetter, 1968; Rodey and Good, 1969).

Lymphocytes which are transformed by PHA are considered to be derived from the thymus (Lischner, Punnett and Di George, 1967; Davies, Festenstein, Leuchars, Wallis and Doenhoff, 1968; Greaves, Roitt and Rose, 1968). Although responsiveness to PHA has been taken as a measure of "cell-mediated" immunity (Solomon, 1970; Stites, Wybran, Carr and Fudenberg, 1972), responding cells need not be immunologically competent (Coifman, Good and Meuwissen, 1971) and it has recently been recognized that B-lymphocytes can respond to PHA in the presence of a minor population of normal or irradiated T-lymphocytes (Bullock, 1974).

Human lymphocytes obtained from cord blood at term, when incubated with ^{51}Cr labelled CRBC in the presence of PHA were found to lyse the labelled cells (Carr, Lieber and Fudenberg, 1970), as did thymocytes from human foetuses at 16 weeks gestation (Hayward and Soothill, 1972). The physiological significance of these results is difficult to perceive. Lymphocytes from the spleen of a 13 week old human foetus gave local GVH reactions when injected under the capsules of rat kidneys. By 18 to 23 weeks gestation the reactivity of such human foetal cells was similar to cells from adult humans. Thymocytes from foetuses were less reactive than spleen cells in this assay (Asantila, Sorvari, Hirvonen and Toivanen, 1973).

In vitro reactivity to allogeneic lymphocytes in the one-way mixed lymphocyte reaction (MLR) has also been considered to specify a function of thymus-derived cells (Meuwissen, Van Alten, Bach and Good,

1968) and appears to correlate with "cell-mediated" immune competence *in vivo* (Oppenheim, 1968; Daguillard and Richter, 1969). Liver cells from a 10 week old human foetus have been reported to respond *in vitro* to mitomycin-treated allogeneic lymphocytes from adults (Carr *et al*, 1973). Thymocytes from a foetus of 12 weeks gestation were also capable of giving this response (Hayward and Soothill, 1972). Cells responsive in a one way MLR have been found in the spleen and blood of foetuses, but never in the bone marrow (Carr *et al*, 1973). While liver cells from a human foetus responded in a MLR *in vitro*, they did not respond to PHA; this may indicate that a MLR responsive, PHA non-responsive population of cells develops in the liver during gestation. The level of reactivity of cells from the thymus of foetuses between 12 and 26 weeks gestation in MLR assays remained fairly constant (Hayward and Soothill, 1972).

In the mouse, GVH reactivity as tested by the Simonsen spleen weight assay, first appeared in thymocytes from newborn to 3 day old mice (Cohen *et al*, 1963), then in cells from the spleen of 6 to 8 day old mice (Bortin, Rimm and Saltzstein, 1969). Cells from the bone marrow of newborn or adult mice (Bortin *et al*, 1969) or liver haematopoietic cells (Bortin and Saltzstein, 1968) did not respond in this assay.

Without going into the concept that functional maturation of both T and B cells may be regulated in foetal animals and newborns by "suppressor" T cells (Gershon, 1974; Mosier and Johnson, 1975) it seems that B cells become functional in the mouse (Spear, Wang, Rutishauser and Edelman, 1973; Gelfand *et al*, 1974; Owen *et al*, 1974) before T cells (Spear and Edelman, 1974), if the assessment is made from *in vitro* tests. The proposal leading from this is that subsequent developments in immune function reflect the maturation of T cell function. There

are, however, several other ways in which the immune response may undergo maturation without implicating any involvement of T cells and as yet there is no experimental evidence to prove that thymus-derived lymphocytes regulate the development of immune responsiveness in foetal animals.

(d) Hormones and the Development of Immunological Competence

(i) Rodents

The parallel development of the immune and endocrine systems in newborn and young rodents (Pierpaoli, Fabris and Sorkin, 1970) suggests that there may be some cause and effect relationship in the development of the two systems. A single injection of anti-whole pituitary serum in young mice caused thymic atrophy and a syndrome similar to the wasting disease seen after thymectomy (Pierpaoli and Sorkin, 1967); the antiserum had antibodies against the growth hormone producing cells in the anterior pituitary (Pierpaoli and Sorkin, 1969).

Hereditary pituitary dwarf mice (Snell-Bagg mice) have poorly developed thymuses and peripheral lymphoid tissues (Baroni, 1967; Baroni, Fabris and Bertoli, 1967) and these can be restored to normal by treatment with somatotrophic hormone and thyroxine (Pierpaoli, Baroni, Fabris and Sorkin, 1969). The delayed antibody response to SRBC and delayed rejection of allografts in Snell-Bagg mice could also be restored to normal by hormone treatment. These experiments suggested that somatotrophic hormone affects thymic growth and function (Pierpaoli *et al*, 1970). In mice somatotrophic hormone and thyroxine potentiate the GVH reactivity of thymus cells from newborn or adult mice or of spleen cells from adult mice. If the recipients were thymectomized the effect of the hormone treatment was not reduced. This indicated that the hormones may have a direct effect on the donor thymocytes.

Somatotrophic hormone may act directly on the thymus or may

mediate the release of other factors from the thymus, such as thymosin (Trainin, 1974). The immune responsiveness of thymectomized mice can be largely restored by implanting into them cell-impermeable diffusion chambers which contain thymus tissue (Osoba and Miller, 1963), or by injecting a cell-free, lymphocytopoietic factor extracted from the thymus of calves (Trainin and Linker - Israeli, 1967; Goldstein, Asanuma, Battisto, Hardy, Quint and White, 1970). The failure of thymectomized mice to reject allografts can be prevented by thymosin, although the defective PFC and haemagglutination antibody responses to SRBC can only be restored partially by thymosin.

(ii) Foetal sheep

Growth hormone and thyroxine, both implicated in the development of the immune system, have been found in the circulation of foetal sheep at 50 days gestation (Bassett, Thorburn and Wallace, 1970; Thorburn and Hopkins, 1972). The level of thyroxine in the circulation increases from 50 to 95 days gestation and after this time the concentration remains relatively steady until just prior to parturition. Thyroidectomy retards the growth and maturation of foetal sheep (Hopkins and Thorburn, 1972) and if performed at 81 days gestation, prevents secondary wool fibre growth which would normally appear around 86 days gestation (Hardy and Lyne, 1956). Growth hormone appears in the pituitary of foetal sheep between 43 and 53 days gestation (Stokes and Boda, 1968). The concentration of growth hormone in foetal plasma is 50 to 59 ng/ml at 100 to 110 days gestation, reaches 110 to 120 ng/ml at 130 to 140 days gestation and then falls to approximately 5 ng/ml after birth.

Pancreatic islets with β-cell granulation and insulin have been detected in foetal sheep at 42 days gestation (Alexander, Britton, Cohen, Nixon and Parker, 1968) although the concentration of insulin in the

plasma was unrelated to the age of the foetus and did not change much during gestation (Alexander *et al*, 1968; Basset and Thorburn, 1971). Adrenaline and noradrenaline were detected in the plasma of foetal sheep at 80 days gestation. The concentration of adrenaline and noradrenaline rose throughout the remainder of gestation, increasing sharply near term (Comline and Silver, 1961), as did the concentration of corticosteroids (Basset and Thorburn, 1969). To date, however, there has been no direct evidence which relates the appearance of these various hormones to the development of the immune mechanisms of foetal sheep, as such experiments have not been done.

Within the limits of this introduction it has not been possible to discuss in any detail the effect on the outcome of an immune response brought about by the nature and dose of antigen or its route of administration. The role of enzymes in the degradation of antigens (Levine and Benacerraf, 1964), the significance of a fully functional complement system in the opsonization of antigens (Rice and Silverstein, 1964; Dlabac *et al*, 1970; Adinolfi, 1972; Geiger, Day and Good, 1972) or the part played by regulatory proteins in the development of the foetal immune system has not been mentioned. The multitude of factors other than macrophages, T cells, B cells and opsonins, which are inevitably involved in determining whether or not a foetal animal gives a detectable immune response, highlights the difficulty of implicating any single factor in the failure of a particular antigen to induce an immune response.

It is correct to say from the available evidence that immune responses to various antigens become detectable at different stages of foetal development (Silverstein and Prendergast, 1970; 1971). The reason why this happens may depend on the late maturation of any one of the factors which we know are involved in an immune response, or even

some other factors as yet undescribed. Since the reported sequential appearance of various aspects of immune responsiveness cannot be related directly to the activity of the lymphocytes which carry the genetic information for antibody formation or for specific "cell-mediated" immunity, the data cannot be interpreted to have any bearing on either the germ-line (Dreyer and Bennett, 1965; Smith, Hood and Fitch, 1971; Garver and Hilschmann, 1972), somatic mutation (Cohn, 1971; Jerne, 1971) or somatic recombination (Edelman and Gally, 1967; Gally and Edelman, 1970) theories for the generation of diversity.

The importance of studying the ontogeny of immunity relates in part to the possible application of such knowledge to the problem of treating immunodeficiency diseases, although a much wider application could be envisaged for any data that expanded our understanding of the factors which control, either by stimulation or suppression, the development of immune function. Certain experimental models, and the foetal sheep is one of these, present an opportunity of studying virgin immune responses as they develop, together with the factors which regulate the magnitude, character and duration of the response. An understanding of such factors has an obvious application to transplantation and tumour immunology as well as to immunization and vaccination.

The sheep is one of the most convenient animals in which to study the *in utero* development of immunological competence as the foetus develops in an essentially antigen-free, germ-free environment, in the absence of maternal immunoglobulin (Sterzl and Silverstein, 1967; Brambell, 1970; Solomon, 1971). The foetal sheep is amenable to surgical manipulation for almost the last two-thirds of gestation and does not present the same logistic problems as do larger animals.

This thesis describes experiments undertaken to investigate the development of the antibody response in the foetal sheep. The

experimental results represent the phenotypic outcome of antigenic challenge of the foetus as measured by the production of humoral antibody. The magnitude, character and duration of these responses has been recorded, together with the histological changes observed in the lymphoid tissues as a result of antigenic experience. Some of the factors which may influence the development of immunocompetence in the foetus have also been investigated.

CHAPTER 2

MATERIALS AND METHODS

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(a) Experimental Animals

Merino and Merino-Border Leicester cross, two to four year old, virgin ewes were mated with Merino, Border Leicester or Dorset Horn rams. To ensure accurate recording of conception dates, each ram was fitted with a Sire Sine harness and crayon (Hortico Ltd., Port Melbourne, Victoria). The ewes were maintained on pasture until 1 to 2 weeks before surgery when they were brought indoors and fed lucerne-chaff and a supplement of grain oats.

Outbred White Leghorn hens were used as a source of red blood cells.

(b) Antibiotics

(i) Crystapen (Glaxo, Australia) - Crystalline sodium penicillin G (5×10^6 units/3 gram).

(ii) Bicillin (Wyeth) - Aqueous suspension of benzathine penicillin G (600,000 units), procaine penicillin G (300,000 units) and potassium penicillin G (300,000 units) - injected intramuscularly (i.m.) into ewes 24 hours after surgery.

(iii) Streptopen (Glaxovet) - Aqueous suspension of procaine penicillin G (250,000 units/ml) and dihydrostreptomycin (250 mg/ml) - 5 ml injected i.m. daily when required.

(c) Anticoagulants

(i) Heparin B.P. (Evans Medical) - 5,000 U/5 ml ampoule, without preservative. The heparin was diluted 1:4 in sterile 0.9% NaCl (saline).

(ii) Alsever's Solution - Glucose, 20.5 g; sodium citrate, 8 g; citric acid, 0.8 g; sodium chloride, 4.2 g made up to 1 litre with distilled water, pH adjusted to 6.1 with 10% ($^{w}/v$) citric acid and autoclaved.

(d) Antigens

(i) Chicken Red Blood Cells (CRBC)

Blood was withdrawn from the wing vein in a sterile manner, into an equal volume of Alsever's solution. The blood was washed three times with sterile saline and the cells resuspended to a concentration of 5×10^{9} cells/ml.

(ii) Polymeric Flagellin (POL)

Polymeric flagellin was prepared from *Salmonella typhimurium* Strain SL 870, according to the method of Ada, Nossal, Pye and Abbot (1964). Professor Ada (Department of Microbiology, JCSMR) kindly provided the polymeric flagellin used in these experiments. POL was sterilized by ultra-violet irradiation for 30 minutes.

(iii) Monomeric Flagellin (MON)

Monomeric flagellin was prepared by the acid hydrolysis of POL. One tenth of a ml of 1M HCl was added to 10 mg of POL (0.6 ml) and the mixture stood at room temperature for 30 minutes, before 0.1 ml of 1M NaOH was added to restore the pH to 7.0. To prevent the monomer from repolymerizing (Parish and Stanley, 1972), the solution was treated with 0.003 M chloramin T (Fluka A.G., Buchs S.G., Switzerland) for 15 minutes at room temperature. The chloramin T was inactivated by 0.01 M potassium metabisulphite (Analar - BDH).

The monomer was purified by centrifugation in a sucrose density gradient (10% to 30%) on a Spinco Model L at 58,000 g for 15 hours. Fractions were collected, their optical density read and the peak samples pooled, dialysed against phosphate-buffered saline (PBS) for 6

hours at 4°C and diluted to 1 mg/ml. The solution was sterilized by filtration through a 0.22 μ Millipore filter (Millipore Filter Corp., USA).

(iv) *Salmonella typhimurium* - whole organisms

Whole *S. typhimurium* (SL 870) organisms were prepared essentially by the method described by Campbell, Garvey, Cremer and Sussdorf (1970). One litre of nutrient broth, dispensed in 4 Erlenmeyer flasks, was seeded with an overnight nutrient broth culture and incubated at 37°C for 18 hours in a shaking water bath. After incubation the flasks were boiled for 2.5 hours and the cells centrifuged down at 2,000 g for 30 minutes. The cells were resuspended in sterile 0.3% ($^{v}/v$) formal-saline and recentrifuged. The concentration of the resuspended cells was estimated using Brown's opacity tubes (Burroughs Wellcome and Co.) and the suspension diluted in formal-saline to 1×10^{10} organisms/ml. The cells were reboiled for 2 hours, dispensed into 20 ml sterile McCartney bottles and stored at -20°C. Portions were tested for sterility by incubation in nutrient broth at 37°C for 2 days, the broth then being plated onto nutrient agar and horse-blood agar for 24 hours at 37°C.

(v) *Salmonella typhimurium* - Lipopolysaccharide (LPS)

Lipopolysaccharide was prepared by a modification of the method described by Halliday and Webb (1965). A 4 hour culture of *S. typhimurium* (SL 870) was used to innoculate 12 Roux bottles of nutrient agar which were incubated at 37°C for 18 hours. The cells were harvested into saline, centrifuged and washed 3 times with saline. The cells were diluted to 1×10^{10} organisms/ml (compared with Brown's opacity tubes). An equal volume of 0.04M NaOH in saline was added, causing rapid lysis of the bacterial cells and an increase in viscosity. The suspension was incubated at 37°C for 24 hours. The pH was adjusted

to 7.0 with 1M HCl and the suspension centrifuged for 20 minutes. One half volume of acetone (Univar, Ajax Chem., Aust.) was added to the clear supernatant which had been collected and the solution stored at 4°C for 18 hours. The precipitate which formed was removed by centrifugation and a further half volume of acetone added to the supernatant (a final concentration by volume of 50% acetone). Again the solution was stored at 4°C for 18 hours and then centrifuged. The precipitate was collected, dissolved in 50 ml of saline and reprecipitated with 50 ml of acetone at 4°C for 18 hours. The solution was again centrifuged and the precipitate was collected, dissolved in a small volume of distilled water, lyophilized and stored under nitrogen in small bottles at room temperature. A solution of LPS was prepared by dissolving the precipitate in saline to give a final concentration of 1 mg/ml. The solution was autoclaved and stored at -20°C.

(vi) Ferritin (FER)

Cadmium-free ferritin was prepared from horse spleens by the ammonium sulphate precipitation method of van Heyningen and was kindly provided by Dr. Gutta Schoefl (Department of Experimental Pathology, JCSMR). Ferritin was dissolved in saline to a concentration of 10 mg/ml; the solution being sterilized by filtration through a 0.22μ Millipore filter.

(vii) Ovalbumin (OA)

Crystallized, lyophilized, salt-free ovalbumin (Grade V) was obtained commercially (Sigma Chem. Co.). The OA was approximately 99% pure as judged by electrophoresis and was dissolved in saline to a concentration of 10 mg/ml; the solution was sterilized by filtration through a 0.22μ Millipore filter.

(viii) Chicken Gamma Globulin (CγG)

Normal chicken serum (CSL, Melbourne) was precipitated with

33% saturated $(NH_4)_2SO_4$ twice. The precipitate was redissolved in water between precipitations. The second precipitate was washed twice with 50% saturated $(NH_4)_2SO_4$ before being dissolved in saline and dialysed against several changes of saline over 24 hours at 4°C. Any precipitate which formed was removed by centrifugation. The optical density (OD) was measured at 280 mμ, and the solution diluted to 10 mg/ml ($E_{280\ m\mu}$ = 14.0). The CγG was sterilized by filtration through a 0.22 μ Millipore filter.

(e) Antisera

(i) Rabbit anti-whole-sheep-serum

Three rabbits were injected twice subcutaneously (s.c.) at an interval of 4 weeks with 0.25 ml of sheep serum emulsified in 0.25 ml of Freund's complete adjuvant (FCA - Difco). Two and 4 weeks later the rabbits were injected i.m. with 1 ml of whole serum. The animals were bled 1 week after each injection and the antisera tested against sheep serum by immunoelectrophoresis. Two rabbits were selected and subsequently received 1.0 ml of serum i.m. at 6 monthly intervals. After 18 months the rabbits were injected s.c. with 1 mg of IgM in 0.5 ml of FCA and the animals bled 1 week later. The anti-IgM component of the antisera from these rabbits was enhanced and the antisera compared favourably with commercially obtained antisera (Behringwerke A.G.).

(ii) Monospecific rabbit anti-sheep immunoglobulin sera

Monospecific anti-IgG_1, anti-IgG_2, anti-IgM and anti-IgA were prepared as described by Brandon, Watson and Lascelles (1971). The antisera were provided by Dr. M.R. Brandon (Department of Experimental Pathology, JCSMR).

(iii) Rabbit anti-whole-chicken serum

A rabbit was injected twice with 0.25 ml of normal chicken serum in 0.25 ml of FCA given s.c. at an interval of 2 weeks. The

antiserum obtained 1 week later was tested against chicken serum by immunoelectrophoresis. The rabbit was subsequently boosted 6 months later with 0.5 ml of chicken serum in 0.5 ml of FCA and bled 1 week later.

(iv) Chicken anti-sheep red blood cells (SRBC)

An adult hen was injected intravenously (i.v.) with 5×10^9 sterile washed SRBC. Two injections were given, 2 weeks apart and the hen bled out 1 week after the second injection.

(v) Chicken gamma-globulin anti-SRBC

Fifteen ml of chicken anti-SRBC serum was precipitated with saturated $(NH_4)_2SO_4$ solution, as described previously. The globulin solution was dialysed against 0.05M phosphate buffer pH 8.0 for 24 hours and loaded onto a 2.5 cm x 20 cm DEAE cellulose column and eluted with 0.05M phosphate buffer. Eluates with 0.10M, 0.15M and 0.20M buffer were collected and the peak from each (as assessed by OD measurements at 280 mμ) was concentrated by Diaflo filtration (Amicon Corp., USA) using an XM 100A membrane. Each fraction was titrated against 1% ($^V/_v$) packed SRBC and the most active fractions pooled (the 0.10M and 0.15M eluates), dispensed in small portions and stored at -20°C. The original serum had a titre of $\log_2$ 10.0, whilst the purified CγG anti-SRBC (22 ml, 1.8 mg/ml) gave a titre of 5.0 (equivalent to a titre of 7.3 if compared on an equal volume basis with original serum). The final product appeared pure by immunoelectrophoresis when developed with rabbit anti-whole-chicken serum.

(f) Haemagglutinating Antibody Assays

All antibody assays were performed in Cook Engineering Co. (Alexandria, Virginia) Microtitre V bottomed trays, except the direct anti-CRBC haemagglutination assay which was performed in U bottomed trays. Serial 2 fold dilutions were made with 25 μl Microtitre diluters and

25 µl Microtitre droppers.

All antibody titres have been expressed as $\log_2$ of the reciprocal of that dilution of serum which showed partial agglutination.

(i) Anti-CRBC Antibody

A 0.5% ($^{v}/v$) suspension of washed CRBC was added to serial dilutions of sera in 0.25% ($^{w}/v$) gelatin - saline solution, mixed and incubated at 37°C for 1 hour.

(ii) Anti-*Salmonella typhimurium* - whole organisms and LPS antibody

Fresh sheep red blood cells (SRBC), collected in Alsever's solution were washed 3 times with saline and resuspended to 5% ($^{v}/v$). An equal volume of LPS (100 µg/ml) was added and the mixture incubated at 37°C for 2 hours with occasional shaking. The minimal concentration of LPS which ensured complete sensitization of the SRBC was determined by testing a standard antiserum against cells sensitized with different concentrations of LPS. After incubation the cells were washed 3 times with saline and resuspended to a final concentration of 1% ($^{v}/v$). Serial dilutions of sera were made in 1% ($^{v}/v$) heat-inactivated foetal calf serum (FCS: CSL, Melbourne) - saline solution, an equal volume of sensitized cells added to each well and the plates incubated at 37°C for 2 hours.

(iii) Anti-MON and anti-POL antibody

The $CrCl_3$ method described by Langman (1972) was employed to couple POL to SRBC. One hundred µl of POL (1 mg/ml in saline) and 100 µl of 0.1% ($^{v}/v$) $CrCl_3$ (Univar, Ajax Chem., Aust.) were added to 2 ml of a 10% ($^{v}/v$) suspension of washed SRBC, which had been warmed to 30°C. The mixture was incubated at 25°C for 30 minutes without any further mixing. After incubation 8 ml of Alsever's solution was added to each tube. The tubes were centrifuged for 5 minutes at 1,000 g, the supernatant discarded and the cells washed with 10 ml of 0.25% gelatin

- saline, centrifuged and washed with 10 ml of saline, before finally being centrifuged and resuspended to 1% (^{v}/v) in saline. An equal volume of sensitized cells was added to each well containing serial dilutions of sera in saline and the plates mixed and stored at 4°C for 2 to 4 hours.

(iv) Anti-OA and anti-FER antibody

OA and FER were coupled to washed SRBC with $CrCl_3$ by the technique described by Poston (1974). One hundred µl of a 0.15% solution of $CrCl_3$ was added to 100 µl of SRBC which had been suspended in 600 µl of piperazine buffer (pH 6.5) containing 100 µl of antigen solution. The optimal concentration of FER for coupling was 1 mg/ml and for OA was 2 mg/ml. The mixture was agitated and kept at 25°C for 5 minutes. The reaction was stopped with 30 ml of saline and the cells washed 3 times in saline by centrifugation, resuspended to 1% (^{v}/v) in saline and stored at 4°C.

Serial dilutions of sera were made in 0.25% gelatine-saline and an equal volume of sensitized cells added to each well, mixed and stored at 4°C for 2 to 4 hours.

During the course of these studies both the tannic acid and bisdiazobenzidine methods of coupling OA and FER to SRBC, described by Campbell *et al* (1970), were examined, but both proved much less sensitive and reproducible than the above technique.

(v) Anti-CγG antibody

Antibodies to CγG were detected by a modification of the method described by Miller and Warner (1971). SRBC were coated with CγG by incubating an equal volume of 1% SRBC with a 1:100 dilution of CγG anti-SRBC (a 1:3 dilution of the minimum agglutinating concentration). The cells were incubated at 37°C for 1 hour, washed 3 times with saline and resuspended to 1% (^{v}/v). Sensitized cells were added to wells

containing serial dilutions of sera in 1% (^{v}/v) heat-inactivated FCS - saline. Plates were incubated at 37°C for 1 to 2 hours.

(vi) Anti-Dinitrophenyl (DNP) antibody

SRBC were coated with trinitrophenyl (TNP) as described by Rittenberg and Pratt (1969) to assay anti-DNP antibody which cross reacts with TNP. Sixty mg of 2, 4, 6 - trinitro - benzene sulfonic acid (Sigma) was dissolved in 21 ml of cacodylate buffer (pH 6.9) and 3 ml of packed SRBC added dropwise with slow stirring. The reaction mixture was stirred for 10 minutes at 25°C and then poured into 25 ml of barbital buffer (pH 7.3). The cell suspension was centrifuged and the cells resuspended in barbital buffer containing 22 mg of glycyl-glycine. The cells were centrifuged and resuspended in barbital buffer before being washed twice in saline. An equal volume of a 1% (^{v}/v) suspension of TNP - SRBC was added to serial dilutions of foetal sera in 0.25% gelatin-saline, mixed and stored at 4°C for 2 to 4 hours.

(vii) Anti-P815 mastocytoma antibody

P815 mastocytoma cells were harvested from the peritoneal cavity of DBA/2J mice and labelled by incubating 5 x 10^{6} P815 cells with 100 µC of ^{51}Cr (Radiochemical Co., Amersham) in Eagles medium, pH 7.4 (Gibco-F15) containing 10% (^{v}/v) heat-inactivated FCS at 37°C for 30 minutes. The cells were washed by centrifugation in cold medium and resuspended to a concentration of 1 x 10^{6} cells/ml. Heat-inactivated foetal sheep sera were serially diluted in Hank's balanced salt solution (BSS: CSL, Melbourne) and an equal volume (25 µl) of ^{51}Cr-P815 cells added. The plates were incubated at 37°C for 30 minutes in a CO_2/O_2/air atmosphere before the addition of 25 µl of rabbit complement (diluted 1:3). The plates were reincubated at 37°C for 1 hour and 25 µl of 0.1M EDTA in PBS (pH 7.5) added to inactivate the complement. The plates were centrifuged and 50 µl of the supernatant removed for

counting on a Packard Gamma Scintillation Spectrometer (Model 3002). The radioactivity in the supernatant was plotted as a percentage of that released from the cells by successive freeze-thawings. The end point was that dilution of foetal serum which caused the specific release of 50% of the ^{51}Cr from the cells.

(viii) Controls

When SRBC were used in passive haemagglutination assays for LPS and CγG a 1% ($^{v}/v$) suspension of the same cells was used to test the sera for non-specific agglutinins; these were never detected in foetal sera. When cells were coated with POL, MON, OA or FER, cells treated with $CrCl_3$ in the presence of gelatin were used as control cells. Occasionally non-specific agglutinins were detected in foetal sera with these cells; this could be overcome by obtaining fresh SRBC from a different donor sheep. All cells, including the CRBC suspensions, were titrated against known standard antisera before use and diluent controls were always included for each batch of cells.

(ix) 2-Mercaptoethanol (ME) treatment of sera

The 2-ME sensitivity of sera was determined by incubating 25 µl of sera with 25 µl of 0.2 M 2-ME (Eastman Organic Chemicals) in the Microtitre trays at 37°C for 1 hour. The sera were then diluted in the appropriate diluent and the assay performed as above.

(g) Surgical Procedures

Foetal sheep were exposed by the technique described by Smeaton, Cole, Simpson-Morgan and Morris (1969) and by Cole and Morris (1971a).

(i) Intravenous cannulation

The external jugular vein, the facial vein and the lateral saphenous vein of foetuses have been cannulated. Clear vinyl cannulae (Dural Plastics, N.S.W., Aust.) were used, the diameter of the cannula

being matched against the vein being cannulated. The most commonly used sizes were SV35, (I.D. 0.50 mm: O.D. 0.90 mm), SV45 (I.D. 0.58 mm: O.D. 0.96 mm) and SV55 (I.D. 0.80 mm: O.D. 1.20 mm). Only one jugular vein was cannulated in foetal sheep up to 75 days gestation, and either an SV35 or SV45 cannula was used. From 80 to 100 days gestation 1 jugular vein (SV45 or SV55) and 1 saphenous vein (SV35) were cannulated while older foetuses were cannulated through the facial vein (SV45 or SV55). It was possible to cannulate both facial veins in late term foetuses; cannulating both jugular veins proved fatal for young foetuses.

As it was difficult to keep venous cannulae in place in young foetuses, the carotid artery was cannulated. The carotid artery was mobilized from the surrounding connective tissue and the vagus nerve and sympathetic trunk carefully dissected free. Employing this technique the number of foetuses surviving surgery was less than that achieved with jugular vein cannulations, probably due to inadvertent damage to the vagus nerve during mobilization of the artery.

The cannula was secured in the vein with two silk ligatures and anchored to the foetus with a further 3 to 4 ligatures, the distal one being secured to the ear, nostril or hock, depending on the vein being cannulated, to provide as solid a support as possible for the cannula.

Before the cannula was led through the purse-string suture used to reconstitute the foetal membranes, a short length of it was left inside the amniotic cavity to allow for foetal movement. The cannula was further anchored to the outside of the uterus before the uterus was returned to the peritoneal cavity of the ewe. The cannula was led out through a stab incision high in the abdominal wall of the ewe.

The cannula was filled with heparinized-saline (250 U/ml)

and sealed by tying a knot in the cannula. The cannula was coiled and secured with a silk ligature to the wool on the back of the ewe.

(ii) Thymectomy

The operation was that of Cole and Morris (1971a), which was a modification of the method described by Kraner (1965). The cervical thymus was removed by blunt dissection with glass probes through an incision extending from the manubrium of sternum to the epiglottis and laterally along each angle of the mandible. The thoracic thymus was approached by removing the fourth rib on the left hand side of the thorax. The thymus was removed by blunt dissection and the thoracic inlet inspected for thymic remnants, using a small spotlight to illuminate the region.

(iii) Lumbar Trunk Cannulation

The lumbar trunks of 120 day old foetuses were cannulated essentially as described by Smeaton *et al* (1969). The foetus was positioned with its left hand side uppermost and an incision made from the last rib to the coxal tuber parallel to and approximately 3 cm ventral to the spine. The musculature was separated by blunt dissection, great care being taken not to rupture the peritoneal lining. The lumbar trunk lymphatic could then be approached retroperitoneally and dissected free from the aorta. An SV35 cannula was introduced 4 to 5 mm into the lymphatic and securely anchored in place. The cannula was led out through a stab wound in the side of the foetus, anchored to the foetal skin and then brought outside the ewe as previously described. When there was more than one duct present these were tied off; on occasions the duct was found on the right hand side of the aorta.

Lymph was collected into plastic bottles containing approximately 100 U of powdered heparin (Pularin, Evans), secured to the side of the metabolism cage at a level below that of the foetus when the ewe

was lying down. This aided flow of the lymph and reduced the resistance to flow imposed by the long cannula.

After foetal surgery approximately 250 mg of Crystapen was added to the amniotic fluid before it was returned and a further 500 mg of Crystapen dusted into the peritoneal cavity of the ewe before the abdominal incision was closed. The ewes routinely received 1.2×10^6 units of penicillin (Bicillin) 24 hours after surgery.

(h) Injection of Antigens

In experiments in which several antigens were used only one antigen was injected at each site, the four limbs being the only sites injected. POL, MON and *S. typhimurium* (whole organisms and LPS) were injected i.m., usually in a volume of 0.1 ml, while the same volume of CRBC was injected s.c. FER, OA and CγG were usually emulsified in an equal volume of FCA and 0.2 ml injected s.c.

(i) Collection and Counting Foetal Blood Samples

The end of the cannula was washed with 70% ethanol-Hibitane (I.C.I.) and cut. The heparinized-saline filling the cannula was drawn into a syringe through a needle fitted tightly into the end of the cannula. A blood sample, usually 2 ml, was then withdrawn into a fresh sterile syringe and the cannula refilled with sterile heparinized-saline. The volume of the cannula had been determined previously to minimize the amount of heparin reaching the foetus.

Cell counts were performed on a Model Fn Coulter Counter (Coulter Electronics Ltd, Dunstable, England). Total leucocyte and large leucocyte counts were made on a 1:100 dilution of fresh blood in 20 ml of saline or Isoton (Coulter Electronics - N.S.W.; a modified Eagle's solution). The blood was mixed with 0.1 ml of 2% ($^{W}/v$) Saponin (BDH) and left at room temperature for 10 minutes to lyse the

erythrocytes before counting. Total erythrocyte counts were performed on blood diluted 1:40,000. The packed cell volume of the blood was recorded by centrifuging blood in Heparinized Micro-Hematocrit tubes (Clay Adams, N.J., U.S.A.) in a Readacrit centrifuge (Clay Adams) for 2 minutes. Blood smears were air-dried, fixed and stained with Leishman stain (0.15% in methanol) and differential cell counts done. Finally the remaining blood was centrifuged at 2,000 g for 10 minutes and the plasma collected and stored at -20°C.

(j) Histology

Foetal sheep were obtained by hysterotomy and the wet weight and curved crown-rump length of the foetus measured. The popliteal, prescapular and axillary lymph nodes draining the sites of the injection were excised together with the mesenteric lymph nodes, the spleen, the liver and the thymus. Excessive connective tissue was removed and the weight of the draining nodes recorded. The tissues were fixed in Carnoy's solution (ethanol:chloroform:glacial acetic acid; 6:3:1) at 4°C for 24 to 48 hours, embedded in paraffin and sections cut and stained with Haematoxylin-Eosin and Methyl Green-Pyronin.

(k) Immunochemical Analysis

(i) Immunoelectrophoresis

Samples were analysed on a Shandon electrophoresis apparatus Model 477 (Shandon Scientific Co. Ltd, London). Glass slides 2.5 cm x 7.5 cm were precoated with 0.1% agar and allowed to dry. Eight slides were loaded onto a level tray and overlayered with 1% ($^{w}/v$) agar (Oxoid-L28) in veronal buffer (LKB - Ionic Strength 0.1, pH 8.6) diluted 1:4. When set, patterns were cut in the gels with a Shandon Pattern Cutter (SAE 2580), the agar removed and 3 µl samples loaded into the wells. Full strength buffer was added to the tanks

of the electrophoresis apparatus and the circuit completed with paper wicks (Whatman 3MM) soaked in buffer. The samples were electrophoresed at a constant 200 volts for 1.25 to 1.50 hours. Approximately 1 µl of a 1% bromophenol-blue solution was added to a serum sample to mark the migration of albumin and thus determine the time of the run. After electrophoresis, the gel in the troughs was removed and 40 µl of the appropriate antisera added. The slides were developed for 24 hours at 4°C in a humidified atmosphere. The slides were washed for 24 hours in saline and distilled water, dried and stained with 0.2% ($^{w}/v$) Ponceau S (Gurr Ltd, London) in 5% ($^{w}/v$) trichloracetic acid for 2 hours at room temperature. The slides were washed in 5% ($^{v}/v$) acetic acid and distilled water, dried and photographed.

(ii) Double diffusion

Precoated 2.5 cm x 7.5 cm glass slides were covered with 3 ml of 1% electrophoresis agar in quarter strength veronal buffer. Wells were punched in the gel using a mould which had six holes arranged concentrically around and 5 mm from the central well. The volume of each well was 10 µl. The reagents were allowed to diffuse for 24 hours at 4°C in a humidified atmosphere. The slides were then dried and stained by the method described for immunoelectrophoresis.

(iii) Isoelectric focusing

Analysis of sera was performed on an LKB 2117 Multiphor apparatus (LKB-Produkter AB, Sweden). The method employed was that described in the LKB application note 75, including the recommended alterations. Five percent ($^{w}/v$) acrylamide gels containing 5% ($^{v}/v$) Ampholine in the pH ranges 3.5 to 9.5 and 5.0 to 8.5 were polymerized with riboflavin. The gels were used to analyse the IgG component of the immune responses. The gels were formed between two thin glass plates (12.5 x 12.5 cm) separated by a distance of 1.5 mm. Samples of

sera (10 to 50 µl) were loaded onto 8 mm wide Whatman 3MM paper wicks, placed at the anodal end of the plate. The voltage across the plate was slowly built up to 1,000 volts, always keeping the current below 10 mA/plate. The application wicks were removed when constant voltage was reached and the plates run for 3 hours. The gels were fixed in a sulphosalicylic acid, methanol, water solution (1:2:18) for 30 minutes at 60°C, stained with 0.11% Coomassie Brilliant Blue (Sigma) dissolved in a solution of acetic acid, ethanol and water (1:3:8) for 30 minutes at 60°C, then washed extensively in the same solution before being photographed.

(1) Quantitation of Sheep Immunoglobulins

(i) Single radial immunodiffusion technique

The technique developed by Mancini, Carbonara and Heremans (1965) was employed using monospecific antisera as described by Brandon *et al* (1971). Essentially the method involved covering level 2.5 cm x 7.5 cm glass slides with 3 ml of an agar - antiserum solution. The agar (Special Agar Noble - Difco) was at a final concentration of 1.5% in barbiturate buffer (9.0 g sodium dimethyl barbiturate, 65 ml of 0.1M HCl and 0.5 g sodium azide made up to 1 litre with distilled H_2O). The amount of antiserum added was proportional to the expected level of immunoglobulin in the sample but never exceeded 10% of the total volume. After the agar had solidified, 12 wells were punched and 5 µl samples were assayed by placing them in the wells. The four corner wells were always filled with the secondary reference standard which was a large batch of frozen or freeze dried normal sheep serum, the immunoglobulin levels of which had been accurately estimated against pure immunoglobulin primary reference standards. The reaction between the sample and the antiserum in the gel was allowed to proceed for exactly 24 hours at room

temperature in a humidified atmosphere. The slides were extensively washed before being dried and stained for 10 to 60 seconds with Amido black (6 g/litre of rinsing solution) and rinsed four times in a solution of methanol, acetic acid and distilled water (9:2:9), each rinse lasting 2 minutes.

After staining, the precipitin rings were magnified using an overhead projector and the boundaries outlined on paper of constant thickness and weight. The paper circles of the magnified precipitin rings, minus the area of the well, were weighed accurately and used to estimate the concentration in µg/ml from standard regression equations.

(ii) Estimation of specific anti-POL antibody

An immunoadsorbent was prepared from POL and CNBr-activated Sepharose 4B (Pharmacia, Sweden). One gram of gel was swollen and washed with 0.001M HCl (200 ml) for 30 minutes at room temperature to wash away the dextran and lactose used to stabilize the gel. The aqueous phase was removed by filtration and the gel mixed with 10 mg of POL dissolved in 5 ml of 0.1M $NaHCO_3$ solution containing 0.5M NaCl. The slurry was rotated gently at 4°C for 48 hours, before being loaded onto a glass filter and the uncoupled POL washed through with coupling buffer. Any remaining active groups on the Sepharose were reacted with 1M ethanolamine at pH 8.0 for 2 hours. The gel was then washed 3 times with 0.1M acetate buffer containing 1M NaCl, pH 4.0 and 0.1M borate buffer containing 1M NaCl, pH 8.0 to remove non-covalently absorbed POL. The coupling procedure had an efficiency of 20% and the eluted POL was concentrated and stored for reuse. The gel was stored at 4°C in PBS containing 0.05% sodium azide.

Sera to be adsorbed (0.4 ml) were trace labelled with ^{125}I-rat albumin (0.1 ml of a 1 mg/ml solution) and a 0.25 ml sample loaded onto a 0.1 g gel column prepared in a Pasteur pipette. The serum was

run into the column and left for 30 minutes at room temperature before being eluted with PBS. Virtually 100% of the radioactivity could be recovered from the column in the first 2 ml of eluate. Half the eluate was loaded into a Minicon macrosolute concentrator (Amicon Corp. U.S.A.) and reduced to approximately 0.1 ml. The remaining eluate was incubated at 37°C for 30 minutes with 0.1 ml (packed cell volume) of POL coupled to SRBC. After adsorption the supernatant collected by centrifugation was concentrated as described above.

The dilution factor was calculated from the radioactivity in 50 µl samples of the pre and post adsorption sera and the haemagglutination titres and immunoglobulin levels determined as already described.

(m) Separation of Serum Components

(i) DEAE Cellulose column chromatography

DEAE Cellulose (Whatman DE 22) was prepared by stirring 50 g of cellulose in 1 litre of 0.5M NaOH for 30 minutes, removing the solutes by vacuum filtration on a sintered glass funnel and washing the cellulose extensively with distilled water. The cellulose was then suspended in 1 litre of 0.3M NaH_2PO_4, stirred for 30 minutes, filtered and left overnight resuspended in the same buffer. The cellulose was then washed on a glass filter with distilled water to neutral pH before being resuspended in 0.5M NaOH for 30 minutes. The cellulose was washed until the pH of the effluent was about 9.0 and then resuspended in 1 litre of 0.01M phosphate buffer pH 7.8. Washing was continued until the pH of the effluent stabilized at 7.8. The DEAE cellulose was stored in phosphate buffer under nitrogen at 4°C.

Rabbit anti-sheep immunoglobulin sera and foetal and adult sheep sera were fractionated by DEAE cellulose chromatography using essentially the same procedures. A 5 to 10 ml sample was dialysed against 0.01M phosphate buffer pH 7.8, overnight, then loaded onto

a 2.5 cm x 20 cm cellulose column. The first peak was eluted from the column with 0.01M buffer before a gradient was established by slowly adding 0.3M phosphate buffer to the reservoir containing the starting buffer. Fractions (5 to 7 ml) were collected on a LKB Minirac 17000 and the peaks, which had been continuously monitored by a LKB Uvicord 4700 connected to an LKB Recorder, pooled and concentrated by Diaflo filtration using an XM100A membrane. The fractions were stored at -20°C.

(ii) G200 Sephadex column chromatography

G200 Sephadex (Pharmacia, Sweden) was prepared by stirring 35 g of G200 in 1 litre of 0.2M NaCl in 0.1M Tris (Sigma) buffer pH 8.0 for 2 to 3 days at room temperature. The slurry was packed into a 1.8 cm x 60 cm descending column and 3 to 4 ml of serum loaded. The fractions were eluted with the same buffer at a flow rate of approximately 12 ml/hour. Three to 4 ml fractions were collected and their $OD_{280\ m\mu}$ determined. Fractions were concentrated and stored as described above.

(iii) Rivanol separation of sheep immunoglobulins

A crude preparation of total sheep immunoglobulins was prepared by precipitation with Rivanol (2-ethoxy - 6, 9 diaminoacridine lactate-monohydrate: Koch-Light). Three hundred ml of 0.4% Rivanol (pH 7.6) was added dropwise over 1 hour to 100 ml of sheep serum, with constant stirring. The suspension was stirred for a further 1 hour, centrifuged at 5,000 g for 30 minutes and the supernatant removed.

Activated charcoal (20 g) was added to the supernatant which was stirred for 30 minutes and then centrifuged to remove the charcoal. The colourless supernatant was freeze dried and redissolved in PBS as required.

(n) Miscellaneous Methods

(i) Adsorption of Forssmann antibodies from rabbit complement

Fresh rabbit serum, to be used as a source of complement in haemolytic reactions was adsorbed with CRBC and/or P815 cells before use. One ml of packed cells was added to 5 ml of fresh rabbit serum to which 0.5 ml of 0.1M EDTA had been added. The mixture was incubated at 4°C for 60 minutes, the serum collected by centrifugation (2,000 g for 10 minutes) and 0.25 ml of 0.2M $CaCl_2$ added. Portions of the serum were stored at -20°C.

(ii) Plaque forming cell assay (PFC)

Two clean 2.5 cm x 7.5 cm glass slides were used to make PFC chambers by placing transverse strips of double coated tape (Scotch Brand No. 4010) at the ends and in the middle of one slide, pressing the second slide on top. The lymph cells were washed once with Alsever's solution and twice with Eagles medium, pH 7.4 before being resuspended to 1 x 10^6 and 1 x 10^7 cells/ml in Eagles medium containing 10% heat-inactivated FCS. The plaquing mixture was prepared by mixing 0.5 ml of cell suspension, 0.05 ml of adsorbed rabbit complement and 0.1 ml of a 20% ($^v/_v$) suspension of CRBC in Eagles medium. The mixture was run into the PFC chambers which were then sealed with warm paraffin. The chambers were incubated at 37°C for 1 hour and the plaques counted under a microscope (25 x magnification). The number of PFC/10^6 cells was calculated, knowing the calibrated volume of the PFC chambers and concentration of lymphocytes.

(iii) Anti-CRBC haemolysin antibody titration

Serial dilutions of sera were made in 0.25% gelatin - Dulbecco's PBS (containing Ca++ and Mg++) as previously described for anti-CRBC haemagglutination titrations. One drop (25 µl) of a 1:10 dilution of adsorbed rabbit complement was added to each well and the

plates incubated for 30 minutes at 37°C. One drop of CRBC (0.5% ($^{V}/v$) in Dulbecco's PBS) was then added to each well and the plates reincubated for 1 hour at 37°C.

(iv) Preparation of anti-immunoglobulin immunoadsorbent - (Avrameas and Ternynck, 1969)

Bovine serum albumin (1.6 g - Armour) and Rivanol extracted sheep immunoglobulin (400 mg) were added to 40 ml of 0.2M acetate buffer and the pH readjusted to 5.0. Eight ml of 2.5% ($^{V}/v$) glutaraldehyde was added dropwise into the stirred solution and the solution allowed to stand at room temperature for 3 hours. The gel which formed was dispersed in 50 ml of 0.1M phosphate buffer, pH 7.4, by passing it through an 18 gauge needle. The slurry was centrifuged at 5,000 g for 15 minutes, the supernatant discarded and the gel redispersed in 50 ml of phosphate buffer. The gel was washed by centrifugation until the $OD_{280m\mu}$ of the supernatant was less than 0.1 units, then dispersed in 200 ml of 0.2M HCl-glycine (pH 2.8) and stirred at room temperature for 15 minutes. After centrifugation the gel was dispersed in 40 ml of 1M $Na_2H\ PO_4$, diluted to 200 ml with distilled water and centrifuged. The gel was washed with PBS and centrifuged until the $OD_{280\ m\mu}$ of the supernatant was less than 0.05. Sodium azide (0.1%) was added to the gel which was stored in PBS at 4°C.

(v) Isolation of the specific antibody component of anti-whole sheep serum - (Avrameas and Ternynck, 1969)

Rabbit IgG anti-sheep-immunoglobulin serum, prepared from 5 to 10 ml of serum by DEAE cellulose chromotography, was added to the immunoadsorbent in a volume of 10 ml and stirred for 1 hour at room temperature; all subsequent operations were then carried out at 4°C.

The slurry was centrifuged and the supernatant kept for testing. The immunoadsorbent was washed by successive centrifugations in PBS until the $OD_{280m\mu}$ of the supernatant was less than 0.05. Fifty ml of 0.2M HCl-glycine (pH 2.8) was added to the gel which was stirred for 15 minutes, centrifuged and the supernatant collected. The supernatant was neutralized with 10 ml of 1M Na_2HPO_4. The elution from the immunoadsorbent was repeated with 0.2M HCl-glycine (pH 2.2) and the supernatant collected and neutralized. The supernatants were pooled, concentrated to 2.5 mg/ml by Diaflo filtration (PM10 membrane), dialysed against PBS overnight and stored frozen at -20°C.

(vi) Coupling of horseradish peroxidase to antibody preparations - (Avrameas and Ternynck, 1971)

An excess of glutaraldehyde (0.2 ml of a 1.25% solution of glutaraldehyde in 0.1M phosphate buffer, pH 6.8) was added to 10 mg of horseradish peroxidase (HRP - Type VI: Sigma) and left at room temperature for 18 hours. The antibody preparation (5 to 10 mg) to be conjugated with HRP was dialysed against 2 changes of 0.15M NaCl at 4°C and concentrated down to 1 ml. Excess glutaraldehyde was removed from the activated HRP by passing the solution through a G-25 Sephadex column (1cm x 60cm); the brown coloured fractions eluted with 0.15 M NaCl were pooled and concentrated to 1 ml. The activated HRP was added to the antibody solution in the presence of 0.2 ml of 0.5M bicarbonate buffer, pH 9.5. The solution was kept at 4°C for 24 hours, then dialysed against PBS at 4°C for 24 hours. The HRP labelled protein was kept at 4°C for several days before portions were frozen at -20°C.

(vii) Detection of cells having membrane bound immunoglobulin

Foetal lymphocytes were washed twice with Hank's BSS containing 2% ($^{W}/v$) BSA by centrifugation at 1,000 g for 5 minutes and

then passed through a glass wool column to remove any cell clumps. Fifty million viable cells, as assessed by trypan blue exclusion, were centrifuged to form a pellet, then gently resuspended in 8 drops of Hank's BSS + BSA and 2 drops of HRP labelled anti-whole sheep serum gamma-globulin. The cells were reacted for 1.5 hours at 4°C with occasional shaking, before being washed 4 times with Hank's BSS + BSA and centrifuged. The final pellet was resuspended in a drop of foetal calf serum and smears prepared and air dried. Control cell preparations were treated in exactly the same manner, but HRP conjugated to normal rabbit IgG replaced the antisera. The dried smears were fixed in 4% ($^{w}/v$) para-formaldehyde in 0.1M phosphate buffer for 15 minutes at room temperature, washed with 3 changes of PBS and while still wet the smears were overlayed with staining solution for 3 minutes. The slides were then washed with distilled water and dried. The staining solution was prepared by dissolving 4 mg of 3 amino-9-ethyl carbazole(Grade 2 - Sigma) in 1 ml of dimethyl formamide (BDH), then adding 19 ml of 0.05 M acetate buffer (pH 5.0) and 4 drops of 3% ($^{v}/v$) hydrogen peroxide (100 volume - Univar) just before use. The staining solution was always tested by mixing a few drops with a drop of HRP solution. If the staining solution was active a deep brown precipitate formed.

(viii) Radioiodination of proteins

Rat albumin (Fraction V - Sigma) was labelled with ^{125}I by the Olins and Edelman (1964) modification of the method of McFarlane (1963). From 0.2 to 1.0 mC of ^{125}I in dilute NaOH solution (Radiochemical Co., Amersham) was added to 100 to 500 µg of protein in 0.5 ml of 0.15M NaCl solution in 0.2M borate buffer (pH 8.0). Non-protein bound ^{125}I was removed from the rat albumin by passing it through a 7 ml Dowex AG1-X8 anion exchange column, after the column had been treated with unlabelled rat albumin diluted in borate buffer. Protein bound ^{125}I was determined by precipitation with an equal volume of 10%

(^{w}/v) trichloracetic acid and counting the radioactivity remaining in the supernatant after centrifugation.

(ix) ^{3}H-Thymidine incorporation by cells *in vitro*

A portion of foetal lymph containing 1 x 10^{8} lymphocytes was centrifuged and the cell pellet resuspended in 1 ml of autologous cell-free lymph. Ten µC of ^{3}H-thymidine (methyl ^{3}H-thymidine; Radiochemical Co., Amersham) was added to the cell suspension, which was then incubated at 37°C for 1 hour. The cell suspension was washed 3 times with Hank's BSS containing 1% heat-inactivated FCS. The cell pellet was resuspended in a drop of FCS and smeared on glass slides. The smears were fixed in methanol for 15 minutes, dipped in a 0.5% gelatin - 0.05% (^{w}/v) potassium chromium sulphate solution and dried. The slides were covered with stripping film (Kodak 10AR) and stored at 4°C in a dry, dark box for 4 to 7 days. The films were developed (Kodak - D19) and fixed before the cells were stained with 0.1% Azur A in acetate buffer (pH 5.2) for 1 minute.

CHAPTER 3

HUMORAL ANTIBODY RESPONSES IN FOETAL SHEEP

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HUMORAL ANTIBODY RESPONSES IN FOETAL SHEEP

INTRODUCTION

Silverstein and his colleagues (Silverstein *et al*, 1963a, 1963b, 1964; Silverstein and Kraner, 1965) reported over 10 years ago that foetal sheep develop the capacity to respond immunologically to different antigens over a considerable period of *in utero* life. They also claimed that the ability to respond to any one antigen is established at a closely defined stage of development. Intuitively, it would be surprising if the ability to respond to all antigens appeared at one particular stage of development. The discrete onset of competence to each antigen at different times suggests that certain components that are required for each specific response become functional at fixed stages of development.

Although the formal proof that immunological competence matures sequentially rests largely on experiments with foetal sheep, results from studies on newborn opossums (*Didelphis virginiana*) in the pouch, have been interpreted as showing a similar sequential development of immune reactivity (Rowlands, Blakeslee and Angala, 1974). The interpretation of the results obtained in the opossum was confounded, however, by the fact that maternal antibody transferred to the young in the milk could have influenced the development of the immune system. Experimental results from studies in foetal dogs (Jacoby *et al*, 1969) and foetal cattle (Schultz, 1973) also tend to support the original conclusions reached by Silverstein.

Reconstitution of lethally irradiated adult mice with syngeneic liver cells from foetal animals is also thought to provide

a model system for studying the ontogeny of immunity. Sherwin and Rowlands (1974) used this model and reported that antibodies to eight different antigens appeared sequentially in these lethally irradiated, reconstituted mice. Unfortunately the contribution of the host animal, recovering from irradiation, to the observed response was not determined.

The claims made by Silverstein (1972) and Silverstein and Prendergast (1970) that "once the young animal is able to respond at all to a given antigen, its response appears to be adult-like with respect both to heterogeneity of immunoglobulin product and to the appearance of both humoral and cellular immune components" and further that there is a "remarkable precision with which the foetus develops competence to a given antigen at a given stage of gestation" have important implications for immunological theory and practice. In view of this, experiments were designed to study the way in which the foetal sheep responds to different antigens during development.

RESULTS

The results in this Chapter were obtained by injecting foetal sheep with up to four antigens simultaneously. Each antigen was injected either subcutaneously (s.c.) or intramuscularly (i.m.) into a different limb of the animal. A cannula was inserted into the blood circulation and regular blood samples taken for as long as the cannula remained patent; the first sample was collected before antigen was injected. Great care was taken during surgery to avoid any contamination of the foetal environment with maternal blood.

(a) Antibody Response of Foetal Sheep to Chicken Red Blood Cells

Thirty-six foetal sheep from 49 to 126 days gestation were successfully challenged with CRBC. Usually 5×10^8 CRBC was injected

s.c. into each animal in a volume of 0.1 to 0.2 ml. Several of the older foetuses received 1 x 10^9 cells to see if a larger dose of antigen would increase the magnitude of the response to CRBC. The antibody responses in these foetuses were no different from those in foetuses injected with 5 x 10^8 cells. In only three of the sixteen animals challenged before 65 days gestation was it possible to obtain blood samples sequentially, therefore the results from these young foetuses have been obtained largely from blood samples taken at hysterotomy, 14 days after the injection of antigen. It is possible that a short-lived immune response had occurred in these animals before day 14 but had disappeared by the time the blood sample was taken, although no evidence of this was found in the blood samples taken sequentially from three 60 day old foetuses. In fact the three positive serum samples which were obtained from this age group were all obtained by hysterotomy. The positive sera were examined by radial immunodiffusion to preclude the possibility that the antibody detected came from the maternal circulation at hysterotomy. Only IgM was detected in the sera from these animals (Chapter 4); IgG would also have been present if the antibodies had come from the mother.

Around 70 days gestation successful cannulations were made more regularly and serial blood samples were taken for periods of up to 4 weeks. Positive sera were obtained within 2 weeks of the injection of antigen from all but one of the five animals tested. The one animal which failed to respond to the first challenge with CRBC was infused through the cannula with 1 x 10^9 CRBC and did respond subsequently. This indicated that the foetus had not been rendered tolerant by the first injection of antigen. From 80 days gestation onwards all the foetuses injected with CRBC produced specific antibody.

All antibody formed within the first 14 days of the response

by foetuses less than 80 days gestation was sensitive to incubation with 2-mercaptoethanol (ME). One animal injected at 80 days gestation initially gave a good response to CRBC and all this antibody was sensitive to 2-ME: subsequently this foetus produced small amounts of 2-ME resistant antibody at day 18 and this antibody could be detected for a further 18 days. Not until 110 days gestation however, did the foetuses consistently produce 2-ME resistant antibody within the first 14 days. In these older foetuses antibody appeared by day 4 and the first 2-ME resistant antibody by day 6. Peak titres of total antibody occurred between 6 and 10 days after the injection of antigen, while 2-ME resistant antibody did not reach a peak in many cases until 14 to 16 days after challenge.

Due to between animal variation it was difficult to conclude that the magnitude of the anti-CRBC response increased between 70 and 120 days gestation. There was a tendency for antibody to appear earlier and for the maximum titre of antibody to be higher the later in gestation the foetuses were challenged. Taking into account the five fold increase in the blood volume of foetuses during this 50 day period (Barcroft, 1946), it can be concluded that the older foetuses made more antibody molecules than the younger animals in response to a given antigenic challenge.

(b) Antibody Responses of Foetal Sheep to Flagellin

(i) Polymerized flagellin (POL)

Twenty-two foetal sheep between 49 and 120 days gestation were injected with POL. The antigen was injected i.m. in a volume of 0.1 ml. Foetuses of 80 days gestation or more were injected with 100 µg of POL: this dose killed younger foetuses. Foetuses younger than 80 days gestation were usually injected with 50 µg of POL. Two foetuses, one 50 days and one 70 days gestation, were injected with

25 µg of POL and two 70 day old foetuses received 10 µg POL. The 70 day old foetus which was injected with 25 µg of POL gave a similar response to a foetus which was injected with 50 µg of POL. The responses in the two animals which had received 10 µg of POL were delayed in onset and the titres were significantly lower.

Only one of five animals injected between 56 and 60 days gestation had detectable levels of antibody in serum collected by hysterotomy 14 days later. It is possible however, that a short-lived response may have gone undetected in 60 day old foetuses as sequential samples from a 70 day old foetus showed a peak titre at day 4 which was undetectable by day 14. After 59 days gestation all but one of 12 foetuses which were bled every second day after antigenic challenge, produced detectable antibody by day 4 and maximum titres between days 4 and 8. The remaining foetus produced antibody by day 6.

As with CRBC, foetal sheep of up to approximately 80 days gestation produced 2-ME sensitive antibody to POL, while from 100 days gestation all produced significant levels of 2-ME resistant antibody during the first 14 days of the response. Although the time at which antibody was first detected did not vary, the duration of the responses was longer in older foetuses. The mean titres of two 70 day old foetuses injected with 25 µg and 50 µg of POL respectively and four 120 day old foetal animals injected with 100 µg of POL are shown throughout the responses in Figure 3-i. In general, both the duration of the response and the production of antibody resistant to 2-ME increased throughout gestation. The implications of these results will be discussed more fully in Chapter 5.

(ii) Monomeric flagellin (MON)

The response of fifteen foetal sheep between 60 and 123 days gestation to MON was tested by injecting 100 µg of MON (1 mg/ml) i.m.

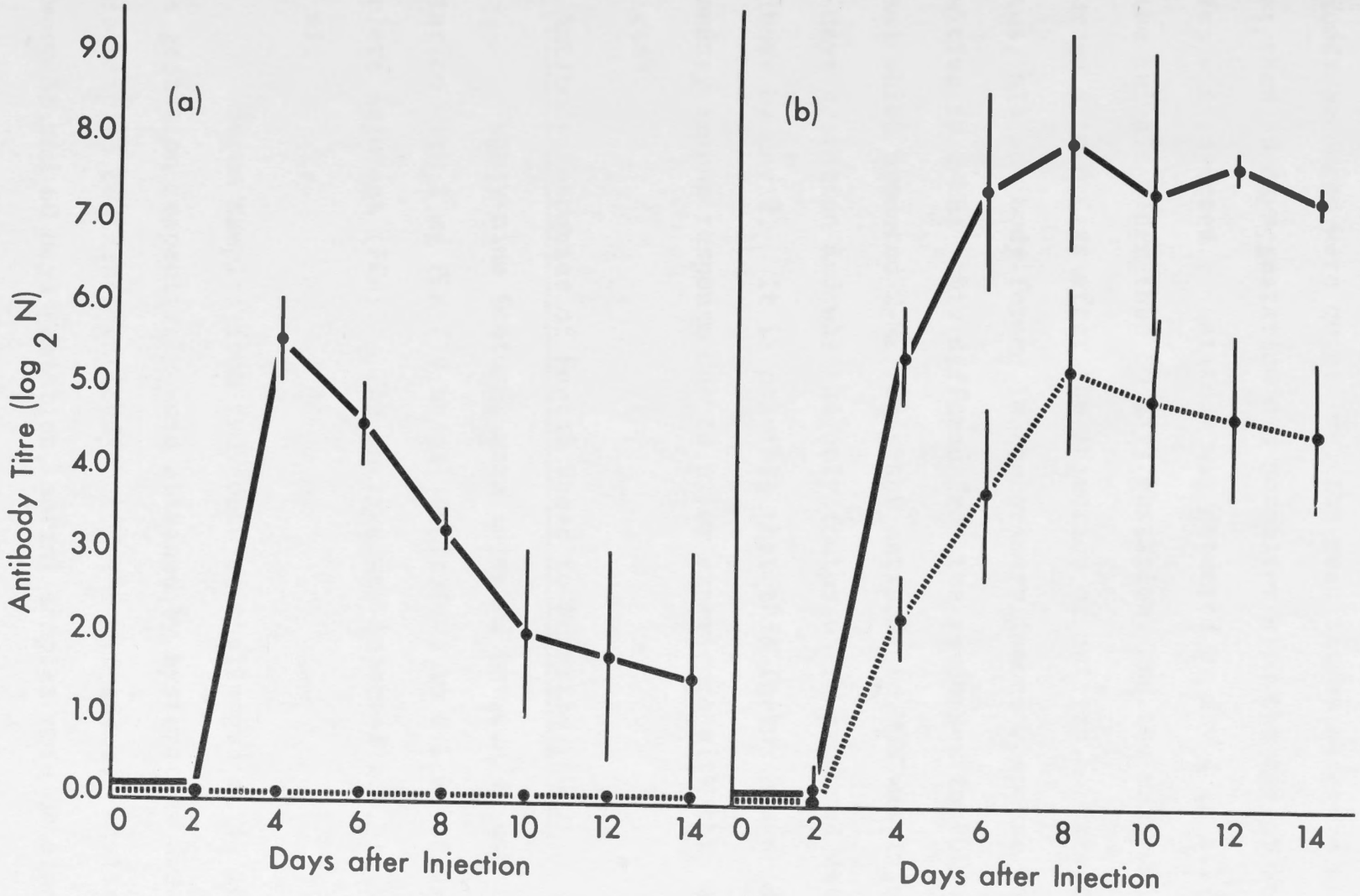


Figure 3-i: Mean haemagglutinating antibody response of (a) two 70 day old foetal sheep and (b) four 120 day old foetal sheep to POL. Total antibody (▬▬▬▬▬▬); 2-ME resistant antibody (▪▪▪▪▪▪▪▪▪▪▪▪▪▪▪▪▪▪▪▪▪▪). Standard errors indicated by vertical bars.

Although the onset of the antibody responses detected in two animals challenged at 71 and 72 days gestation was delayed and the titres of antibody produced were quite low, the mean titres observed in foetuses older than 80 days gestation did not alter with the age at which the foetus was injected. Antibody was detected by day 4 in all but one of the animals older than 80 days gestation, and the maximum titres occurred 4 to 6 days after the injection of antigen. Except for one foetus, all antibody formed in the primary immune response to MON was sensitive to 2-ME; this differed from the responses to POL. The one animal which produced 2-ME resistant antibody to MON was injected at 107 days gestation and was the only foetus which produced detectable antibody by day 2. It is possible that this foetus had produced a secondary immune response due to prior experience with this or related antigens.

(c) Antibody Responses of Foetal Sheep to Ferritin (FER)

Twenty-nine foetuses were injected between 49 and 126 days gestation with 1 mg FER (10 mg/ml) emulsified in 0.1 ml of Freund's complete adjuvant (FCA). The antigen was injected s.c. in a volume of 0.2 ml.

Serum samples from two foetuses challenged at 49 and 50 days gestation respectively were obtained by hysterotomy and one of these samples contained antibody. Of the six animals challenged between 58 and 60 days gestation, serial samples were obtained from two animals; one of these did not produce any detectable antibody for 22 days after the antigen was injected. Three positive and one negative serum samples were obtained by hysterotomy. From 70 days gestation regular blood samples were obtained from nearly all the foetuses tested. Four of six animals challenged between 69 and 70 days gestation produced detectable antibody, as did all animals challenged on or after

79 days gestation.

The majority of animals produced 2-ME sensitive antibodies during the first 14 days of the response and the titres observed in animals challenged after 100 days gestation often persisted at very high levels. Many of the older animals produced good levels of 2-ME resistant antibody 10 to 20 days after challenge. The duration of the immune response was not the only factor which influenced the production of 2-ME resistant antibody however, for three animals between 79 and 81 days gestation were followed for 26, 28 and 36 days without them ever producing 2-ME resistant antibody. One 101 day old foetus which maintained good titres of anti-FER antibodies throughout the 37 days it was followed, never produced 2-ME resistant antibody.

Again the magnitude of the anti-FER response increased the later in gestation the foetuses were challenged. In foetuses older than 70 days gestation antibody was usually detected 4 days after the injection of antigen and peak titres occurred between days 8 and 12. Two negative samples were obtained at day 6 from foetuses injected at 60 days gestation but these same animals subsequently produced antibody at day 12 and 14 respectively. This result suggested that the response to FER may be delayed in animals of this age.

The mean total antibody titres of four animals at 70 days gestation, three at 100 days gestation and three at 120 days gestation are shown in Figure 3-ii. The response appears to increase in magnitude and duration with increasing foetal age. The presence of non-responders, slow-responders or irregularities in the times at which the samples were obtained, make the construction of valid response curves difficult for other age groups.

(d) Antibody Responses of Foetal Sheep to Chicken Gamma Globulin (CγG)

Twenty foetal animals between the ages of 59 and 123 days

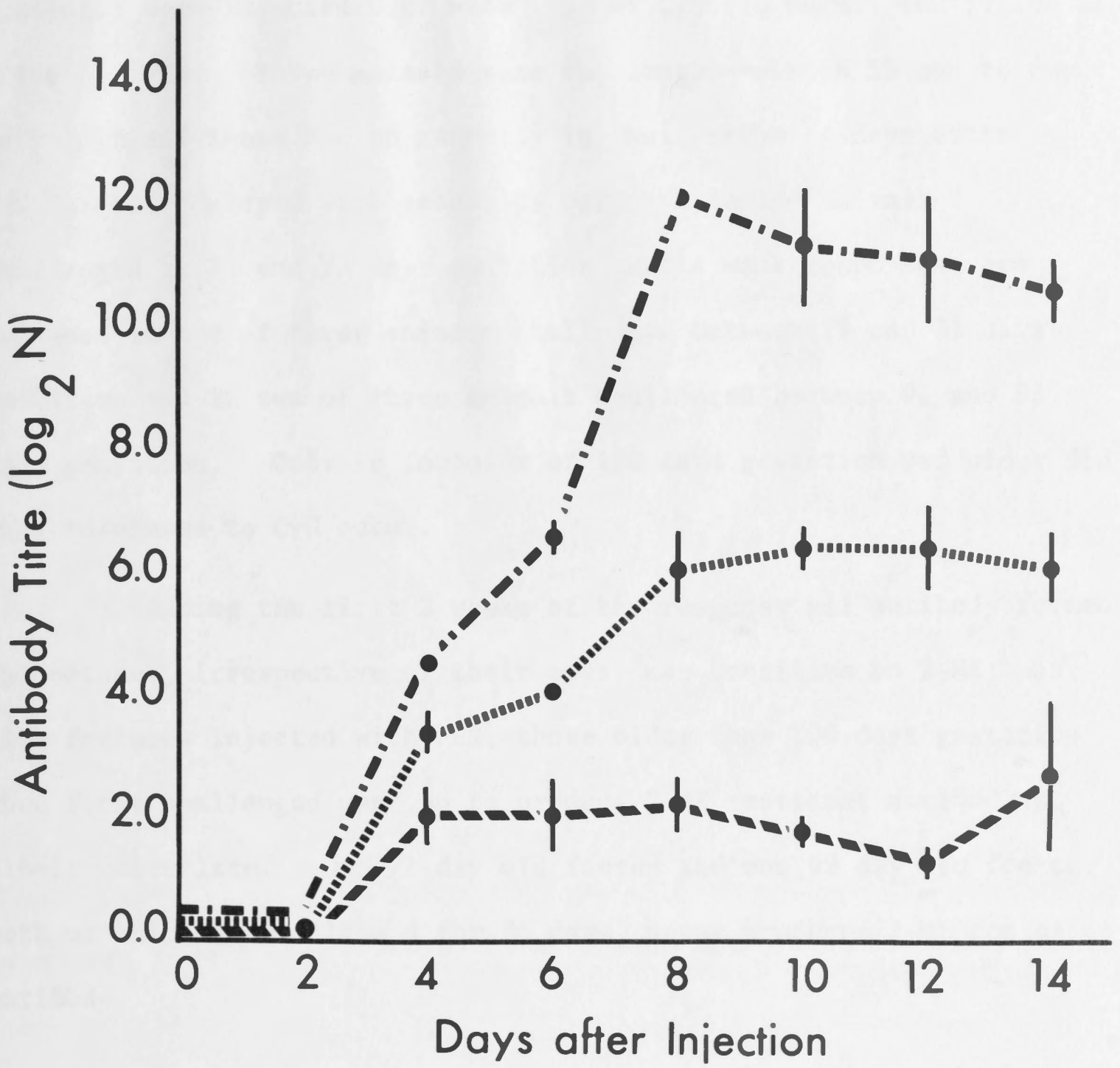


Figure 3-ii: Mean total haemagglutinating antibody responses of foetal sheep to 1 mg FER in FCA.
70 days gestation (▬▬▬▬); 100 days gestation (▮▮▮▮▮▮▮▮▮▮▮▮); 120 days gestation (▮▮▬▮▬▮▮).
Standard errors indicated by vertical bars.

gestation were injected s.c. with 1 mg of CγG (10 mg/ml) emulsified in 0.1 ml of FCA. Three animals were challenged between 59 and 60 days gestation and these had no antibody in their serum 14 days after challenge. Delayed weak responses occurred in two animals challenged at 71 and 72 days gestation, while weak responses were obtained in one of three animals challenged between 78 and 81 days gestation and in two of three animals challenged between 91 and 93 days gestation. Only in foetuses of 100 days gestation and older did good responses to CγG occur.

During the first 2 weeks of the response all antibody formed by foetuses, irrespective of their ages, was sensitive to 2-ME; as with foetuses injected with FER, those older than 100 days gestation when first challenged went on to produce 2-ME resistant antibodies, albeit quite late. One 71 day old foetus and one 92 day old foetus, both of which were followed for 36 days, never produced 2-ME resistant antibody.

As already mentioned, the responses observed in animals before 100 days gestation were quite weak. Antibody responses to CγG were stronger in foetuses challenged closer to term. In Figure 3-iii a comparison is made between the responses which occurred in three foetuses around 100 days gestation and three others around 120 days gestation. Except for one foetus antibody was never detected at day 4, while peak antibody titres often occurred on day 8, only two days after the first antibody was normally detected.

(e) The Antibody Response of Foetal Sheep to Ovalbumin (OA)

Thirty foetal sheep from 69 days to 126 days gestation were injected s.c. with 0.1 ml of OA (10 mg/ml) emulsified in 0.1 ml FCA. It was very difficult to interpret the responses observed after the injection of OA due to variations in the time that antibody first

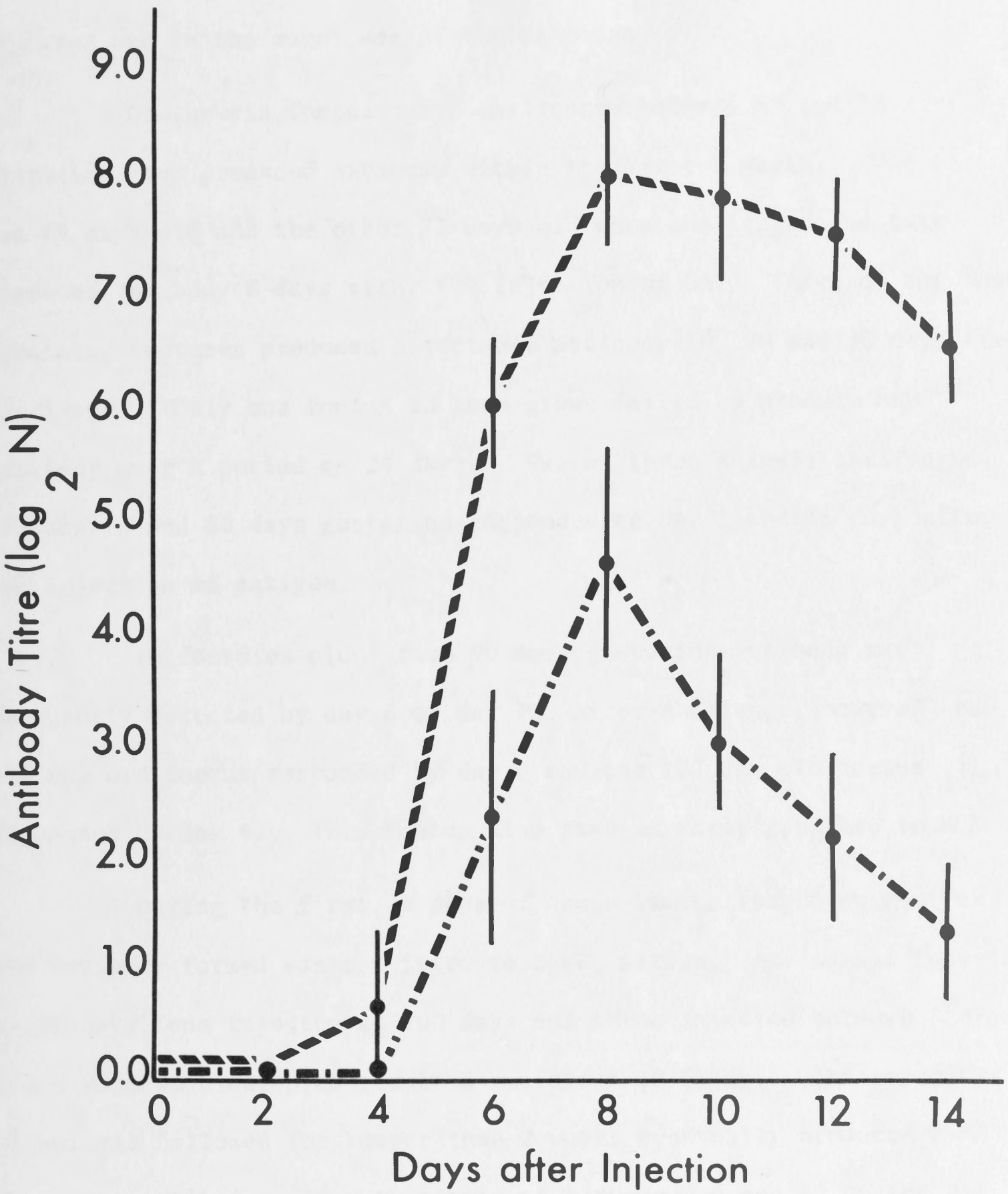


Figure 3-iii: Mean total haemagglutinating antibody response of foetal sheep to 1 mg CγG in FCA.
100 days gestation (▬ ▮ ▬ ▮ ▬); 120 days gestation (▬▬▬▬).
Standard errors indicated by vertical bars.

appeared and in the magnitude of the response.

Of the six foetal sheep challenged between 69 and 72 days gestation, two produced antibody within the first 2 weeks. One foetus was 68 days old and the other 72 days old when challenged and both produced antibody 8 days after the injection of OA. Three of the four remaining foetuses produced detectable antibody 16, 20 and 30 days after challenge. Only one foetus in this group failed to produce any antibody over a period of 24 days. Two of three animals challenged between 78 and 80 days gestation responded to OA, 8 and 16 days after the injection of antigen.

In foetuses older than 90 days gestation antibody was frequently detected by day 8 or day 10 after challenge, however one 100 day old foetus responded by day 6 and one 107 day old foetus responded by day 4. This foetus also gave an early response to MON.

During the first 14 days of these immune responses most of the antibody formed was sensitive to 2-ME, although one animal injected at 91 days, one injected at 100 days and three injected between 119 and 122 days gestation, produced 2-ME resistant antibody. The majority of animals followed for longer than 2 weeks eventually produced 2-ME resistant antibody, often to titres of between 8.0 and 12.0 ($\log_2 N$). Because of the variability in the responses, comparing mean antibody titres to OA at different stages of development was not possible. Foetuses at all ages tested gave maximum antibody titres between 3.0 and 8.0 at variable times after challenge and it was not possible to relate the character of the response to the age of gestation. Three animals injected around 100 days gestation highlight the variability encountered between foetuses; their maximum titres during the 2 weeks after the injections of OA were 3.0, 5.5 and 12.0 respectively.

(f) Antibody Responses of Foetal Sheep to Somatic "O" Antigens of *Salmonella typhimurium* SL 870

(i) Whole boiled organisms of *S. typhimurium*

Nine foetal sheep 81, 92, 93, 100, 101, 107, 108, 122 and 123 days old were injected i.m. with 1×10^9 whole boiled organisms of *S. typhimurium*. Some of these foetuses, particularly the older ones, were followed for 20 to 30 days. No animal produced any detectable haemagglutinating antibody.

(ii) Lipopolysaccharide extract of *S. typhimurium* (LPS)

Nine foetal sheep 80, 90, 100, 100, 111, 113, 119, 119 and 121 days old were injected i.m. with 100 µg of LPS (1mg/ml). Again several of these animals were sampled for over 30 days, and at no time did any animal produce detectable haemagglutinating antibody to LPS.

(g) Miscellaneous Antigens

At various times during these studies antigens other than those already described were injected into a limited number of foetuses. Although the studies on these antigens are incomplete, the following results were obtained.

(i) Dinitrophenyl (DNP) coupled to MON

Six animals aged 59, 69, 70, 78 and 100, and 119 days gestation were injected i.m. with 50 to 100 µg of MON-DNP (1mg/ml). The responses observed were very weak to the DNP hapten and totally absent for the MON carrier. The youngest foetuses which gave a response were 69 and 70 days gestation when they were injected. The response to DNP was very short-lived, being over in 4 to 6 days and the antibody titre never exceeded 3.0. The preparation of MON-DNP used was a gift from Dr. S. Kirov (Department of Microbiology, JCSMR) and was highly immunogenic in mice (Kirov, 1974).

(ii) Mastocytoma P815

Mastocytoma P815 cells harvested from the peritoneal cavity of DBA/2J mice were injected into foetuses to study their responses to another cellular antigen. Due to difficulties in obtaining sterile preparations of sufficient numbers of tumour cells, the experiments with this antigen were discontinued. However, two foetal sheep at 60 days, one at 70 days and one at 78 days gestation were injected s.c. with from 3×10^7 to 1×10^8 viable P815 cells. One of the animals injected at 60 days gestation produced detectable antibodies by day 14 and the foetus injected at 70 days gestation produced antibody by day 18. Antibody was detected by the complement mediated release of ^{51}Cr from P815 cells. The peak antibody titres were again quite low, being 3.5 and 2.5 for the 60 and 70 day old foetuses respectively.

(h) The Appearance of 2-Mercaptoethanol Resistant Antibody in Foetal Sheep

The appearance of 2-ME resistant antibody to various antigens in foetal sheep has already been described. There was a remarkable consistency in the age of the foetuses in which this type of antibody first appeared.

In Table 3-1 the results obtained from several foetuses injected with different antigens have been collated to give an estimate of the age at which foetuses first produced 2-ME resistant antibody. In these relatively young foetuses 2-ME resistant antibody did not appear until 16 to 22 days after the injection of antigen, by which time the foetuses were between 92 and 98 days gestation.

(i) The Antibody Responses of Lambs to Antigens Employed in Foetal Studies

FER, CRBC, POL, CγG, OA, *S. typhimurium* (whole organisms and LPS) were injected into 3 to 6 month old lambs at the doses used in

Table 3-1: The earliest appearance of 2-ME resistant antibody in the plasma of foetal sheep challenged with various antigens.

Age of gestation when challenged (days)	Antigen	Age when 2-ME resistant antibody was detected (days)
70	OA	92
79	POL	95
80	OA	96
80	CRBC	98

Mean 95 days

foetal experiments and all, including the two forms of the somatic antigens of *S. typhimurium* induced haemagglutinating antibody responses.

The responses of four lambs to 1 mg of FER or 1 mg of OA, both emulsified in FCA, appeared on days 4 and 7 respectively, as did the responses observed in foetal sheep. The titres of anti-FER antibody in the circulation of these lambs were similar to those detected in foetuses near term, except that most of the antibody was resistant to 2-ME. The maximum titres of anti-OA antibody were detected on day 10 and ranged from 10.0 to 14.0; after day 10 the anti-OA antibody was almost entirely resistant to 2-ME. The anti-CγG responses were also similar to those detected in foetuses near term as were the anti-POL responses detected in four lambs, except that the anti-POL antibody formed by lambs was largely resistant to 2-ME. The responses of two lambs to 5×10^8 CRBC however, were less than those detected in some late-term foetuses, which may suggest that the dose of CRBC was sub-optimal in the larger lambs. Four lambs injected i.m. with 1×10^9 *S. typhimurium* organisms all produced specific antibody by day 4 and reached maximum titres between 5.5 and 7.0 on day 7. The antibody was sensitive to 2-ME. Similarly, two lambs injected i.m. with 100 µg of LPS synthesized antibody which was first detected on day 4 and reached titres of 3.5 and 5.0 by day 7.

(j) Antibody Production in Pregnant Ewes Following Antigenic Challenge of Foetuses

The pregnant ewes were routinely bled prior to surgery, then at fortnightly intervals throughout the experiment. The sera obtained were titrated to determine the antibody levels to the antigens injected into the foetus.

The ewes in this study had significant levels of antibody before their foetuses were challenged. Anti-CRBC antibody titres

varied between 2.0 to 6.5 and anti-POL titres between 4.0 to 12.0. The ewes had only low or undetectable levels of antibody to FER (range 0.0 to 2.5) and OA (range 0.0 to 3.0). The level of antibody to these various antigens remained stable throughout the experiment, usually not increasing or decreasing by more than 1 or 2 fold. The exception to this was POL, where in about 30% of ewes tested, there was a 3 to 4 fold increase after the foetus was challenged. Due to the relatively high levels of anti-POL antibodies in adult sheep and to the variation that occurred between animals, it could not be stated that the increase observed was due to the antigen injected into the foetus, rather than to some other cause.

(k) Cross-reacting Antibodies in Foetal Sheep

Prior to antigenic challenge the foetal sheep were agammaglobulinaemic, with the exception of a proportion of the late term foetuses (Chapter 4). No antibodies to any of the antigens employed in this study were ever detected in the plasma obtained at the time of surgery.

It was possible that some of the responses detected in the foetal sheep were due to the induction of antibody by one antigen which cross-reacted with one of the other antigens being tested, although analysis of the responses in the foetuses indicated that this was probably not occurring. Some animals were found which produced antibody to one antigen, but not to others. For example, several foetuses were positive for anti-FER antibody while being negative for anti-CRBC antibody. One foetus was positive for anti-CRBC antibody and negative for anti-FER antibody. Similarly, plasmas from foetuses which contained anti-POL antibody were uniformly negative in assays for anti-CRBC, anti-FER, anti-CγG and anti-OA antibody, while anti-CRBC antibody did not give detectable titres in assays for anti-POL, anti-FER, anti-CγG

and anti-OA antibody. Anti-FER antibody, anti-CγG antibody and anti-OA antibody were also shown to be negative in assays for antibody to other antigens and the somatic antigens of *S. typhimurium* could be excluded from all cross-reactivity as no antibody was ever detected for these antigens.

(l) The Variation in the Immune Response Between Foetuses

As in all physiological experiments carried out in sound outbred animals there was a significant degree of variation between animals, even when studied at the same stage of development. Exceptions could be found in this study to any generalization that was made.

As a rule a foetus injected with both FER and OA would produce antibody to FER before antibody to OA could be detected. Of 13 animals injected with this combination of antigens and in which responses were observed to both antigens, one was found which did not conform to this statement. Similarly, antibody was never detected before 4 days after the injection of antigen, with one exception which has already been discussed. With one exception for each antigen, all antibodies formed to MON or CγG during the first two weeks of the response were sensitive to 2-ME.

One generalization was true, however, for all the foetuses injected with either MON or POL, together with other antigens; no other antibody ever preceded the appearance of anti-MON or anti-POL antibody.

(m) Development of Lymphoid Tissues Following the Injection of Antigen

The lymph nodes draining the sites of injection, namely the popliteal, prescapular and axillary lymph nodes, together with the thymus, spleen, liver and mesenteric lymph nodes were removed from the foetuses 14 to 28 days after the injection of antigen and examined histologically. A comparison between normal unchallenged foetal tissues

and those obtained from the challenged animals can be seen in Figures 3-iv and 3-v.

Lymph nodes from normal foetuses showed little structural maturation until quite late in gestation when they became increasingly cellular; at this time primary follicles could be found in the developing cortex [Figure 3-iv (a), (c) and (e)]. Following the injection of antigen, the degree of cellularity in the node increased, particularly in the cortex. Cortico-medullary differentiation and follicle formation became evident and germinal centres were found [Figure 3-iv (b), (d) and (f)]. Germinal centres were even found in lymph nodes from a 73 day old foetus which had been injected with antigen at 59 days gestation [Figure 3-iv (b)].

The spleen of normal foetuses is largely a haematopoietic organ until late in gestation. At 70 days gestation the periarteriolar region of the spleen is only sparsely populated with small lymphocytes. The periarteriolar sheath is apparent by 100 days gestation and by 140 days gestation numerous medium-sized lymphocytes are evident in the white pulp [Figure 3-v (a), (c) and (e)]. Following the injection of antigen the amount of white pulp increased, periarteriolar lymphoid development became conspicuous and germinal centres appeared [Figure 3-v (b), (d) and (e)]. The cells in the periarteriolar sheath were predominantly large reticular like cells which had a pale-staining nucleus containing prominent nucleoli. Numerous pyroninophilic "blast" cells could be found in the white pulp.

The effects of antigenic challenge were least obvious in the thymus and this could be expected from observations made in other species. The thymus was lymphoid by 44 days gestation [Figure 3-vi (a)] and cortico-medullary differentiation was evident by 63 days gestation [Figure 3-vi (b)]. Hassal's corpuscles were first detected at 73 days

Figure 3-iv:

Histological appearance of lymph nodes from normal and antigenically stimulated foetal sheep.

Magnification x 100 : Stained with Haematoxylin-Eosin

73 days gestation

(a) Normal; undifferentiated lymph node containing few lymphocytes.

(b) Stimulated; increased cellularity with germinal centre in cortex. Numerous pyroninophilic cells.

105 days gestation

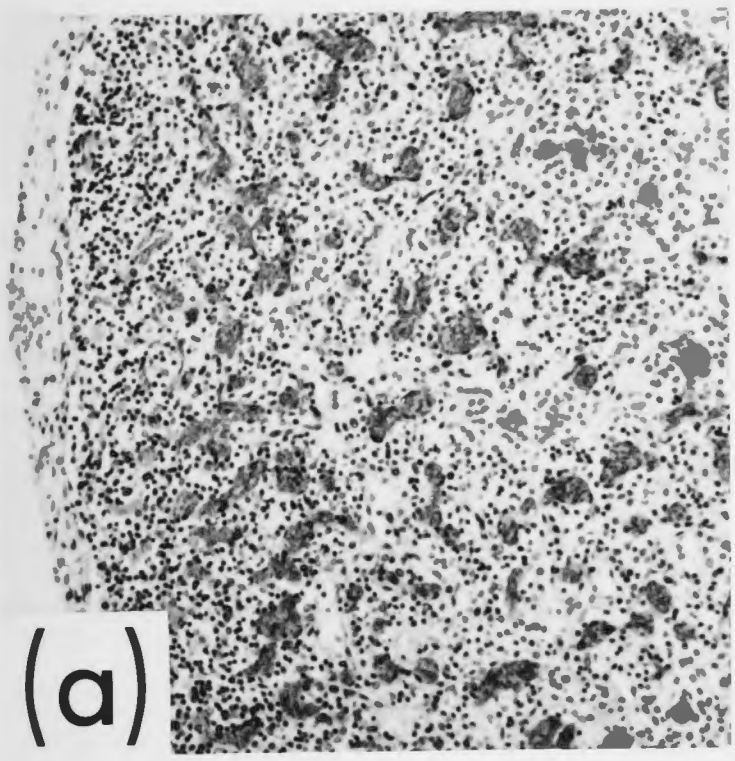
(c) Normal; primitive primary follicles in cortex.

(d) Stimulated; primary follicles and germinal centres in densely populated cortex.

140 days gestation

(e) Normal; follicles well developed in cortex and an increased cellularity in medulla.

(f) Stimulated; germinal centres in cortex.



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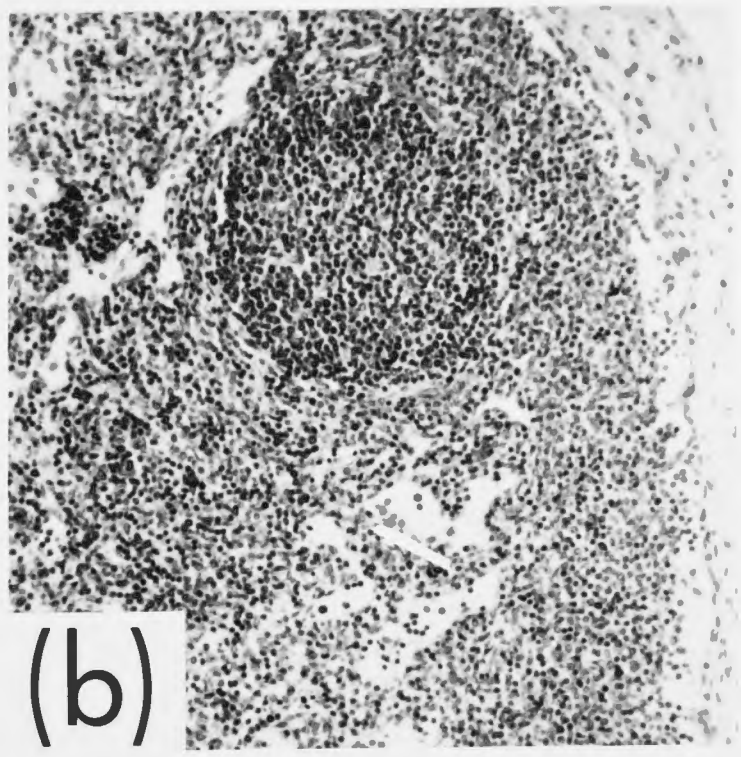

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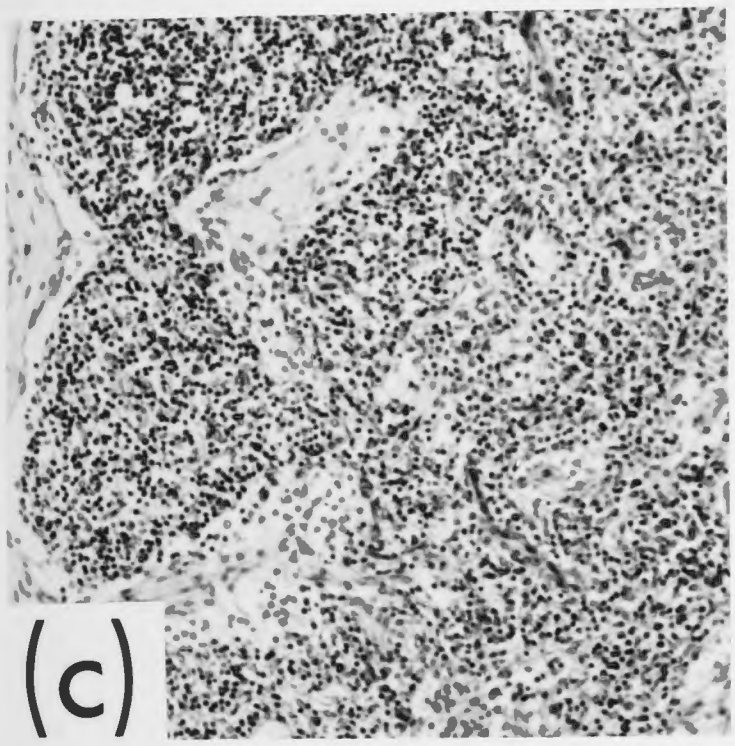

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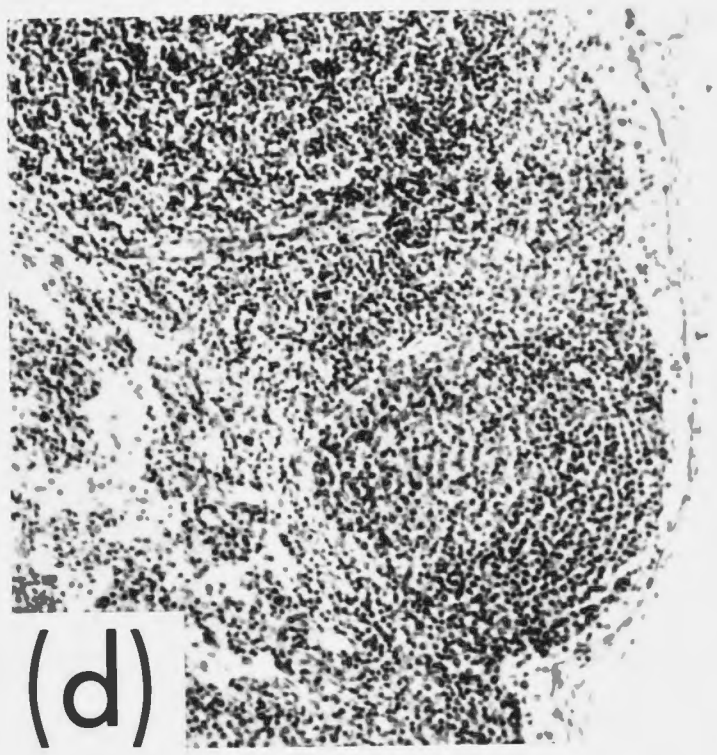

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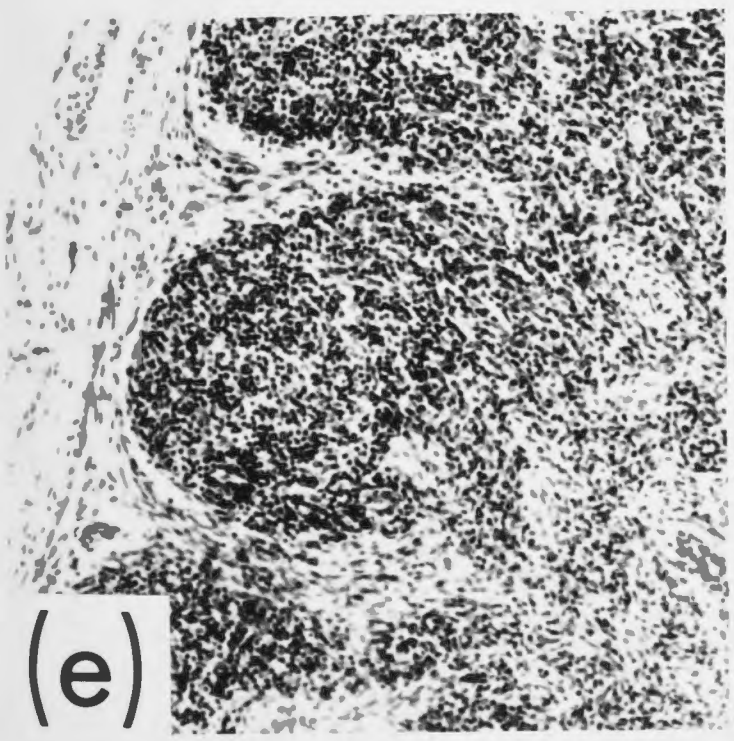

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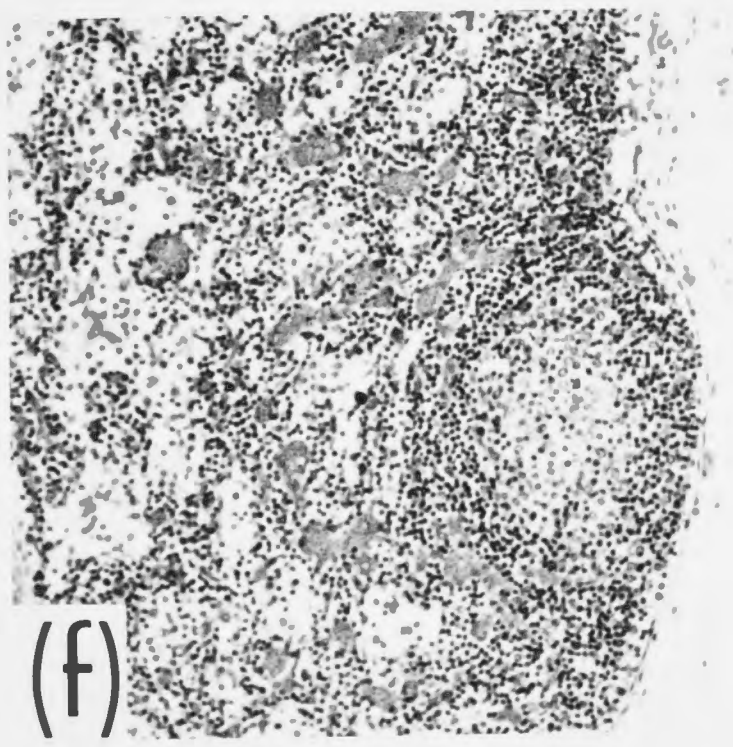


(f)

Figure 3-v:

Histological appearance of spleens from normal and antigenically stimulated foetal sheep.

Magnification x 250 : Stained with Haematoxylin-Eosin

73 days gestation

(a) Normal; few lymphocytes in periarteriolar region.

(b) Stimulated; enlarged periarteriolar sheath containing pyroninophilic blast cells and reticular cells with pale staining nuclei and prominent nucleoli.

105 days gestation

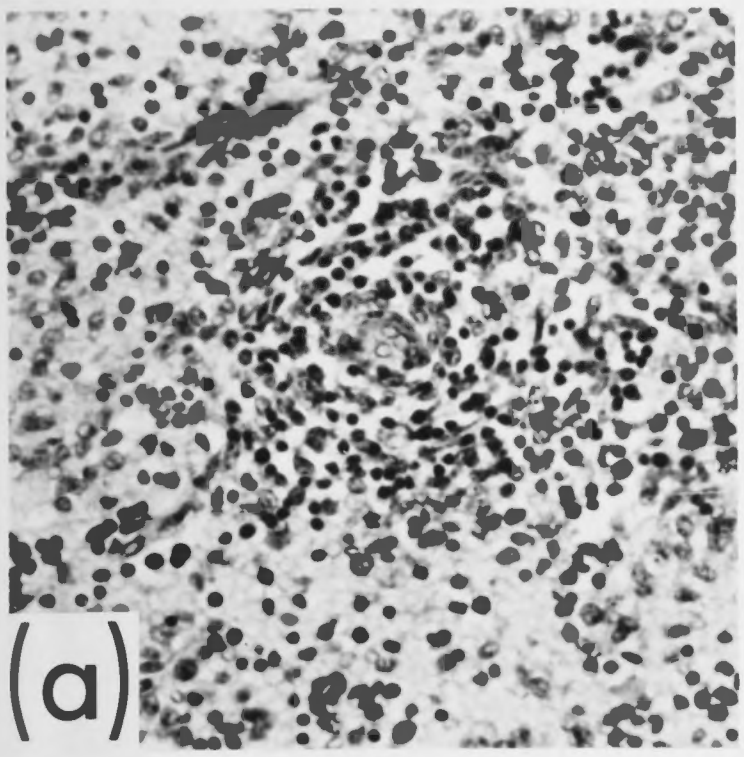
(c) Normal; developed periarteriolar sheath.

(d) Stimulated; as for (b).

140 days gestation

(e) Normal; periarteriolar sheath is well developed and contains small and medium sized lymphocytes.

(f) Stimulated; germinal centre and eccentrically positioned arteriole.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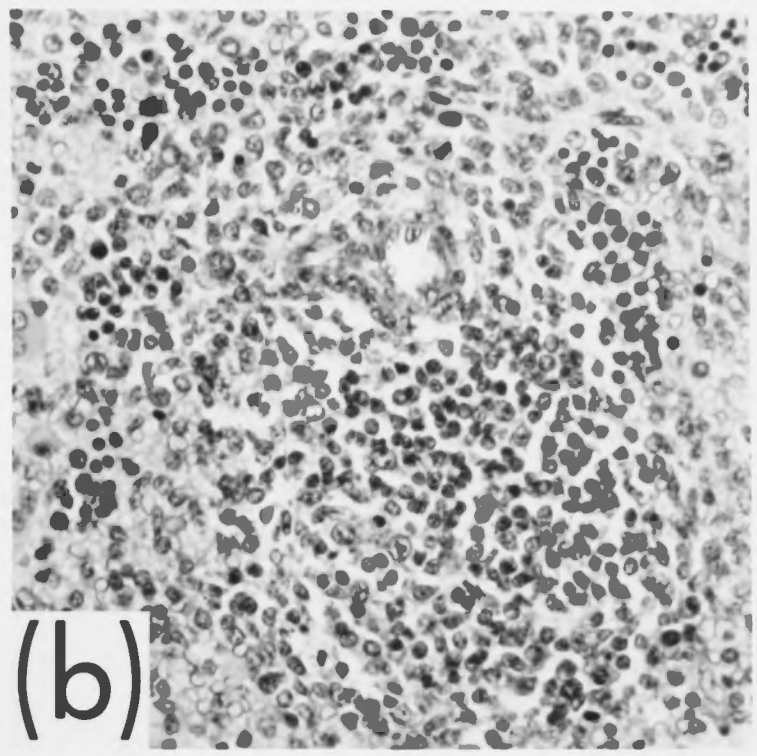
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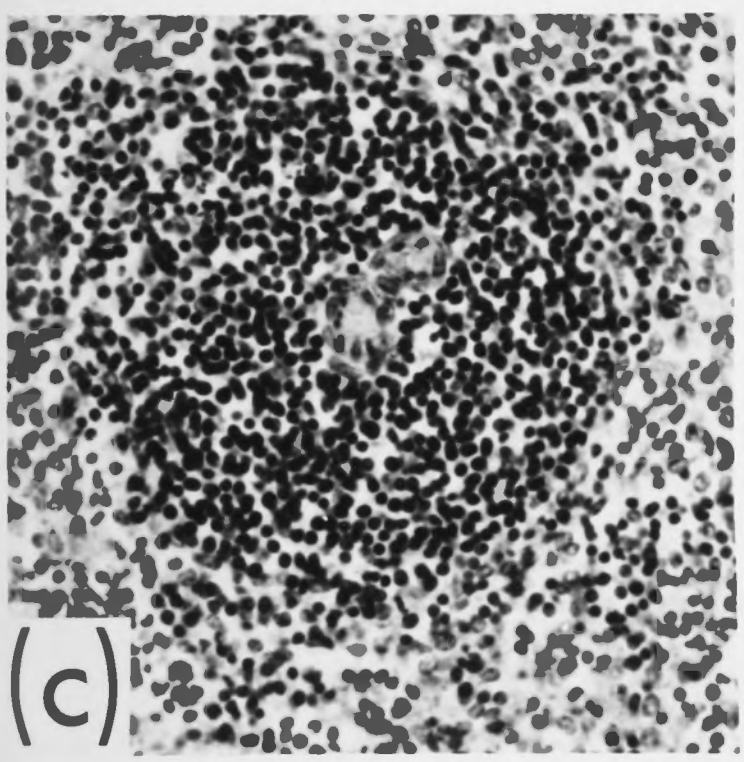
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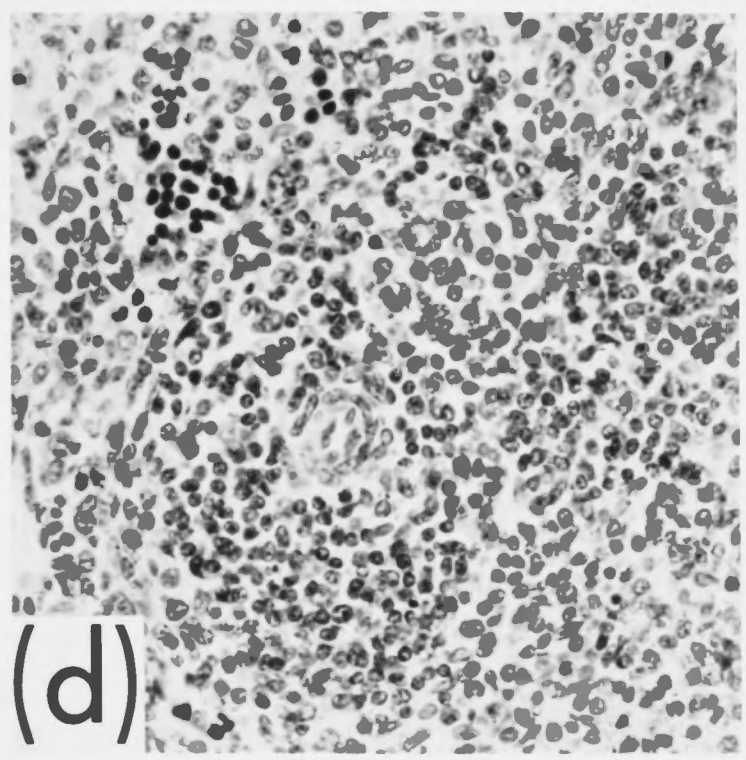
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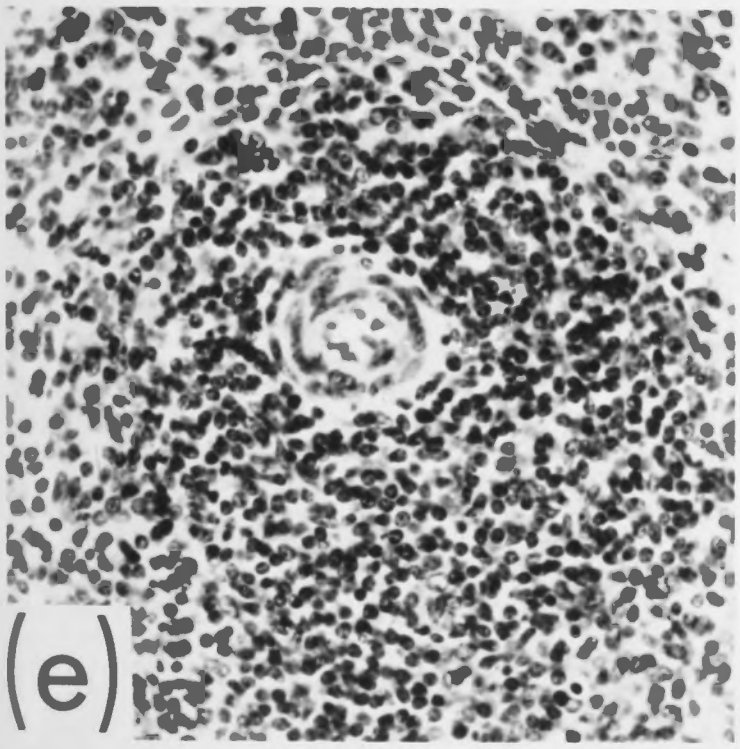
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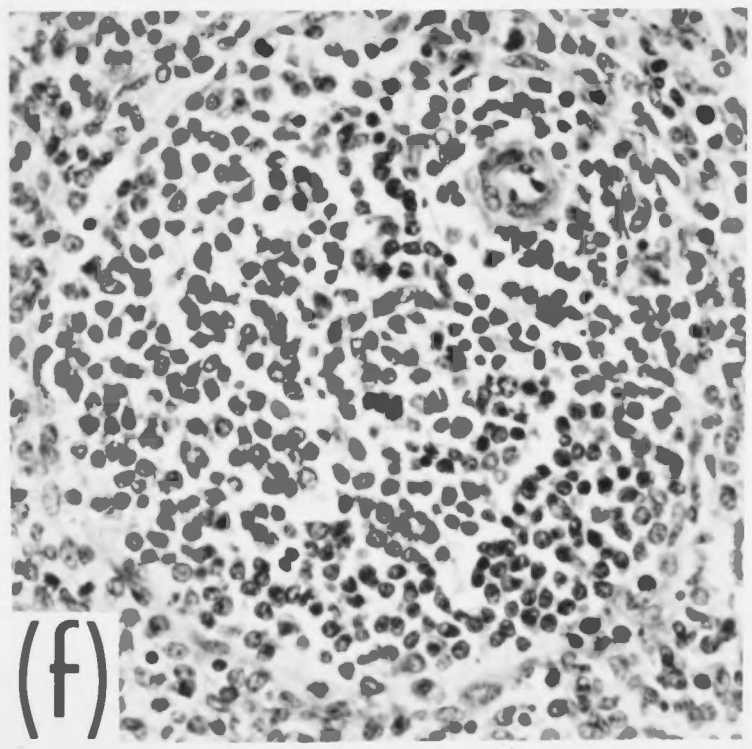

(f)

Figure 3-vi:

Histological appearance of the thymus and liver in antigenically stimulated foetal sheep.

Magnification x 100 : Stained with Haematoxylin-Eosin

Thymus

(a) 44 days gestation; lymphocytes present in thymus, but no cortico-medullary differentiation.

(b) 63 days gestation; cortico-medullary differentiation and increased cellularity.

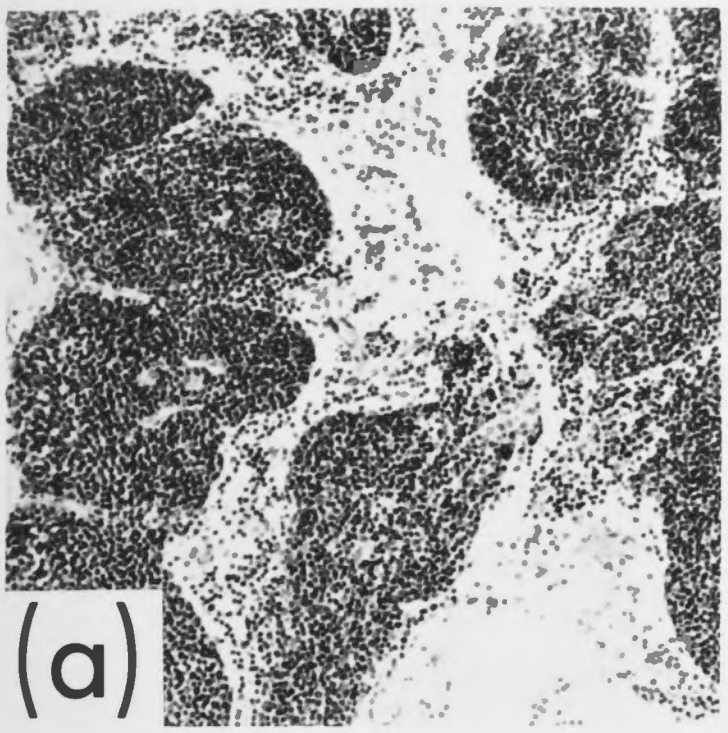
(c) 73 days gestation; earliest appearance of Hassal's corpuscles.

Liver

(d) 63 days gestation.

(e) 100 days gestation.

(f) 139 days gestation.



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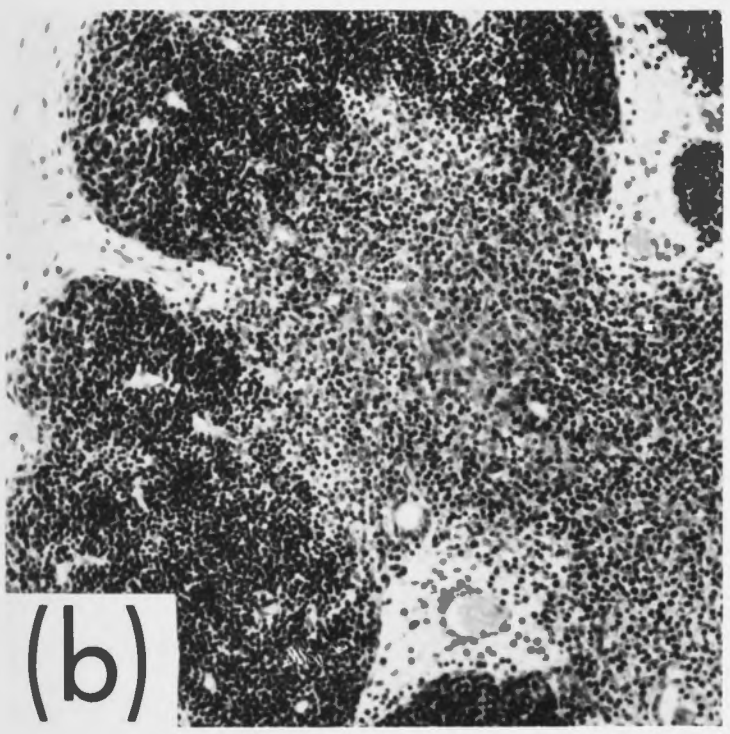

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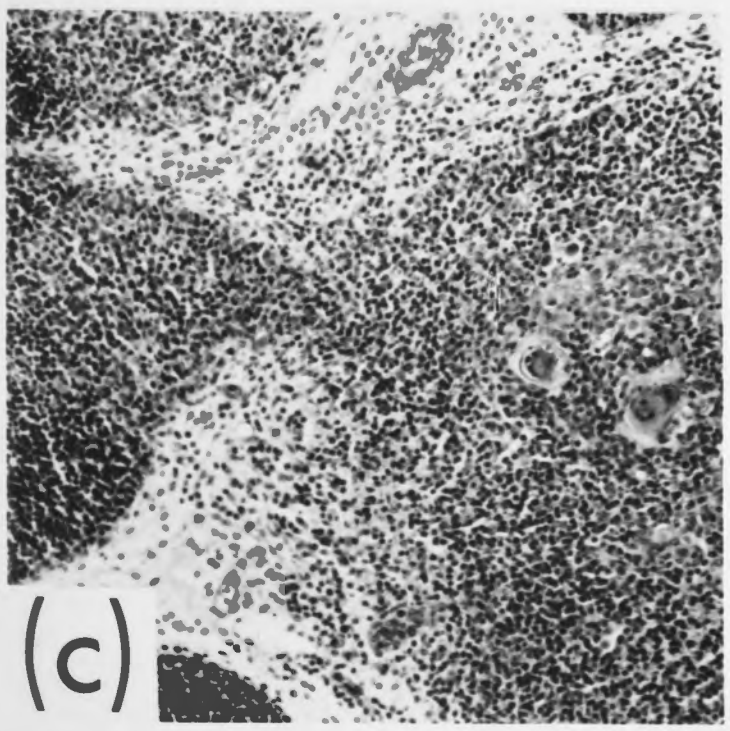

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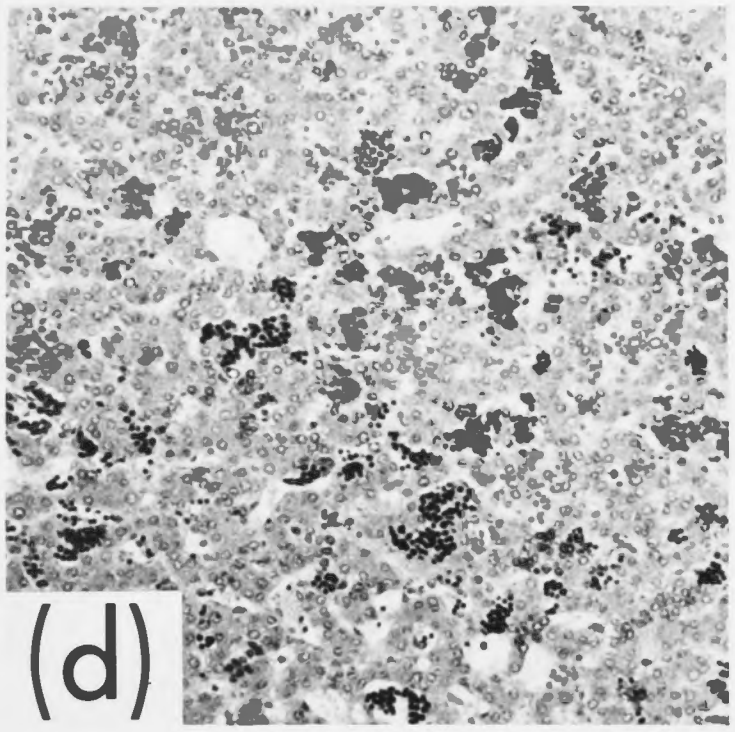

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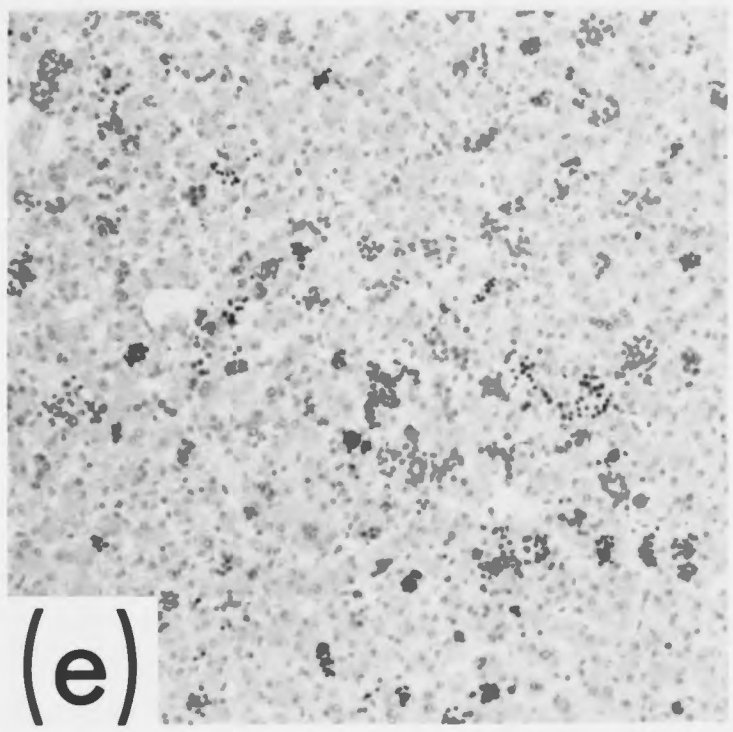

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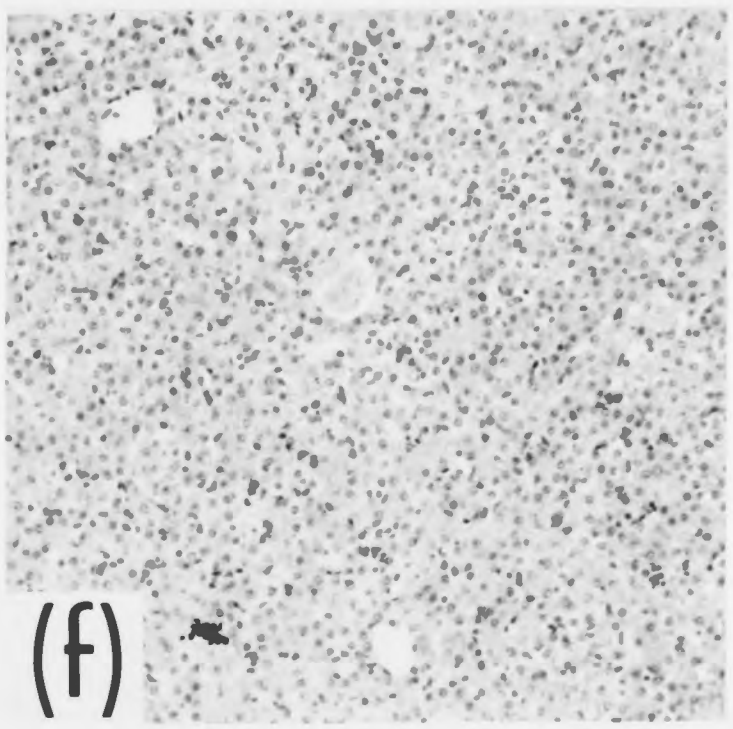

(f)

gestation [Figure 3-vi(c)]. The liver became less haematopoietic with increasing gestational age, and no sign of immune activity was manifest in this organ [Figure 3-vi (d), (e), (f)].

DISCUSSION

Is there a sequential development of immunological competence in foetal sheep and is the immune response fully developed from the moment it first occurs? These were the questions the experiments described in this Chapter set out to answer. Before discussing these questions, however, it needs to be stipulated that any answers that can be derived from data presented in this Chapter relate only to the response observed to a single dose of antigen, injected by one route, in one physical form and given simultaneously with several other antigens. Beyond these boundary conditions, no extrapolation can be assumed valid.

The data presented in this Chapter have been pooled in Table 3-2. The Table divides the gestation period of the sheep into 10 day intervals from 46 to 126 days. The number of animals at each time interval which responded during the first 2 weeks after the injection of a particular antigen, is given as a fraction of the number of animals tested. The 2 week interval was an arbitrary period, which was chosen to allow some judgement to be made on the competence of the foetus when it was first injected, or shortly thereafter.

The order the antigens are listed represents an attempt to depict an order in the development of competence, but the differences described between the onset of competence to CRBC, POL and MON is arbitrary. FER has been placed first due to the fact that one 50 day old foetus did respond to this antigen and also because of the animals challenged at or before 60 days gestation, four produced antibodies

Table 3-2: The proportion of foetal sheep which responded to various antigens at different stages of gestation.

Antigen / Days Gestation	FER	CRBC	POL	MON	CγG	OA	*S. tym.* Whole Orgs.	*S. tym.* LPS
46 to 55	1/2*	0/4	0/3	NT	NT	NT	NT	NT
56 to 65	4/6	3/12	1/5	0/2	0/3	NT	NT	NT
66 to 75	4/6	4/5	2/2	2/2	2/2**	2/6	NT	NT
76 to 85	3/3	2/2	2/2	2/2	1/3	1/3	0/1	0/1
86 to 95	3/3	4/4	3/3	3/3	2/3	5/5	0/2	0/1
96 to 105	3/3	1/1	NT	2/2	3/3	3/4	0/2	0/2
106 to 115	3/3	2/2	2/2	2/2	2/2	3/5	0/2	0/2
116 to 126	3/3	5/5	5/5	2/2	4/4	7/7	0/2	0/3

* $\frac{\text{No. of animals producing antibody within 14 days}}{\text{No. of animals tested}}$

** Weak responders

N.T.: Not tested

S. tym: *S. typhimurium*

to FER but not to CRBC, while only one foetus was positive for CRBC while negative for FER.

If one interprets Table 3-2 in terms of when the majority of foetal animals tested can respond to a particular antigen it can be seen that for FER this would be around 60 days, for CRBC, POL, MON and CγG around 70 days, for OA around 90 days and for the somatic antigens of *S. typhimurium*, not at all. These results could be interpreted to show that the ability to react to different antigens occurs in a sequential fashion.

On the other hand if it is more important to decide the age at which a foetus was first found to produce detectable antibody to a given antigen the results have a different emphasis (Table 3-3). The figures for DNP and P815 are derived from limited observations but are added for completeness.

The first antibody detected to 7 different antigens and 2 different forms of 1 of these antigens, appeared in foetuses whose age varied only by 18 days. Leaving aside the failure of the foetuses to respond to *S. typhimurium* and considering the limitations of the experimental protocol, it would not be unreasonable to interpret the data as showing that there is a relatively short period of development during which the foetus acquires the ability to respond to a quite wide range of antigens.

Both lines of argument can be interpreted as indicating a sequential development of immunological competence, although when the data are seen from the second viewpoint the time scale for this development is foreshortened substantially.

Quite apart from the question of whether or not there is a "hierarchy" of immunological competence, several other points can be

Table 3-3: The first appearance of haemagglutinating antibody to antigens injected into foetal sheep older than 50 days gestation.

Antigen	FER	CRBC	POL	P815	OA	MON	DNP	CγG	*S. tym.*
Age of gestation at which antibody first detected	64	72	74	74	76	78	78	82	>143

S. tym: *S. typhimurium*

made from the results.

Contrary to observations made by Silverstein and Prendergast (1970) on immune responses in foetal sheep the capacity to produce antibody against three different antigens (FER, POL and CγG) increased significantly during foetal development. Additionally, whereas foetuses could only produce antibodies which were sensitive to 2-ME before 90 days gestation, after this time an increasing number of animals produced 2-ME resistant antibody to CRBC, POL, FER and OA.

Although the maximum titres of antibody to CRBC, MON and OA did not increase to the same extent in older foetuses as the responses to other antigens, the total amount of antibody formed by the older foetuses was much greater when the increase in the size of the extracellular fluid pool was taken into account. From the results reported it can be concluded that as the foetus develops there is a significant increase in the amount of antibody formed and that changes occur in the characteristics of the immune responses during *in utero* development.

The results summarized in Table 3-2 demonstrate that responses to both forms of flagellin (MON and POL) occur with certainty after 69 days gestation. The results obtained with several other antigens, particularly CγG and OA, gave a much less precise indication of the onset of immune competence to these antigens. The appearance of antibody to OA was most irregular, for although two of six foetuses injected around 70 days gestation responded to the antigen within 14 days, it was not until after 120 days gestation that all animals gave responses within this period. Silverstein and Prendergast (1970) stated that they examined the responses to OA in 50 to 60 foetal animals and never observed a response prior to 118 days gestation, while only one of 20 to 30 foetal sheep injected after 125 days

gestation failed to produce antibody. Excluding differences between breeds of sheep or antigen preparations, their results seem incompatible with those presented in this Chapter.

There did not seem to be any correlation between the appearance of a particular immune capability and any specific stage in the development of the lymphoid apparatus. A wide range of responsiveness could be induced in a foetus at a time when histologically, the lymphoid tissues with the possible exception of the thymus, were very immature. Foetal tissues were sparsely populated with lymphoid cells and lacked recognizable structural differentiation at the time when the majority of animals could respond to 7 of 8 different antigens, and to different forms of the same antigen.

Following antigenic challenge the structure of the lymphoid tissues changed and this occurred particularly in the lymph nodes draining the injection site and the spleen. Structures which were not usually found until near to or after parturition appeared in the lymph nodes and spleen of the youngest foetus examined after antigenic challenge (73 days gestation). These structural changes were accompanied by a substantial increase in the cellularity of the tissues.

There are several obvious criticisms of the experiments described in this Chapter. The first is that the methods used to detect antibody were too insensitive and may vary in their sensitivity for the different antigens. There are certainly more sensitive assays for antibody than the haemagglutination assay. However, no matter what the assay, a level of activity by the foetus has to be decided upon which will be accepted as the threshold for deciding that an immune response to that particular antigen has taken place. Whether extremely sensitive assays would substantially alter the time sequence

of the appearance of immunological competence remains unresolved. Using the techniques described, from 30 to 100 ng/ml of antibody can be detected in the circulation of foetal sheep and these levels must be considered as approaching the lower level of physiological significance. An attempt has been made to minimize the differences in sensitivity of the assays used for the different antigens by using similar techniques wherever possible, for example $CrCl_3$ has been used as the coupling agent between SRBC and OA, FER and POL, while all passive haemagglutination techniques employed a 1% SRBC suspension.

The second major objection would be that "antigenic competition accounted in part for the sequential development of competence. Again there is no proof that antigenic competition does not play a role in the responses observed in these animals, although by injecting antigens simultaneously into different sites, the likelihood of competition should have been greatly reduced (Taussig, 1973). Analysis of the data from individual foetal animals showed that after 90 days gestation the vast majority of animals responded to all the antigens injected, with the exception of the somatic antigens of *S. typhimurium*. Since animals at all ages received similar treatments it may be said that competition does not operate in foetuses from 90 days gestation onwards and it may be inferred that it is not a major consideration in the unresponsiveness of younger foetuses. Antigenic competition will be discussed again in Chapter 5 in relation to the responses of foetal sheep to the injection of a single antigen.

CHAPTER 4

THE AMOUNT AND IDENTITY OF SERUM IMMUNOGLOBULINS PRODUCED BY NORMAL AND ANTIGENICALLY STIMULATED FOETAL SHEEP

CHAPTER 4

THE AMOUNT AND IDENTITY OF SERUM IMMUNOGLOBULINS PRODUCED BY NORMAL AND ANTIGENICALLY STIMULATED FOETAL SHEEP

INTRODUCTION

Sheep, pigs and cattle are members of a group of mammals in which no maternal immunoglobulin is transferred to the foetus *in utero* (Brambell, 1970; Sterzl, Rejnek and Travnicek, 1966). Maternal immunoglobulin is only transferred to the young of these species when they ingest colostrum during the first few days after birth (Brambell, 1970). In most other species of mammals maternal immunoglobulins pass through the placenta and enter the foetal circulation (Brambell, 1970; Solomon, 1971).

The idea that the foetal sheep does not receive immunoglobulins from the mother arose from studies done on lambs born to immunized ewes. Pregnant ewes hyperimmunized with formalized lamb dysentery toxin (Mason, Dalling and Gordon, 1930) or vaccinated against enterotoxaemia (Oxer, 1936) gave birth to lambs with no detectable levels of antibody in their circulation before they had suckled. Similar observations have been made with typhoid bacteria and diphtheria toxoid (Schneider and Szathmary, 1939) and with *Salmonella pullorum* (Cummings and Bellville, 1963). These results have been confirmed in Chapter 3, where numerous foetal sera, obtained at various stages of gestation, were titrated for haemagglutinating antibodies against CRBC and the somatic and flagellar antigens of *Salmonella typhimurium*. Although many pregnant ewes had very high levels of circulating antibodies to these antigens, no foetus was found at the time of surgery to have antibodies in its blood. Similar results have also been reported in pigs (Hoerlin, 1952, 1957; Sterzl,

Kostka, Mandel, Riha and Holub, 1962) and cattle (McAlpine and Rettger, 1925; Mason *et al*, 1930; Graves, 1963).

It is easier to analyse the endogenous synthesis of immunoglobulin during *in utero* development when there is no exogenous immunoglobulin present and because of this the pig has been studied extensively. Usually germ-free, colostrum-deprived neonatal pigs have been used which are obtained by caesarean section. Despite reports that germ-free, colostrum-deprived piglets did not have circulating immunoglobulin (Kim, Bradley and Watson, 1966a) it now seems well established that there is immunoglobulin present in these animals which has the properties of a 4S IgG molecule (Prokesova, Rejnek, Sterzl and Travnicek, 1969; Bourne, 1974). 4S IgG was found to be present in the circulation at a concentration of 10 to 40 µg/ml, to be antigenically and electrophoretically identical with 7S IgG and to possess both L and γH polypeptide chains. No IgM was detected in the sera of these foetal pigs (Prokesova *et al*, 1969). More recently it was reported that the IgG in ceasarean-derived piglets had three different molecular weights; 160,000; 75 to 95,000 and 40 to 55,000 (Rejnek and Prokesova, 1973).

IgG has also been found in the sera of foetal pigs (112 days gestation), by the technique of radial immunodiffusion, at a concentration of 15 to 190 µg/ml (Travnicek and Mandel, 1971). Neither IgM nor IgA was found in these foetal pigs, although IgA has been shown to be synthesized in germ-free, colostrum-deprived piglets, reaching a serum concentration of about 0.2 to 2.0 µg/ml (Prokesova and Rejnek, 1971).

In the bovine, IgM has been found in the circulation of foetuses at 130 days gestation and IgG has been found at 145 days gestation. IgA has not been detected in the circulation of the bovine foetus (Schultz, Dunne and Heist, 1971b).

Silverstein *et al* (1963b) found IgM by immunoelectrophoresis and low levels of IgG (approximately 5 µg/ml) by agar diffusion analysis in the circulation of nearly all the normal foetal sheep that they examined. Unfortunately the quantity of IgM was not estimated, while the quality of the antiserum used in the quantitation of IgG seemed questionable.

Antigenic stimulation of germ-free, colostrum-deprived piglets with MSP-2 actinophage reportedly induced the synthesis of immunoglobulin with physicochemical properties similar to 19S IgG (Kim, Bradley and Watson, 1966b). The IgG had a sedimentation coefficient of 18.5S and a molecular weight of 900,000; it was not thought to be aggregated 7S IgG (Kim, Bradley and Watson, 1968). Both 19S IgG and 7S IgG were synthesized by antigenically stimulated piglets before IgM or IgA.

Prokesova *et al* (1969) and Prokesova, Rejnek, Sterzl and Travnicek (1970) found that the first immunoglobulin formed by germ-free, colostrum-deprived piglets injected with 2×10^{10} SRBC was 19S IgM followed later by 7S IgG. They found no 19S IgG. IgM serum haemagglutinins were produced in 74 day old foetal pigs in response to SRBC and identified by immunoelectrophoresis and sensitivity to 2-ME (Schultz, Wang and Dunne, 1971a) while 2-ME sensitive neutralizing antibodies to ϕX174 were produced by 70 day old foetal pigs (Hajek *et al*, 1969). Unfortunately, neither immunoelectrophoresis nor 2-ME sensitivity excludes the presence of 19S IgG as the latter is sensitive to 2-ME and on immunoelectrophoresis appears in an almost identical position to 19S IgM (Kim *et al*, 1968). The question can really only be answered using monospecific antisera for IgM and IgG (Prokesova *et al*, 1969). A recent report by Bourne *et al* (1974) showed that following the injection of pregnant sows with porcine parvovirus, which crosses the placenta, 19S IgM and lesser amounts of 7S IgG,

19S IgG, 4S IgG and 7S IgA were present in the serum of foetuses at term. Provided the immunoglobulin detected was not due to contamination from the mother it seems that, at best, 19S IgG is only a small component of the immunoglobulins produced in an immune response in the foetal pig.

Less controversy seems to surround the production of immunoglobulins by foetal sheep in response to antigenic stimulation. Silverstein *et al* (1963b) reported IgM as the first immunoglobulin formed after the injection of a mixture of antigens, IgG_1 being detected 10 to 20 days later. Although no IgA was found in the circulation of foetal sheep, IgG_2 regularly appeared in serum samples taken from older foetuses 31 to 44 days after stimulation. The sequential appearance of IgM followed by IgG during the primary response was later confirmed in 100 to 135 day old foetal sheep injected with ϕX174 (Silverstein, Parshall and Uhr, 1966). The classification of immunoglobulin classes in this work relied on their electrophoretic migration rather than precipitation with monovalent antisera. Osburn and Hoskins (1969) found IgM was produced in foetuses of 80 days gestation and IgG by 98 day old foetuses infected with *Brucella ovis;* antibody was detected by complement fixation at 91 days gestation. No haemagglutinating antibody was found in this study and precipitating antibody only appeared late in gestation.

Silverstein *et al* (1963b) postulated that adjuvant was essential to induce the synthesis of IgG. They found that three animals, injected with ϕX174 and diphtheria toxoid without FCA, failed to produce IgG within 10 to 11 days of challenge. The results obtained in foetal sheep injected with ϕX174 with or without adjuvant however, showed no correlation between the appearance of IgG immunoglobulin in the sera and the resistance of the antibody formed to 2-ME. In fact, 2 of 3 animals which had not been given adjuvant produced 2-ME resistant

antibody, while the 2 animals injected with adjuvant produced only 2-ME sensitive antibody (Silverstein *et al*, 1963a; 1963b). Varela-Diaz and Soulsby (1972) reported that in colostrum-deprived lambs studied for 3 to 4 weeks after birth IgG_1 was produced first followed by IgG_2.

Similar sequences for immunoglobulin production have been reported in other mammals, including man. The normal human foetus is competent to synthesize IgM (Gitlin and Biasucci, 1969) and IgA_1 and IgA_2 from 15 to 17 weeks gestation (Cederqvist and Litwin, 1974). Martensson and Fudenberg (1965) by detecting Gm allotypes coded for by the foetus, found IgG synthesized by the foetus in cord blood collected at term. Premature infants respond to the injection of ϕX174 first by producing IgM antibodies and then, some 2 to 6 weeks later, by producing IgG antibodies (Uhr *et al*, 1962a).

RESULTS

This Chapter records the results of studies on the nature of immunoglobulins appearing normally in foetal sheep during gestation and in response to antigens injected at different stages of gestation.

(a) The Appearance of Immunoglobulins in the Blood of Foetuses During Gestation

Blood samples were obtained from ninety-one normal foetuses of different ages by surgical exposure of the foetus. These blood samples were assayed for immunoglobulin content using the radial immunodiffusion technique with monospecific rabbit antiserum to sheep IgM, IgG_1, IgG_2 and IgA. The results presented in Table 4-1, show the number of the foetuses tested which gave positive reactions with the monospecific antisera and the mean concentration of immunoglobulin in the positive samples.

IgM was detected in the plasma of only one of thirty-four

Figure 4-1: Appearance of immunoglobulin in the serum of normal foetal sheep.

Mean age (days of gestation)	Range (days)	No. of foetuses tested	No. positive foetuses (mean concentration µg/ml±S.E.)			
			IgM	IgG_1	IgG_2	IgA
49.5	49 → 50	2	0	0	0	0
59.2	57 → 61	13	0	0	0	0
70.1	68 → 72	19	1 (5)	0	0	0
79.5	76 → 82	10	2 (62 ± 5)	0	0	0
89.4	87 → 92	8	1 (12)	0	0	0
99.5	96 → 101	13	4 (47 ± 30)	1 (146)	0	0
111.2	108 → 113	6	2 (32 ± 17)	0	0	0
119.7	118 → 121	20	12 (42 ± 8)	2 (36 ± 5)	0	0

foetuses between 49 and 72 days gestation. The percentage of normal foetal sheep with IgM in their circulation increased after 76 days gestation from around 20% to 60% at 120 days gestation. The concentration of IgM never exceeded 100 µg/ml except in one 100 day old foetus which had 136 µg/ml of IgM; this foetus also had 146 µg/ml of IgG_1. IgG_1 was detected in only three of the ninety-one foetuses tested. Two of the positive samples came from foetuses at 120 days gestation. IgG_2 and IgA were not detected by the radial immunodiffusion assay in any foetus up to 122 days gestation.

It seems that after mid-gestation some foetal sheep can synthesize IgM and the percentage of animals which have detectable IgM in their blood increases during *in utero* development.

(b) The Synthesis of Immunoglobulins Following Antigenic Challenge

(i) The concentration of immunoglobulin produced in the primary immune response

The haemagglutinating antibody responses of foetal sheep injected simultaneously with up to four antigens, some emulsified in FCA, have been described (Chapter 3). The immunoglobulin levels in the plasma and serum samples obtained from forty-five of these foetuses during the course of the primary immune response were assayed by radial immunodiffusion.

The concentration of immunoglobulin produced by individual animals showed considerable variability and this was possibly related to the competence of the animal to respond to the antigens at the time it was injected. An estimate of the potential of foetuses to synthesize the various immunoglobulin classes was obtained by comparing groups of animals injected at about the same stage of gestation, that is, animals injected around 63 days, 86 days, 108 days and 121 days gestation.

The levels of IgM and IgG_1 detected in the circulation of

foetuses between 49 and 72 days gestation. The percentage of normal foetal sheep with IgM in their circulation increased after 76 days gestation from around 20% to 60% at 120 days gestation. The concentration of IgM never exceeded 100 µg/ml except in one 100 day old foetus which had 136 µg/ml of IgM; this foetus also had 146 µg/ml of IgG_1. IgG_1 was detected in only three of the ninety-one foetuses tested. Two of the positive samples came from foetuses at 120 days gestation. IgG_2 and IgA were not detected by the radial immunodiffusion assay in any foetus up to 122 days gestation.

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The levels of IgM and IgG_1 detected in the circulation of

foetal sheep injected with antigen between 57 and 126 days gestation are shown in Table 4-2 (a), (b), (c) and (d). The levels of IgG_2 and IgA are not recorded as no foetus produced these immunoglobulins during the first 14 days of primary immune responses to the various antigens injected in this study.

The synthesis of immunoglobulin by a group of 13 foetal sheep injected with antigen between 57 and 70 days gestation (mean age 63 days) is shown in Table 4-2 (a). While very low levels of IgM could be detected 6 to 8 days after the injection of antigen, no IgG_1 was detected during the 2 weeks subsequent to the injection of antigen.

In the groups of older foetuses [Table 4-2 (b), (c) and (d)] IgM first appeared by day 4 after the injection of antigen; peak concentrations regularly occurred at day 8. Although the mean concentrations of IgM in animals injected before 70 days gestation were significantly lower than in the older animals, the levels of IgM produced by groups of foetuses injected around 86, 108 and 121 days of gestation were not statistically different from each other at any time interval after challenge. IgG_1 appeared regularly in the circulation of animals injected after 79 days gestation and was often detected 6 to 8 days after the injection of antigen. The concentration of IgG_1 usually continued to increase throughout the first 14 days of the response. The group of animals injected around 86 days gestation had lower concentrations of IgG_1 at most time intervals after challenge, than did the animals injected around 121 days gestation.

(ii) Onset of IgG_1 synthesis

Looking for the earliest production of IgG_1 by individual foetuses injected with a variety of antigens, several animals between 69 and 81 days gestation were found which had all produced IgG_1 by the time they had reached 90 days gestation (Table 4-3). The mean age of

Figure 4-2: Synthesis of immunoglobulin during the primary immune responses in foetal sheep

(a) Mean age 63 days - 13 animals from 57 to 70 days gestation

Time after injection (days)	No. of samples	IgM		IgG_1	
		µg/ml±S.E.	Range	µg/ml±S.E.	Range
0	13	0	-	0	-
2	8	0	-	0	-
4	7	0.6 ± 0.6	0 → 4	0	-
6	7	18 ± 10	0 → 73	0	-
8	6	26 ± 11	4 → 78	0	-
10	5	21 ± 5	6 → 34	0	-
12	5	17 ± 6	3 → 34	0	-
14	12	35 ± 7	3 → 80	0	-

(b) Mean age 86 days - 8 animals from 79 to 92 days gestation

Time after injection (days)	No. of samples	IgM		IgG_1	
		µg/ml±S.E.	Range	µg/ml±S.E.	Range
0	8	8 ± 5	0 → 37	0	-
2	8	8 ± 5	0 → 42	0	-
4	8	31 ±10	0 → 78	0	-
6	8	79 ±15	0 →123	12 ± 8	0 → 53
8	8	151 ±47	20 →386	33 ± 11	0 → 86
10	7	93 ±19	0 →147	68 ± 15	0 → 106
12	6	92 ±10	69 →126	62 ± 16	0 → 103
14	7	66 ±11	32 →104	70 ± 9	48 → 103

(c) Mean age 108 days - 7 animals from 100 to 113 days gestation

Time after injection (days)	No. of samples	IgM		IgG_1	
		µg/ml±S.E.	Range	µg/ml±S.E.	Range
0	7	17 ± 9	0 → 55	0	-
2	7	20 ± 8	0 → 48	0	-
4	7	41 ± 14	0 → 86	0	-
6	7	67 ± 15	0 → 109	0	-
8	7	109 ± 28	16 → 235	30 ± 11	0 → 63
10	6	110 ± 29	34 → 213	62 ± 26	0 → 149
12	5	110 ± 16	78 → 160	68 ± 32	0 → 152
14	7	88 ± 12	55 → 135	134 ± 31	80 → 259

(d) Mean age 121 days - 6 animals from 119 to 126 days gestation

Time after injection (days)	No. of samples	IgM		IgG_1	
		µg/ml±S.E.	Range	µg/ml±S.E.	Range
0	6	34 ± 16	0 → 90	0	-
2	6	34 ± 13	0 → 73	0	-
4	6	50 ± 13	0 → 86	0	-
6	6	112 ± 24	36 → 198	28 ± 17	0 → 106
8	5	172 ± 44	58 → 310	106 ± 33	40 → 200
10	5	144 ± 35	45 → 243	87 ± 15	53 → 148
12	3	122 ± 30	66 → 166	117 ± 16	77 → 146
14	4	124 ± 51	32 → 235	128 ± 14	84 → 164

Table 4-3: First appearance of IgG_1 in the plasma of antigenically stimulated foetal sheep

Age of gestation when challenged (days)	Antigens injected	Age when IgG_1 was detected (days)
69	POL, CRBC, FER, OA	89
79	POL, CRBC, FER, OA	87
79	POL, CRBC, FER	87
80	CRBC, FER, OA, LPS	90
81	MON, FER, CγG, *S. tym.*	89

Mean 88.5 days

S. tym: *S. typhimurium*

gestation at which IgG_1 was first detected in these animals was 88.5 days; in the youngest animal, injected at 69 days gestation, IgG_1 did not appear until 20 days after challenge. The appearance of IgG_1 in the circulation of these animals following antigenic challenge, occurred slightly earlier than 2-ME resistant antibody (Table 3-1).

(iii) Influence of adjuvant on the synthesis of immunoglobulins

IgG synthesis was not dependent on the injection of antigens in FCA (Table 4-4). The production of IgM and IgG_1 by four foetuses injected with antigens without FCA was similar to that observed in animals of comparable age injected with antigens in FCA [c f. Tables 4-2 (b), (c) and (d)].

(c) Immunochemical Characterization of Foetal Immunoglobulins

(i) Immunoelectrophoresis

Plasma from foetuses was analysed by immunoelectrophoresis and the patterns developed with multivalent rabbit anti-whole sheep serum, monospecific rabbit anti-sheep IgM and monospecific anti-sheep IgG serum. Only in those foetuses injected relatively late in gestation were the levels of immunoglobulin sufficiently high for the various fractions to be identified by this technique. Figures 4-i and 4-ii show the appearance of immunoglobulins in the circulation during an immune response.

A 100 day old foetus was injected with MON-DNP, LPS and OA in FCA and its response followed for 32 days. A weak anti-DNP response appeared on days 4 and 6. Anti-OA antibody appeared on day 8 and until day 18 this antibody was sensitive to 2-ME (titres 3.5 to 5.5); after day 18 all anti-OA antibody was resistant to 2-ME (titres 6.0 to 10.5). The LPS did not induce an antibody response. The immunoelectrophoretic patterns obtained with plasma collected on days 0, 8, 14, 20, 26 and 32 after the injection of antigen are shown in Figure 4-i. When the plasma

Table 4-4: Synthesis of immunoglobulin in foetal sheep injected with antigens without Freund's complete adjuvant

Mean age 91.5 days - 4 animals from 79 to 113 days gestation

Time after injection (days)	No. of Samples	IgM		IgG_1	
		µg/ml±S.E.	Range	µg/ml±S.E.	Range
0	4	0	0	0	0
2	4	4 ± 4	0 → 15	0	0
4	3	52 ± 26	0 → 78	0	0
6	3	62 ± 36	0 → 123	15 ± 15	0 → 44
8	4	73 ± 32	20 → 159	45 ± 8	29 → 65
10	3	84 ± 52	0 → 178	97 ± 20	59 → 127
12	2	89 ± 16	73 → 104	106 ± 47	59 → 152
14	2	51 ± 19	32 → 69	129 ± 68	61 → 197

Figure 4-i:

Immunoelectrophoretic analysis of plasma from a foetal sheep following antigenic challenge; a 100 day old foetus injected with MON-DNP, LPS and OA/FCA.

Days after Injection

anti-sheep serum

anti-sheep IgM

anti-sheep IgG

samples were reacted with anti-whole sheep serum after electrophoresis, IgM and IgG_1 were found to be present in samples from day 20 onwards. The concentration of both these immunoglobulins appeared to increase after day 20. The presence of these foetal immunoglobulins was confirmed by reacting the plasma after electrophoresis with anti-sheep IgM serum and anti-sheep IgG serum. IgG_2 and IgA were not detected in the plasma of this foetus by immunoelectrophoresis.

A second foetus was injected at 101 days gestation with MON, *S. typhimurium*, FER AND CγG, the last two antigens being emulsified in FCA. The foetus produced haemagglutinating antibody to MON, FER and CγG during the first 2 weeks of the response, all of which was sensitive to 2-ME The foetus was rechallenged with *S. typhimurium* on day 19 and recannulated. After day 21 the antibody to CγG was entirely resistant to 2-ME (titres 4.0 to 5.5), although the anti-FER antibody remained sensitive to 2-ME (titres 6.0 to 7.0). No antibody was formed to *S. typhimurium*. The sera obtained on days 0, 6, 12, 21, 25, 29, 33 and 37 after challenge were analysed by immunoelectrophoresis (Figure 4-ii). IgM was detected in the plasma collected from day 12 onwards, while IgG_1 was not detected until 25 days after challenge. The presence of IgM in the sample collected on day 12 was confirmed with monospecific anti-IgM serum; anti-IgG serum revealed that low concentrations of IgG_1 were also present on days 12 and 21, increasing to easily detectable levels by day 25. Traces of IgG_2 were detected on days 33 and 37 after antigenic challenge.

The immunoglobulins detected in both these foetuses had similar electrophoretic mobilities to immunoglobulins from adults. The technique of immunoelectrophoresis appeared much less sensitive than radial immunodiffusion, as samples giving positive reactions in the latter assay failed to produce detectable reactions by immunoelectro-

Figure 4-ii:

Immunoelectrophoretic analysis of plasma from a foetal sheep following antigenic challenge; a 101 day old foetus injected with MON, *Salmonella typhimurium*, FER/FCA and CγG/FCA.

Days after Injection

Adult serum

anti-sheep serum

anti-sheep IgM

anti-sheep IgG

phoresis.

(ii) Gel filtration and double-diffusion

A number of foetal and adult serum and plasma samples were fractionated by Sephadex G200 column chromatography. Two millilitres of whole adult serum or 3 to 4 ml of foetal serum were concentrated to 1 ml for loading onto the column. The $OD_{280m\mu}$ was read for each fraction and from a plot of OD against tube numbers the foetal samples were divided into 5 fractions:

#1 - the ascending slope and top of the first peak

#2 - the trough before the second peak

#3 - the second peak

#4 - the trough before the third peak and the ascending slope of the third peak

#5 - the top and descending slope of the third peak.

An additional fraction was prepared from the serum sample of adult sheep because of their higher protein concentrations:

#4 - the trough before the third peak

#5 - the ascending slope of the third peak including the peak

#6 - the descending slope of the third peak.

The two sera from adult sheep gave very similar profiles [Figure 4-iii (a) and (b)]. The protein in each fraction was concentrated by Diaflo and Minicon filtration to 10 mg/ml, as estimated from the OD. Double-diffusion analysis against monospecific antisera showed IgM in fractions 1 and 2, IgG_1 and IgG_2 in fractions 2, 3 and 4 and IgA in fraction 2. The double-diffusion analysis of one of the fractionated serum samples from an adult sheep is shown in Figure 4-iv (a), while the immunoelectrophoretic analysis is shown in Figure 4-iv (b); the fractions separated by electrophoresis were developed against anti-whole sheep serum, anti-sheep IgM and anti-sheep IgG.

Figure 4-iii (a) and (b):

Elution profiles of Sephadex G200 chromatography analysis of sera from adult sheep.

Figure 4-iv (a):

Double-diffusion analysis of fractions, obtained by Sephadex G200 chromatography, of serum from an adult sheep.

1 → 6: Fraction Numbers.

Figure 4-iii(a) and (b)

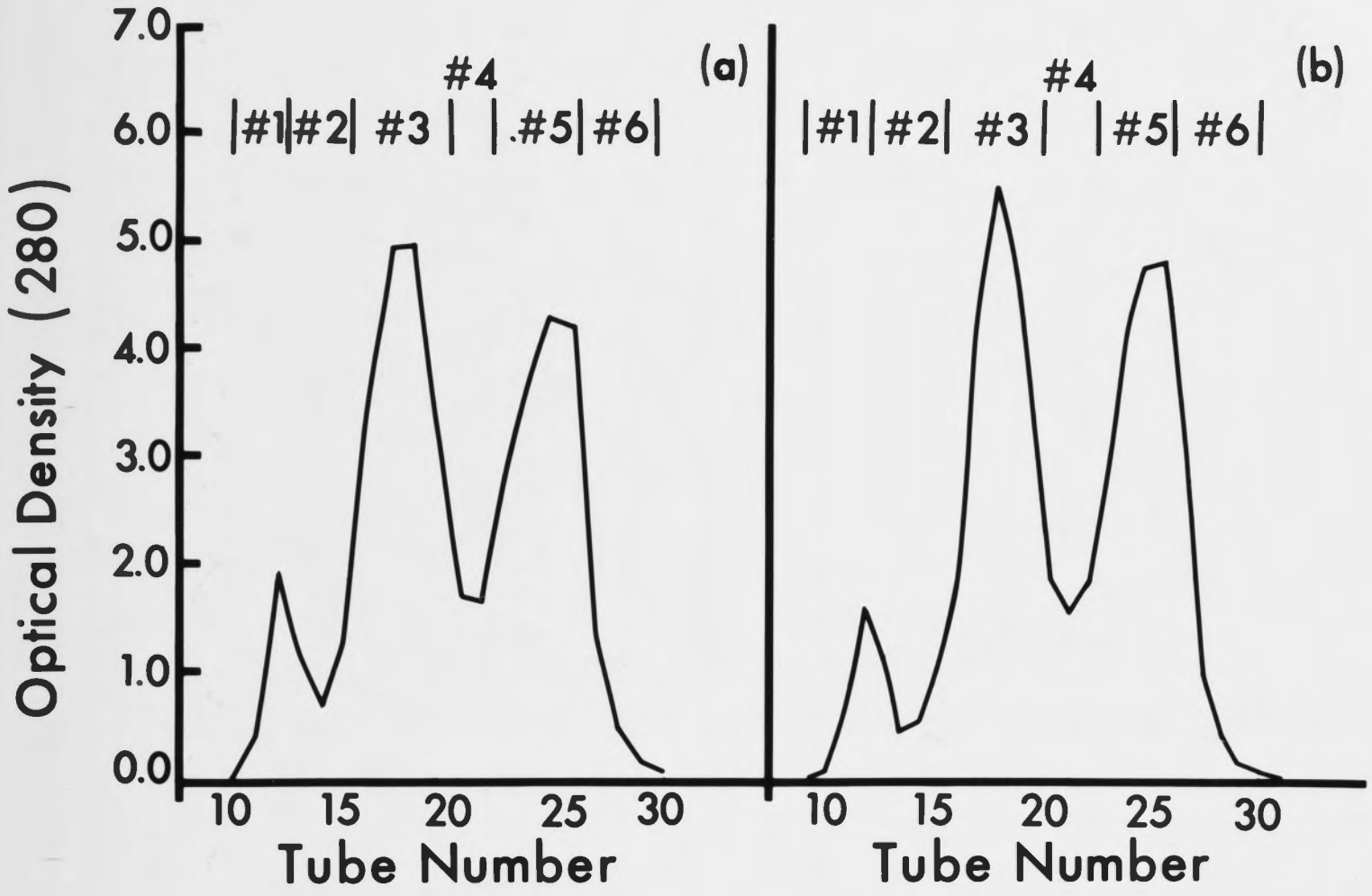
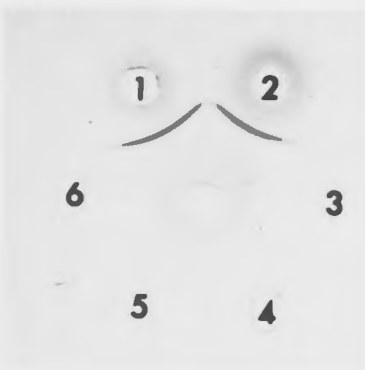


Figure 4-iv(a)

anti-IgM



anti-IgG$_1$

anti-IgG$_2$

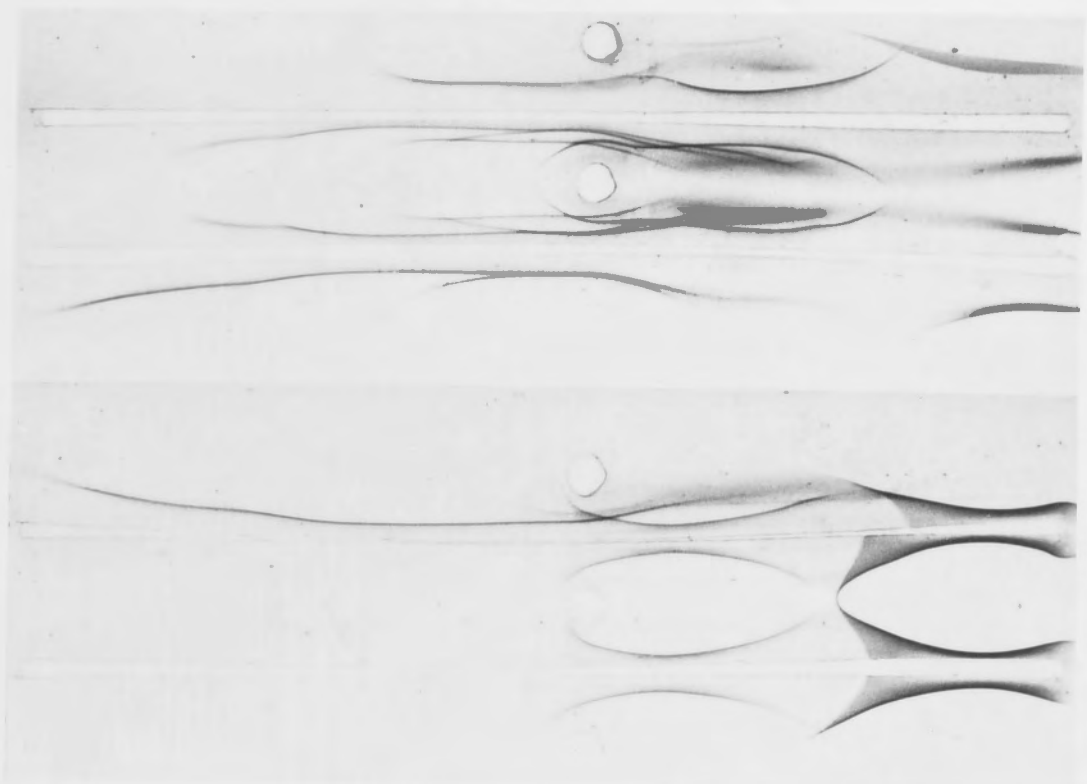
anti-IgA

Figure 4-iv (b):

Immunoelectrophoretic analysis of fractions, obtained by Sephadex G200 chromatography, of serum from an adult sheep.

Fraction Number

#1
#2
#3
#4
#5
#6



anti-sheep serum

#1
#2
#3

anti-sheep IgM

#1
#2
#3
#4
#5
#6

anti-sheep IgG

Plasma obtained from a 58 day old foetus by pooling samples taken on days 6, 8, 10, 12 and 14 after challenge and serum obtained 14 days after the injection of antigen into a 60 day old foetus were chromatographed and divided into 5 fractions, (the two fractionations are shown in Figure 4-v (a) and (b) respectively). Fractions 1 to 3 were concentrated to 0.5 ml, while fractions 4 and 5 were concentrated to 10 mg/ml. When the samples were analysed by double-diffusion IgM was seen only faintly in fractions 1 and 2, while IgG_1 was detected in fractions 3 and 4 of the serum obtained 14 days after challenging a 60 day old foetus; the fractions from the other foetus were negative (Figure 4-vi). Neither IgG_2 nor IgA was detected by double-diffusion analysis.

The sera from two older foetuses were also analysed. These sera had been obtained 15 days after challenging a 113 day old foetus and 28 days after challenging a 79 day old foetus. The fractionation profiles for the two sera are shown in Figures 4-vii (a) and (b). Fraction 1 of both serum samples gave a strong reaction with anti-IgM on double diffusion while fraction 2 gave a faint reaction with anti-IgM. A strong reaction with anti-IgG_1 was detected in fractions 2 and 3, while a very faint precipitate was detected with anti-IgG_2 in fraction 3 of the serum from the 113 day old foetus. Fractions 4 and 5 were free of immunoglobulin. The double-diffusion patterns of the older foetuses are shown in Figure 4-viii (a), while the immunoelectrophoretic analysis with anti-whole sheep serum and anti-sheep IgG are shown in Figure 4-viii (b). Although IgM was not detected by this latter technique, IgG_2 was detected in fraction 3.

IgA was not detected in foetal serum or plasma even though each fraction was concentrated 6 to 8 fold. IgG_1 was never detected in the first peak obtained by G200 column chromatography (fraction 1), while

Figure 4-v (a) and (b):

Elution profiles of Sephadex G200 chromatography analysis of (a) pooled plasma from a foetus injected with antigen at 58 days gestation and (b) serum obtained on day 14 from a foetus injected with antigen at 60 days gestation.

Figure 4-vi:

Double-diffusion analysis of fractions of serum, obtained by Sephadex G200 chromatography, from the foetus injected with antigen at 60 days gestation.

1 → 5: Fraction Numbers.

0 : Original Serum.

Figure 4-v(a) and (b)

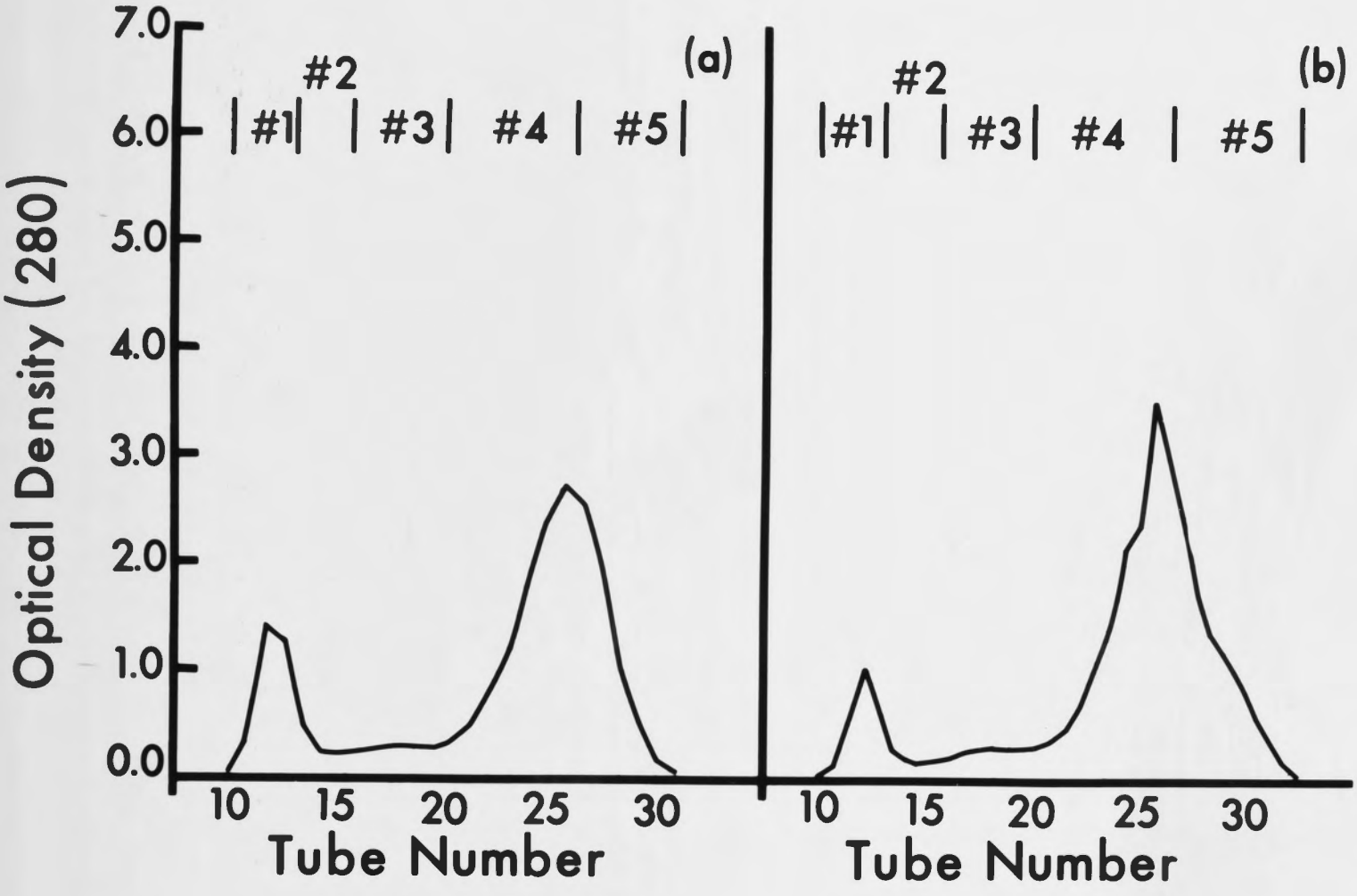
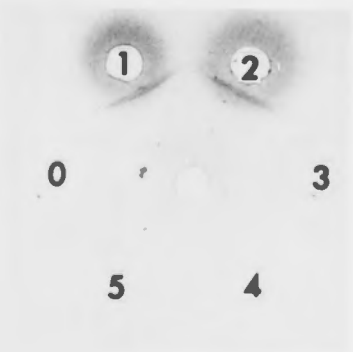


Figure 4-vi

anti-IgM



anti-IgG_1

Figure 4-vii (a) and (b):

Elution profiles of Sephadex G200 chromatography analysis of (a) serum obtained 15 days after antigenically challenging a 113 day old foetus and (b) serum obtained 28 days after challenging a 79 day old foetus.

Figure 4-viii (a):

Double-diffusion analysis of fractions of serum, obtained by Sephadex G200 chromatography, from a foetus injected with antigen at 113 days gestation.

1 → 5: Fraction Numbers.

0 : Original Serum.

Figure 4-vii(a) and (b)

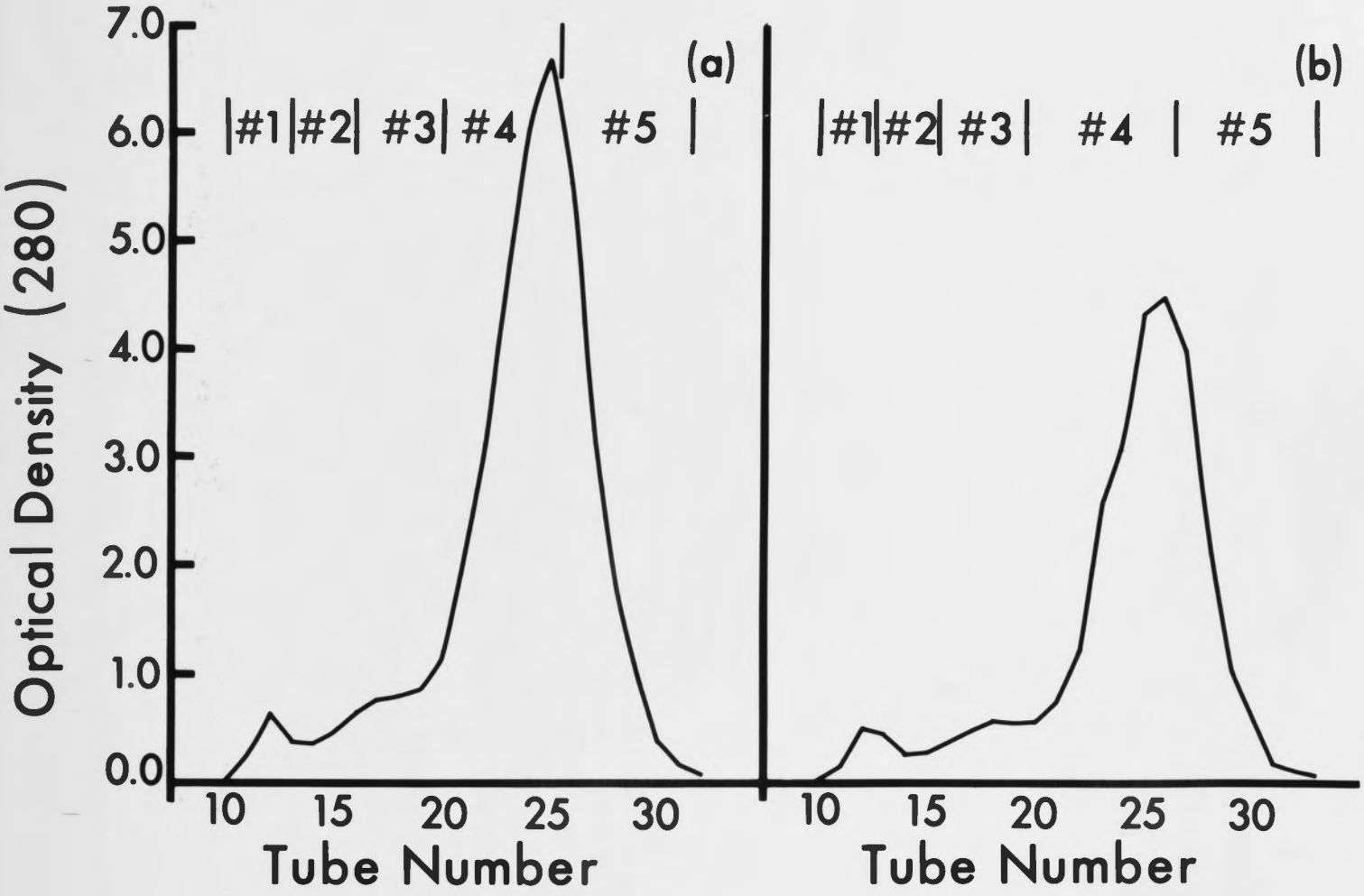


Figure 4-viii(a)

anti-IgM

anti-IgG$_1$

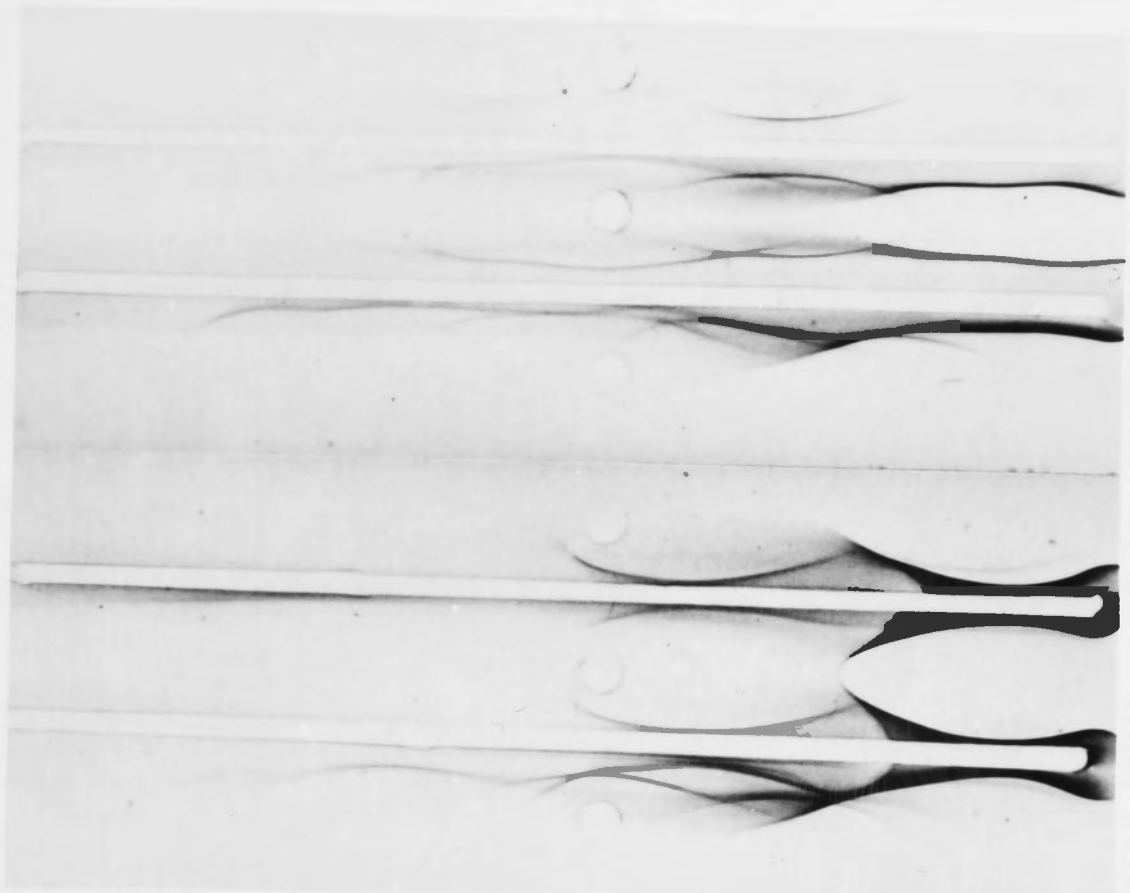
anti-IgG$_2$

Figure 4-viii (b):

Immunoelectrophoretic analysis of fractions, obtained by Sephadex G200 chromatography, from a foetus injected with antigen at 113 days gestation.

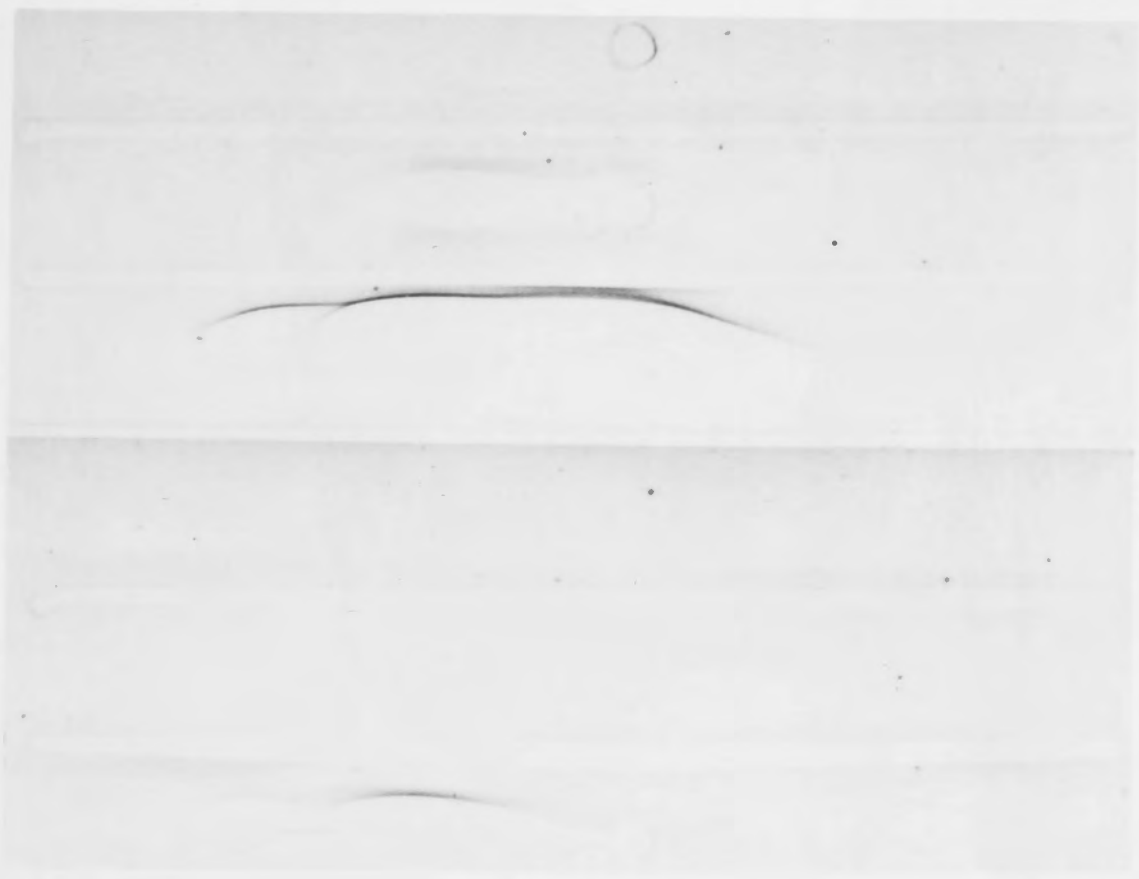
anti-sheep serum

#1
#2
#3
#4
#5
Orig.



anti -sheep IgG

#1
#2
#3
#4
#5
Orig.



IgM was never detected in the second peak (fraction 3). IgG_1 was detected in the fraction incorporating the trough and ascending portion of the albumin peak (fraction 4). Immunoglobulin was not found in the descending portion of the albumin peak (fraction 5).

(iii) Isoelectric focusing

Changes occurring in the proteins present in foetal serum during an immune response were characterized further by isoelectric focusing (IEF). Plasma samples (40 µl) from a 101 day old foetus challenged with MON, LPS, FER/FCA and CγG/FCA, which had been analysed by immunoelectrophoresis (Figure 4-ii), were subjected to IEF on a pH 3.5 to 10.0 gradient; samples of adult sheep IgG_1 and IgG_2 were analysed together with the foetal plasma to identify the regions where these immunoglobulins characteristically focused. The sample of plasma collected before the injection of antigen (Figure 4-ix) was devoid of proteins in the IgG_1 or IgG_2 regions. By day 12 faint bands of protein were found in the IgG_1 region and the number and intensity of bands in this region increased until day 33. From day 29 onwards a protein band was detected in the IgG_2 region of the plate. At day 37 some of the bands in the IgG_1 region of the plate decreased in intensity or disappeared.

The fractions of adult and foetal serum obtained by G200 column and DEAE cellulose chromatography were analysed by IEF, to characterize further the immunoglobulins detected in foetal serum during immune responses.

Forty µl samples of G200 fractions of serum collected on day 15 from a foetus challenged at 113 days gestation were focused on a pH gradient from 3.5 to 10.0 [Figure 4-x (a)]. These fractions had been analysed by double-diffusion (Figure 4-viii). Fraction 1 failed to penetrate the gel, while fraction 2 contained protein which migrated

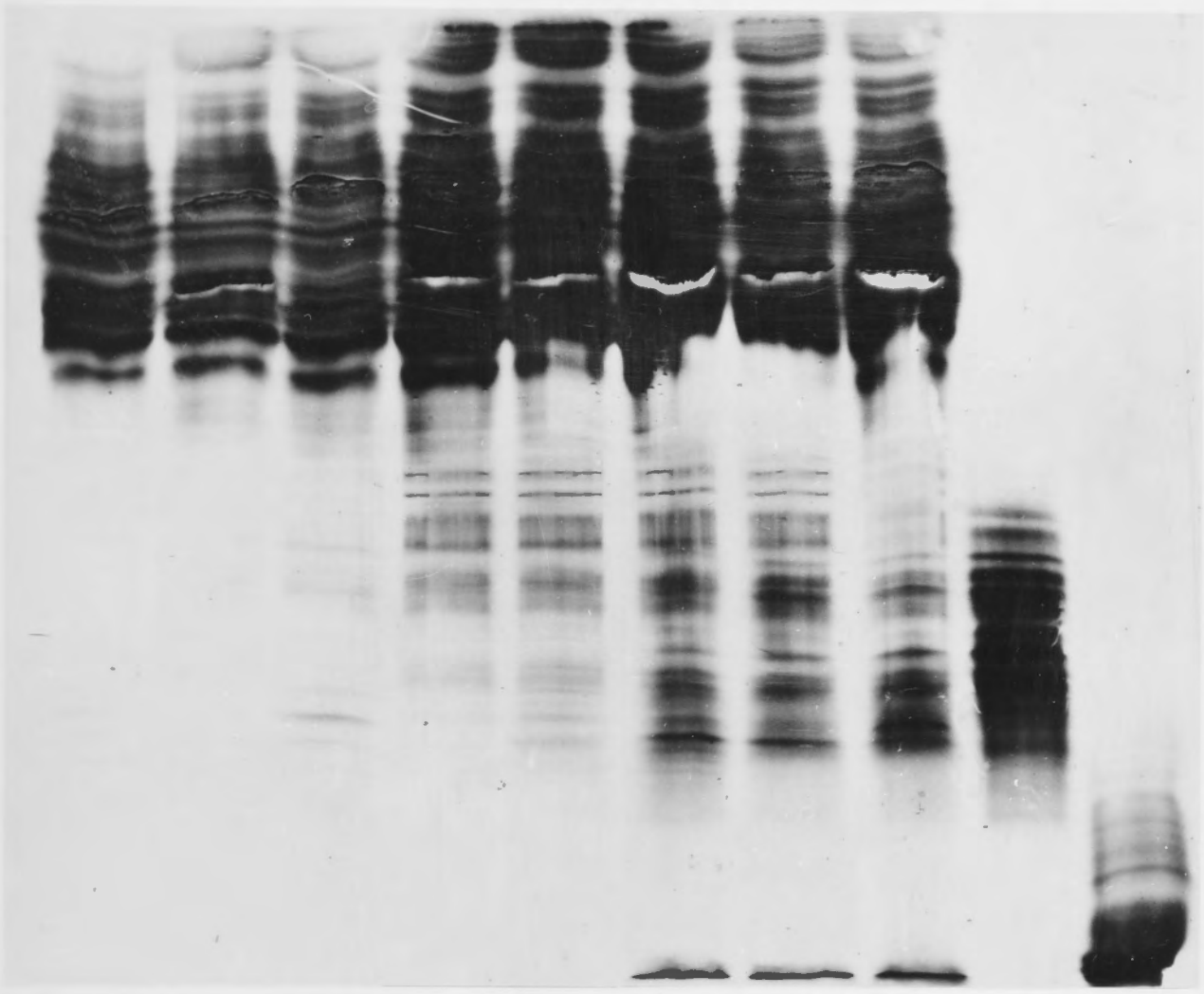
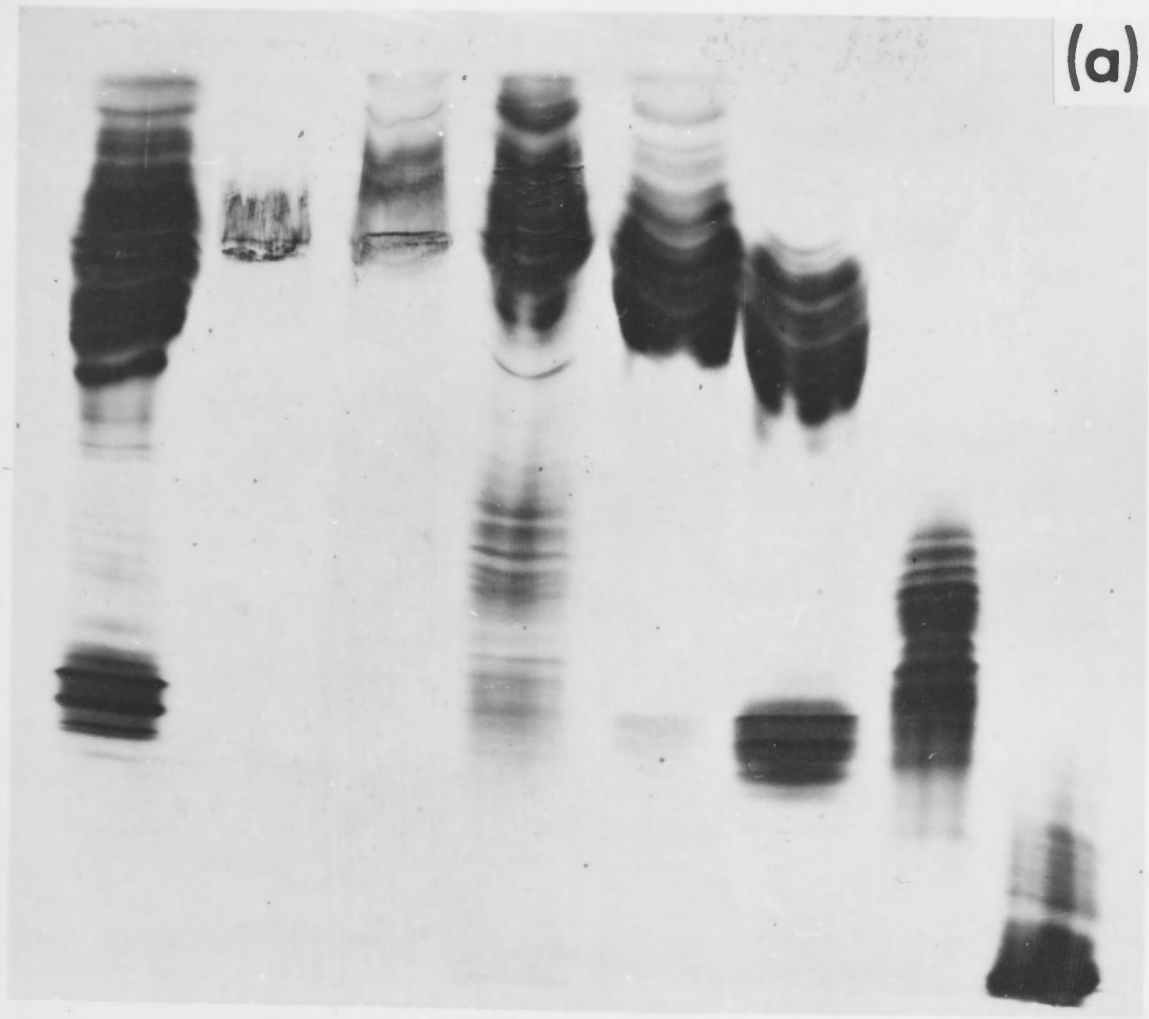
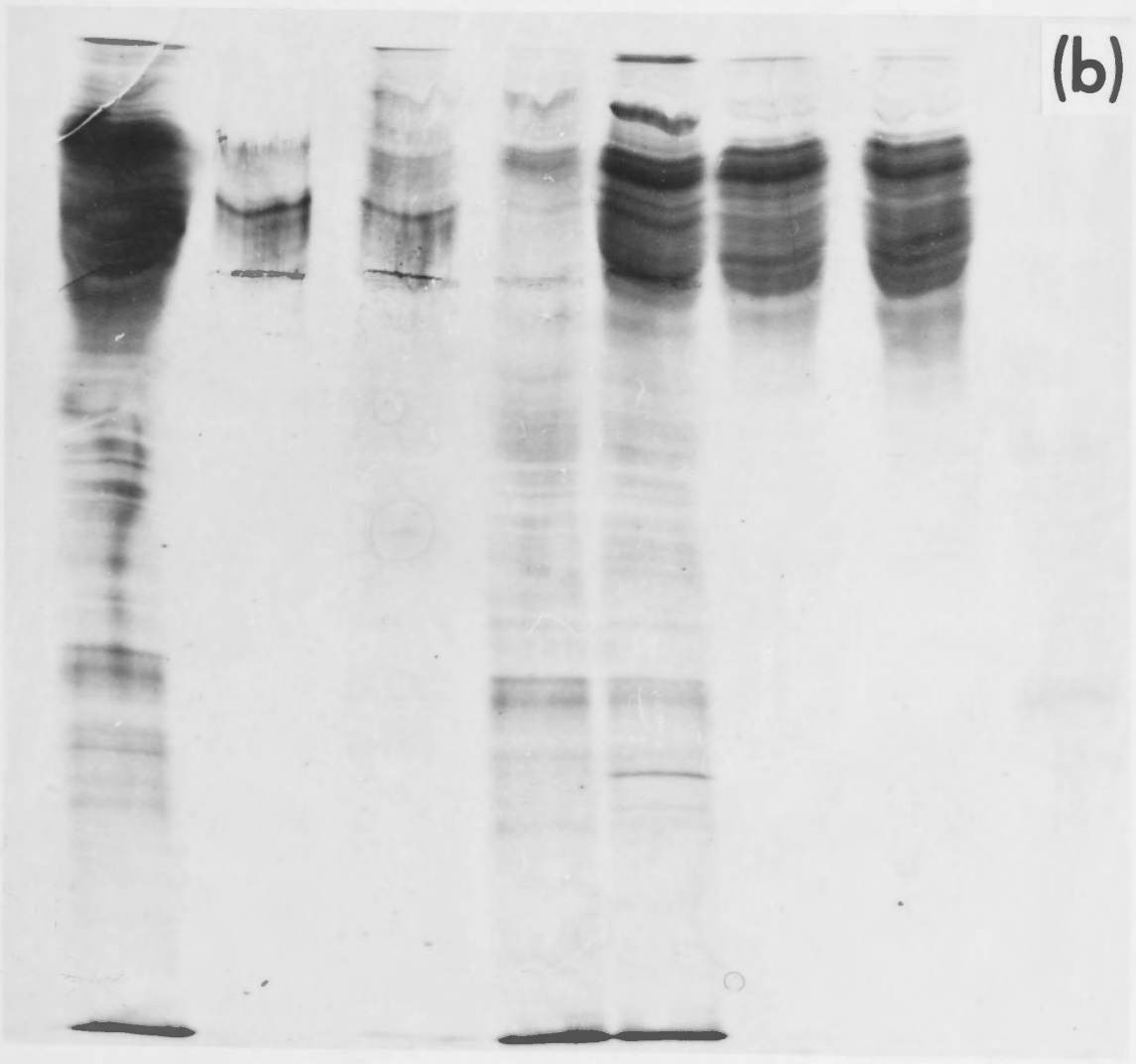


Figure 4-ix: Isoelectric focusing of plasma obtained from a foetal sheep following antigenic stimulation at 101 days gestation.

Figure 4-x (a) and (b):

Isoelectric focusing of fractions, obtained by Sephadex G200 chromatography, of (a) serum from a foetus challenged with antigen at 113 days gestation and (b) serum from an adult sheep. The IgG_1 and IgG_2 were obtained by DEAE cellulose chromatography of serum from an adult sheep.


(a)
pH
3.5
10.0
Orig.
#1
#2
#3
#4
#5
IgG1
IgG2


(b)
5.0
9.0
Orig.
#1
#2
#3
#4
#5
#6
IgG1

towards the anode. Fraction 3 contained the bulk of IgG_1 together with proteins which migrated anodally or remained at the application point. Fractions 4 and 5 contained protein which focused cathodally to the application point. Fraction 5 contained most of the haemoglobulin from the original serum.

G200 fractions of serum from an adult sheep were similarly analysed except that the pH range of the plates was 5.0 to 9.0 and 20 µl samples were loaded onto the plates. These fractions had been analysed by double-diffusion [Figure 4-iv (a)]. The IEF patterns [Figure 4-x (b)] were similar to those of the fractions of foetal serum except that proteins focusing in the IgG_1 and IgG_2 regions were detected in fractions 3 and 4. Proteins focusing in the region of albumin were detected in fractions 4, 5 and 6.

DISCUSSION

Only the IgM class of immunoglobulin appeared during the primary immune response in foetuses injected with antigen before 70 days gestation. After 80 days gestation the majority of animals produced both IgM and IgG_1 in response to antigenic stimulation.

Although the sequential expression of different classes of immunoglobulins during development may be deduced from published data, this is the first formal demonstration that such a sequence occurs in the developing foetus. This finding may have some significance in terms of the postulated "switch" from IgM to IgG synthesis during an immune response and also in the role of "cell collaboration" in immunoglobulin synthesis. The results obtained may in part reflect a quantitative phenomenon, since IgG_1 was detected in concentrated fractions of sera obtained 14 days after antigenically stimulating 60 day old foetal sheep (Figure 4-vi). This cannot be the complete explanation, however, as the ratio of IgM to IgG_1 production was significantly less in foetuses

challenged late in gestation, particularly when the response was analysed 14 days after the administration of antigen.

There seems no doubt that a proportion of normal foetal sheep synthesize IgM and to a lesser extent IgG_1 during gestation and this is also the case in foetal pigs and cattle. The percentage of foetuses which have detectable levels of IgM increases from 70 to 120 days gestation, at which time 60% of the foetuses had IgM in their circulation. A more sensitive analytical technique, for example, radioimmunoassay would almost certainly show that a higher percentage of normal foetuses have detectable immunoglobulin levels. Using the technique of radial immunodiffusion, only a small percentage of late term foetuses had IgG_1 in their circulation, while none was detected with IgG_2 or IgA. The stimulus, if in fact a stimulus is necessary for this low level synthesis of immunoglobulin, is not known. It may be endogenous antigen, for example, meconium (Ruebner, Kanayama, Bronson and Blumenthal, 1974) or exogenous antigen reaching the foetus as a result of intermittent breakdown of the placental barrier, possibly due to infection.

Foetuses older than 80 days gestation when challenged with antigen all reached similar levels of IgM in their circulation, irrespective of their age. While this represents a greatly increased production of IgM by the older animals due to their much greater extracellular volume, it seems to indicate that there is some control which regulates the concentration of immunoglobulins in the serum of foetal sheep.

The immunochemical and physicochemical analysis of immunoglobulins produced by foetuses in response to antigenic challenge suggested that these immunoglobulins were similar in character to immunoglobulins from adults. Foetal IgM migrated electrophoretically and on column chromatography in a manner similar to adult 19S IgM,

while foetal IgG_1 appeared to be the 7S monomer. The trace amounts of IgG_2 produced by older foetuses also appeared to be 7S by comparison with adult IgG_2. No evidence of 19S IgG or 7S IgM molecules was found during these analyses. A 4S IgG molecule has not been totally excluded, although it seems unlikely as IgG was not found throughout the albumin peak.

The appearance of trace amounts of IgG_2 late in the immune response of foetal sheep, may indicate that its synthesis follows IgG_1 although this is quite speculative. The failure of antigen to stimulate IgA synthesis in the foetus could be interpreted to indicate that competence to produce this class of immunoglobulin appears sequentially after IgM, IgG_1 and IgG_2 synthesis occurs. However, the route of injection of the antigen may be important in determining the synthesis of IgA, for this class of immunoglobulin appears to be intimately involved with the gut associated lymphoid tissues (Beh, Watson and Lascelles, 1974).

CHAPTER 5

THE RESPONSE OF FOETAL SHEEP TO THE INJECTION OF A SINGLE ANTIGEN

CHAPTER 5

The Response Of Foetal Sheep To The Injection Of A Single Antigen

INTRODUCTION

The results obtained from experiments in which foetuses were injected with several antigens simultaneously suggested that some generalized interpretations could be made about the qualitative and quantitative competence of foetal sheep to respond to various antigenic challenges. In order to characterize further the immune capability of foetal sheep, it was necessary to study their response to the injection of a single antigen. It was judged that experiments of this type would enable an analysis to be made of the relationship between antibody titre and the synthesis of immunoglobulin. The amount of specific and non-specific immunoglobulin synthesized in response to a single antigen, and any changes that occurred in the circulating blood leucocyte levels and fixed lymphoid tissues could also be studied.

Since the beginning of the century it has been known that the concentration of gamma-globulin in the serum increases during an immune response (Glaessner, 1905-6; Moll, 1906). The first attempt to quantitate the relationship between the amount of immunoglobulin and the amount of antibody produced during an immune response (Boyd and Bernard, 1937) resulted in the concept of "non-specific" synthesis of immunoglobulin. The production of non-specific immunoglobulin would seem anomolous in a physiological response based on the specificity of the product for the inducing agent. Urbain-Vansanten (1970) studied the production of immunoglobulin and antibody to tobacco mosaic virus (TMV). After injecting rabbits with relatively large amounts of TMV (60 mg in the primary response and 15 mg in the secondary response) she

found that the majority of IgG formed in the primary response had no demonstrable antibody activity, while in the secondary response nearly all IgG synthesized had antibody activity. Similarly de Vos-Cloetens, Minsart-Baleriaux and Urbain-Vansanten (1971) using large doses of serum albumins from various species (180 mg in the primary response) found non-specific IgG produced during the primary response of rabbits to the antigens, but little or no non-specific IgG formed during the secondary or tertiary responses. Roszman, Folds and Stavitsky (1971) obtained similar results by injecting alum-precipitated keyhole limpet haemocyanin into rabbits, but pointed out the possibility that "hidden" antigenic determinants may stimulate the synthesis of immunoglobulins which would not be detected in the antibody assay they used.

Recently Kolb, Di Pauli and Weiler (1974) injected newborn mice with *Salmonella anatum* and found that the mice showed a 10 to 30 fold increase in IgG synthesis, although no haemolytic antibody was produced. A 2 fold increase in spleen weight was also recorded in newborn mice which received *S. anatum*, but not in mice injected with other antigens. English (1974) found that the majority of IgM and IgG formed by adult sheep given a primary stimulus of salmonella LPS or swine influenza virus could not be adsorbed to either antigen. The role played by "hidden" antigenic determinants in the induction of non-specific immunoglobulin synthesis could not be estimated in these experiments. These observations in mice and sheep suggest that LPS may be an antigen which non-specifically stimulates the synthesis of immunoglobulin. In this regard LPS has been found to have certain activities not shared by other antigens. It has been found to be mitogenic for lymphocytes and stimulate IgM synthesis *in vitro* (Andersson, Sjoberg and Moller, 1972), to be a potent adjuvant (Neter, 1969) and to be able to substitute for thymus-derived lymphocytes in

certain immune responses (Jones and Kind, 1972; Coutinho and Moller, 1973; Watson, Epstein, Nakoinz and Ralph, 1973).

Experiments carried out by Miller, Ternynck and Avrameas (1974; 1975) and Avrameas (1975) demonstrated a 4 to 5 fold discrepancy, at the cellular level, between the number of immunoglobulin producing cells and the number of antibody producing cells in animals injected with horseradish peroxidase (HRP) in FCA or HRP in saline; only immunoglobulin synthesizing cells were detected following primary stimulation with HRP in saline. Although histochemical techniques were used to detect both immunoglobulin and antibody synthesizing cells, the sensitivity of the two techniques may not be comparable as one test depends on the affinity of a hyperimmune anti-immunoglobulin sera, while the other depends on the affinity of the antibody formed within the cell for the antigen. However, the observation that immunoglobulin-forming cells increase exponentially with a doubling time of 11.7 hours, whereas antibody-forming cells increase exponentially with a doubling time of 4.3 hours (Miller *et al*, 1975) cannot simply be explained by differences in the sensitivity of the assays. Using the same techniques Casenave, Ternynck and Avrameas (1974) found that some cells synthesizing immunoglobulin which had no demonstrable antibody activity, contained immunoglobulin of the same idiotype as some antibody producing cells. This result could occur if the technique was unable to demonstrate low affinity antibody in the cells synthesizing immunoglobulin. The authors interpreted the result as demonstrating the antigen specific synthesis of non-antibody immunoglobulin.

The issue of non-specific immunoglobulin synthesis really rests on the question of whether epitopes on antigens can stimulate the synthesis of immunoglobulin molecules which cannot subsequently be shown by the assay procedures employed, to have antibody activity. Whether

the assay techniques are capable of detecting low affinity antibodies or whether the assays detect antibodies directed to "hidden" determinants on the antigen would seem to be criticisms of all experiments which have shown non-specific immunoglobulin synthesis, except if specificity is defined in the functional terms dictated by the assay. Specific antibody assays rest on the assumption that antigenic determinants capable of specifically stimulating immunoglobulin synthesis will interact with the product that results from this stimulation. However, the capacity of an antigenic determinant to trigger immunoglobulin synthesis may be different from its capacity to react *in vitro* with the immunoglobulin synthesized. Similarly, the biological activity of the immunoglobulin produced in response to antigen and the assay procedure used to detect antibody *in vitro* may have no relationship and consequently the assay may have little value in deciding the specificity of the product resulting from antigenic stimulation, at least in any physiological terms.

RESULTS

In this Chapter two antigens have been selected for further investigation: POL because it was a very good immunogen in the foetus and *S. typhimurium* because it did not stimulate antibody synthesis in the foetus at all. Many of the studies undertaken in this chapter may best have been done with more defined antigenic determinants, however, the foetal sheep did not respond well to the haptenic determinant DNP (Chapter 3).

1. STUDIES ON THE IMMUNE RESPONSE OF FOETAL SHEEP TO POLYMERIZED FLAGELLIN (POL)

The characteristics of the immune response of foetal sheep to

POL have been studied extensively. POL induced specific antibody formation when injected into foetuses throughout the last half of gestation, did not require adjuvant to be effective and stimulated both IgM and IgG_1 synthesis. Polymerized flagellin is composed of numerous identical subunits referred to as flagellin or monomeric flagellin. The monomer has a molecular weight of 41,000, is approximately 16 nm long, 5 nm wide and 2 nm thick (Bode, Engel and Winklmair, 1972). From an estimated length of 15 µm (Iino, 1969) the polymer with a mass/length ratio of 68,000/nm (Bode *et al*, 1972) would have a molecular weight between 100 to 1,000 million. Functionally, polymeric and monomeric flagellin have the same antigenic determinants (Parish, Wistar and Ada, 1969).

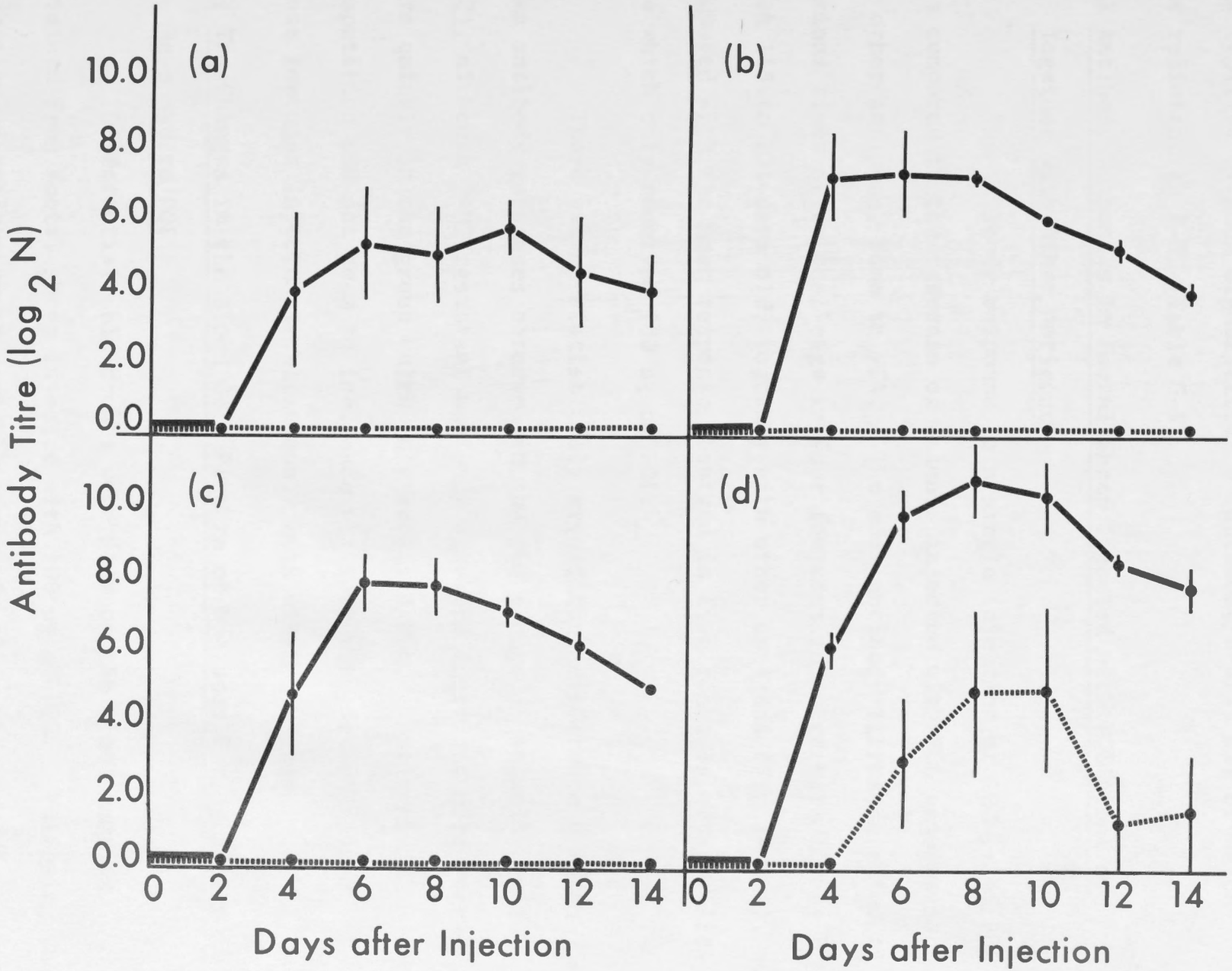
(a) Antibody Responses of Foetal Sheep to POL

Three foetuses between 70 and 71 days gestation, three foetuses between 79 and 87 days gestation, four foetuses between 97 and 100 days gestation and five foetuses between 119 and 120 days gestation were injected i.m. with 100 µg of POL and blood samples obtained every second day through indwelling venous cannulae. The total haemagglutinating antibody response and the 2-ME resistant antibody response of each group of foetuses are shown in Figure 5-i. The magnitude of the antibody response increased with increasing mean gestational age for each group of foetuses. Antibody was detected in the plasma of all foetuses by 4 days after challenge. The peak antibody response occurred in younger animals 4 to 6 days after the injection of antigen; in older foetuses the peak of the response did not occur until 6 to 8 days after challenge. Only animals challenged around 120 days gestation produced 2-ME resistant anti-POL antibody during the primary immune response [Figure 5-i (d)].

The response to a second injection of 100 µg of POL was

Figure 5-i:

Passive haemagglutinating antibody response of groups of foetal sheep at different stages of gestation to 100 µg POL. (a) mean age 70 days, (b) mean age 82 days, (c) mean age 99 days and (d) mean age 119 days. Total antibody - solid lines: 2-ME resistant antibody - broken lines. Standard errors indicated by vertical bars.


(a)
(b)
(c)
(d)
Antibody Titre ($\log_2$ N)
10.0
8.0
6.0
4.0
2.0
0.0
0 2 4 6 8 10 12 14
Days after Injection
Days after Injection

studied in three foetuses between 111 and 114 days gestation, 27 to 32 days after receiving the first injection of antigen. The response was typified by much higher titres of antibody, nearly all of which was resistant to 2-ME (Table 5-1).

(b) Antibody Responses in Foetal Sheep Injected with POL Alone or Together with Other Antigens

The antibody response to a single injection of 100 µg of POL was compared to the response of animals injected with POL accompanied by other antigens, some in FCA. The mean antibody titres detected at various times after challenge in four foetuses injected with 100 µg POL when 118 to 121 days old, together with other antigens (Figure 3-i), was compared with the mean response observed in five foetuses of a similar age which only received 100 µg of POL.

There was no statistically significant difference between the mean antibody responses observed in the two groups of animals (Table 5-2), although 2-ME resistant antibody appeared later and disappeared more quickly in the group which only received POL. Antigenic competition did not seem to influence the responses detected to POL in those foetuses injected simultaneously with other antigens.

(c) The Changes in the Blood Cell Picture of Foetuses During an Immune Response to POL

Differential cell counts were done on the blood samples obtained from foetal sheep injected with 100 µg of POL. Following the injection of antigen great variability was found in the blood leucocyte responses of different animals at the same stage of development and in fact between twin foetuses receiving identical treatment.

Two foetuses challenged at 79 and 80 days gestation both showed a 2 fold increase in the concentration of circulating lymphocytes following the injection of antigen. The peak concentration of

Table 5-1: Antibody response of foetal sheep to a second injection of 100 µg of POL

Haemagglutinating antibody	Anti-POL antibody titre on day							
	0	2	4	6	8	10	12	14
Total	3.2±1.2*	3.3±0.7	10.0±2.4	12.7±1.1	12.7±0.6	13.2±0.2	12.7±0.2	12.2±0.2
2-ME resistant	2.0±0.8	3.0±1.1	10.0±2.0	12.7±1.0	13.0±1.5	13.0±1.0	13.0±0.5	12.0±0.5

* Standard error

Table 5-2: Primary antibody response of 120 day old foetal sheep to 100 μg POL injected alone or with other antigens

Antigenic challenge	Haemagglutinating antibody	Anti-POL antibody titre on day							
		0	2	4	6	8	10	12	14
Multiple	Total	<1.0	0.3±0.3	5.4±0.6	7.4±1.2	8.0±1.3	7.4±1.7	7.7±0.2	7.3±0.2
	2-ME resistant	<1.0	<1.0	2.3±0.5	3.8±1.1	5.3±1.0	4.9±1.0	4.7±1.0	4.5±0.9
Single	Total	<1.0	<1.0	6.1±0.5	9.8±0.7	10.8±1.2	10.3±1.0	8.5±0.3	7.8±0.6
	2-ME resistant	<1.0	<1.0	<1.0	3.0±1.8	4.9±2.3	5.0±2.4	1.3±1.3	1.7±1.7

lymphocytes in the blood of one foetus occurred only 1 day after the injection of antigen and in the other foetus 4 days after injection. The level of lymphocytes in both animals then fell before slowly returning to prestimulation levels. The changes in the level of neutrophils in the blood followed a similar pattern to the lymphocyte response, except that initially the concentration rose by at least 10 fold. Both animals received a second injection of antigen 32 days after the first injection. The lymphocyte level remained unchanged in one and showed a 2 fold increase in the other, while the concentration of neutrophils again showed a transitory 10 fold increase. A plot of one of these two animals is shown in Figure 5-ii.

The blood picture of foetal sheep injected with POL at 87, 100 and 120 days and twin foetuses injected at 99 days gestation was also studied. While the response observed in individual animals was quite variable, it was possible to draw some general conclusions. Following the injection of 100 μg of POL the level of circulating lymphocytes increased 2 to 3 fold. Peak lymphocyte levels usually occurred in the first 4 days of the response although in some foetuses the levels continued to increase up until day 10. Occasionally a decrease in the concentration of lymphocytes was recorded immediately following the injection of antigen. Such a response is shown in Figure 5-iii (a) which is from one of the twins injected at 99 days gestation. The other twin showed no decrease in lymphocyte concentration [Figure 5-iii (b)].

In contrast to the level of lymphocytes in the blood, the changes in the level of neutrophils following antigenic challenge were usually short-lived. A 10 to 20 fold increase in the concentration of neutrophils in the circulation was recorded in all foetuses and this usually occurred 1 to 2 days after the injection of antigen. The level

Figures 5-ii, iii and iv:

The changes in blood leucocyte levels in foetal sheep injected with 100 µg POL.

Figure 5-ii:

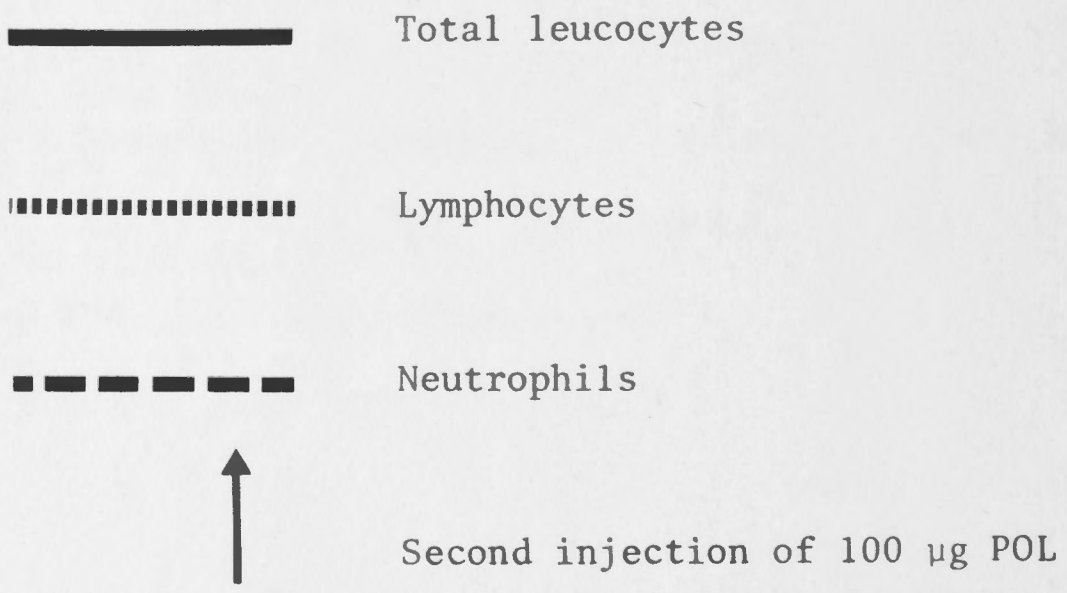
An 80 day old foetus.

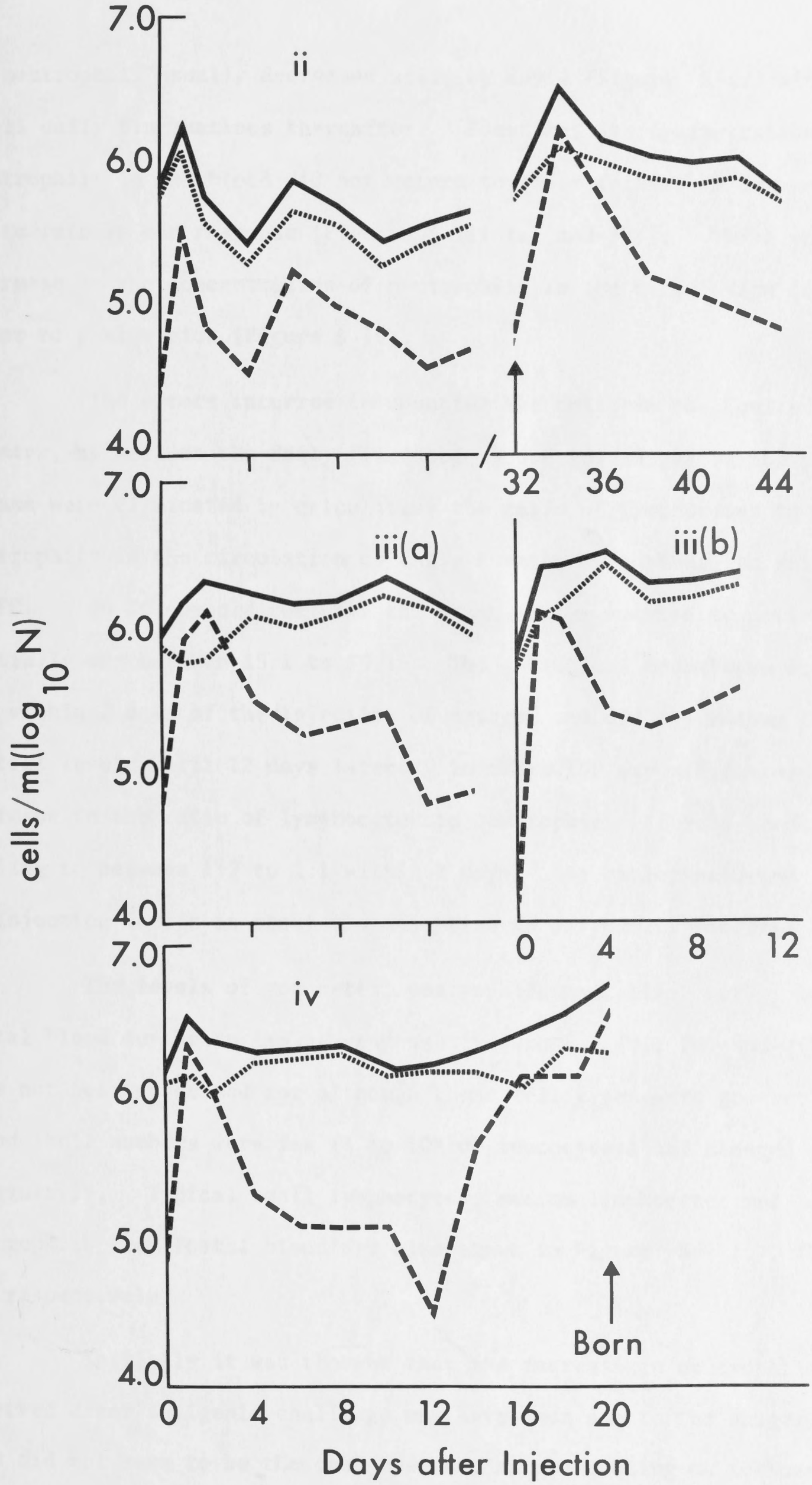
Figure 5-iii (a) and (b):

Twin 99 day old foetuses.

Figure 5-iv:

A 120 day old foetus.




ii
iii(a)
iii(b)
iv
7.0
6.0
5.0
4.0
32
36
40
44
0
4
8
12
16
20
Born
cells/ml(log10 N)
Days after Injection

of neutrophils usually decreased again by day 4 (Figure 5-iv) with small daily fluctuations thereafter. Sometimes the concentration of neutrophils in the blood did not return to their initial values until quite late in the response [Figures 5-iii (a) and (b)]. There was an increase in the concentration of neutrophils in the circulation just prior to parturition (Figure 5-iv).

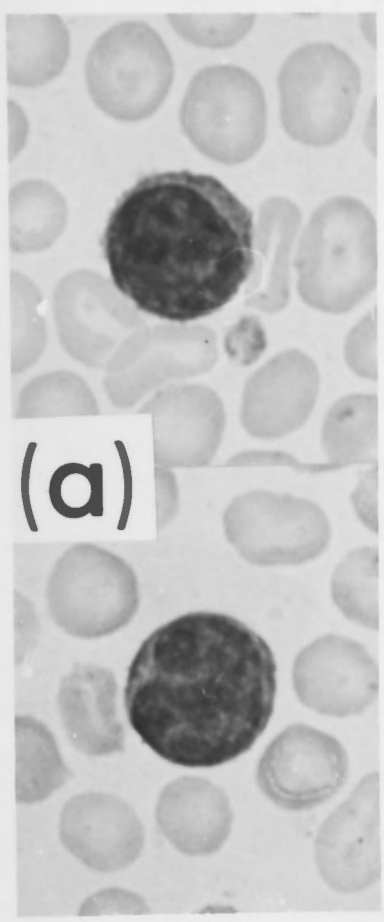
The errors incurred in counting the cells on the Coulter Counter, as well as the daily fluctuations due to changes in the plasma volume were eliminated by calculating the ratio of lymphocytes to neutrophils in the circulation of these animals throughout the response to POL. In 80 day old foetuses the ratio of lymphocytes to neutrophils initially was between 15:1 to 30:1. The ratio fell to between 3:1 to 4:1 within 2 days of the injection of antigen and did not return to the initial levels until 12 days later. In three 100 day old foetuses the decrease in the ratio of lymphocytes to neutrophils was even greater, falling to between 1:2 to 2:1 within 2 days; the ratios returned to preinjection levels in about the same time as in younger foetuses.

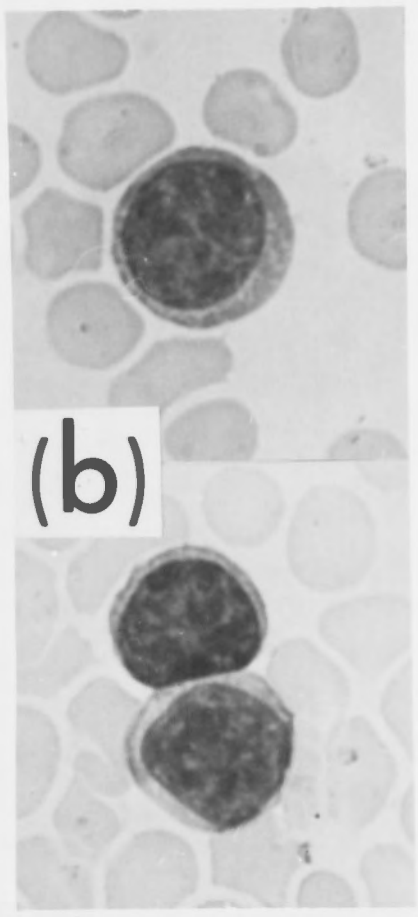
The levels of monocytes, eosinophils and blast cells in foetal blood during an immune response [Figure 5-v (d), (e) and (f)] have not been discussed for although those cell types were present in the blood their numbers were few (1 to 10% of leucocytes) and changed quite irregularly. Typical small lymphocytes, medium lymphocytes and neutrophils from foetal blood are also shown in Figure 5-v (a), (b) and (c) respectively.

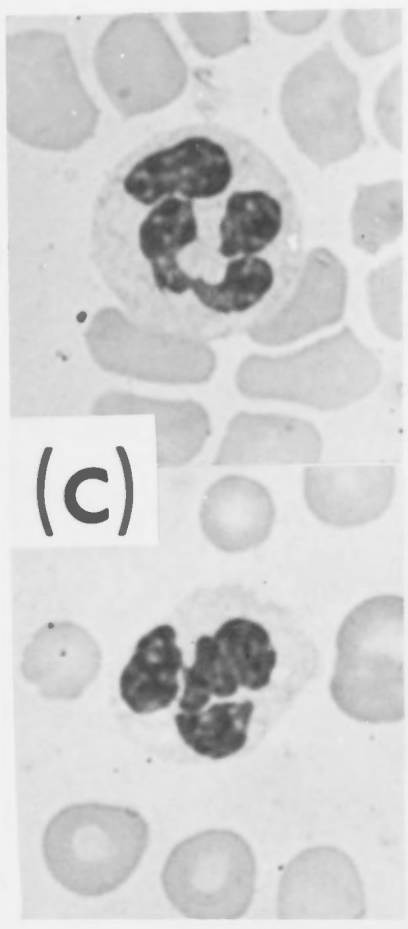
Initially it was thought that the increase in neutrophils observed after antigenic challenge may have been due to the surgery. This did not seem to be the case however, as reoperating on foetuses during the course of an immune response to replace venous cannulae or challenging foetuses with non-immunogenic antigens (see later) did not

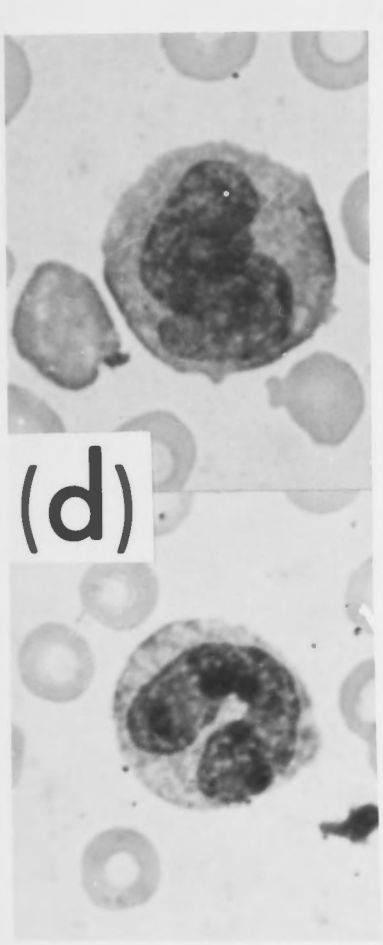
Figure 5-v:

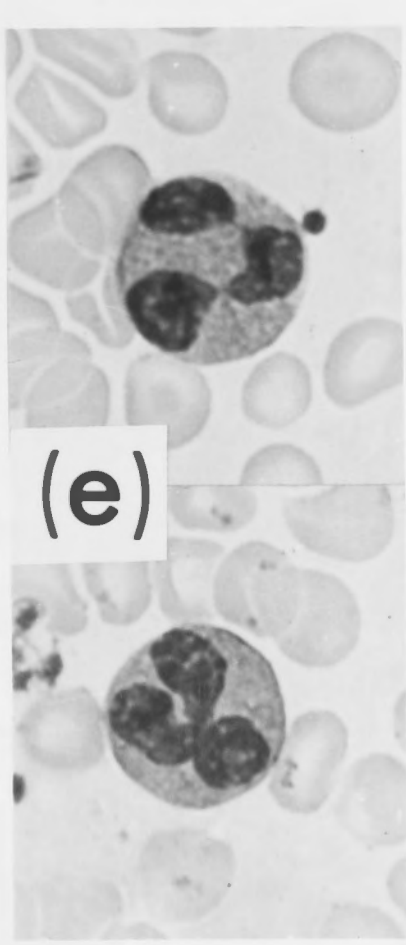
Appearance of blood leucocytes in normal foetal sheep (Leishman stain). (a) small lymphocytes, (b) medium lymphocytes, (c) neutrophils, (d) monocytes, (e) eosinophils and (f) blast cells. Magnification x 400.

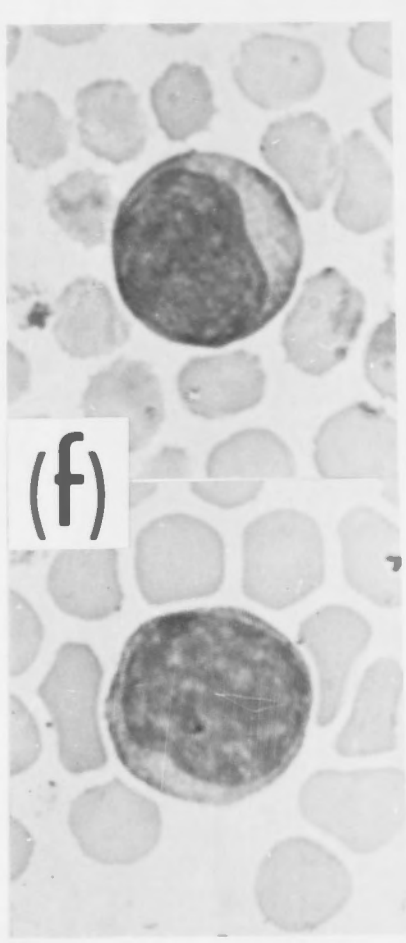

(a)


(b)


(c)


(d)


(e)


(f)

significantly alter the level of circulating neutrophils.

(d) Production of Immunoglobulin Following the Injection of POL

The immunoglobulin levels in the fifteen foetal sheep injected with POL were analysed by single radial immunodiffusion with monospecific rabbit anti-sheep immunoglobulin sera. IgM was synthesized by all animals injected with 100 µg of POL. The mean concentrations of IgM detected at 2 day intervals in foetuses injected at a mean age of 70, 82, 99 and 119 days gestation are shown in Table 5-3. Although the maximum concentration of IgM reached during the response did increase with the age of the animals when challenged, the variation between foetuses of the same age made it difficult to draw meaningful conclusions.

The concentrations of IgM in animals injected at 70 days gestation were lower than those in animals injected at an older age and at some times during the response the differences between the various groups of animals were statistically significant. However, the animal to animal variation both in onset of detectable IgM synthesis and in the quantity of IgM synthesized, made it impossible to make any biologically significant interpretations of the results. The higher mean concentration of IgM in the group of animals challenged around 119 days gestation was almost entirely due to one foetus; the concentration of IgM in this animal reached 1 mg/ml on days 8 and 10.

IgG_1 was detected in one foetus challenged at 79 days gestation and one foetus challenged at 100 days gestation, 10 days after the injection of POL (maximum concentrations of 130 and 678 µg/ml respectively). All animals challenged around 119 days gestation synthesized IgG_1 at some time during the first 2 weeks of the primary response and the mean concentrations detected during the response are shown in Table 5-3. Neither IgG_2 nor IgA was detected by radial immunodiffusion during the primary response of foetal sheep to

Table 5-3: Synthesis of immunoglobulin by foetal sheep following the injection of 100 µg POL

(a) Mean age - 70 days

IgM µg/ml	Days after injection							
	0	2	4	6	8	10	12	14
Mean ± S.E.	0	0	7 ± 7	25 ± 8	22 ± 12	35 ± 35	57 ± 57	-
Range	-	-	0 → 21	10 → 39	0 → 42	0 → 70	0 → 144	-
No. samples	3	3	3	3	3	2	2	-

(b) Mean age - 82 days

IgM * µg/ml	Days after injection							
	0	2	4	6	8	10	12	14
Mean ± S.E.	0	10 ± 10	12 ± 7	54 ± 31	62 ± 21	60 ± 20	42 ± 18	39 ± 18
Range	-	0 → 29	0 → 23	19 → 115	37 → 104	34 → 100	10 → 73	8 → 70
No. samples	3	3	3	3	3	3	3	3

*One foetus had detectable IgG_1 on days 10 to 14 (maximum concentration 130 µg/ml)

(c) Mean age - 99 days

IgM* µg/ml	Days after injection							
	0	2	4	6	8	10	12	14
Mean ± S.E.	9 ± 9	29 ± 18	35 ± 21	58 ± 25	95 ± 30	92 ± 22	62 ± 23	61 ± 9
Range	0 → 34	0 → 73	0 → 86	0 → 104	39 → 172	48 → 118	39 → 84	52 → 70
No. samples	4	4	4	4	4	3	2	2

* One foetus had detectable IgG_1 on days 10 to 14 (max. concentration 678 µg/ml)

(d) Mean age - 119 days

IgM µg/ml	Days after injection							
	0	2	4	6	8	10	12	14
Mean ± S.E.	39 ± 22	42 ± 12	50 ± 13	63 ± 12	276 ± 174	304 ± 189	108 ± 74	122 ± 95
Range	0 → 120	0 → 73	0 → 66	37 → 104	48 → 954	39 → 1050	23 → 256	27 → 217
No. samples	5	5	5	5	5	5	3	2
IgG_1								
Mean ± S.E.	6 ± 6	14 ± 9	14 ± 10	19 ± 12	93 ± 43	115 ± 51	86 ± 48	95 ± 47
Range	0 → 31	0 → 46	0 → 51	0 → 51	0 → 255	0 → 302	24 → 180	48 → 142

100 µg of POL.

(e) Production of Specific and Non-Specific Immunoglobulin by Foetal Sheep Injected with POL

Plasma samples taken from foetal sheep during the primary and secondary immune response to 100 µg of POL were assayed for their immunoglobulin concentrations by radial immunodiffusion before and after all detectable antibody had been removed by adsorption.

(i) Proportion of specific antibody formed during the primary immune response to POL

Plasma samples obtained from foetuses challenged at 79, 80 and 100 days gestation, at a time when either the antibody titre to POL had reached its peak or when the levels of IgM or IgG_1 had reached their maximum concentrations, were assayed for immunoglobulin concentrations before and after the removal of all detectable antibody by adsorption to POL. The antibody titre, both total and 2-ME resistant, and the concentration of immunoglobulin in each sample before adsorption are shown in Table 5-4. After adsorption the immunoglobulin concentrations were corrected for the dilution factor and the percentage of immunoglobulin removed by adsorption calculated.

One foetus, which produced a very good antibody response to POL, had 34% "specific" IgM 4 days after challenge and 42% 16 days after challenge. In the remaining two foetuses there was little "specific" IgM. The majority of IgG_1 synthesized by all three foetuses during the primary immune response also appeared to be "non-specific", including the foetus which had unusually high concentrations of IgG_1. This atypical foetus may have received an inadvertent antigenic stimulus at the time of surgery.

(ii) Proportion of specific antibody formed during the secondary immune response to POL

Table 5-4: Specific immunoglobulin synthesis to a primary injection of 100 µg of POL

Foetal age when challenged (days)	Time after injection (days)	Antibody titre and immunoglobulin concentration before adsorption			% Specific immunoglobulin	
		Titre (2ME-R*)	IgM	IgG_1	IgM	IgG_1
79	0	0.0	0	0	-	-
	10	6.0 (<1.0)	227**	94	0	0
	16	4.0 (<1.0)	100	214	8	3
80	0	0.0	0	0	-	-
	4	9.0 (<1.0)	132	0	34	-
	16	2.0 (<1.0)	73	109	42	15
100	0	0.0	0	0	-	-
	10	6.0 (<1.0)	21	180	0	0
	14	5.0 (<1.0)	57	554	9	5
	16	4.0 (<1.0)	32	523	22	0

* 2-ME resistant antibody

** µg/ml

The synthesis of "specific" and "non-specific" immunoglobulin by three foetuses given a second injection of POL at 111, 112 and 114 days gestation was analysed with the same techniques. The plasma obtained from each foetus, before the second injection of POL, was adsorbed to remove the low titres of anti-POL antibody persisting from the primary challenge and the concentration of non-antibody immunoglobulin determined. This was then subtracted from the concentration of immunoglobulin detected throughout the secondary response, before the percentage of specific immunoglobulin was calculated. The plasma samples were taken at the time when either the antibody titre or immunoglobulin concentrations had reached maximum values.

The concentration of IgM in the plasma of two of the foetuses decreased throughout the secondary response (Table 5-5). The third foetus had 8% "specific" IgM at day 8, although no "specific" IgM could be detected at day 18. All foetuses produced a maximum of between 42% and 100% "specific" IgG_1 during the secondary response to POL. The percentage of "specific" IgG_1 in one foetus was 96% 6 days after challenge and 100% 8 days after challenge and then decreased to 43% 12 days later. The level of "specific" IgG_1 remained fairly constant in the other two foetuses, being 35% and 62% respectively 18 days after the injection of antigen.

(f) Molecular Heterogeneity of Anti-POL Synthesized by Foetal Sheep

Following the observation that the immunoglobulin formed by one clone of cells produced a restricted banding pattern when subjected to isoelectric focusing (Williamson and Askonas, 1972; Askonas and Williamson, 1972) an attempt was made to analyse the specific immunoglobulins produced in response to POL by this technique. It was thought that an estimate of the number of clones of cells stimulated to produce antibody to POL in the foetus could be made if anti-POL antibody

Table 5-5: Specific immunoglobulin synthesis to a second injection of 100 µg of POL

Foetal age when challenged (days)	Time after injection (days)	Antibody titre and immunoglobulin concentration before adsorption			% Specific immunoglobulin	
		Titre (2ME-R*)	IgM	IgG_1	IgM	IgG_1
111	0	1.0 (<1.0)	58**	252	0	4
	6	11.0 (11.0)	36	457	0	96
	8	13.0 (13.0)	26	615	0	100
	20	12.5 (12.5)	29	419	0	43
112	0	5.0 (3.0)	74	124	7	13
	10	13.0 (13.0)	34	716	0	42
	18	12.0 (12.0)	39	519	0	35
114	0	3.5 (3.0)	63	0	0	0
	8	13.5 (13.5)	68	272	8	60
	18	11.0 (11.0)	21	245	0	62

* 2-ME resistant antibody

** µg/ml

could be identified on an isoelectric focusing (IEF) plate. Several different techniques were tried including overlayering the gels with ^{125}I labelled POL prior to exposing the plates to X-ray film (Williamson, 1971), overlayering the plates with POL coupled to HRP before developing the plates with diamino-benzidine, and finally using a complement-mediated lytic reaction (Phillips and Dresser, 1973) with SRBC coated with POL by the $CrCl_3$ technique. The use of ^{125}I labelled POL was not successful because the background binding of the iodinated antigen to the plate was very difficult to remove. Similarly the lytic technique did not produce discrete bands of lysis, although lysis in the region of the application wicks was observed. The use of HRP seemed most promising, as anti-HRP antibody in the lymph of an adult sheep undergoing a secondary immune response to HRP, could be identified on IEF plates. Coupling HRP to POL however, proved to be quite difficult, and only light coupling was ever achieved. It was not possible to identify the antibody-antigen complex over the background precipitation.

The samples obtained following the removal of anti-POL antibody from foetal plasma by adsorption were analysed by IEF. All samples were from secondary immune responses (Table 5-5), as little IgG_1 was formed during the primary response and the IEF technique was really only suitable for the analysis of IgG.

The proteins in the plasma of two of the three foetuses, which banded in the IgG_1 region of the IEF plates, were very faint and diffuse. However, plasma from the third foetus, obtained 8 days after the second injection of antigen, had one dense line in the IgG_1 region when analysed by IEF. This line totally disappeared following the removal of the antibody (Figure 5-vi). It seemed that at least in this foetus the 96% to 100% of IgG_1 which was removed by adsorption to POL

Figure 5-vi: Isoelectric focusing of plasma before (a) and after removal of anti-POL antibody by adsorption with sepharose-POL (b) and SRBC-POL (c). Note the disappearance of a single isoelectric band in IgG_1 region after adsorption.

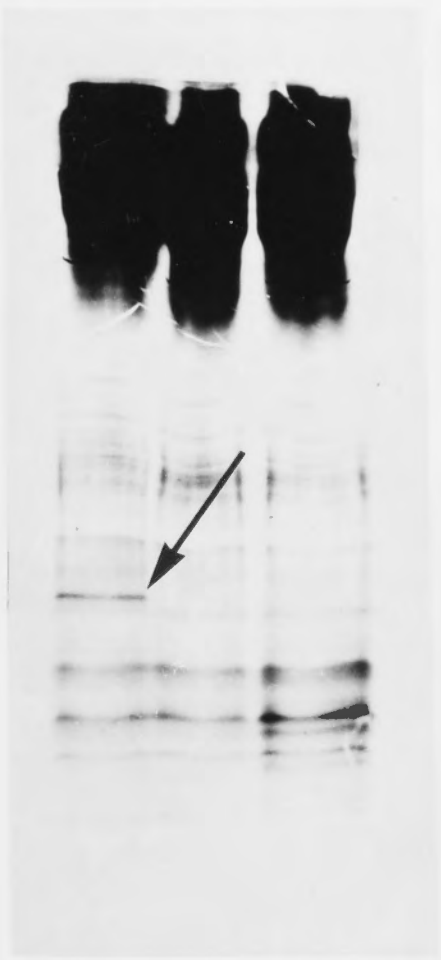
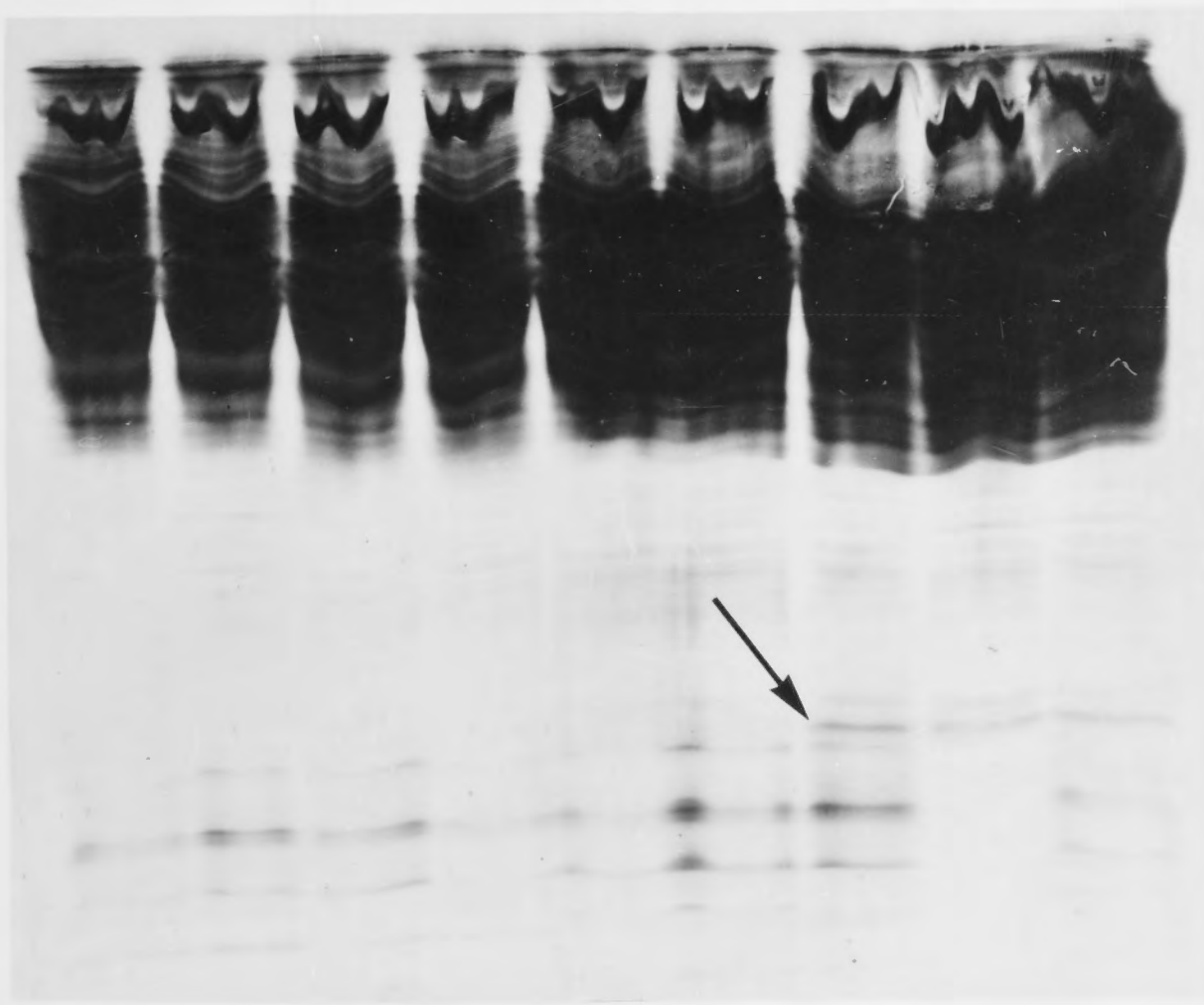


Figure 5-vii: Isoelectric focusing of plasma obtained during a primary and secondary response of a foetal sheep to 100 µg POL. Note the appearance of anti-POL IgG_1, 6 days after the second injection of antigen.



was represented by a single major IEF line. Several faint lines may also have disappeared after adsorption, but positive identification was beyond the limits of resolution of the technique. When the plasma from this foetus was analysed further, no line comparable with the anti-POL line was present in samples obtained during the primary immune response, or in the samples obtained during the first 4 days of the secondary response. The anti-POL line appeared 6 days after the second injection of antigen and persisted for at least 10 days (Figure 5-vii).

2. STUDIES ON THE IMMUNE RESPONSES OF FOETAL SHEEP AND LAMBS TO *SALMONELLA TYPHIMURIUM* WHOLE BOILED ORGANISMS OR LIPOPOLYSACCHARIDE (LPS)

The somatic antigens of *S. typhimurium* are a constitutive part of the bacterial cell wall and are a complex of lipid, polysaccharide and protein. The lipopolysaccharide, prepared by alkaline extraction, which was used for coating red blood cells, consists of the O specific polysaccharide coupled to lipid A. The O specific polysaccharide of *S. typhimurium* is a polymer of the trisaccharide, mannose-rhamnose-galactose, with abequose attached as a side chain to mannose. Lipid A, composed of N-acetylglucosamine, phosphoric acid ester and β-hydroxy myristic acid, is attached through galactose - 1 - phosphate to the polysaccharide. The complex has a molecular weight of 280,000 (Luderitz, Staub and Westphal, 1966; Horecker, 1966). There is no response to either *S. typhimurium* or LPS when given as a primary challenge to foetal sheep in conjunction with other antigens (Chapter 3).

(a) Secondary Challenge of Foetal Sheep Previously Challenged with *Salmonella typhimurium*

Three foetal sheep which were injected with 1×10^9

S. typhimurium i.m. simultaneously with other antigens, at 87, 92 and 101 days gestation, were challenged subsequently when they had reached 120 days gestation, with a second i.m. injection of 1×10^9 *S. typhimurium* organisms. The foetuses primed at 81 and 101 days gestation failed to produce detectable antibody, while the foetus primed at 92 days and rechallenged at 120 days produced a good haemagglutinating antibody response (Table 5-6), a component of which was resistant to treatment with 2-ME.

By comparison with the response in lambs (Tables 5-7 and 5-8) the response of the foetus to the second injection of *S. typhimurium* seemed to have the kinetics of a primary immune response.

(b) Competence of Young Lambs, Previously Challenged with the Antigen *in utero*, to respond to *Salmonella typhimurium*

The immunological status of 5 lambs, 4 of which had failed to produce detectable antibody to either one or two injections of *S. typhimurium* during gestation, was assessed following a further challenge with the same organism. All the lambs were weaned from their mothers 6 to 7 weeks after birth and raised on milk supplement (Denkavit) and lucerne chaff for 40 to 100 days to allow maternal colostral antibodies to be catabolized.

Three lambs previously challenged *in utero* with *S. typhimurium*, including the one that had produced antibody to a second injection of antigen (Table 5-6), were injected i.m. at 80 to 83 days of age with 1×10^9 organisms of *S. typhimurium*. A normal lamb of the same age was injected similarly. All lambs (Table 5-7) mounted good immune responses of a primary nature to the antigen, including the animal which had previously produced antibody *in utero*. The remaining two lambs were challenged at 139 and 141 days of age with 2×10^9 organisms of

Table 5-6: Haemagglutinating antibody response of a foetal sheep to a second injection of *S. typhimurium*

Day after injection	0	2	4	6	8	10	12	14
Total antibody	<1	<1	2.0	8.0	8.0	7.0	6.0	6.0
2-ME resistant antibody	<1	<1	<1	3.0	4.0	4.0	3.5	2.0

Table 5-7: Haemagglutinating antibody response to *S. typhimurium* of a normal lamb and lambs previously challenged *in utero*

In utero history			Antibody titre on day							
1^{o}	2^{o}	Response to *S. tym*	0	2	4	7	10	14	18	21
+	-	-	<1	<1	2.0	6.5	4.5	4.5	4.0	3.5
+	-	-	<1	<1	3.0	5.5	4.0	3.5	2.5	2.0
+	+	+	<1	<1	2.0	6.0	4.5	4.5	4.0	3.0
-	-	-	<1	<1	3.0	6.5	5.0	4.5	3.5	3.0

S. tym: S. typhimurium

S. typhimurium. Both animals responded in a similar manner to the younger lambs (Table 5-8), antibody first being detected on day 4, with peak titres occurring at day 7. The *in utero* challenge with *S. typhimurium* did not seem to affect the post-natal responsiveness of lambs to this antigen, either by sensitizing them or rendering them unresponsive to the antigen. The dose of *S. typhimurium* used in the foetuses was quite immunogenic in lambs 2 to 3 months after birth.

(c) The Response of Foetal Sheep to *Salmonella typhimurium* - Whole Boiled Organisms

Foetal sheep were injected with one or two doses of 1×10^9 organisms of *S. typhimurium* to study their immune responses. Five foetuses between 97 and 101 days gestation, including one set of twins, were injected i.m. with *S. typhimurium*. The youngest animal was followed for 6 days, while the remainder were followed for 12 to 17 days. At no stage was haemagglutinating antibody detected following the primary injections. The synthesis of immunoglobulin in these animals was assessed by radial immunodiffusion and the results shown in Table 5-9. All foetal sheep synthesized IgM following the injection of *S. typhimurium* organisms.

Three foetal sheep, including the twins, were rechallenged at 118 days gestation, 17 to 18 days after the first injection of *S. typhimurium*. The response was followed for a further 10 days in the twins and for 24 days in the remaining animal before the experiments were terminated and the lymphoid tissues removed for histology. Again no haemagglutinating antibody could be demonstrated in the circulation of these animals. The synthesis of immunoglobulin by the foetus that was followed for 24 days is shown in Table 5-10.

Serial dilutions of the plasma obtained from these animals following the primary and secondary challenge were incubated with the

Table 5-8: Haemagglutinating antibody response to *S. typhimurium* of lambs previously challenged *in utero*

In utero history			Antibody Titre on Day							
1^o	2^o	Response to *S. tym*	0	2	4	7	10	14	17	21
+	-	-	<1	<1	4.0	9.0	7.0	5.5	4.5	4.0
+	-	-	<1	<1	4.5	7.0	6.0	5.0	4.5	3.5

S. tym: S. typhimurium

Table 5-9: Synthesis of immunoglobulin following the injection of 1×10^9 organisms of *S. typhimurium*

Days after injection	No. of samples	IgM		IgG_1
		Mean concentration ±S.E. (µg/ml)	Range of concentration	Mean concentration (µg/ml)
0	4	5 ± 4	0 → 15	0
2	4	12 ± 8	0 → 34	0
4	4	30 ± 9	15 → 55	0
6	3	38 ± 11	16 → 55	0
8	3	36 ± 11	17 → 55	0
10	3	35 ± 18	15 → 70	0
12	3	31 ± 9	21 → 48	0
14	2	28 ± 6	22 → 34	0

Table 5-10: Synthesis of immunoglobulin by one foetus following a second injection of 1×10^9 organisms of *S. typhimurium*

Immunoglobulin concentration (µg/ml)	Days after secondary challenge							
	0	2	4	8	12	16	20	24
IgM	21	22	23	23	66	46	55	34
IgG_1	0	0	0	0	36	51	111	120
IgG_2	0	0	0	0	0	0	0	0
IgA	0	0	0	0	0	0	0	0

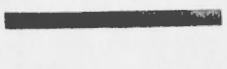
whole boiled organisms. Although no visible agglutination was observed, microscopic examination showed that weak agglutinating antibody was present in the plasma obtained during the secondary response of the foetus followed for 24 days; the only foetus to synthesize IgG_1. The antibody was present between days 4 and 14 of the secondary response at serum dilutions of up to 1:4.

The lymphoid tissues of the foetuses injected with *S. typhimurium* showed little evidence of antigenic stimulation. The cortico-medullary development was similar to that observed in normal animals at the same stage of development; no germinal centres could be found. Differential cell counts were performed on the blood samples obtained from two animals followed throughout the primary and secondary responses to the antigen. Both animals showed a 2 fold increase in the concentration of lymphocytes in the circulation by days 2 to 4 of the primary response [Figure 5-viii(a) and (b)]. The increased concentration of lymphocytes in the circulation persisted throughout the response and was not affected by the second injection of antigen. The concentration of neutrophils in the circulation of these foetuses did not increase after challenge with *S. typhimurium* as it did with other antigens. A transient increase in the level of neutrophils in the circulation was observed after the second injection of antigen into the foetus which subsequently produced agglutinating antibody. Surprisingly one of the two foetuses followed throughout the primary immune response had significant levels of blast cells in the circulation from the second day of the response. This was not observed in the other foetus challenged with *S. typhimurium* and this result was similar to that obtained in foetuses injected with POL.

(d) The Response of Foetal Sheep to *Salmonella typhimurium* LPS on Carrier Red Blood Cells

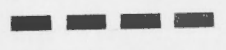
Figure 5-viii(a) and (b):

The changes in the blood leucocyte levels in foetal sheep injected with 1 x 10^9 *Salmonella typhimurium* organisms at 100 days gestation.



Total leucocytes

Lymphocytes

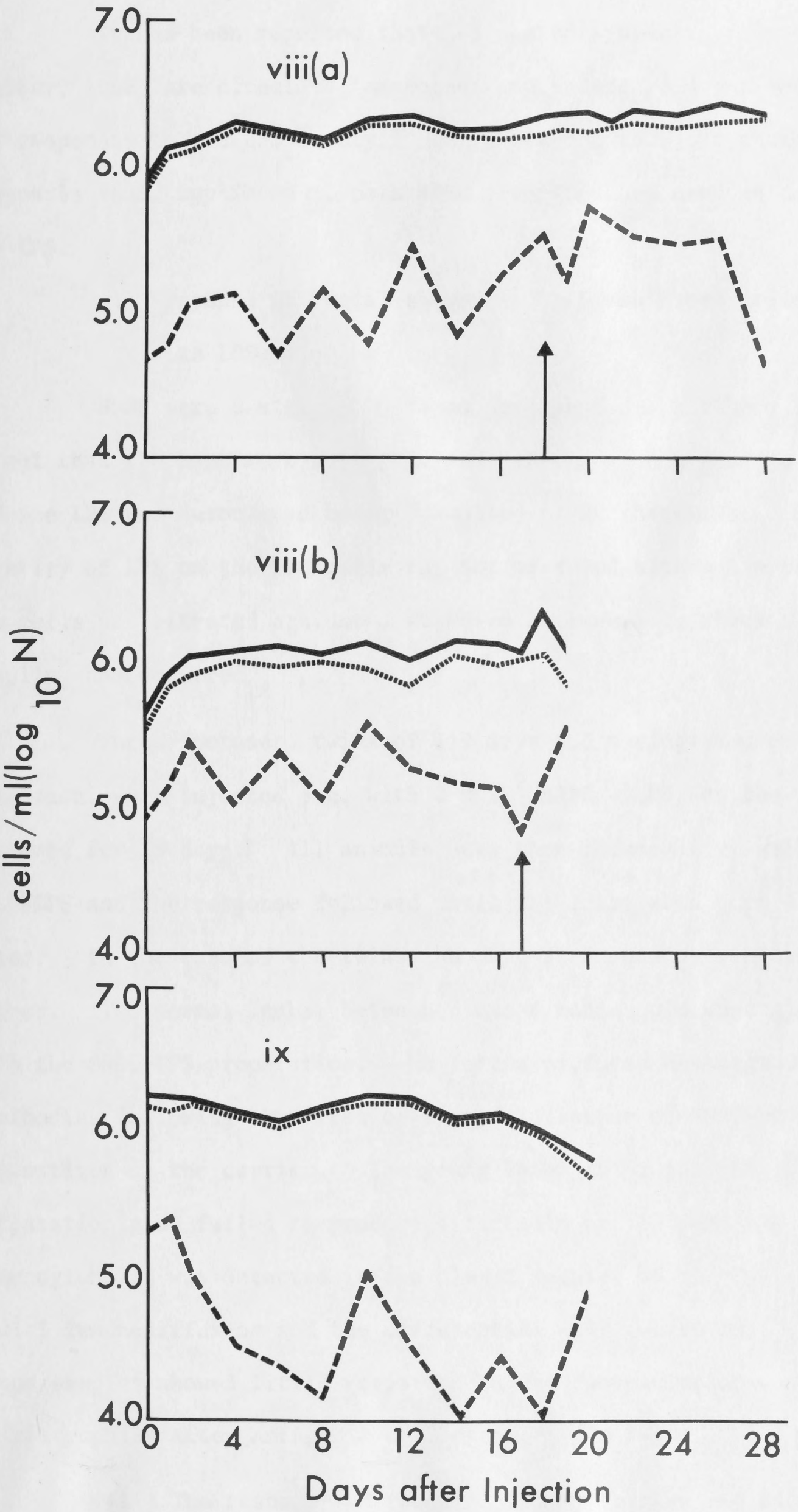


Neutrophils

Second injection of 1 x 10^9 *Salmonella typhimurium*

Figure 5-ix:

The changes in the blood leucocyte levels in a foetal sheep injected with 2 x 10^9 LPS coated SRBC at 120 days gestation.


viii(a)
viii(b)
ix
7.0
6.0
5.0
4.0
cells/ml(log 10 N)
0
4
8
12
16
20
24
28
Days after Injection

It has been reported that LPS coated syngeneic red cells (Moller, 1965) are effective immunogens and induce good antibody and PFC responses to the LPS moiety. In an attempt to evoke antibody responses to *S. typhimurium*, both SRBC and CRBC were used as carriers for LPS.

(i) Response of foetal sheep to sheep red blood cells coated with LPS

SRBC were coated with LPS as described for antibody titration, except that the SRBC were collected and processed in a sterile manner and the LPS was autoclaved before coupling it to the cells. The quantity of LPS on the red cells was not measured although a portion of the cells was titrated against a standard antiserum to check that coupling had occurred.

Three foetuses, twins of 119 days and a singleton of 120 days gestation, were injected s.c. with 2×10^9 SRBC - LPS and the response followed for 18 days. All animals were then infused i.v. with 2×10^9 SRBC-LPS and the response followed until the lambs were born 4 to 8 days later. In the case of the twins the SRBC used were obtained from the mother. Two normal lambs, between 3 and 4 months old were also injected with the SRBC-LPS preparation. No foetus produced haemagglutinating antibodies following the first or second injection of SRBC-LPS to either the antigen or the carrier. The young lambs injected with the SRBC-LPS preparation also failed to produce detectable antibody to LPS. No immunoglobulin was detected in the plasma samples of the foetal sheep, by radial immunodiffusion and the differential cell counts performed on the blood samples showed little variation in the concentration of lymphocytes or neutrophils after antigenic challenge (Figure 5-ix).

(ii) The response of foetal sheep to chicken red blood cells coated with LPS

Two foetal sheep, 122 and 123 days old were injected with LPS coupled to CRBC. Each foetus received a s.c. injection of 2×10^9 CRBC-LPS and the responses followed for 14 to 16 days. After this time each animal was infused i.v. with a further 2×10^9 CRBC-LPS and the response followed until the lambs were born. Two normal lambs between 3 and 4 months old, were also injected with CRBC-LPS.

Again no haemagglutinating antibody was formed to the LPS moiety, although both animals did give normal immune responses to the CRBC carrier (Table 5-11). The normal lambs also gave antibody responses to CRBC, but not to the LPS. It seemed from these experiments that injecting LPS on a CRBC carrier, gives rise to an immune response to the carrier, but not to the LPS.

(e) Antibody Response of Foetal Sheep at 120 days Gestation to 1×10^9 CRBC

Three foetal sheep, between 120 and 121 days gestation were injected with 1×10^9 CRBC to see if the antibody produced could be analysed by IEF, using a complement mediated lysis system. In addition, the proportion of "specific" antibody synthesized by these foetuses could also be estimated by adsorption of the antibody to CRBC. Because of the multiplicity of potential antigens on or inside CRBC, they were subsequently considered unsuitable for estimating specific antibody synthesis, while the relative resistance of CRBC to lysis made it difficult to detect specific antibody on IEF plates.

The antibody responses obtained in these animals, both total and 2-ME resistant were determined by direct haemagglutination and in Table 5-12 the mean antibody response detected every second day in these animals is compared with the mean response in 3 foetal sheep of similar age, injected with 1×10^9 CRBC together with other antigens (Chapter 3).

Table 5-11: Primary antibody response of two foetal sheep to 2×10^9 LPS coated CRBC

Test antigen	Haemagglutinating antibody response on day								
	0	2	4	6	8	10	12	14	16
CRBC	<1	<1	3.0	7.0	7.0	7.0	7.5	6.5	6.5
CRBC-LPS	<1	<1	2.5	7.0	7.5	7.0	6.5	6.0	6.0
SRBC	<1	<1	<1	<1	<1	<1	<1	<1	<1
CRBC	<1	<1	1.5	5.5	6.0	5.0	4.0	4.0	-
CRBC-LPS	<1	<1	1.5	5.0	5.5	4.5	4.0	4.0	-
SRBC-LPS	<1	<1	<1	<1	<1	<1	<1	<1	-

Table 5-12: Primary antibody response of 120 day old foetal sheep to 1 x 10^9 CRBC injected alone or with other antigens

Antigenic challenge	Haemagglutinating antibody	Anti-CRBC antibody titre on day							
		0	2	4	6	8	10	12	14
Multiple	Total	<1.0	<1.0	0.8±0.2	6.0±0.6	6.5±0.3	6.7±0.3	5.8±0.8	5.5±0.5
	2-ME resistant	<1.0	<1.0	<1.0	1.3±0.3	2.5±0.6	2.8±0.8	1.5±0.5	2.3±1.3
Single	Total	<1.0	<1.0	1.2±0.2	6.7±1.3	7.8±1.2	8.3±1.4	8.0±1.0	7.3±0.8
	2-ME resistant	<1.0	<1.0	<1.0	1.7±0.3	2.2±0.7	2.3±0.6	3.2±0.7	4.6±0.6

Again there was no significant difference between the responses. The same conclusion was reached for CRBC as had already been reached for POL; that antigenic competition was not a factor in the response observed to CRBC in sheep simultaneously challenged with several different antigens.

DISCUSSION

The magnitude of the haemagglutinating antibody response to POL increased with the age at which the foetus was challenged. Furthermore the number of different classes of immunoglobulin synthesized by the foetuses following antigenic challenge was greater in older foetuses. Young foetuses only synthesized IgM, while foetuses challenged during the last two months of gestation synthesized both IgM and IgG_1. Neither IgG_2 nor IgA was detected following a primary or secondary systemic challenge with POL.

The mean concentration of IgM reflected the mean total antibody titres of the various groups of animals. There was a good correlation between the antibody titre and the immunoglobulin level in individual foetuses, although this correlation was not absolute. The foetus with the lowest maximum antibody titre (5.0) had the lowest concentration of IgM (10 µg/ml), while the foetus with the highest titre (15.0) had the highest concentration of IgM (1 mg/ml). Other animals, however, with similar antibody titres had IgM concentrations which differed by a factor of 1 to 2 fold, while animals with similar IgM concentration had titres which differed by 1 to 3 doubling dilutions. One explanation of these anomalies could be differences in the affinity of the antibody synthesized by different animals, although the synthesis of "non-specific" IgM or inadvertent stimulation of IgM synthesis as a result of surgical intervention could also explain the results.

The quantity of IgG_1 detected by radial immunodiffusion did not always reflect the titre of 2-ME resistant anti-POL antibody in the plasma. Foetuses less than 79 days old did not synthesize IgG_1 or 2-ME resistant anti-POL antibody, although one 79 day old, one 100 day old and one 119 day old foetus did synthesize IgG_1 in the absence of detectable 2-ME resistant antibody. Putting aside the explanation that the IgG_1 may have been induced by inadvertent stimulation of the foetus at surgery, this result could be explained if the antigen stimulated either low affinity IgG_1 antibody or "non-specific" IgG_1. In the other four foetuses challenged between 119 and 120 days gestation the presence of 2-ME resistant antibody did indicate IgG_1 synthesis.

A contributing factor to the lack of correlation between immunoglobulin concentrations and antibody titres could be the two assay procedures employed. The radial immunodiffusion assay estimates the concentration of immunoglobulin, while the haemagglutination assay measures both the quantity and the quality of the immunoglobulin (affinity). The serial dilution of plasma carried out in the haemagglutination assay is not adequate for accurate estimation of antibody concentration.

The secondary response to POL was characterized by very high titres of total antibody, all of which was resistant to 2-ME, and relatively high concentrations of IgG_1 in the circulation. The concentration of IgM did not increase significantly during the secondary response.

Estimations of the levels of "specific" and "non-specific" immunoglobulin synthesized during the primary and secondary immune response to POL were based on the adsorption of antibody onto POL coated sepharose or SRBC. These estimates were complicated in the secondary responses by the fact that several foetuses had detectable levels of

antibody, IgM and IgG_1 in their circulation prior to the second antigenic challenge.

During the primary response to POL, most of the immunoglobulin synthesized was IgM and most of this appeared to be "non-specific". There was little "specific" IgG_1 synthesis during the primary antibody response. The percentage of the total IgM that was "specific" increased in all foetuses towards the end of the primary response, even though the absolute concentration of IgM decreased. Following the second injection of POL, the major class of immunoglobulin synthesized was IgG_1, and at the peak of the response from 42% to 100% of it could be removed from the plasma by adsorption to POL. The percentage of "specific" IgG_1 decreased towards the end of the response in two of the three foetuses studied. During the secondary response to POL, a high proportion of the IgG_1 synthesized was "specific" for the antigen.

The analysis of "specific" and "non-specific" antibody synthesis during a primary immune response to POL in foetal sheep is similar to that of Asofsky, Ikari and Hylton (1968) who found an increase in "non-specific" IgM in germ-free mice immunized with ferritin. Similarly, Nashe, Crabbe, Bazin, Eyssen and Heremans (1969) found all classes of non-antibody immunoglobulin were synthesized by germ-free mice challenged with ferritin, although proportionally more non-antibody was detected in the IgM class than in the other immunoglobulin classes.

The analysis of the molecular heterogeneity of anti-POL IgG_1 synthesized during the secondary immune response indicated that at least in one foetus, the IgG_1 antibody formed was of a single molecular species, indicative of a very restricted clonal response. Similar restricted IEF patterns in immature animals have been reported by Montgomery and Williamson (1970). They found that the IEF spectrum of an anti-DNP response in several newborn rabbits had the characteristics of a single

clone (monoclonal antibody); these clones appeared to be stable and could be detected for up to 92 days, following intermittent antigenic challenge. Goidl and Siskind (1974) also concluded from the results of experiments in which foetal liver cells were transferred to irradiated, syngeneic adult mice, that foetal mice are restricted with respect to the catalogue of different anti-DNP antibodies they can produce. Recently Roelants and Goodman (1974) found that in guinea pigs once a clone of antibody producing cells dominated an anti-DNP response, it remained stable during the primary response and was the only clone to reappear after secondary stimulation. Both the restricted clonal response of neonates and the dominance of an established clone could explain the restricted anti-POL IEF spectrum found in the foetal sheep.

The finding that in all foetal sheep tested no primary response occurred to whole boiled *S. typhimurium* organisms while only one of six foetuses rechallenged with the antigen produced any haemagglutinating antibody cannot be reconciled with the optimism of Bennett (1964) that: "an investigator in almost any biological field is likely to obtain a 'positive' result if he tries endotoxin in the experimental system he is using."

All of the foetal sheep which were injected with *S. typhimurium* synthesized low levels of non-antibody IgM (maximum 38 μg/ml), while one of three foetuses synthesized IgG_1 following a secondary challenge. The plasma from this foetus did agglutinate whole organisms, albeit very weakly. The finding of IgM synthesis in the absence of detectable antibody is very similar to the finding *in vitro* of IgM synthesis in LPS stimulated lymphocyte cultures (Anderson *et al*, 1972). IgG was not detected in these *in vitro* cultures, although it has recently been reported to be synthesized in newborn mice, but not adult mice, injected with *S. anatum* or its Lipid A component (Kolb *et al*, 1974). The IgG

synthesized by these mice had no demonstrable antibody activity. The finding of "non-antibody" IgM synthesis in foetal sheep injected with *S. typhimurium* would probably be ascribed to the B-lymphocyte mitogenic properties of LPS or Lipid A (Coutinho and Moller, 1973), although the synthesis of antibody to "hidden" antigenic determinants must also be considered.

It may be that the enzymes which are necessary to degrade whole *S. typhimurium* organisms or LPS to an immunogenic form, are not fully functional until after birth. This could explain the failure of foetal animals to respond to these antigens. However, nothing is known of the role of opsonization in antibody responses in foetal sheep, nor in fact, of other factors which may be crucial in the production of antibody, so speculation on this question is unwarranted. The fact that one foetus did produce antibody to a second challenge of *S. typhimurium* indicates that the developing foetus has the genetic information necessary to synthesize antibody. This is supported further by the observation that foetuses which failed to respond to a primary or secondary challenge *in utero* subsequently gave good immune responses as young lambs. Their *in utero* experience did not seem to have changed their immune competence, as they responded subsequently in a similar manner to lambs which had not received *S. typhimurium* organisms *in utero*.

Attempts to modify the immunogenenicity of LPS of *S. typhimurium* by injecting it passively attached to erythrocytes proved unsuccessful. Young lambs similarly challenged also failed to produce antibody, indicating that the dose of LPS given in this manner may have been sub-optimal as lambs do respond to 100 µg of LPS injected i.m. (Chapter 3).

A good correlation exists between antibody synthesis and elevated neutrophil levels after the injection of antigen. The foetal

animals which received SRBC-LPS did not show either the transient neutrophil response or an increase in concentration of lymphocytes in the blood after the injection of antigen. Conversely "immunogenic" antigens such as POL, caused a transient 10 fold increase in neutrophils shortly after injection, while a slower but pronounced increase in blood lymphocytes was invariably recorded at or before the time antibody was detected in the circulation.

Finally, the humoral antibody responses to POL or CRBC did not seem to be significantly different in animals which received these antigens alone or as part of a multiple antigenic challenge. The possibility of antigenic competition affecting the antibody responses obtained by injecting foetal sheep simultaneously with several antigens has been discussed (Chapter 3). The antibody responses detected to POL or CRBC injected alone support the conclusion that antigenic competition was not a complicating factor in experiments in which several antigens were used, possibly because the antigens were injected simultaneously at different anatomical sites (rev. Taussig, 1973). One interesting observation was that the synthesis of 2-ME resistant antibody to POL appeared earlier in gestation in animals receiving multiple antigenic challenges which included POL, than in animals which received POL alone. This observation suggested that some form of enhancement may occur between antigens.

CHAPTER 6

MODIFICATION OF THE IMMUNOLOGICAL COMPETENCE OF FOETAL SHEEP: THE EFFECT OF THYMECTOMY

CHAPTER 6

MODIFICATION OF THE IMMUNOLOGICAL COMPETENCE OF FOETAL SHEEP: THE EFFECT OF THYMECTOMY

INTRODUCTION

Although the thymus takes no overt role in immune reactions (Thorbecke and Keuning, 1953; Askonas and White, 1956), observations on its role as a generator of cells directly involved in many manifestations of immune reactivity are legion (rev. Miller and Osoba, 1967; Hess, 1968; Katz and Benacerraf, 1972).

The reduction in peripheral blood leucocytes following thymectomy in the guinea pig was reported by Paton and Goodall (1904). Miller (1962) confirmed this observation in mice and showed that the reduction occurred in the lymphocyte population. A similar reduction in the lymphocyte level in the thoracic duct lymph of neonatally thymectomized mice was also recorded (Miller, Mitchell and Weiss, 1967). Thymectomy of neonatal mice reduced the number of lymphocytes in the diffuse cortices of lymph nodes and periarteriolar sheaths in the spleen, but not the number or the cell population of germinal centres or lymphoid follicles or the number of plasma cells (Parrot, de Sousa and East, 1966).

The observation that neonatal thymectomy delayed the rejection of skin allografts on rodents implicated the thymus in what is termed "cell mediated immunity" (Miller, 1961). However, to generalize that thymectomy invariably results in delayed graft rejection, even in rodents, is not possible. The effects of thymectomy depend on the strain of rodent used as graft recipient, the degree of histocompatibility between the donor and recipient and whether or not

the recipients are maintained in a specific pathogen-free environment (rev. Hess, 1968).

The effect of neonatal thymectomy on antibody and plaque forming cell (PFC) production also varies from one species to another and from antigen to antigen. The degree of suppression depends on whether it was a primary or secondary response, whether the animals were reared in conventional or germ-free environments and at what time after thymectomy the animals were challenged (rev. Miller and Osoba, 1967; Hess, 1968).

Much of the evidence implicating the thymus as the organ giving rise to a population of cells with specific immunological functions, both in "cell mediated" and "humoral" immunity, stems from *in vitro* experiments and reconstitution experiments in which cells were transferred to neonatally thymectomized, irradiated, bone marrow reconstituted animals (rev. Feldman and Nossal, 1972; Katz and Benacerraf, 1972; Dutton and Hunter, 1974).

Cole and Morris (1971a) reported that the *in utero* thymectomy of foetal sheep between 62 and 76 days gestation resulted in a reduction in the concentration of small lymphocytes in the lymph and blood. The cellularity of lymphoid organs, such as the spleen, lymph nodes and Peyer's patches was also reduced in thymectomized (Tx) lambs, although the number of germinal centres or plasma cells did not appear to change (Cole and Morris, 1971a). Thymectomy did not affect the autologous synthesis of immunoglobulin in colostrum-deprived lambs, while the cellular and antibody responses in the popliteal lymph of Tx lambs challenged with swine influenza virus, *S. muenchen* organisms or CRBC were similar to responses in normal lambs (Cole and Morris, 1971b). Tx lambs were also fully capable of rejecting skin allografts and giving an immediate-type hypersensitivity reaction to ferritin

(Cole and Morris, 1971c). However, Cole and Morris (1971d) did find that the delayed-type hypersensitivity reaction to tuberculin and the normal lymphocyte transfer reaction were both depressed in Tx lambs. The normal lymphocyte transfer reaction was depressed when cells from Tx lambs were transferred to normal lambs or when cells from normal lambs were transferred to Tx lambs. Virtually no response could be detected in the skin of Tx lambs injected with lymphocytes from Tx donors.

Silverstein and Prendergast (1970) also reported that thymectomy at 50 days gestation resulted in a state of lymphopenia which persisted into adulthood. The sequential attainment of competence to different antigens at discrete stages of gestation remained unaltered in these foetuses. Subsequent treatment of Tx foetal sheep with anti-lymphocyte serum resulted in the almost complete ablation of lymphoid tissues and circulating lymphocytes. Soon after birth however, lymphocytes started to appear and by 2 to 3 months of age these lambs could reject skin allografts and give antibody responses to ferritin, ovalbumin and bovine-γ-globulin (Silverstein and Prendergast, 1973).

Recent reports suggest that the thymus plays a critical role in both the development of immunological competence and the synthesis of IgG in rodents (Taylor and Wortis, 1968; Mitchell, Grumet and McDevitt, 1972; Pritchard, Riddaway and Micklem, 1973; Spear and Edelman, 1974), although not all data support the absolute "T-dependence" of IgG synthesis (Grewther and Warner, 1972; Hucket and Feldman, 1974; Jacobson, Caporale and Thorbecke, 1974). The immunological reactivity of thymectomized foetal sheep has been investigated in an attempt to define the role of the thymus in the development of immunoglobulin and specific antibody synthesis in sheep.

RESULTS

Twelve foetal sheep were thymectomized (Tx) between 55 and 60 days gestation. The foetuses were returned to the uterine environment where they continued to develop prior to being challenged with antigen. The antigens employed in this study were POL, a so called "T-independent" antigen (Brooke, 1965,; Davies, Carter, Leuchars, Wallace and Dietrich, 1970) and CRBC and MON, so called "T-dependent" antigens (Feldman and Basten, 1971; Dennert and Lennox, 1972). Labelling MON and POL "T-dependent" and "T-independent" is itself dependent on the strain of bacteria employed and the species of animal studied (Lind, 1970; Langman, Armstrong and Diener, 1974; Steward, 1971).

Two of the Tx foetal sheep were challenged at 100 days gestation and four between 119 and 120 days gestation with POL and CRBC. Two foetuses at 120 days gestation were challenged with CRBC together with MON, while four foetuses of the same age were injected with CRBC. The lumbar lymphatic trunks of three of the four foetuses challenged with CRBC were cannulated and lymph was collected for 1, 9 and 15 days respectively; the PFC response to CRBC in the lymph was followed in the two latter animals. Antibody and immunoglobulin production was measured in blood samples obtained through indwelling vascular cannulae.

(a) The Effect of Thymectomy on Foetal Development

When the Tx foetal sheep were exposed subsequently *in utero* for antigenic challenge they appeared to have developed quite normally. The animals were of a comparable size to normal foetuses of the same age and wool growth seemed typical. When the peripheral blood leucocyte counts were determined using the Coulter Counter, nine of the

twelve Tx foetuses had reduced levels compared to normal foetuses at the same stage of development. Figure 6-i shows the levels of white cells in the blood of normal foetuses at the time of surgery. The mean levels in two 100 day old Tx foetuses and seven 120 day old Tx foetuses were about a third that of normal foetal sheep of the same age. The three remaining Tx foetuses had blood leucocyte levels which were similar to those found in normal foetuses.

Blood samples obtained from the Tx animals at 120 days gestation, immediately prior to antigenic challenge, had significantly fewer lymphocytes than normal (Table 6-1). The concentration of lymphocytes in the blood of Tx foetuses was less than a quarter of that in normal foetuses, although the concentration of neutrophils appeared normal.

(b) Humoral Antibody Response of Tx Foetal Sheep to Antigenic Challenge

(i) Haemagglutinating antibody response to CRBC

All twelve Tx foetal sheep were injected s.c. with 1.0×10^9 CRBC between 100 and 120 days gestation. The haemagglutinating antibody titres before and after the plasma samples had been treated with 2-ME were determined, as were the haemolysin antibody titres of several foetuses.

Three Tx foetuses failed to produce antibody during the primary immune responses, which could be detected by either the haemagglutination or haemolysin assays. The blood leucocyte levels in all three foetuses, prior to challenge, were lower than normal. Of the remaining six foetuses with reduced blood leucocyte levels, five gave very weak responses to CRBC (haemagglutination titres of 1.0 to 2.0), while one gave a moderately good response (peak titre 5.0). All antibody formed by these foetuses was sensitive to 2-ME. Two of the three Tx foetuses with near normal blood leucocyte levels

Figure 6-i:

The concentration of blood leucocytes in normal foetal sheep (circles) and foetal sheep thymectomized between 55 and 60 days gestation (squares). Standard errors indicated by vertical bars.

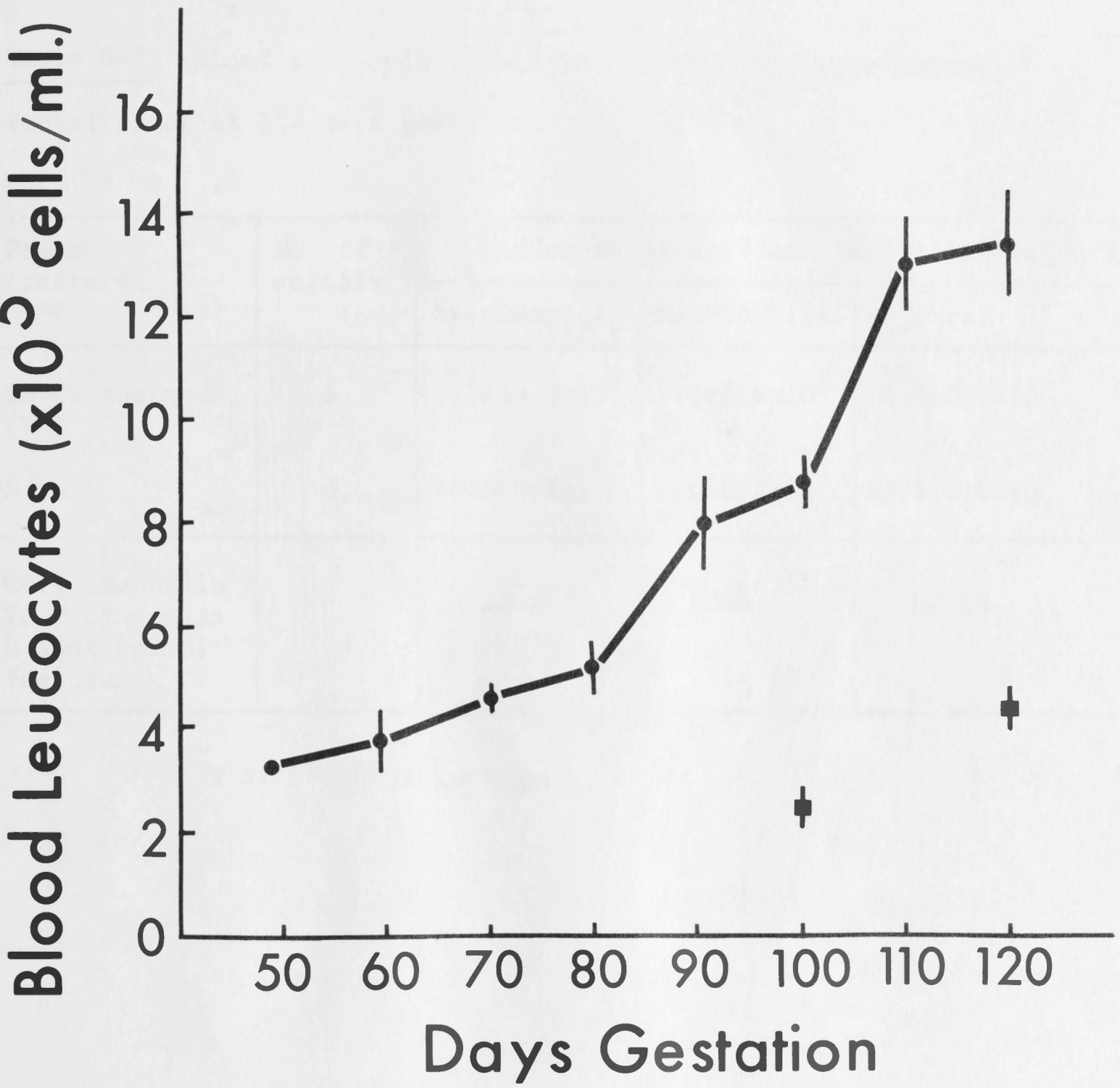

Blood Leucocytes (x10^5 cells/ml.)
0
2
4
6
8
10
12
14
16
50
60
70
80
90
100
110
120
Days Gestation

Table 6-1: Blood leucocyte levels in normal and thymectomized foetal sheep at 120 days gestation

Prior treatment	No. of animals	Differential cell counts (cells/ml±S.E.)		
		Lymphocytes	Neutrophils	Total
Thymectomized* (Tx)	6	$2.4\pm0.6\times10^5$	$1.6\pm0.5\times10^5$	$4.3\pm0.5\times10^5$
Normal	6	$10.6\pm0.8\times10^5$	$1.6\pm0.2\times10^5$	$12.6\pm0.8\times10^5$
Cell counts in Tx foetuses as a % of normal foetuses	-	22.6%	100%	34.1%

*Tx at ≤60 days gestation

gave responses which were only marginally less than responses produced by six normal foetal sheep of a similar age. Some of the haemagglutinating antibody synthesized by these two Tx foetuses was resistant to 2-ME.

The haemagglutinating antibody responses of the six Tx foetuses with low blood leucocyte levels, the three Tx foetuses with normal levels and six normal foetal sheep injected with CRBC are presented in Figure 6-ii (a), (b) and (d) respectively, together with the appearance of 2-ME resistant antibody. The haemolysin titres determined for some of these animals substantially reflected the haemagglutination titres, but were occasionally 1 to 2 fold higher.

Five of the six Tx foetuses which gave a reduced anti-CRBC response [Figure 6-ii (a)] were subsequently infused i.v. with 1.0×10^9 CRBC, 14 to 16 days after the primary challenge. The mean haemagglutinating antibody titres, detected at 2 day intervals, are shown in Figure 6-ii (c) and resemble the primary response in the normal animals [Figure 6-ii (d)]. Four of the five animals produced good titres of 2-ME resistant antibody to the second challenge with CRBC.

(ii) Haemagglutinating antibody responses to POL and MON

Eight Tx foetuses were challenged simultaneously with CRBC and 100 µg POL or MON injected i.m. into one limb. Two of the animals which failed to respond to CRBC were amongst this group and again they failed to produce anti-POL antibody even though they were followed for 20 days after challenge. Three Tx foetuses with low blood leucocyte levels gave reduced anti-POL responses compared with those of three normal foetuses receiving the same challenge; all antibody formed by these Tx animals was sensitive to 2-ME.

Of the three Tx foetuses with normal blood leucocyte levels

Figure 6-ii:

Haemagglutinating antibody responses of normal and thymectomized foetal sheep to 1 x 10^9 CRBC.

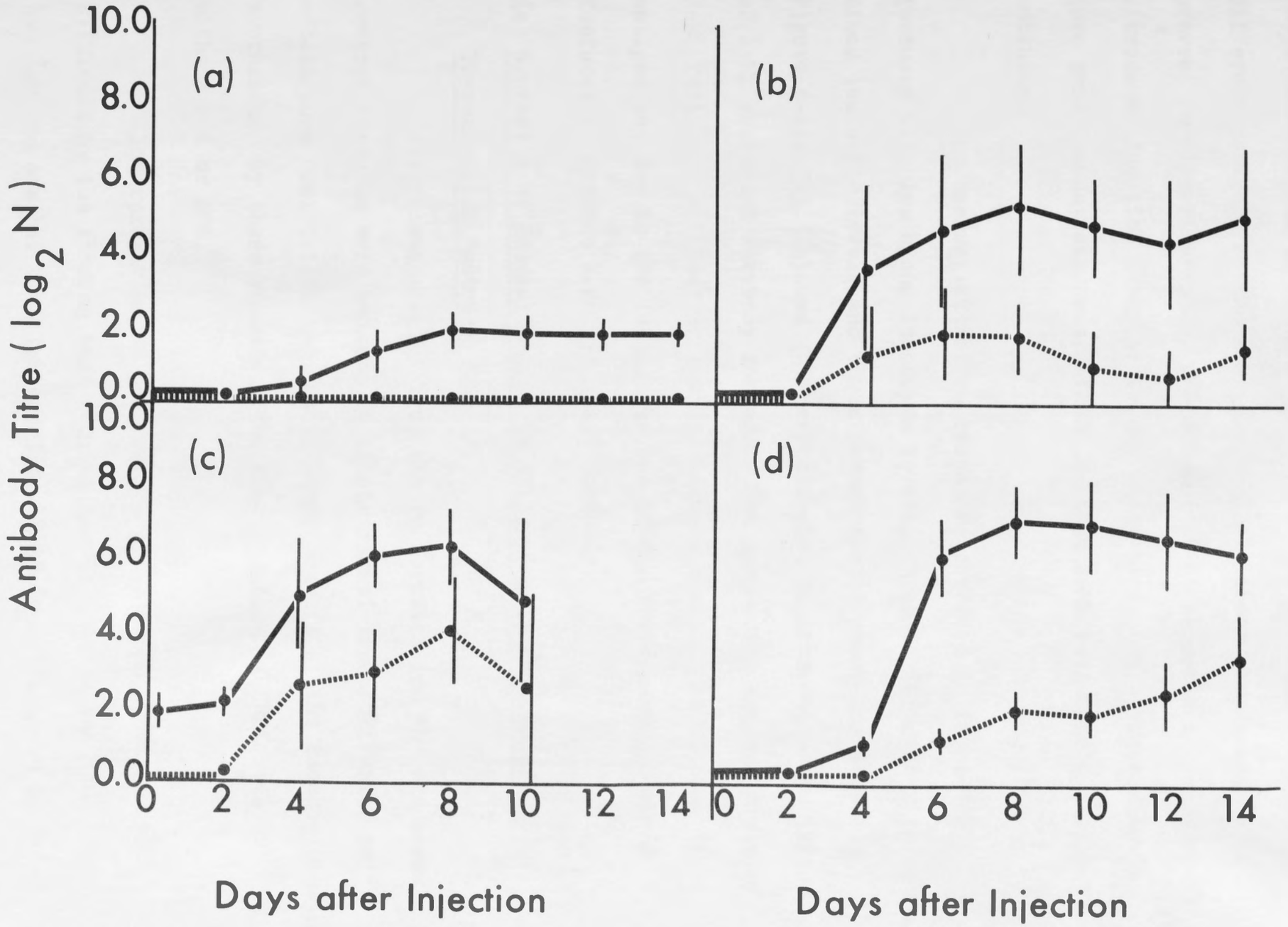
(a) 1^o response of Tx foetuses with low blood leucocyte levels;

(b) 1^o response of Tx foetuses with normal blood leucocyte levels;

(c) 2^o response of Tx foetuses with low blood leucocyte levels; and

(d) 1^o response of normal foetal sheep.

Total antibody - solid line: 2-ME resistant antibody - broken line: Standard errors indicated by vertical bars.


(a)
(b)
(c)
(d)
Antibody Titre (log2 N)
10.0
8.0
6.0
4.0
2.0
0.0
0 2 4 6 8 10 12 14
Days after Injection
Days after Injection

prior to challenge, two received MON and one received POL. The responses observed in these animals have been pooled despite the different antigens, as the MON was prepared before the necessity to prevent repolymerization of the monomer or to separate the monomer by ultracentrifugation (Chapter 2) was understood. All these animals gave good immune responses, producing both 2-ME resistant and sensitive antibody.

The haemagglutination responses observed in three Tx foetuses with low blood leucocyte levels, three Tx foetuses with normal blood leucocyte levels and three normal foetal sheep are shown in Figure 6-iii (a), (b) and (c) respectively, together with the titres of 2-ME resistant antibody formed. The apparently higher levels of 2-ME resistant antibody in the Tx foetuses that responded well to antigen was due to the failure by one of the three normal control foetuses to produce 2-ME resistant antibody.

(c) Synthesis of Immunoglobulin by Antigenically Challenged Thymectomized Foetuses

All plasma samples from the Tx foetuses and the six normal control foetuses were assayed by single radial immunodiffusion to obtain some qualitative and quantitative measure of the immunoglobulin synthesized by these animals after the injection of CRBC with or without POL or MON.

Interpretation of the results of these analyses was made difficult by the finding that four of the Tx animals had IgG_1, two had IgM and one had both IgM and IgG_1 in their circulation prior to challenge. In these foetuses the quantity of immunoglobulin synthesized after antigenic challenge was obtained by subtracting the level present in the circulation prior to challenge. Both the Tx and normal foetuses have been divided into groups depending on the

Figure 6-iii:

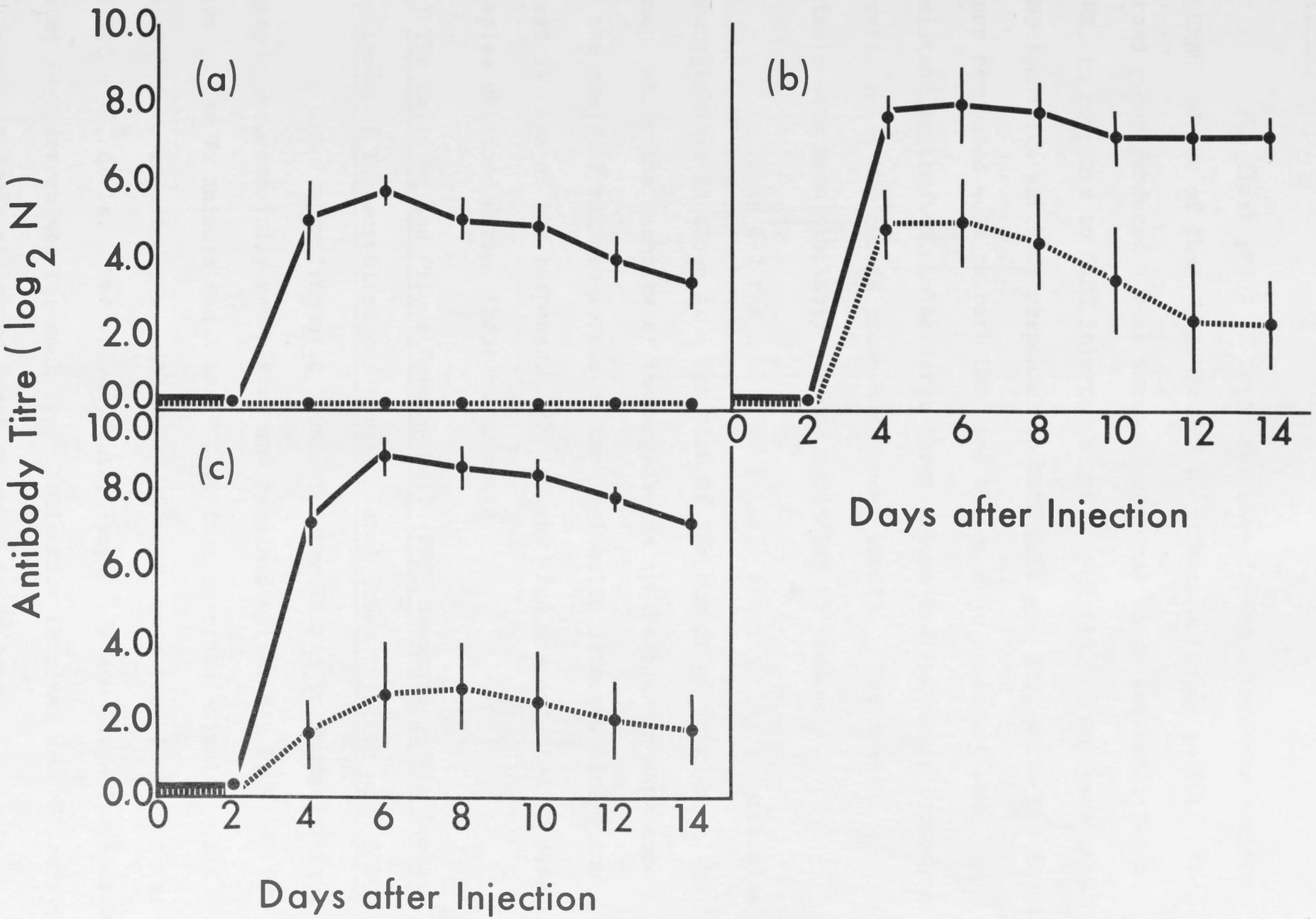
Passive haemagglutinating antibody responses of normal and thymectomized foetal sheep to 100 µg POL or MON.

(a) 1^{o} response of Tx foetuses with low blood leucocyte levels;

(b) 1^{o} response of Tx foetuses with normal blood leucocyte levels; and

(c) 1^{o} response of normal foetal sheep.

Total antibody - solid line: 2-ME resistant antibody - broken line: Standard errors indicated by vertical bars.


(a)
(b)
(c)
10.0
8.0
6.0
4.0
2.0
0.0
0
2
4
6
8
10
12
14
Antibody Titre (log2 N)
Days after Injection
Days after Injection

antigens injected and the nature and magnitude of the antibody response detected.

The first group of three foetuses failed to produce antibody to CRBC and two of these also failed to produce antibody to POL; the second group produced a weak antibody response which was sensitive to 2-ME, in response to CRBC injected alone. The third group gave weak 2-ME sensitive antibody responses to both CRBC and POL, while the fourth group responded well to both CRBC and POL or MON, producing 2-ME resistant antibodies. The first three groups had low blood leucocyte levels, while the fourth group had normal levels. The control animals have been similarly divided according to treatment.

In Table 6-2 the number of animals synthesizing a particular immunoglobulin is shown as a fraction of the number of animals in the group, while the quantity of immunoglobulin synthesized is expressed as the range of maximum levels of immunoglobulin detected during the first 14 days of the response. No IgG_2 or IgA was detected in plasma samples obtained during these experiments.

(d) The Cellular and Plaque Forming Cell (PFC) Response in the Lumbar Lymph of Thymectomized and Normal Foetal Sheep Injected with CRBC

The lumbar lymphatic trunks of three Tx and two normal foetal sheep were successfully cannulated and remained patent for 1, 9 and 15 days in the Tx animals and 5 and 7 days in the normal animals. All animals were injected s.c. with 2.5×10^8 CRBC into each limb, a total of 1.0×10^9 cells. The volume, total lymphocyte and large lymphocyte count were determined for each lymph collection obtained and PFC assays performed on cells collected fresh every 12 to 24 hours.

The Tx foetus followed for 9 days did not give either a PFC or humoral antibody response to CRBC. The cell output from this

Table 6-2: Synthesis of immunoglobulin by antigenically challenged thymectomized foetal sheep

Magnitude of the response of Tx animals to antigen	Antigenic challenge	IgM		IgG_1	
		No. responders / No. animals	Range (µg/ml)	No. responders / No. animals	Range (µg/ml)
*Tx non responders	CRBC; CRBC/POL	0/3	Nil	1/3	0 to 20
*Tx low responders	CRBC	3/3	21 to 566	1/3	0 to 77
*Tx low responders	CRBC/POL	3/3	70 to 115	1/3	0 to 56
Tx responders	CRBC/POL/MON	3/3	180 to 2200	3/3	60 to 1675
Normal controls	CRBC	3/3	190 to 490	3/3	111 to 168
Normal controls	CRBC/POL	3/3	92 to 141	3/3	64 to 136

* Low PBL levels prior to challenge

foetus varied from 0.62 to 1.22 x 10^6 cells/hour during the first 4 days of the response, increasing to 1.42 to 1.76 x 10^6 cells/hour between days 5 to 7 of the response. The output then fell to about 0.4 x 10^6 cells/hour by day 9 when the foetus was aborted. During the period of lymphocyte drainage the blood leucocyte level fell from an initial level of 4.3 x 10^5 to 4.0 x 10^4 cells/ml on day 8. The percentage of blast cells (large lymphocytes) in the lumbar lymph of this foetus fluctuated between 5.9 and 10.0% before falling to 2.9% just before the foetus died. There was no significant blast cell response following the injection of antigen.

The second Tx foetus did give a PFC response to CRBC. The appearance of blast cells in lumbar lymph of this foetus together with the appearance of PFC, expressed as PFC/10^8 nucleated cells, is shown in Figure 6-iv. The blast cell response reached a peak of 23.6% on day 5. Significant numbers of PFC were detected after 5.5 days, a peak of 27 PFC/10^8 cells being reached after 7.5 days. The cell output from this foetus fell from an initial level of 2.5 x 10^6 cells/hour to 1.3 x 10^6 cells/hour on day 2 of the response. The output steadily increased after day 2 to 6.9 x 10^6 cells/hour on day 7; during this period the PFC response was increasing rapidly. The cell output then fell to reach the original level by day 9 when the PFC response could no longer be detected.

The percentage of lymphocytes in the lumbar lymph which had membrane-bound immunoglobulin was determined on lymphocytes collected during the first and second days of the response. Using a peroxidase labelled anti-whole sheep serum 12.4% and 18.0% [Figure 6-v (a)] positively labelled lymphocytes were found in the respective samples. In addition the percentage of lymphocytes collected on days 1, 3, 5 and 7 of the response which incorporated ^{3}H-thymidine when incubated *in vitro* was

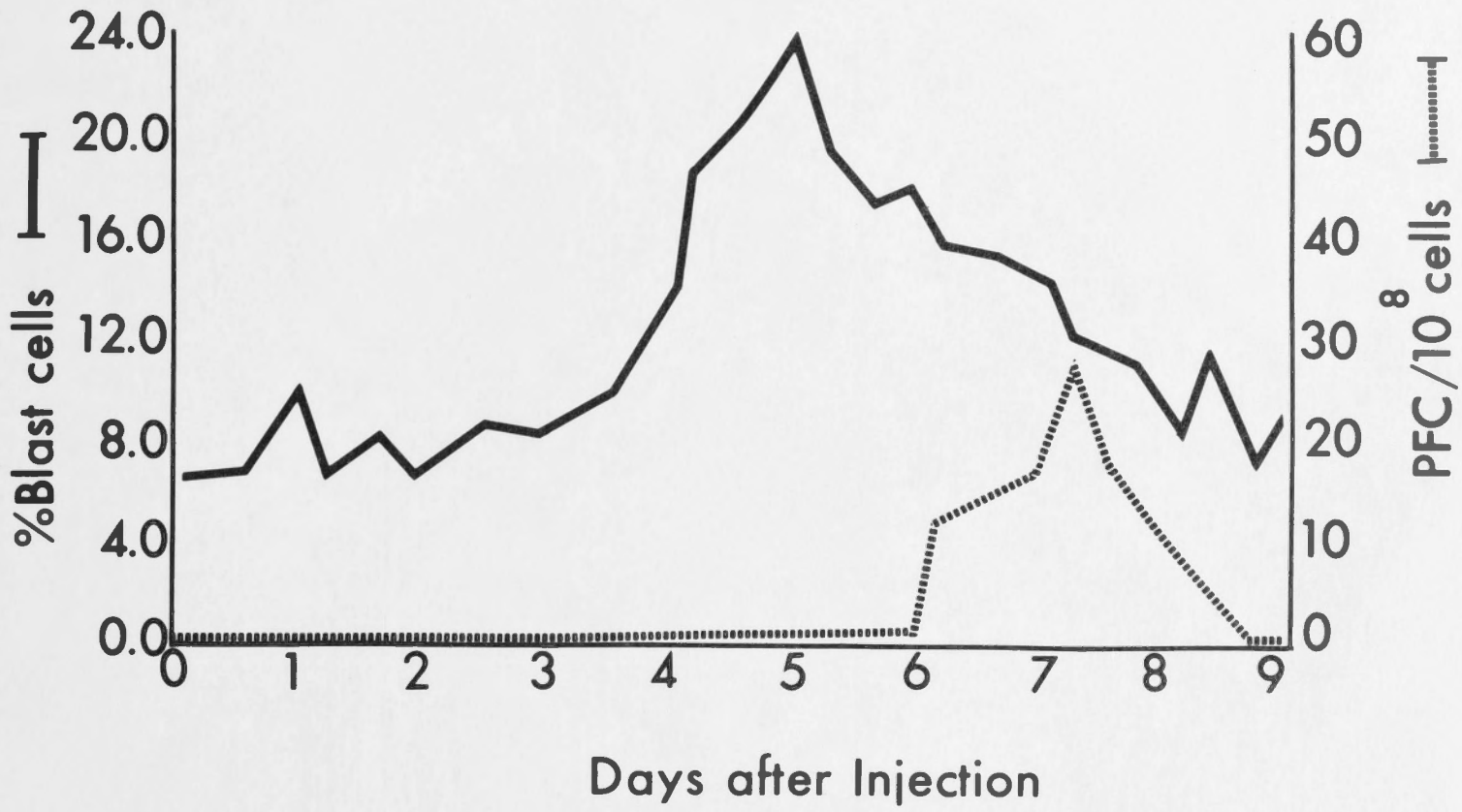
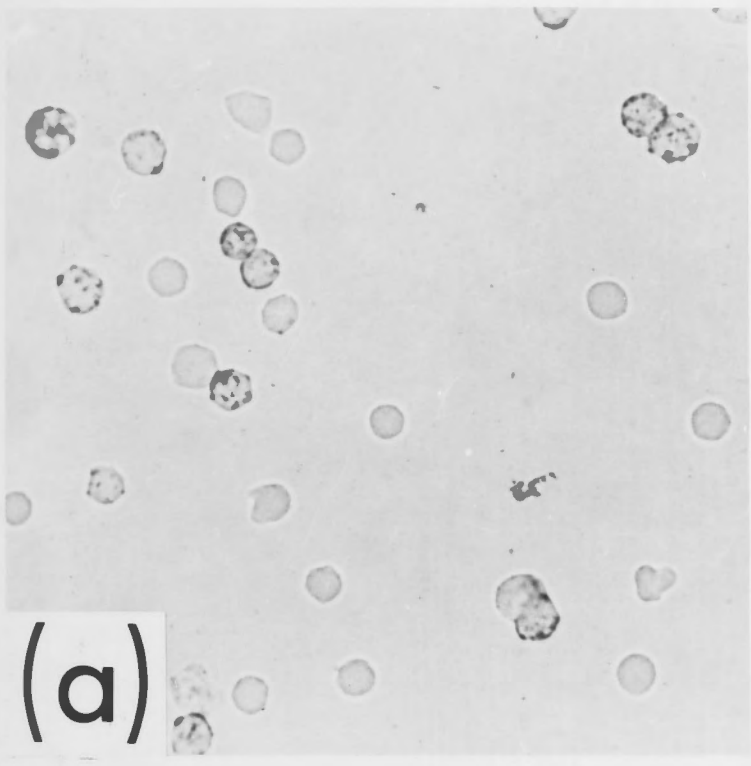


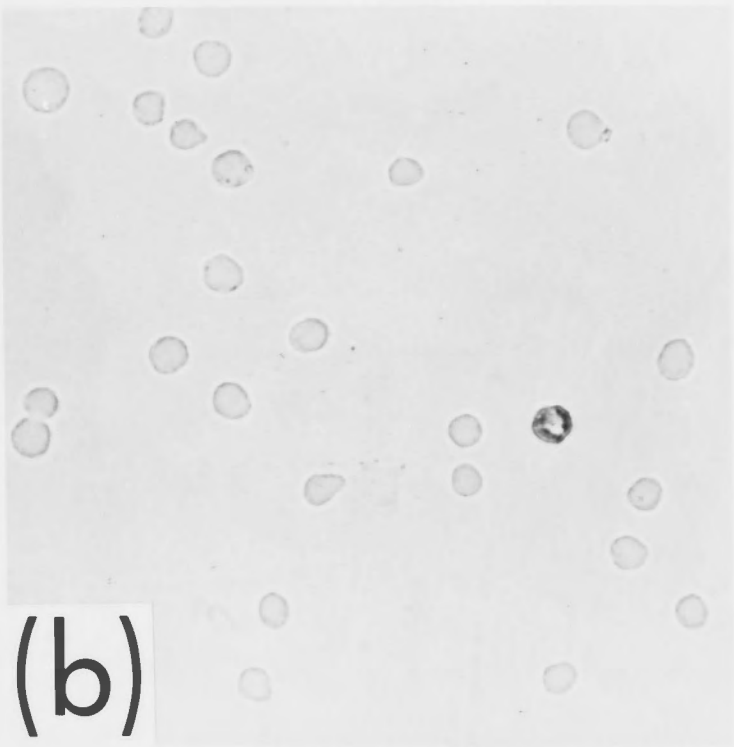
Figure 6-iv:

The "blast" cell and PFC response in the lumbar lymph of a Tx foetal sheep injected with CRBC.

Figure 6-v:

Appearance of lymphocytes from Tx [(a) and (b)] and normal [(c) and (d)] foetal sheep when reacted with HRP labelled rabbit anti-sheep immunoglobulin sera [(a) and (c)] or HRP labelled normal rabbit IgG [(b) and (d)].


(a)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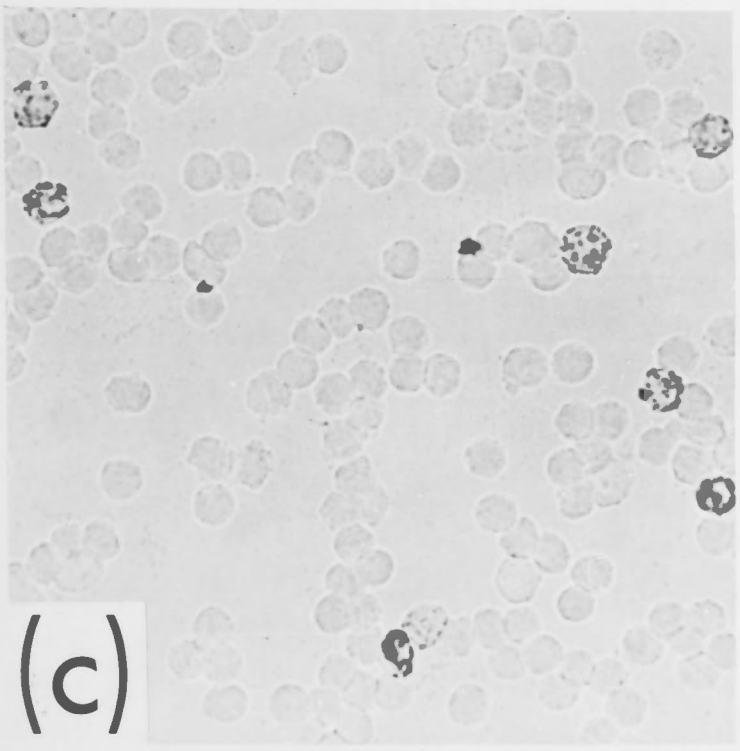
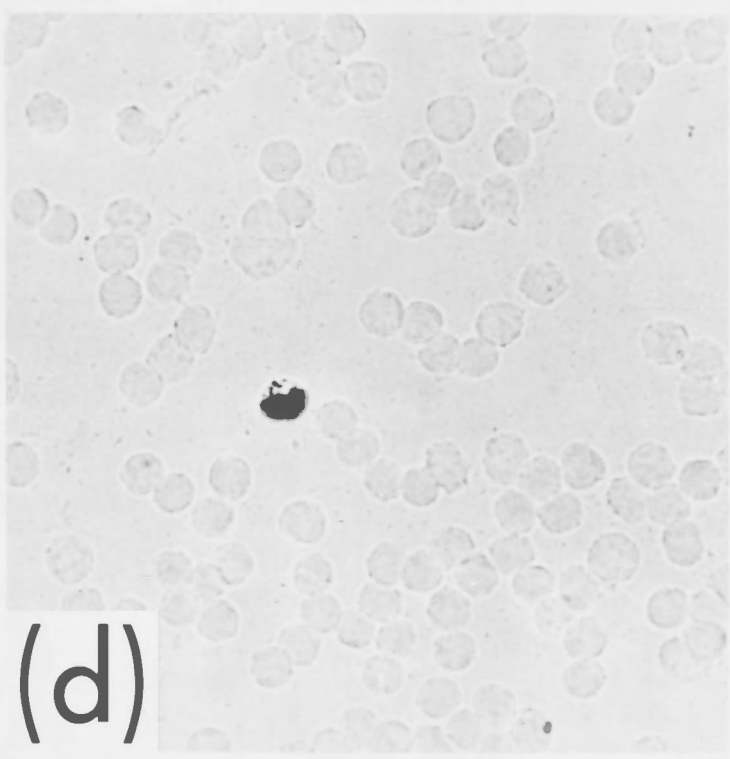

(c)


(d)

determined and essentially reflected the blast cell response, being 0.3%, 5.9%, 15.5% and 8.0% respectively.

The lumbar lymphatic cannula in the third Tx foetus remained patent for only 24 hours. The cell output during this period was 0.81×10^6 cells/hour of which 5.0% were blast cells; 58% of these cells had membrane-bound immunoglobulin. The foetus went on to produce a quite low antibody response to the first injection of CRBC, but a good response to the second injection of CRBC which included 2-ME resistant antibody.

The cannula in the first normal foetus remained patent for 5 days during which time the percentage of blast cells had increased from about 2% to 15% (Figure 6-vi). The first plaques were detected at day 4 and by day 5 had reached 27 PFC/10^8 nucleated cells. The cell output from this foetus was 24.0×10^6 cells/hour during the first 7 hours and rose to 86.6×10^6 cells/hour on day 1, before slowly decreasing to 30 to 35 $\times 10^6$ cells/hour on day 5.

The cannula in the second normal foetus (Figure 6-vii) remained patent for 7 days which allowed the entire PFC response to be followed. The blast cell response in this foetus increased from around 2% at the time antigen was injected to 22% on day 4, slowly decreasing thereafter. Significant numbers of PFC were first detected on day 4 reaching a peak of 121 PFC/10^8 nucleated cells on day 6. The cell output from this foetus rose from an initial level of 13.0×10^6 cells/hour during the first 4 hours to 49.0×10^6 cells/hour on day 4 of the response, decreasing to 23.0×10^6 cells/hour by day 7.

The percentage of lymphocytes with membrane-bound immunoglobulin in the lumbar lymph of the first control foetus was determined on samples collected during the first 7 hours and on days 1

Figure 6-vi and 6-vii:

The blast cell and PFC response in the lumbar lymph of normal foetal sheep injected with CRBC.

Figure 6-vi:

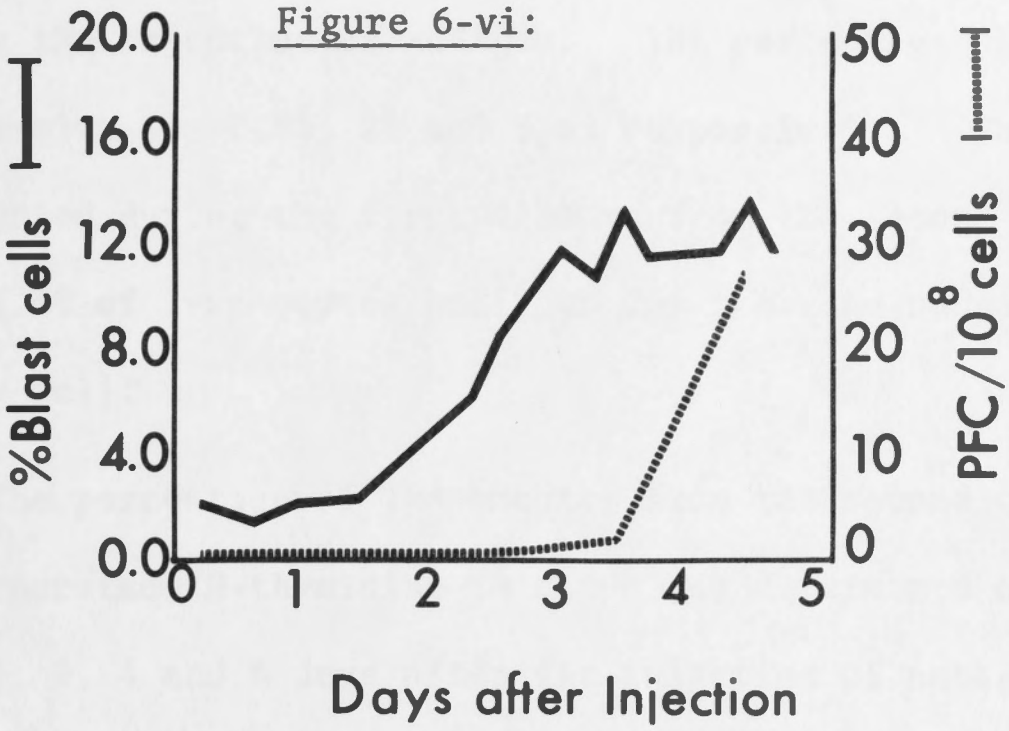
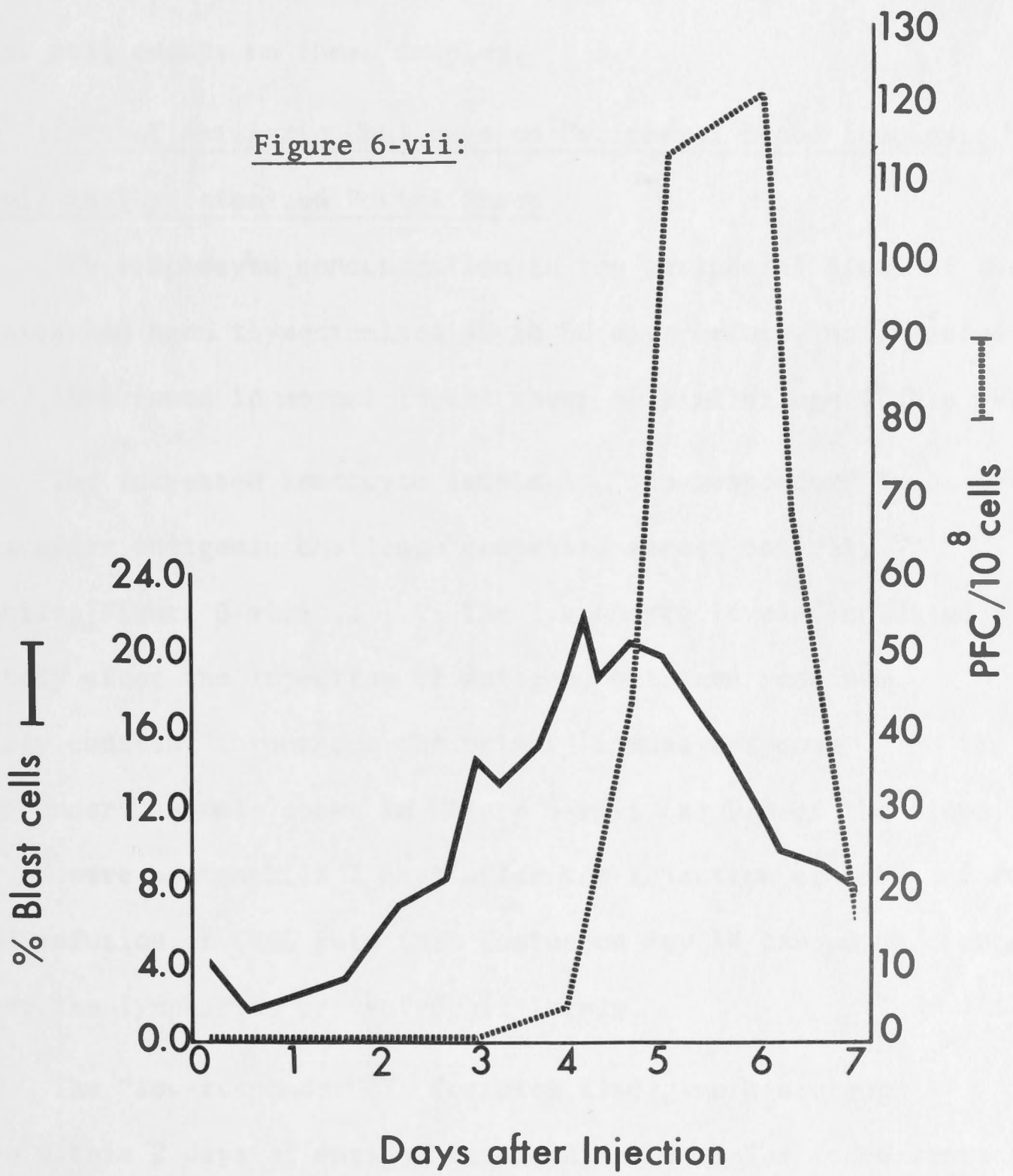


Figure 6-vii:



and 5 after the injection of antigen. The percentage of positive cells in these samples was 2.5%, 2% and 3.6% respectively. The lumbar lymph collected during the first 4 hours from the second normal foetus contained 1.6% of lymphocytes positive for membrane-bound immunoglobulin [Figure 6-v (c)].

The percentage of lymphocytes from the second control foetus which incorporated ^{3}H-thymidine *in vitro* was determined on lymph samples collected 1, 2, 4 and 6 days after the injection of antigen and found to be 0.5%, 0.6%, 20% and 13.5% respectively, essentially confirming the blast cell counts on these samples.

(e) The Effect of Antigenic Challenge on Peripheral Blood Leucocyte Levels in Thymectomized Foetal Sheep

The lymphocyte concentration in the peripheral blood of foetal sheep which had been thymectomized 40 to 60 days before, was reduced to 23% of that found in normal foetal sheep of similar age (Table 6-1).

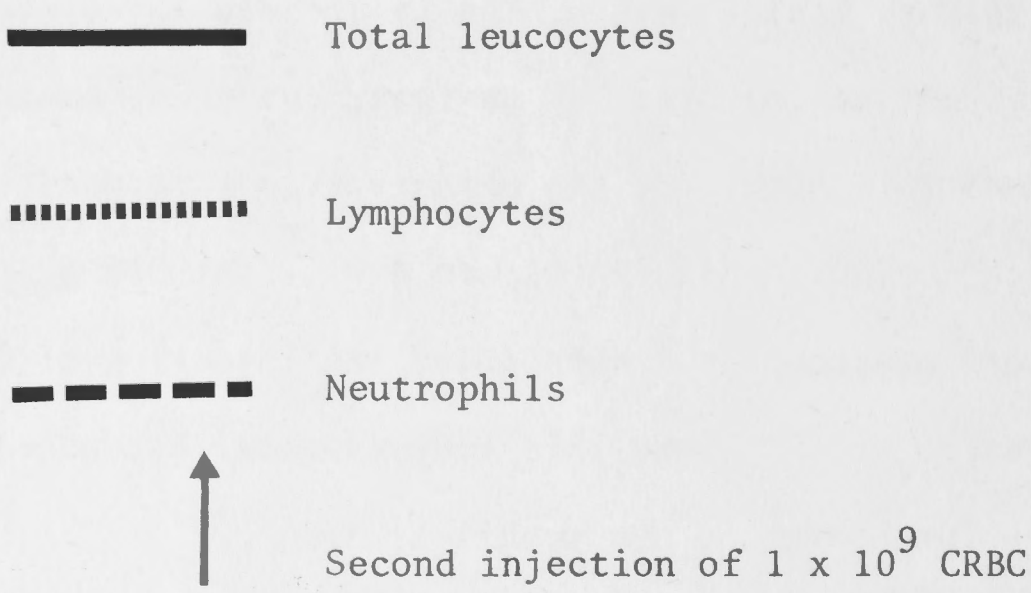
The increased leucocyte levels in "non-responder" Tx foetuses after antigenic challenge comprised almost entirely neutrophils [Figure 6-viii (a)]. The lymphocyte level fluctuated immediately after the injection of antigen, but then remained relatively constant throughout the primary immune response. In the "non-responder" animals shown in Figure 6-viii (a) 97% of the blood leucocytes were neutrophils 2 days after the injection of CRBC and POL. A second infusion of CRBC into this foetus on day 14 caused no change in either the lymphocyte or neutrophil levels.

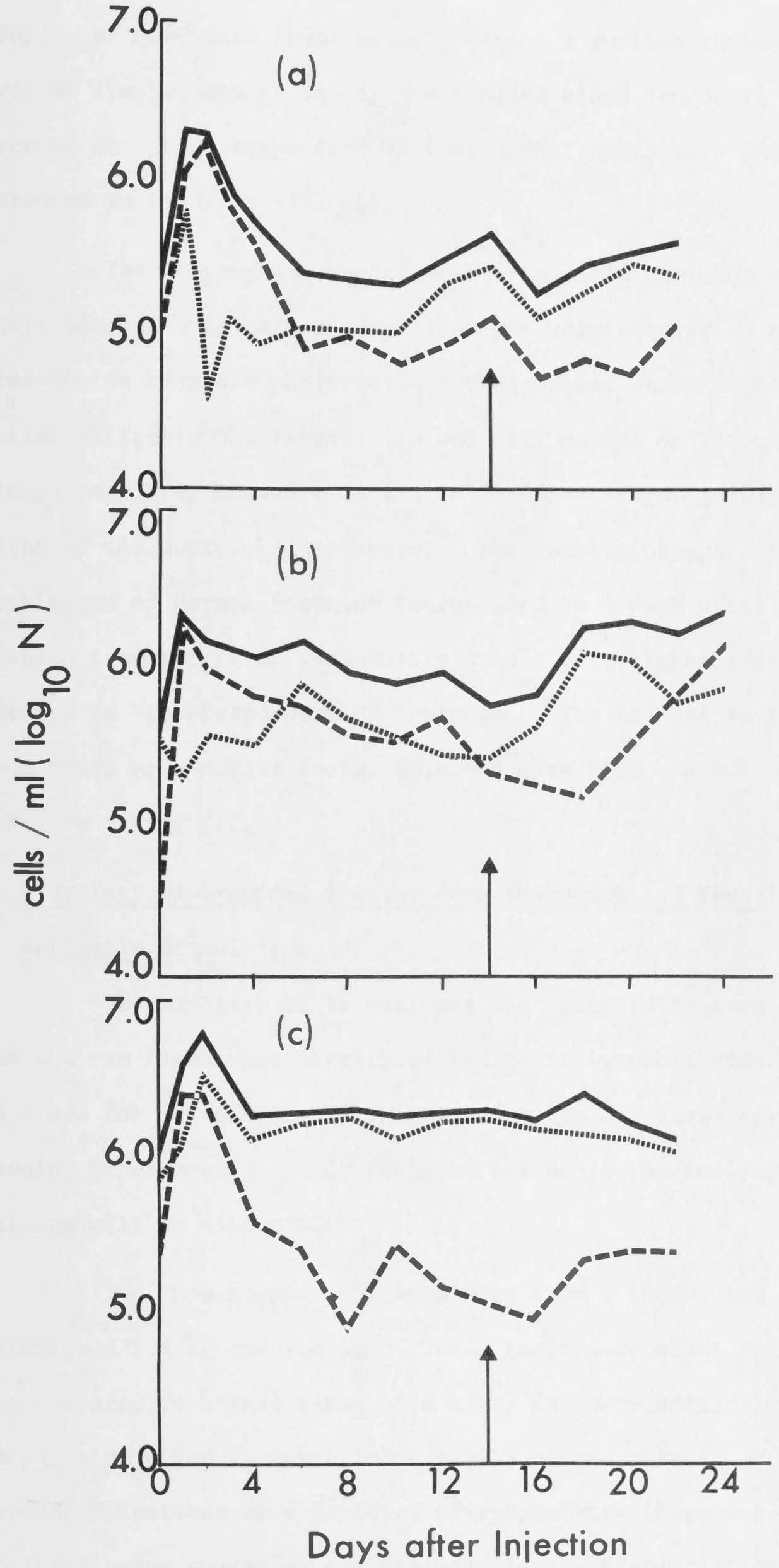
The "low-responder" Tx foetuses also gave a neutrophil response within 2 days of antigen being injected. The concentration of lymphocytes in the blood of these animals again fluctuated immediately after the injection of antigen, but by day 6 of the response it had increased 2 to 7 fold from its initial level. A second

Figure 6-viii:

The changes in the blood leucocyte levels in normal and thymectomized foetal sheep injected with 1 x 10^9 CRBC and 100 µg POL.

(a) "Non-responder" Tx foetus, (b) "low-responder" Tx foetus and (c) normal foetus.




7.0
6.0
5.0
4.0
(a)
(b)
(c)
cells / ml (log10 N)
0
4
8
12
16
20
24
Days after Injection

infusion of CRBC into these animals caused a further increase in the level of lymphocytes by day 4. A typical blood leucocyte response observed in a "low-responder" Tx foetus challenged with CRBC and POL is presented in Figure 6-viii (b).

The neutrophil response observed in Tx foetuses within 1 to 2 days after the injection of antigen was very similar in absolute terms to the response observed in normal foetal sheep receiving similar antigenic challenges. Blood cell counts on Tx and normal animals regularly showed 1 to 2 x 10^6 neutrophils/ml of blood during the height of the neutrophil response. The level of lymphocytes in the circulation of normal foetuses increased 2 to 4 days after antigen, at which time the level was usually 3 to 4 fold higher than that observed in "low-responder" Tx foetuses. The changes in the white blood cells of a normal foetus injected with CRBC and POL are shown in Figure 6-viii (c).

(f) Histology of Lymphoid Tissues from Thymectomized Foetal Sheep after Antigenic Stimulation

The majority of Tx foetuses was carried to term at which time the ram lambs were sacrificed and their lymphoid tissues removed and fixed for histological examination. The ewe lambs were kept for breeding experiments. Only foetuses not subjected to lymphatic drainage will be discussed.

Two "non-responder" sheep were born 5 to 10 days prematurely and weighed 1.4 kg and 1.6 kg. These lambs were about 1 kg underweight when compared to normal lambs born after *in utero* antigenic challenge. The white pulp and periarteriolar region of the spleens of "non-responder" foetuses were depleted of lymphocytes [Figure 6-ix (a)] and the lymph nodes showed no cortico-medullary differentiation, lymphoid follicles or germinal centres [Figure 6-ix (b)]. Pyroninophilic cells

Figure 6-ix:

Histological appearance of lymphoid tissues from thymectomized foetal sheep following antigenic challenge.

(a) and (b) a "non-responder" Tx foetus at birth;

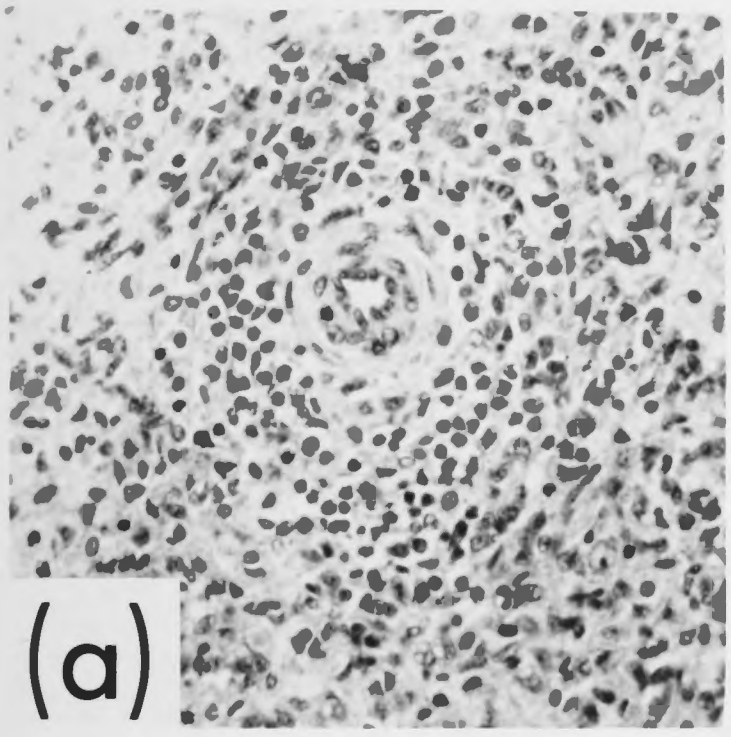
(c) and (d) a "low-responder" Tx foetus at birth;

(e) and (f) a "low-responder" Tx lamb 1 month after birth.

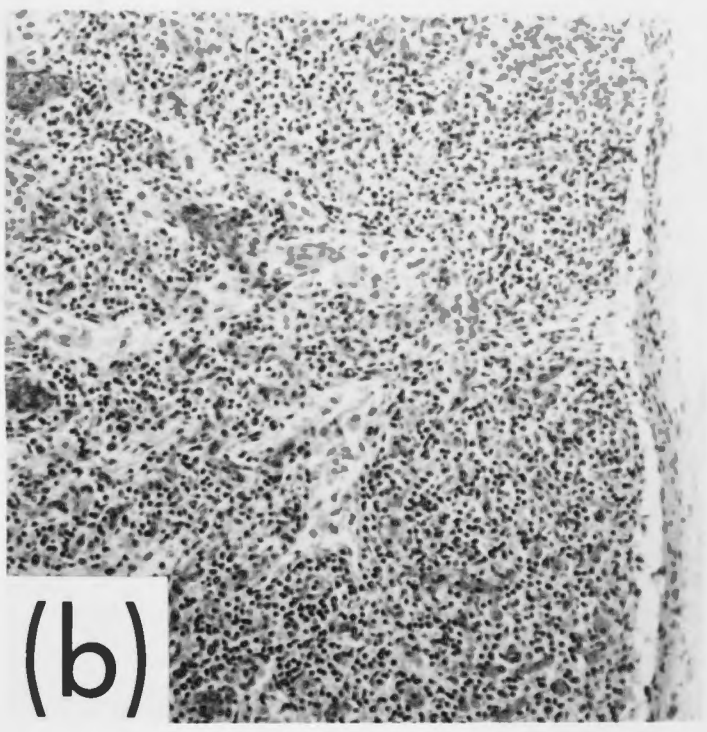
(a), (c) and (e) periarteriolar region of the spleen (x 250);

(b), (d) and (f) lymph nodes (x 100).

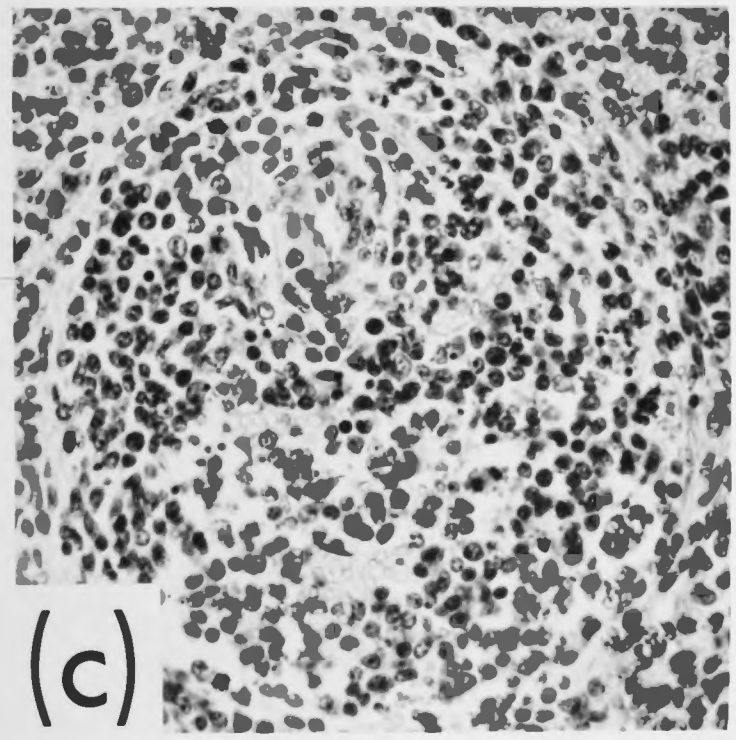
Stained with Haematoxylin-Eosin.



(a)



(b)



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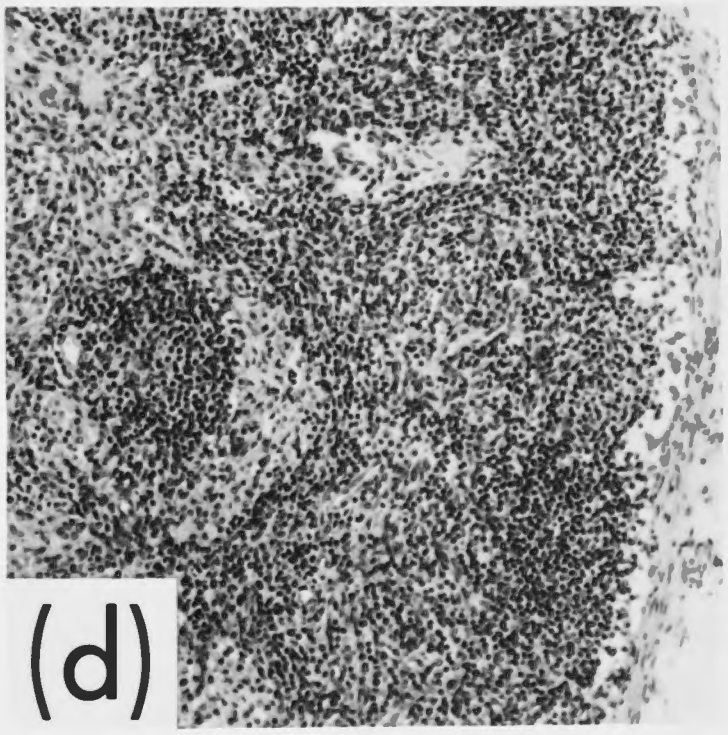

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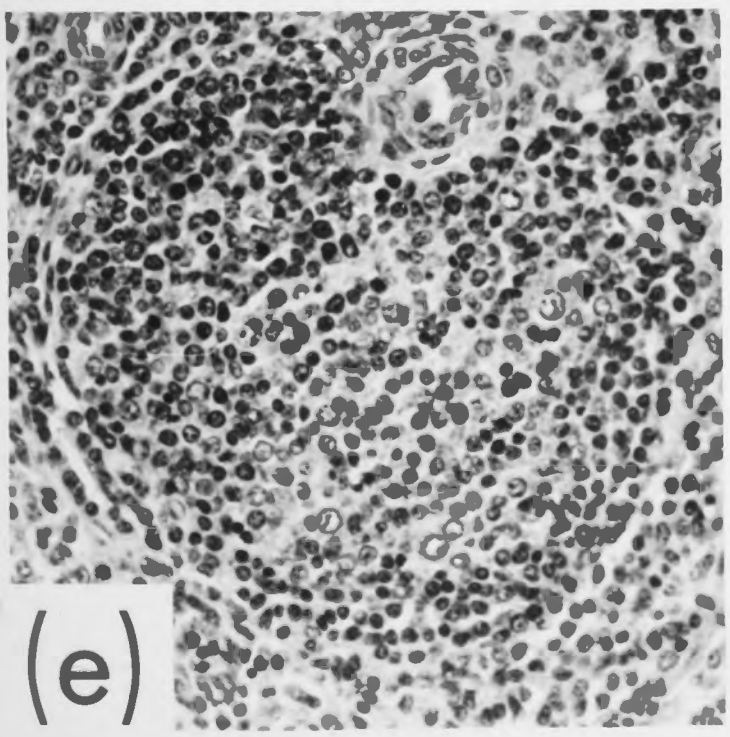
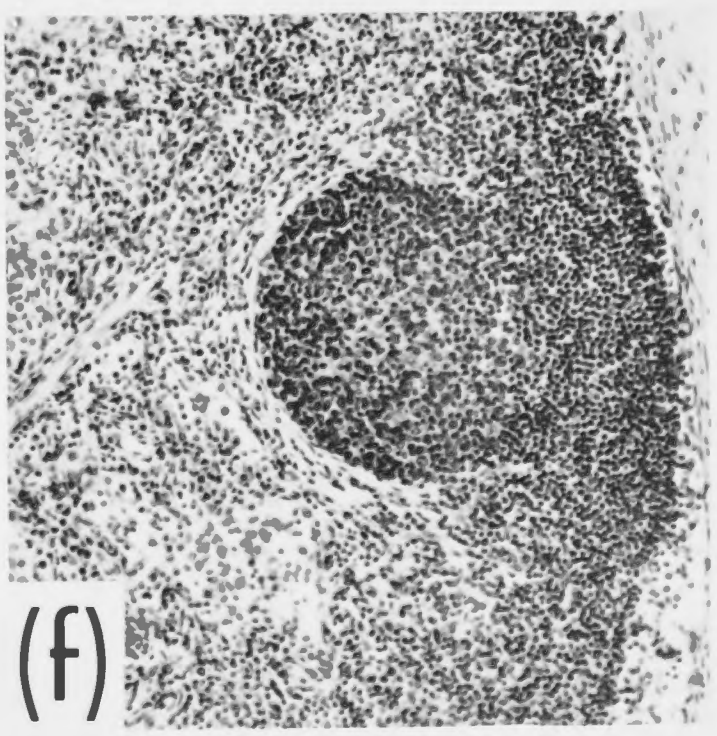


(e)



(f)

and plasma cells were difficult to detect. Interestingly a thymic remnant (of about 100 mg) was found in one of the three animals in this group; this foetus aborted 9 days after antigenic challenge. The two animals that were carried to birth did not have thymic remnants.

The "low-responder" Tx lambs had birth weights between 2.5 and 3.0 kg and suckled immediately after birth. All the females from this group of Tx lambs survived and have been successfully mated. The lymphoid tissues from two "low-responder" foetuses sacrificed at birth were only sparsely populated with lymphocytes by comparison with normal newborn animals. Lymphoid follicles were found in the white pulp in the spleen, although the periarteriolar sheaths were depleted of lymphocytes [Figure 6-ix (c)]. The lymph nodes had a defined cortex and primary follicles could be found, sometimes deep in the depleted parcortical region of the nodes [Figure 6-ix (d)]. Pyroninophilic cells and plasma cells were found in both the spleen and lymph nodes. One "low-responder" Tx lamb was sacrificed 1 month after birth. Again the spleen was depleted of lymphocytes, particularly in the periarteriolar regions, although well developed germinal centres containing many pyroninophilic cells were found in the spleen [Figure 6-ix (e)]. The lymph nodes from this lamb had well defined cortical development, and they contained numerous germinal centres [Figure 6-ix (f)]. The lymphocyte mantles around the germinal centres were absent and the paracortex only sparsely populated with lymphocytes.

There was no residual thymic tissue in the "low-responder" animals examined. Conversely, both "responder" Tx lambs did have remnants of thymic tissue, approximately 200 to 300 mg in weight. The remnants were located at the manubrium of sternum.

Lymphoid tissues from the "responder" Tx lambs were histologically similar to tissues from antigenically stimulated late term foetuses. The lymph nodes were densely populated with cells and the cortico-medullary areas were well defined; germinal centres were present in the lymph nodes and spleen and the white pulp was well developed.

DISCUSSION

The results obtained in Tx foetal sheep challenged *in utero* with CRBC or CRBC and POL or MON have been presented and discussed as though they came from three distinct groups of animals. This arbitrary division was made following analysis of the humoral immune responses given by the foetuses. Of the twelve Tx foetal sheep successfully challenged, three failed to produce any detectable antibody during the 2 weeks the primary response was followed. These animals have been designated as "non-responders". A second group of six foetuses gave reduced antibody responses to CRBC or POL and did not produce 2-ME resistant antibody to either antigen. These animals have been described as "low-responders". The remaining three animals gave good responses to CRBC, POL or MON and all produced detectable levels of 2-ME resistant antibodies to at least one of the two antigens injected; two animals producing 2-ME resistant antibody to both antigens. These animals have been described as "responder" animals.

Having made these divisions it was necessary to explain the differences in the immune responses found in these groups. The first piece of supportive data came from the total and differential cell counts performed on the blood of the Tx animals immediately prior to antigenic challenge. The leucocyte levels in the "non" and "low-responder" foetuses were reduced to about a third of those in the "responder" or

normal control foetuses of the same age (Figure 6-i). The differential cell counts performed on these animals confirmed that this was due to fewer blood lymphocytes (Table 6-1). It may be that the differences in blood leucocyte levels and in immune responsiveness between different Tx animals was due to the presence of residual thymic tissues. This would not seem to be the whole answer however, as one "non-responder" foetus had a thymic remnant. Also it was difficult to conceive that a piece of thymus representing at best a few per cent of the thymic mass present in a normal foetus at birth, could be totally responsible for the immune competence of these animals.

The histological examination of the Tx animals showed a correlation with their ability to mount humoral immune responses. The "non-responders" showed little histological development of their lymphoid tissues even after antigenic challenge. The "low-responder" Tx animals had sparsely populated lymphoid tissues, but did respond to antigen by the generation of germinal centres and periarteriolar sheaths in the spleen. The lymphoid tissues of "responder" Tx foetuses could not be distinguished histologically from normal foetuses receiving similar antigenic challenge.

The data obtained by cannulating the lumbar trunk of Tx and normal foetuses demonstrated the depletion of recirculating lymphocytes which followed thymectomy. Similar results have recently been published for thoracic duct lymph from normal and Tx foetal sheep by Pearson, Simpson-Morgan and Morris (1976). Conversely, there was an increase in the percentage of lymphocytes which had membrane-bound immunoglobulin in the lymph of Tx animals compared to the percentage found in normal foetal sheep (12.4% and 58% compared to 1.6% and 2.5%). This observation could indicate that cells without surface immunoglobulin had been specifically depleted by thymectomy or that

the maturation of cells with membrane-bound immunoglobulin was specifically or non-specifically accelerated as a result of thymectomy. Similar results have been reported in athymic (nude) mice, the percentage of thoracic duct lymphocytes with membrane-bound immunoglobulin increasing from 26% in conventional mice to 87% in nude mice (Bankhurst and Warner, 1972). The percentage of cells with surface IgM increased 3 fold, while the percentage of cells with surface IgG and IgA both showed a 2 fold increase in nude mice.

The peak of the blast cell response in the lumbar lymph of normal foetuses challenged with CRBC occurred on about day 4 and in the Tx foetuses on about day 5. The maximum percentage of blast cells ranged between 15% and 22% in both groups of animals. PFC were detected in the Tx foetus 36 hours later than in the two normal foetal sheep, while the peak PFC response also occurred 36 hours later in the Tx foetus than in the normal foetus. The peak PFC response, on a cell to cell basis, was reduced in the Tx foetus compared with the normal foetus (27 compared to 121 PFC/10^8 nucleated cells). If the reduced cellular output in the Tx foetus was considered together with the reduced PFC response, the difference from the response in the normal animal was even more evident. Initially and at the peak of the respective PFC responses the cell output in the Tx foetuses was about 20% of that in the normal foetus. The difference between the peak of the PFC response in the two animals was about 25 fold if calculated in terms of PFC output per hour.

The difficulty in evaluating the *in vivo* results obtained in Tx animals lay in distinguishing between the quantitative and the qualitative effects of thymectomy. Was the decreased competence observed simply due to the marked lymphoid depletion following thymectomy or were some immune functions specifically depressed in these animals?

Serum antibody titres are a quantitative measurement of immune competence, while differences in PFC responses could reflect a qualitative difference between populations of cells. However, as a PFC response is the end result of a complex interaction of cells and molecules, a reduction in PFC numbers in thymectomized animals could be due to a quantitative change in any of the components of the reaction, particularly if the thymus has an endocrine function (Osoba and Miller,1963), as well as being a generator of lymphocytes.

Analysis of antibody synthesis following antigenic challenge, particularly the response to CRBC, indicated that the response in Tx animals, excluding the 3 "responder" foetuses, was either absent or significantly depressed. The foetuses did not discriminate between POL and CRBC as "T-dependent" or "T-independent" antigens as they either responded to both or to neither.

As no 2-ME resistant antibody was synthesized by the "low-responder" Tx foetuses, it could be suggested that these animals could not synthesize IgG antibody. However, if the observed 8 fold decrease in the anti-POL titre found in Tx animals compared with normal animals was purely quantitative then a comparable reduction in the 2-ME resistant antibody response to POL detected in normal animals would render the antibody undetectable using the haemagglutination technique. Similar reasoning could be used to explain the failure of Tx foetuses to produce detectable 2-ME antibody to CRBC.

An argument against the effect of thymectomy being qualitative was the finding that "low-responder" Tx foetuses subsequently mounted good antibody responses to a second injection of CRBC [Figure 6-ii (c)], a large component of which was resistant to 2-ME. This result suggests that the reduced competence of Tx foetuses can be renovated by antigenic stimulation and that all cellular components of

a secondary response to CRBC, including the production of IgG, are present 50 to 70 days after the removal of the thymus. Sinclair (1967) made a similar observation in neonatally Tx Swiss mice which, while giving a much reduced primary antibody response to sheep erythrocytes, gave a good 19S and 7S haemolysin antibody response to a second injection of antigen.

The restoration of immune competence in Tx foetal sheep that followed antigenic stimulation could account for the findings of Cole and Morris (1971a, b, c and d) that *in utero* thymectomy, whilst causing depletion of lymphoid tissues and a reduction in circulating lymphocyte levels, did not affect the ability of lambs to mount both "cellular" and "humoral" immune responses when tested several months after birth.

The analysis of immunoglobulin synthesis following antigenic challenge did not totally resolve the question of whether Tx foetal sheep could synthesize IgG_1. With the exception of all "non-responder" Tx foetuses, all foetuses synthesized IgM, some to concentrations comparable to those produced by normal foetuses. Two of the six animals described as "low-responders" synthesized IgG_1, which was detected in these animals on days 4 and 8 respectively; both produced less than 80 µg/ml. The fact that any synthesis was detected could be taken to indicate that the capacity to synthesize IgG_1 was not specifically ablated, although the failure by the remaining four foetuses would then have to be explained as a quantitative reduction in IgG_1 synthesis to an undetectable level, despite the synthesis of normal levels of IgM. Sephadex G200 fractionation of pooled plasma from a "low-responder" Tx foetus which did not synthesize detectable levels of IgG_1 following challenge with CRBC, failed to show any detectable anti-CRBC antibody in the IgG region despite a 6 fold concentration from the

initial plasma.

The failure to synthesize IgG_1 could be due to a delay in the maturation of the immune system similar to the findings in normal animals challenged before 80 days gestation. That cells capable of synthesising IgG can differentiate and develop in the absence of the thymus was shown by Tyan, Cole and Hertzenberg (1967). They found foetal liver cells could reconstitute the ability of irradiated Tx mice to synthesize IgG_{2a}. Similarly Huchet and Feldman (1974) found the IgG response in athymic "nude" mice was reduced to about 1/8 to 1/5 of normal levels, but not completely ablated. Schrader (1973; 1974) suggested that "T-independent" antigens such as POL can, acting as adjuvants, enhance antibody synthesis to "T-dependent" antigens in the absence of T cells. In the experiments reported above however, the anti-CRBC response in three "low-responder" Tx foetal sheep injected simultaneously with POL and CRBC was similar to the anti-CRBC response in three "low-responder" foetuses which only received CRBC.

What has been shown is that thymectomy in foetal sheep causes a reduction in lymphoid cells, both in the circulation and fixed tissues. At the same time the synthesis of immunoglobulin, particularly IgG_1 and humoral antibody to CRBC and POL was also reduced. These results however, could be due to quantitative rather than qualitative events. Evidence that thymectomy may selectively effect a particular population of cells rests on the observations that the percentage of cells with membrane-bound immunoglobulin in the lymph of Tx foetal sheep was increased, that the PFC response to CRBC on a cell to cell basis was both delayed in onset and reduced in magnitude and the observation by Pearson *et al* (1976) that cells in the lymph of Tx foetuses recirculate more slowly than cells from normal foetuses (c f. Sprent, 1973).

CHAPTER 7

MODIFICATION OF THE IMMUNOLOGICAL COMPETENCE OF FOETAL SHEEP: ANTIGEN-INDUCED MATURATION OF IgG SYNTHESIS

CHAPTER 7

Modification of the Immunological Competence of Foetal Sheep: Antigen-Induced Maturation of IgG Synthesis

INTRODUCTION

The changes that occur in the kinetics of antibody formation to a secondary challenge with antigen were clearly documented by Glenny and Sudmersen (1921) and Glenny (1925). Studying the response of horses to diphtheria toxoid they found the second injection of antigen induced antibody to appear more quickly, to reach higher levels and to persist longer than antibody induced by the first injection. The primary immune response to many antigens is characterized by an initial synthesis of IgM followed some time later by the synthesis of IgG (Humphrey and Porter, 1956; Bauer and Stavitsky, 1961; Benedict, Brown and Ayengar, 1962; Uhr, Finkelstein and Baumann, 1962b; Bauer, 1963; Roszman, *et al*, 1971). Nossal, Szenberg, Ada and Austin (1964) found that antibody-forming cells in the popliteal lymph node of rats immunized with Salmonella flagella were initially synthesizing IgM, while later in the response, the majority of cells synthesized IgG. Sterzl and Nordin (1971) made a similar observation during the PFC response to SRBC and postulated a gene switch during the multiplication of PFC. Not all antigens investigated however induced the sequential synthesis of IgM and IgG; for some stimulated the simultaneous production of IgM and IgG (Freeman and Stavitsky, 1965; LeFor and Bauer, 1970).

The secondary antibody response frequently had a shorter lag phase, while the antibody formed was less specific (Eisen, Little, Steiner, Simms and Gray, 1969) and of higher affinity (Steiner and Eisen, 1967). The increased affinity was usually associated with IgG

antibodies (Voss and Eisen, 1968; Saryas and Makela, 1970) which were frequently produced in significantly larger amounts than during the primary response (Benedict *et al*, 1962).

In Chapter 4 it was reported that foetal sheep injected with antigen on or before 70 days gestation did not synthesize IgG_1. Three foetal sheep around 70 days gestation were injected with various antigens, some in FCA and their responses followed for 24, 24 and 36 days respectively. Two animals did not synthesize IgG_1 at any stage of the response, while the third animal followed for 24 days had detectable levels of IgG_1 on days 20 to 24. From these animals and others injected around the same stage of gestation, it would appear that foetal sheep cannot synthesize IgG_1 much before 87 days gestation (Table 4-3) even though they can respond to the antigens, by synthesizing IgM. To determine if the time at which IgG_1 was first synthesized could be influenced by antigenic stimulation, foetal sheep were given two injections of antigen well before the stage of development when IgG_1 appears first in primary responses. The antigens chosen for these experiments were POL, CRBC and OA. POL and CRBC were chosen because both stimulate antibody synthesis before mid-gestation and both stimulate IgG_1 synthesis when injected into primed foetuses (Chapters 5 and 6). OA was included in some secondary challenges to determine if the variable onset of competence to this antigen could be modified by antigen induced lymphopoiesis.

RESULTS

HUMORAL ANTIBODY RESPONSES TO A SECONDARY CHALLENGE WITH ANTIGEN BEFORE MID-GESTATION

SERIES 1: Foetal Sheep Primed and Rechallenged with Several Antigens

Three foetal sheep were primed with 50 µg POL and 5×10^8 CRBC

injected between 55 and 56 days gestation. The foetuses developed *in utero* until 70 to 71 days gestation when they were re-exposed and injected with 25 µg of POL, 1 x 10^8 CRBC and 1 mg OA in 0.1 ml FCA. Two unprimed control foetuses of the same age were similarly challenged. An indwelling cannula was introduced into one jugular vein of each foetus to enable serial blood samples to be collected.

One of the three foetuses was found dead in the uterus 8 days after challenge, one was removed from the uterus alive after 8 days and the third was removed alive 34 days after challenge. All foetuses were grossly swollen with massive sub-cutaneous oedma (anasarca). The thoracic duct of the animal that survived for 34 days was no longer patent and the foetus was about twice as large as animals of a similar age. Biochemical analysis of the plasma did not show abnormal levels of urea, creatinine, sodium or potassium. Histological examination of the tissues from the foetus which survived 34 days showed areas of necrosis in the lymphoid tissues.

The two animals that became oedematous within 8 days both had antibody to POL on day 2 and relatively high titres of antibody by day 4; 8.0 and 8.5 ($\log_2$ N). The 2-ME resistant antibody titres to POL in one of these animals was 3.0 on day 4.

The anti-CRBC titres in all primed foetal sheep were low and not significantly different from the control foetuses. Anti-OA responses were not detected in any of the animals receiving a second challenge with antigen and not until day 16 in one of the control animals.

Analysis of the serum from these three foetuses showed that the animal which was found dead on day 8, had very high levels of IgG_1 by day 4 (94 µg/ml). IgG_1 was not detected in either of the other primed foetuses or in the circulation of the control animals.

SERIES 2: Foetal Sheep Primed and Rechallenged with POL

Two foetal sheep were primed by injecting 25 µg of POL into each limb on one side of the animal, at 56 days gestation. Two weeks later the foetuses were re-exposed and 50 µg of POL emulsified in 0.05 ml of Freund's incomplete adjuvant (FIA - Difco), was injected into each limb on the opposite side of the animal. The jugular vein of both animals was cannulated and blood samples collected. A normal 70 day old foetus was also injected with a total of 100 µg POL in FIA.

The cannula in the first experimental foetus remained patent and provided blood samples every second day. This foetus had anti-POL haemagglutinating antibody on day 2, reaching a titre of 6.5 on day 4. Blood samples were obtained from the second experimental foetus on days 0, 6 and 9. The anti-POL titres on days 6 and 9 were 11.0, all of which was resistant to 2-ME. Neither animal showed signs of the syndrome described in the animals of the first series when delivered by hysterotomy on day 9. Antibody was first detected in the normal foetus on day 4 and reached a titre of 7.0 on day 12. All antibody synthesized by this foetus was sensitive to 2-ME.

Quantitation of the immunoglobulin levels in the two experimental and one control foetus showed that the foetus which had produced 2-ME resistant antibody to POL had a relatively high concentration of IgG_1 (152 µg/ml) 6 days after the second injection of POL, while the second experimental animal had low levels of IgG_1 (up to 55 µg/ml). The control foetus only synthesized IgM.

The specificity of the IgG_1 produced by the primed foetus which produced 2-ME resistant antibody to POL was determined by passing the serum collected on day 9 over a Sepharose - POL adsorption column, to remove all antibody. Quantitation of the IgG_1 level in this serum

before and after it had been passed through the column showed 67% of the IgG_1 synthesized by this foetus was specifically adsorbed to POL.

SERIES 3: Specific and Non-Specific Synthesis of IgG_1 in Foetal Sheep

The question of whether the synthesis of IgG_1 by primed 70 day old foetal sheep was antigen "specific" or "non-specific" was investigated by priming the foetuses with POL then rechallenging with POL, CRBC and OA.

Two foetal sheep at 54 and 57 days gestation respectively were injected in both limbs on one side of the animal with a total of 50 µg of POL. Two weeks later one animal was injected s.c. on the opposite side with 50 µg of POL in 0.1 ml FIA and with 5×10^8 CRBC and 1.0 mg of OA in 0.1 ml FCA. Two unprimed normal foetuses (68 and 70 days old) received the same antigenic challenge while the remaining primed foetus (71 days old) only received CRBC and OA in FCA.

The first primed foetus, rechallenged at 68 days gestation, produced good titres of 2-ME sensitive anti-POL antibody (up to a titre of 7.0). Conversely, the response of this foetus to the first contact with CRBC resulted in low titres of haemagglutinating antibody (up to a titre of 3.0), although 2-ME resistant anti-CRBC antibody (titre of 1.0) could be detected from day 10 of the response. The anti-OA response was not detected until day 16.

The foetus challenged with POL at 57 days and CRBC and OA at 71 days gestation, although producing good titres of anti-CRBC antibody (up to a titre of 4.5) by day 8, did not synthesize 2-ME resistant antibody. A response to OA was not detected in this foetus until day 17 and by day 20, 2-ME resistant antibody was detected. The foetus was 91 days old when 2-ME resistant antibody was first detected, which

is very similar to the time at which 2-ME resistant antibody is first produced in normal foetal sheep (Table 3-1).

The two control foetuses produced anti-POL antibody by day 4, reaching peak titres of 5.0 and 6.0 respectively. The response to CRBC appeared in one foetus on day 4 and the other on day 8, but the titre of anti-CRBC antibody in both animals was very low. An anti-OA antibody response was detected in one foetus on day 8 and reached a titre of 7.0 by day 10. The antibody detected in the control foetuses to all these antigens, was sensitive to 2-ME. The anti-OA response in this latter normal foetus appeared substantially earlier than in either of the primed foetuses.

Analysis of the serum from the animals in this series showed that the primed foetuses had detectable concentrations of IgG_1 at 79 and 80 days gestation respectively (48 to 51 µg/ml), while neither control foetus had produced IgG_1 by 84 days gestation.

DISCUSSION

The results of these experiments showed that foetuses challenged with POL at around 56 days and given a second challenge some 14 days later produced anti-POL antibody 2 days after the second challenge; at least 2 days sooner than antibody had ever been detected during primary immune responses in normal foetuses of any age. The earlier onset of anti-POL antibody possibly indicated that the foetuses were giving secondary antibody responses. 2-ME resistant anti-POL antibody was detected in the circulation of two of these six foetuses when they were 74 and 76 days old, respectively. This was at least 16 to 18 days earlier than 2-ME resistant antibody had been detected previously during any primary immune response. Analysis of the immunoglobulin in the circulation of these primed foetuses

after the second injection of antigen showed IgG_1 had been synthesized in four of the six foetuses at or before they had reached 80 days gestation. The IgG_1 synthesized by the foetus which produced the highest titre of 2-ME resistant antibody following the second injection of POL, was almost entirely specific for POL.

It would seem that during the *in utero* development of sheep, the synthesis of IgG occurs substantially later than the synthesis of IgM (Chapters 3 and 4). Why there is sequential synthesis of the two immunoglobulin classes has not been defined, although it can be seen that the onset of IgG synthesis can be accelerated at least in some foetuses by prior antigenic stimulation. Whether this maturation is specific or non-specific has not been finally resolved, although there is evidence to suggest that it may be non-specific. The synthesis of 2-ME resistant antibody to CRBC by a foetus primed with POL may be dependent on the priming antigen being injected simultaneously with the secondary challenge antigen. The finding by Schrader (1973) that antibody production to a "T-dependent" antigen could be enhanced in athymic (nude) mice by the simultaneous injection of POL, a "T-independent" antigen, may be related to the results obtained in foetal sheep. The very high percentage of specific antibody synthesized by the one foetus which was given a second injection of POL, may however suggest that the maturation of IgG_1 synthesis is antigen specific.

The finding that antigenic challenge can change the time at which IgG synthesis occurs in the foetus, is the first report that antigens can effect the maturation of immune competence *in vivo*. Binns and Symons (1974a) found that the injection of antigen into foetal pigs at 59 days gestation resulted in an increase in the percentage of cells with membrane-bound immunoglobulin, while Davie *et al* (1974) suggested that antigen plays a role in the appearance or expression of

cells bearing IgG in the guinea pig. Conversely, Kincade and Cooper (1971) found that antigen did not alter the sequence of development of IgM and IgG containing cells in the bursa of Fabricius in chickens, although antigen did promote the development of immunoglobulin containing cells in peripheral lymphoid tissues. Paul (1974) put forward the proposal that the development of cells with surface IgG, in species lacking a bursa, may depend on antigenic stimulation.

The pathology of the syndrome observed in the first series of experiments has not been resolved. The finding was unexpected as secondary antibody responses to both POL and CRBC have been induced in older foetuses (Chapters 5 and 6) without any overt pathological changes. The syndrome seemed to be antigen specific and could be prevented by giving the second injection of the sensitizing antigen emulsified in Freund's incomplete adjuvant. A purely speculative explanation could be that antigen - antibody complexes prevent the development and growth of the peripheral lymphatics or affect the contractility of the musculature of the lymphatics thereby preventing recirculation of lymph. If such antigen-antibody complexes were formed it is tempting to suggest that the antibody may have been IgG_1, since the foetus which died by day 8 had detectable levels of IgG_1 in the circulation by day 4.

CHAPTER 8

FINAL DISCUSSION AND CONCLUSIONS

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Whether foetal sheep acquire the ability to mount immune responses to different antigens in a sequential manner after 40 days gestation as claimed by Silverstein and Prendergast (1970; 1971) seems doubtful in view of the results of the experiments reported in this thesis. Foetal sheep were found to be capable of producing antibody to seven of the eight antigens studied between 64 and 82 days gestation; a period of 18 days, when the primary immune response was followed by measuring haemagglutinating antibody in serial blood samples. Conclusions based on single blood samples obtained by hysterotomy 6 to 60 days after the injection of antigen (Silverstein *et al*, 1963a) may fail to detect transient antibody responses which occurred frequently in young foetuses injected with CRBC, POL, MON, FER in FCA and OA in FCA, particularly when only IgM antibody was synthesized during the immune response.

Not all foetuses injected at a particular stage of gestation with certain antigens such as OA and CγG mounted detectable antibody responses, even though other animals of the same age could respond. It appeared that for certain antigens the onset of competence did not arise at a precise stage of development. The onset of competence to FER, POL, MON and CRBC appeared to occur over a more restricted period of gestation, after which time all foetuses responded when challenged with these antigens.

The antibody responses to several antigens, especially POL, FER and CγG, increase considerably in magnitude and duration in older

foetuses, although the time at which antibody first appeared during a response did not change substantially with development. One possible exception to this was the response to CRBC, where antibody first appeared sooner in older foetuses. The antibody response of foetuses of the same age to some antigens, particularly OA and CRBC, varied considerably and responses within a group could be absent, weak or relatively strong. The magnitude of the responses to POL, MON, FER and CγG were more uniform within a group of foetuses of the same age. Another parameter of immunological competence, namely the capacity to synthesize 2-ME resistant antibody to several of the antigens studied was also greater in older foetuses.

While the onset of competence to respond to various antigens does not seem to be associated with a particular stage of lymphoid development in the foetal sheep, the subsequent maturation of these antibody responses both in magnitude and character, with increasing gestational age, does parallel the extensive lymphopoiesis going on during the last half of gestation. The increase in the magnitude of the response with increasing foetal age may be directly associated with development of the lymphoid apparatus.

At least two of the classes of immunoglobulin were synthesized sequentially during gestation. Young foetuses synthesized only IgM in response to a primary stimulation with antigen, while older foetuses synthesized both IgM and IgG_1. Trace amounts of IgG_2 were found after prolonged stimulation, but IgA was never detected following systemic antigenic challenge.

Studies on the antibody response of foetal sheep to a single antigen revealed a lack of correlation in individual foetuses between the level of immunoglobulin in the circulation and the antibody titre, when this was measured by haemagglutination. The maximum concentration

of IgM in the circulation of groups of foetuses injected with 100 µg of POL between 70 and 120 days gestation did increase, as did the maximum antibody titre for each group, the later in gestation the foetuses were challenged. The appearance of IgG_1 in the circulation of foetuses challenged at 120 days gestation reflected the presence of 2-ME resistant antibody. Three younger foetuses, however, had detectable levels of IgG_1 in the absence of 2-ME resistant antibody, although in no case was 2-ME resistant antibody ever detected in the absence of IgG_1. The lack of correlation between immunoglobulin levels and antibody titres in individual animals could in part be due to the different parameters being measured. The radial immunodiffusion technique simply determined the quantity of immunoglobulin with a particular antigenic configuration, while the antibody assay also took into account the quality of the immunoglobulin (affinity) as well as the quantity. Neither assay however, measured the biological activity of the immunoglobulin.

During the primary immune response to POL the majority of IgM synthesized did not show specifity for the antigen. This suggested either that the antibody was of very low affinity (Eisen and Siskind, 1964) or that it was "non-specific". During the secondary immune response the majority of IgG_1 synthesised could be adsorbed to POL, indicating that the antibody was "specific" for the stimulating antigen. Being unable to rigorously control either the purity of the antigen preparations used or the possibility of inadvertent stimulation as a result of the surgical procedures, it was not possible to conclude that a major portion of IgM synthesized during the primary immune response, or a minor portion of the IgG_1 synthesized during the secondary response was "non-specific" although the results strongly suggest that this may be so.

Analysis of the IgG_1 synthesized during the secondary immune response to POL suggested that a very limited number of clones of cells were producing anti-POL antibody of the IgG_1 class. In one foetus it was possible that in fact only one clone was producing the homogeneous anti-POL IgG_1 detected.

The synthesis of IgM by all foetal sheep injected with *S. typhimurium* organisms, which had no demonstrable antibody activity, suggested strongly the synthesis of "non-specific" IgM. Contrary to findings in newborn mice (Kolb *et al*, 1974), IgG synthesis was not detected in foetal sheep following the first injection of *S. typhimurium* and was found in only one of three foetal sheep given a second injection; this animal also had agglutinating antibody. *S. typhimurium* seems capable therefore of inducing the synthesis of "non-specific" IgM, but not "non-specific" IgG.

The maturation of immunological competence in foetal sheep was affected by thymectomy (Tx). The magnitude of the antibody response to CRBC and POL was reduced, the synthesis of IgG_1 and to a lesser extent IgM was depressed and no 2-ME resistant antibody to either antigen was produced. Thymectomy greatly reduced the lymphocyte content of the lymph, blood and fixed lymphoid tissues, which could account quantitatively for the reduction in the humoral immune responses which occurred in these animals. Quantitative changes were also detected in the PFC response to CRBC and the percentage of lymphocytes in the lymph of thymectomized animals which had membrane-bound immunoglobulin. Both observations could also be interpreted to show qualitative changes in the lymphocytes present in Tx foetal sheep. The Tx foetal sheep either responded to both CRBC and POL or to neither. These antigens cannot therefore be classified as "T-dependent" or "T-independent" in foetal sheep, although the response to CRBC was more severely depressed

than the response to POL. It seemed that the effects of thymectomy could be reversed by exposure to antigen; Tx foetuses challenged a second time with CRBC synthesized 2-ME resistant antibody and IgG_1.

Antigenic stimulation also caused a maturation of the immune system such that IgG_1 synthesis was initiated in foetuses of a younger age. During primary immune responses IgG_1 was never detected before 87 days gestation. Priming 54 to 57 day old foetuses with POL resulted in IgG_1 synthesis being detected in four of the six foetuses, 2 to 12 days after a second injection of POL, when the foetuses were between 74 and 80 days old; this was 7 to 13 days earlier than it would normally occur in foetuses. Whether the synthesis of IgG_1 by primed foetuses is antigen specific or due to non-specific development of IgG producing cells remains unresolved.

Following surgery and the injection of antigen the level of neutrophils in the blood of foetal sheep rose. At first it was thought that this increase was due to the surgical procedures, however, injection of non-immunogenic antigens such as *S. typhimurium* or SRBC-LPS, did not stimulate a neutrophil response. The transient neutrophil response was associated with the injection of immunogenic antigens and was not affected by thymectomy. In fact, even in Tx foetal sheep which were incapable of producing antibody to CRBC or POL the level of neutrophils rose characteristically after the injection of proven immunogenic antigens.

Foetal lymphoid tissues develop throughout gestation. At mid-term they are sparsely populated with lymphocytes, there is no cortico-medullary differentiation in the lymph nodes and no primary follicles. The spleen is almost entirely a haematopoietic organ with little white pulp. At term, the lymph nodes have a defined cortex and medulla, they are much more densely populated with lymphocytes

and primary follicles are present, although no germinal centres normally develop during *in utero* life. The periarteriolar sheaths in the spleen also enlarge throughout gestation and are quite mature by birth. Antigenic challenge radically alters the histological structure of the draining lymph nodes and spleen producing areas of lymphopoiesis in the white pulp of the spleen and in the cortical region of lymph nodes, even in the youngest animals examined (64 days gestation). Germinal centres were detected by 73 days gestation in a foetus injected with antigen 14 days earlier. The periarteriolar areas in the spleen of this foetus also became greatly enlarged.

The data reported in this thesis have given an impression that although immune responses continue to mature throughout the last two thirds of gestation, many parameters of competence first appear between 65 and 90 days gestation. Foetal sheep can produce detectable levels of antibody to 7 of 8 antigens studied by 82 days gestation, they can synthesize IgG_1 by 87 days gestation and can produce 2-ME resistant antibody by 92 days gestation. In addition, Silverstein *et al* (1964) and Niederhuber *et al* (1971) found that foetal sheep were capable of rejecting allografts at about the same stage of gestation, while the percentage of blood leucocytes in the foetal sheep with membrane-bound immunoglobulin has also been found to reach a maximum level of 15% between 78 and 87 days gestation (Binns and Symons, 1974b).

Current concepts in immunology would explain the ontogeny of immunity to be a reflection of the maturation of thymus-derived lymphocytes (Spear and Edelman, 1974) or to be controlled by "suppressor" T cells (Mosier and Johnson, 1975). The rejection of allografts and the synthesis of IgG, both reportedly "T-dependent" functions, could be cited as evidence that T cell functions mature around 80 days gestation, although a simpler explanation could implicate IgG in allograft

rejection (Pedersen and Morris, 1974). The observation that thymectomy ablated the production of 2-ME resistant antibody and reduced the synthesis of IgG_1 may also demonstrate the role of T-cells in the maturation of immunological competence in foetal sheep, although the effects would have to be demonstrably qualitative, rather than quantitative, to be conclusive.

The fact that antibody synthesis to most antigens first occurred over a relatively short period of development could also be ascribed to T cell function, particularly as CRBC, MON, CγG (Miller and Warner, 1971) and OA are considered to be "T-dependent" antigens, as is DNP on certain carrier molecules (Hucket and Feldman, 1974). Interestingly, the experiments in Tx foetal sheep did not show any difference in the T cell requirement for antibody synthesis to the two antigens used, CRBC and POL. It would be very surprising if the failure of *S. typhimurium* to induce antibody formation in foetal sheep was due to a function of T cells, although it has been suggested that the magnitude of the antibody response of mice to pneumoccal polysaccharide could be regulated by "suppressor" T cells (Baker, Reed, Stashak, Amsbaugh and Prescott, 1973).

If the observed maturation of immunoglobulin synthesis in the foetal sheep is a reflection of T cell maturation then this maturation process must be capable of being influenced by antigenic stimulation. Mitchell *et al* (1972) suggested that the antigen driven "switch" of IgM producing cells to IgG producing cells may require T cells, although Paul (1974) concluded that they were not indispensable. Similarly, it has been postulated that T cells are not involved in secondary immune responses (Sinclair, 1967; Diamantstein and Blitstein-Willinger, 1974) although again Paul (1974) suggested that antigen driven proliferation of IgG producing cells was more dependent on T cells than

antigen driven proliferation of IgM producing cells. It is therefore difficult, at this time, to explain the earlier appearance of IgG_1 synthesis in antigenically primed foetuses in terms of the concepts developed from experiments in rodents.

As well as accelerating the onset of IgG_1 synthesis during foetal development, antigenic stimulation can substantially ameliorate the effects of thymectomy. Whether antigen has a specific or non-specific effect on the maturation of immune responsiveness or the restoration of immune capabilities after thymectomy has yet to be resolved. Antigenic stimulation is certainly an important stimulus to lymphopoiesis.

At this point in time it would be naive to ascribe the maturation of immunological competence in foetal sheep to the development of a particular sub-population of lymphocytes. This is particularly so as the thymus, which is the source of these cells, appears histologically mature well before the foetus acquires the capability to respond to most antigens and more so since we know little of the functional differentiation of lymphocyte populations in the sheep.

The responses detected in foetal sheep following the injection of antigen have raised a number of questions which require further investigation.

What are the ontogenic factors which cause the synthesis of IgM to occur before the synthesis of IgG_1 and how do these factors act on the intracellular "switch" which determines the change from IgM to IgG antibody synthesis, if such a switch occurs during primary immune responses? Why is the antibody response, as assessed at a molecular level, quite restricted in foetal animals, while very heterogeneous in mature animals? Does this infer that the products of a "true" primary

response are relatively homogeneous and that the heterogeneous products of a primary response in a mature animal is a result of prior experience with cross-reacting epitopes? An investigation of the total failure of foetal sheep to respond to a primary challenge with the somatic antigens of *S. typhimurium* may broaden our understanding of antigen handling and the role of cells other than lymphocytes in antibody synthesis. The agammaglobulinaemic foetal sheep could prove very valuable in a study of the synthesis of "specific" and "non-specific" immunoglobulin during "true" primary immune responses to well defined antigens. In addition it may be possible to study factors, other than immunoglobulin, which appear during the immune response and which may be involved in its regulation. The technical possibility of infusing antigens into foetuses *in utero* for long periods could facilitate a study on the induction of tolerance during foetal development or the modification of immunological competence with specific anti-immunoglobulin sera. Although this thesis has concentrated on the development of humoral immunity a similar study of "cell-mediated" immunity could be undertaken employing suitable *in vitro* assays.

The morphogenesis of the foetus has been carefully documented, but understanding the ways in which cells and molecules interact during development to produce an immunologically competent neonate has only just begun.

REFERENCES

REFERENCES

ACKERMAN, G.A. (1966). The lymphocyte: its morphology and embryological origin. *In* The lymphocyte in immunology and haemopoiesis (ed. Yoffey) p. 11.

ADA, G.L., NOSSAL, G.J.V., PYE, J. and ABBOT, A. (1964). Antigens in immunity I. Preparation and properties of flagellar antigens from *Salmonella adelaide*. *Aust. J. exp. Biol. med. Sci.*, *42*, 267.

ADINOLFI, M. (1972). Ontogeny of components of complement and lysozyme. *In* Ontogeny of acquired immunity (Ciba Fndn.) p. 65.

ADLER, W.H., CURRY, J.H. and SMITH, R.T. (1969). Immunoglobulin peptide chain synthesis in the newborn rabbit. *Immunology*, *17*, 457.

ALEXANDER, D.P., BRITTON, H.G., COHEN, N.M., NIXON, D.A. and PARKER, R.A. (1968). Insulin concentration in the foetal plasma and foetal fluids of the sheep. *J. Endocr.*, *40*, 389.

ANDERSSON, B. and BLOMGREN, H. (1970). Evidence for a small pool of immunocompetent cells in the mouse thymus: its role in the humoral antibody response against sheep erythrocytes, bovine serum albumin, ovalbumin and the NIP determinant. *Cell. Immunol.*, *1*, 362.

ANDERSSON, B., SJOBERG, O. and MOLLER, G. (1972). Induction of immunoglobulin and antibody synthesis *in vitro* by lipopolysaccharide. *Eur. J. Immunol.*, *2*, 349.

AOKI, T., HAMMERLING, U., de HARVEN, E., BOYSE, E.A. and OLD, L.J. (1969). Antigenic structure of cell surfaces: an immunoferritin study of the occurrence and topography of H-2, θ, and TL alloantigens on mouse cells. *J. exp. Med.*, *130*, 979.

ARGYRIS, B.F. (1968). Role of macrophages in immunological maturation. *J. exp. Med.*, *128*, 459.

ARGYRIS, B.F. (1969). Transplantation of adult peritoneal cells into newborn mice. *Transplantation*, *8*, 241.

ASANTILA, T., SORVARI, T., HIRVONEN, T. and TOIVANEN, P. (1973). Xenogeneic reactivity of human foetal lymphocytes. *J. Immun.*, *111*, 984.

ASKONAS, B.A. and WHITE, R.G. (1956). Sites of antibody production in the guinea-pig: the relation between *in vitro* synthesis of anti-ovalbumin and γ-globulin and distribution of antibody containing plasma cells. *Br. J. exp. Path.*, *37*, 61.

ASKONAS, B.A. and WILLIAMSON, A.R. (1972). Dominance of a cell clone forming antibody to DNP. *Nature (Lond.)*, *238*, 339.

ASOFSKY, R., IKARI, N.S. and HYLTON, M.B. (1968). The relationship of specific antigenic stimulation to serum IgM levels in germ-free mice. *In* Advances in germ-free research and gnotobiology (ed. Miyakawa and Luckey; CRC Press, Cleveland) p. 219.

ATTIA, M.A., de OME, K.B. and WEISS, D.W. (1965). Immunology of spontaneous mammary carcinomas in mice. II Resistance to a rapidly or a slowly developing tumor. *Cancer Res.*, *25*, 451

AUERBACH, R. (1972). Studies on the development of immunity: the response to sheep red blood cells. *Curr. Top. Develop. Biol. 7*, 257.

AVRAMEAS, S. (1975). Studies on antibody formation with enzyme markers. *Ann. N.Y. Acad. Sci.*, *254*, 175.

AVRAMEAS, S. and TERNYNCK, T. (1969). The cross-linking of proteins with glutaraldehyde and its use for the preparation of immunoadsorbents. *Immunochem.*, *6*, 53.

AVRAMEAS, S. and TERNYNCK, T. (1971). Peroxidase labelled antibody and Fab conjugates with enhanced intracellular penetration. *Immunochem.*, *8*, 1175.

BACHMANN, P.A., SHEFFY, B.E. and VAUGHAN, J.T. (1975). Experimental *in utero* infection of foetal pigs with a porcine parvovirus. *Infect. Immun.*, *12*, 455.

BAKER, P.J., REED, N.D., STASHAK, P.W., AMSBAUGH, D.F. and PRESCOTT, B. (1973). Regulation of the antibody response to type III pneumococcal polysaccharide I. Nature of regulator cells. *J. exp. Med.*, *137*, 1431.

BANKHURST, A.D. and WARNER, N.L. (1972). Surface immunoglobulins on the thoracic duct lymphocytes of the congenitally athymic (nude) mouse. *Aust. J. exp. Biol. Med. Sci.*, *50*, 661.

BARCROFT, J. (1946). Researches on pre-natal life (Blackwell Sci. Pub. Oxford) p. 67.

BARONI, C. (1967). Thymus, peripheral lymphoid tissues and immunological responsiveness of the pituitary dwarf mouse. *Experientia*, *23*, 282.

BARONI, C., FABRIS, N. and BERTOLI, G. (1967). Age dependency of the primary immune response in the hereditary pituitary dwarf and normal Snell-Bagg mouse. *Experientia*, *23*, 1059.

BASSET, J.M. and THORBURN, G.D. (1969). Foetal plasma corticosteroids and the initiation of parturition in sheep. *J. Endocr.*, *44*, 285.

BASSET, J.M. and THORBURN, G.D. (1971). The regulation of insulin secretion by the ovine foetus *in utero*. *J. Endocr.*, *50*, 59.

BASSET, J.M., THORBURN, G.D. and WALLACE, A.L.C. (1970). The plasma growth hormone concentration of the foetal lamb. *J. Endocr.*, *48*, 251.

BAUER, D.C. (1963). The molecular properties of antibodies synthesized in response to simple haptenes. *J. Immun.*, *91*, 323.

BAUER, D.C. and STAVITSKY, A.B. (1961). On the different forms of antibody synthesized by rabbits during the early response to a single injection of protein and cellular antigens. *Proc. natn. Acad. Sci. U.S.A.*, *47*, 1667.

BEH, K.J., WATSON, D.L. and LASCELLES, A.K., (1974). Concentration of immunoglobulins and albumin in lymph collected from various regions of the body of the sheep. *Aust. J. exp. Biol. Med. Sci.*, *52*, 81.

BENEDICT, A.A., BROWN, R.J. and AYENGAR, R. (1962). Physical properties of antibody to bovine serum albumin as demonstrated by haemagglutination. *J. exp. Med.*, *115*, 195.

BENNETT, I.L. (1964). Approaches to the mechanisms of endotoxin action. *In* Bacterial Endotoxins (ed. Landy and Braun) p. 13.

BILLINGHAM, R.E., BRENT, L. and MEDAWAR, P.B. (1953). "Actively acquired tolerance" of foreign cells. *Nature (Lond.)*, *172*, 603.

BINNS, R.M. (1967). Bone marrow and lymphoid cell injection of the pig foetus resulting in transplantation tolerance of immunity, and immunoglobulin production. *Nature (Lond.)* *214*, 179.

BINNS, R.M. and SYMONS, D.B.A. (1974a). The ontogeny of lymphocyte immunoglobulin determinants and their relationship to antibody production in the foetal pig. *Res. vet. Sci.*, *16*, 260.

BINNS, R.M. and SYMONS, D.B.A. (1974b). Ontogeny of immunoglobulin-positive blood lymphocytes in foetal sheep. *J. Int. Res. Commun.*, *2*, 1324.

BLAESE, R.M., HENRICHON, M. and WALDMAN, T.A. (1970). Ontogeny of the immune response: the afferent limb. *Fedn. Proc.*, *29*, 699.

BLOMGREN, H. and ANDERSSON, B. (1969). Evidence for a small pool of immunocompetent cells in the mouse thymus. *Expl. Cell. Res.*, *57*, 185.

BLOMGREN, H. and ANDERSSON, B. (1971). Characteristics of the immunocompetent cells in the mouse thymus: cell population changes during cortisone-induced atrophy and subsequent regeneration. *Cell. Immunol.*, *1*, 545.

BODE, W., ENGEL, J. and WINKLMAIR, D. (1972). A model of bacterial flagella based on small-angle X-ray scattering and hydrodynamic data which indicate an elongated shape of the flagellin protomer. *Eur. J. Biochem.*, *26*, 313.

BORTIN, M.M., RIMM, A.A. and SALTZSTEIN, E.C. (1969). Ontogenesis of immune capability of murine bone marrow cells and spleen cells against transplantation antigens. *J. Immun.*, *103*, 683.

BORTIN, M.M. and SALTZSTEIN, E.C. (1968). Immunologic incompetence of mouse perinatal liver haematopoietic cells against transplantation antigens. *J. Immun.*, *100*, 1215.

BOURNE, F.J. (1974). Gamma globulin in pre-colostral piglet serum. *Res. vet. Sci.*, *17*, 36.

BOURNE, F.J., CURTIS, J., JOHNSON, R.H. and COLLINGS, D.F. (1974). Antibody formation in porcine foetuses. *Res. vet. Sci.*, *16*, 223.

BOYSE, E.A. and OLD, L.J. (1969). Some aspects of normal and abnormal cell-surface genetics. *A. Rev. Genetics*, *3*, 269.

BOYD, W.C. and BERNARD, H. (1937). Quantitative changes in antibodies and globulin fractions in sera of rabbits injected with several antigens. *J. Immun.*, *33*, 111.

BRAMBELL, F.W.R. (1970). The transmission of passive immunity from mother to young. *Frontiers of Biology. Vol. 18.*

BRANDON, M.R., WATSON, D.L. and LASCELLES, A.K. (1971). The mechanism of transfer of immunoglobulin into mammary secretion of cows. *Aust. J. exp. Biol. med. Sci.*, *49*, 613.

BRAUN, R.K., OSBURN, B.I. and KENDRICK, J.W. (1973). Immunologic response of bovine foetus to bovine viral diarrhea virus. *Am. J. vet. Res.*, *34*, 1127.

BRIDGES, R.A., CONDIE, R.M., ZAK,S.J. and GOOD, R.A. (1959). The morphologic basis of antibody formation development during the neonatal period. *J. Lab. clin. Med.*, *53*, 331.

BROOKE, M.S. (1965). The immunological behaviour of mature C57BL/6J mice thymectomized at birth. *Immunology*, *8*, 526.

BRYANT, B.J., SHIFRINE, M. and McNEIL, C. (1973). Cell mediated immune responses in the dog. *Int. Archs. Allergy app. Immun.*, *45*, 937.

BULLOCK, W.W. (1974). Mitogens for T and B cells. *Prog. Immunol. II.*, *2*, 325.

BURCKHARDT, J.J., GUGGISBERG, E. and von FELLENBERG, R. (1974). Thymus immunoglobulin receptors: ontogenic development, response to antigenic stimulation and their possible role in the regulation of "aggressive" immune reactions. *Immunology*, *26*, 521.

BURNET, F.M. and FENNER, F. (1949). The production of antibodies (Pub. Macmillan).

CAMPBELL, D.H., GARVEY, J.S., CREMER, N.E. and SUSSDORF, D.H. (1970). Methods in immunology, 2nd. ed. (Pub. Benjamin N.Y.).

CARR, M.C., LIEBER, E. and FUDENBERG, H.H. (1970). *In vitro* cytolysis by human foetal lymphocytes. *Cell. Immunol.*, *1*, 455.

CARR, M.C., STITES, D.P. and FUDENBERG, H.H. (1973). Dissociation of responses to phytohaemagglutinin and adult allogeneic lymphocytes in human foetal lymphoid tissues. *Nature (New Biol.) 241*, 279.

CASARO, A.P.E., KENDRICK, J.W., and KENNEDY, P.C. (1971). Response of the bovine foetus to bovine viral diarrhea-mucosal disease virus. *Am. J. vet. Res.*, *32*, 1543.

CASTER, P., GARNER, R. and LUCKEY, T.D. (1966). Antigen-induced morphological changes in germ-free mice. *Nature (Lond.)*, *209*, 1202.

CAZENAVE, P.-A., TERNYNCK, T. and AVRAMEAS, S. (1974). Similar idiotypes in antibody-forming cells and in cells synthesizing immunoglobulins without detectable antibody function. *Proc. natn. Acad. Sci. U.S.A.*, *71*, 4500.

CEDERQVIST, L.L. and LITWIN, S.D. (1974). Production of α1 and α2 immunoglobulin heavy chains during foetal life. *J. Immun.*, *112*, 1605.

CHAPMAN, H.A., JOHNSON, J.S. and COOPER, M.D. (1974). Ontogeny of Peyer's patches and immunoglobulin - containing cells in pigs. *J. Immun.*, *112*, 555.

CHASE, M.W. (1959). Immunologic Tolerance. *A. Rev. Microbiol.*, *13*, 349.

CLAMAN, H.N. and BRUNSTETTER, F.H. (1968). The response of cultured human thymus cells to phytohaemagglutinin. *J. Immun.*, *100*, 1127.

COHEN, M.W., THORBECKE, G.J., HOCHWALD, G.M. and JACOBSON, E.B. (1963). Induction of graft-versus-host reaction in newborn mice by injection of newborn or adult homologous thymus cells. *Proc. Soc. exp. Biol. Med.*, *114*, 242.

COHN, M. (1971). The take-home lesson. *Ann. N.Y. Acad. Sci.*, *190*, 529.

COIFMAN, R.E., GOOD, R.A. and MEUWISSEN, H.J. (1971). The function of irradiated blood elements I. Limitations on the response to phytohaemagglutinin as an indicator of immunocompetence in irradiated lymphocytes. *Proc. Soc. exp. Biol. Med., 137,* 155.

COLE, G.J. and MORRIS, B. (1971a). The growth and development of lambs thymectomized *in utero*. *Aust. J. exp. Biol. med. Sci., 49,* 33.

COLE, G.J. and MORRIS, B. (1971b). The cellular and humoral response to antigens in lambs thymectomized *in utero*. *Aust. J. exp. Biol. med. Sci., 49,* 55.

COLE, G.J. and MORRIS, B. (1971c). Homograft rejection and hypersensitivity reactions in lambs thymectomized *in utero*. *Aust. J. exp. Biol. med. Sci., 49,* 75.

COLE, G.J. and MORRIS, B. (1971d). The normal lymphocyte transfer reaction in lambs thymectomized *in utero*. *Aust. J. exp. Biol. med. Sci., 49,* 89.

COMLINE, R.S. and SILVER, M. (1961). The release of adrenaline and noradrenaline from the adrenal glands of the foetal sheep. *J. Physiol. (Lond.), 156,* 424.

CONE, R.E., SPRENT, J. and MARCHALONIS, J.J. (1972). Antigen binding specificity of isolated cell surface immunoglobulin from thymus cells activated to histocompatability antigens. *Proc. natn. Acad. Sci. U.S.A., 69,* 2556.

CONNER, G.H., RICHARDSON, M. and CARTER, G.R. (1973). Prenatal immunization and protection of the newborn: ovine and bovine foetus vaccinated with *Escherichia coli* antigen by the oral route and exposed to challenge inoculum at birth. *Am. J. Vet. Res., 34,* 737.

COOMBS, R.R.A., FEINSTEIN, A. and WILSON, A.B. (1969). Immunoglobulin determinants on the surface of human lymphocytes. *Lancet, 2,* 1157.

COOPER, M.D., LAWTON, A.R. and KINCADE, P.W. (1972a). A two-stage model for development of antibody-producing cells. *Clin. exp. Immunol., 11,* 143.

COOPER, M.D., LAWTON, A.R. and KINCADE, P.W. (1972b). A developmental approach to the biological basis of antibody diversity. *Cont. Top. Immunobiol., 1,* 33.

COUTINHO, A. and MOLLER, G. (1973). B cell mitogenic properties of thymus-independent antigens. *Nature (New Biol.), 245,* 12.

CROSS, A.M., LEUCHARS, E. and MILLER, J.F.A.P. (1964). Studies on the recovery of the immune response in irradiated mice thymectomized in adult life. *J. exp. Med., 119,* 837.

CUMMINGS, J.N. and BELLVILLE, T.P. (1963). Studies on foetal physiology in the sheep: transplacental passage of antibodies and techniques for repeated sampling of the foetal lamb *in situ*. *Am. J. Obstet. Gynec., 86,* 504.

DAGUILLARD, F. and RICHTER, M. (1969). Cells involved in the immune response XII. The differing responses of normal rabbit lymphoid cells to phytohaemagglutinin, goat anti-rabbit immunoglobulin antiserum and allogeneic and xenogeneic lymphocytes. *J. exp. Med., 130,* 1187.

DAVIE, J.M. and PAUL, W.E. (1974). Antigen-binding receptors on lymphocytes. *Cont. Top. Immunobiol., 3,* 171.

DAVIE, J.M., PAUL, W.E., ASOFSKY, R. and WARREN, R.W. (1974). Ontogeny of immunoglobulin bearing lymphocytes and DNP-specific antigen-binding cells in guinea pigs. *J. Immun., 112,* 2202.

DAVIES, A.J.S., CARTER, R.L., LEUCHARS, E., WALLIS, V.J. and DIETRICH, F.M. (1970). The morphology of immune reactions in normal, thymectomized and reconstituted mice. *Immunology, 19,* 945.

DAVIES, A.J.S., FESTENSTEIN, H., LEUCHARS, E., WALLIS, V.J. and DOENHOFF, M.J. (1968). A thymic origin for some peripheral-blood lymphocytes. *Lancet, 1,* 183.

DECKER, J.M. and SERCARZ, E.E. (1974). Early simultaneous appearance of antigen-binding cells in the foetal sheep. *Nature (Lond.), 252,* 416.

DENNERT, G. and LENNOX, E. (1972). Cell interaction in humoral and cell-mediated immunity. *Nature (New Biol.), 238,* 114.

DENNIS, R.A., JACOBY, R.O. and GRIESEMER, R.A. (1969). Development of immunity in foetal dogs: skin allograft rejection. *Am. J. vet. Res., 30,* 1511.

DENT, P.B. and GOOD, R.A. (1965). Absence of antibody production in the bursa of Fabricius. *Nature (Lond.), 207,* 491.

DIAMANTSTEIN, T. and BLITSTEIN-WILLINGER, E. (1974). T Cell independent development of B memory Cells. *Eur. J. Immunol., 4,* 830.

DIXON, F.J. and WEIGLE, W.O. (1957). The nature of the immunologic inadequacy of neonatal rabbits as revealed by cell transfer studies. *J. exp. Med., 105,* 75.

DLABAC, V., MILER, I., KRUML, J., KOVARU, F., and LEON, M.A. (1970). The development and mechanism of bactericidal activity of sera and phagocytosis in pig foetuses and germfree newborn piglets. *In* Developmental aspects of antibody formation and structure (ed. Sterzl and Riha) Vol. 1, p. 105.

DREYER, W.J. and BENNETT, J.C. (1965). The molecular basis of antibody formation: a paradox. *Proc. natn. Acad. Sci. U.S.A.*, *54*, 864.

DROEGE, W., ZUKER, R. and JAUKER, U. (1974). Cellular composition of the thymus: developmental changes and the effect of hydrocortisone. *Cell. Immunol.*, *12*, 173.

DURKIN, H.G., THEIS, G.A. and THORBECKE, G.J. (1971). Homing of cells from the bursa of Fabricius to germinal centres in the chicken spleen. *Adv. exp. Med. Biol.*, *12*, 119.

DUTTON, R.W. and HUNTER, P. (1974). The effects of mitogen-stimulated T cells on the response of B cell, to antigen and the mechanism of T cell stimulation of the B cell response. *In* Cellular selection and regulation of the immune response (ed. Edelman-Raven Press N.Y.) p. 199.

DWYER, J.M. and MACKAY, I.R. (1972). The development of antigen-binding lymphocytes in foetal tissues. *Immunology*, *23*, 871.

DYMINSKI, J.W., FORBES, J., GEBHARDT, B., NAKAO, Y., KONDA, S. and SMITH, R.T. (1974). Relationship between structure and function of human and mouse thymus cell subpopulations. *Prog. Immunol. II*, *3*, 35.

EDELMAN, G.M. and GALLY, J.A. (1967). Somatic recombination of duplicated genes: an hypothesis on the origin of antibody diversity. *Proc. natn. Acad. Sci. U.S.A.*, *57*, 353.

EICHENWALD, H.F. and SHINEFIELD, H.R. (1963). Antibody production by the human foetus. *J. Pediat.*, *63*, 870.

EISEN, H.N., LITTLE, J.R., STEINER, L.A., SIMMS, E.S. and GRAY, W. (1969). Degeneracy in the secondary immune response: stimulation of antibody formation by cross-reacting antigens. *Isr. J. Med. Sci.*, *5*, 338.

EISEN, H.N. and SISKIND, G.W. (1964). Variations in affinities of antibodies during the immune response. *Biochemistry*, *3*, 996.

ELFENBIEN, G.J., HARRISON, M.R. and MAGE, R.G. (1975). Appearance of lymphocyte surface markers and functional responses in neonatal and young rabbit spleens. *Cell. Immunol.*, *15*, 303.

ELLIOT, B.E. and HASKILL, J.S. (1973). Characterization of thymus-derived and bone-marrow derived rosette-forming lymphocytes. *Eur. J. Immunol.*, *3*, 68.

ENGLISH, L.S. (1974). Immunoglobulin synthesis by lymphoid cells in the sheep. PhD Thesis, A.N.U., Australia.

FAHEY, K.J. and MORRIS, B. (1974). Lymphopoiesis and immune reactivity in the foetal lamb. *Ser. Haemat.*, *7*, 548.

FELDMAN, M. and BASTEN, A. (1971). The relationship between antigenic structure and the requirement for thymus-derived cells in the immune response. *J. exp. Med.*, *134*, 103.

FELDMAN, M. and NOSSAL, G.J.V. (1972). Tolerance, enhancement and the regulation of interaction between T cells, B cells and macrophages. *Transplant. Rev.*, *13*, 3.

FENNESTAD, K.L. and BORG-PETERSEN, C. (1962). Antibody and plasma cells in the bovine foetus infected with *Leptospira saxkoebing*. *J. infect. Dis.*, *110*, 63.

FENNESTAD, K.L., BORG-PETERSEN, C. and BRUMMERSTEDT, E. (1968). Leptospira antibody formation by porcine foetuses. *Res. vet. Sci.*, *9*, 378.

FIDLER, J.M., CHISCON, M.O. and GOLUB, E.S. (1972). Functional development of the interacting cells in the immune response. *J. Immun.*, *109*, 136.

FORMAN, M.L. and STIEHM, E.R. (1969). Impaired opsonic activity but normal phagocytosis in low-birth-weight infants. *New Engl. J. Med.*, *281*, 926.

FOWLER, R., SCHUBERT, W.K. and WEST, C.D. (1960). Acquired partial tolerance to homologous skin grafts in the human infant at birth. *Ann. N.Y. Acad. Sci.*, *87*, 403.

FREEMAN, M.J. and STAVITSKY, A.B. (1965). Radio-immunoelectrophoretic study of rabbit anti-protein antibodies during the primary response. *J. Immun.*, *95*, 981.

GALLY, J.A. and EDELMAN, G.M. (1970). Somatic translocation of antibody genes. *Nature (Lond.)*, *227*, 341.

GARVER, F.A. and HILSCHMANN, N. (1972). The primary structure of a monoclonal human λ-type immunoglobulin L-chain of subgroup II (Bence-Jones protein NEI). *Eur. J. Biochem.*, *26*, 10.

GEIGER, H., DAY, N. and GOOD, R.A. (1972). The ontogenic development of the latter complement components in foetal pigs. *J. Immun.*, *108*, 1098.

GELFAND, M.C., ELFENBIEN, G.J., FRANK, M.M. and PAUL, W.E. (1974). Ontogeny of B lymphocytes II. Relative rates of appearance of lymphocytes bearing surface immunoglobulin and complement receptors. *J. exp. Med.*, *139*, 1125.

GERSHON, R.K. (1974). T cell control of antibody production. *Cont. Top. Immunobiol.*, *3*, 1.

GIBSON, C.D. and ZEMJANIS, R. (1973). Immune response of the bovine foetus to several antigens. *Am. J. vet. Res.*, *34*, 1277.

GITLIN, D. and BIASUCCI, A. (1969). Development of γG, γA, γM, β1c/β1A, C1 esterase inhibitor, ceruloplasmin, transferrin, hemopexin, haptoglobin, fibrinogen, plasminogen, α_1-antitrypsin, orosomucoid, β-lipoprotein, α_2-macroglobulin and prealbumin in the human conceptus. *J. clin. Invest.*, *48*, 1433.

GLAESSNER, K. (1905-6). *Ztschr. f. exp. Path. U. Ther.*, *2*, 154.

GLENNY, A.T. (1925). The principles of immunity applied to protective inoculation against diphtheria. *J. Hygiene (Camb.)*, *24*, 301.

GLENNY, A.T. and SUDMERSEN, H.J. (1921). Notes on the production of immunity to diphtheria toxin. *J. Hygiene (Camb.)*, *20*, 176.

GLICK, B. (1961). The influence of dipping eggs in male hormone solutions on lymphatic tissue and antibody response of chickens. *Endocrinology*, *69*, 984.

GLICK, B., CHANG, T.S. and JAAP, R.G. (1956). Bursa of Fabricius and antibody production. *Poult. Sci.*, *35*, 224.

GOIDL, E.A. and SISKIND, G.W. (1974). Ontogeny of B-lymphocyte function 1. Restricted heterogeneity of the antibody response of B-lymphocytes from neonatal and foetal mice. *J. exp. Med.*, *140*, 1285.

GOLDSTEIN, A.L., ASANUMA, Y., BATTISTO, J.R., HARDY, M.A., QUINT, J. and WHITE, A. (1970). Influence of thymosin on cell-mediated and humoral immune responses in normal and in immunologically deficient mice. *J. Immun.*, *104*, 359.

GOLDSTEIN, A.L. and WHITE, A. (1973). Thymosin and other thymic hormones: their nature and role in the thymic dependency of immunological phenomena. *Cont. Top. Immunobiol.*, *2*, 339.

GOOD, R.A., CONDIE, R.M. and BRIDGES, R.A. (1962). Development of the immune response in man and animals. *In* Mechanisms of antibody formation (Czechoslovak Acad. Sci.) p. 118.

GOOD, R.A. and GABRIELSEN, A.E. (1964). The thymus in immunobiology (Pub. Harper and Row).

GRAVES, J.H. (1963). Transfer of neutralizing antibody by colostrum to calves born of foot and mouth disease vaccinated dams. *J. Immun.*, *91*, 251.

GREAVES, M.F. (1971). The expression of immunoglobulin determinants on the surface of antigen-binding lymphoid cells in mice. *Eur. J. Immunol.*, *1*, 186.

GREAVES, M.F. and HOGG, N.M. (1971). Immunoglobulin determinants on the surface of antigen binding T- and B- lymphocytes in mice. *Prog. Immunol.*, *I*, 111.

GREAVES, M.F. and MOLLER, E. (1970). Studies on antigen-binding cells 1. The origin of reactive cells. *Cell. Immunol.*, *1*, 372.

GREAVES, M.F., ROITT, I.M. and ROSE, M.E. (1968). Effect of bursectomy and thymectomy on the responses of chicken peripheral blood lymphocytes to phytohaemagglutin. *Nature (Lond.)*, *220*, 293.

GREWTHER, P. and WARNER, N.L. (1972). Serum immunoglobulins and antibodies in congenitally athymic (nude) mice. *Aust. J. exp. Biol. med. Sci.*, *50*, 625.

GREY, H.M., COLON, S., CAMPBELL, P. and RABELLINO, E. (1972). Immunoglobulins on the surface of lymphocytes V. Quantitative studies on the question of whether immunoglobulins are associated with T cells in the mouse. *J. Immun.*, *109*, 776.

HAJEK, P., KOVARU, F. and KRUML, J. (1969). The antibody response and morphological changes following immunization of pig foetuses with ϕx174 phage. *Folia Microbiol.*, *14*, 492.

HALLIDAY, W.J. and WEBB, M. (1965). A plaque technique for counting cells which produce antibacterial antibody. *Aust. J. exp. Biol. med. Sci.*, *43*, 163.

HARDY, B., GLOBERSON, A. and DANON, D. (1973). Ontogenic development of the reactivity of macrophages to antigenic stimulation. *Cell. Immunol.*, *9*, 282.

HARDY, M.H. and LYNE, A.G. (1956). The pre-natal development of wool follicles in merino sheep. *Aust. J. biol. Sci.*, *9*, 423.

HAYWARD, A.R. and SOOTHILL, J.F. (1972). Reaction to antigen by human foetal thymus lymphocytes. *In* Ontogeny of acquired immunity, (Ciba Fndn. Symp.) p. 262.

HESS, M.W. (1968). Experimental thymectomy: possibilities and limitations, (pub. Springer-Verlag Berlin).

HETCHEL, M., DISCHON, T. and BRAUN, W. (1965a). Influence of oligodeoxyribonucleotides on the immune response of newborn AKR mice. *Proc. Soc. exp. Biol. Med.*, *119*, 991.

HETCHEL, M., DISCHON, T. and BRAUN, W. (1965b). Haemolysin formation in newborn mice of different strains. *Proc. Soc. exp. Biol. Med.*, *120*, 728.

HOERLIN, A.B. (1952). Studies in swine brucellosis I. The pathogenesis of artificial *Brucella melitensis* infection. *Am. J. vet. Res.*, *13*, 67.

HOERLIN, A.B. (1957). The influence of colostrum on antibody response in baby pigs. *J. Immun.*, *78*, 112.

HOPKINS, P.S. and THORBURN, G.D. (1972). The effects of foetal thyroidectomy on the development of the ovine foetus. *J. Endocr.*, *54*, 55.

HORECKER, B.L. (1966). The biosynthesis of bacterial polysaccharides. *A. Rev. Microbiol.*, *20*, 253.

HOWARD, J.C. and GOWANS, J.L. (1972). The role of lymphocytes in antibody formation III. The origin from small lymphocytes of cells forming direct and indirect haemolytic plaques to sheep erythrocytes in the rat. *Proc. R. Soc. (Ser. B)*, *182*, 193.

HUCHET, R. and FELDMAN, M. (1974). Thymus-independent IgM and IgG responses in mice to flagellar antigens. *Int. Archs. Allergy app. Immun.*, *46*, 600.

HUMPHREY, J.H. and PORTER, R.R. (1956). An investigation on rabbit antibodies by the use of partition chromatography. *Biochem. J.*, *62*, 93.

IINO, T. (1969). Genetics and chemistry of bacterial flagella. *Bact. Rev.*, *33*, 454.

JACOBSON, E.B., CAPORALE, L.H. and THORBECKE, G.J. (1974). Effect of thymus cell injections on germinal centre formation in lymphoid tissues of nude (thymusless) mice. *Cell. Immunol.*, *13*, 416.

JACOBY, R.O., DENNIS, R.A. and GRIESEMER, R.A. (1969). Development of immunity in foetal dogs: humoral responses. *Am. J. vet. Res.*, *30*, 1503.

JAROSKOVA, L., TREBICHAVSKY, I., RIHA, I., KOVARU, F. and HOLUB, M. (1973). Immunoglobulin determinants on lymphocytes in germ-free piglets. *Eur. J. Immunol.*, *3*, 818.

JERNE, N.K. (1971). The somatic generation of immune recognition. *Eur. J. Immunol.*, *1*, 1.

JONES, P.P., CRAIG, S.W., CEBRA, J.J. and HERTZENBERG, L.A. (1974). Restriction of gene expression in B-lymphocytes and their progeny. *J. exp. Med.*, *140*, 452.

JONES, J.M. and KIND, P.D. (1972). Enhancing effect of bacterial endotoxins on bone marrow cells in the immune response to SRBC. *J. Immun.*, *108*, 1453.

KARTHIGASU, K., READE, P.C. and JENKIN, C.R. (1965). The functional development of the reticulo-endothelial system III. The bactericidal capacity of fixed macrophages of foetal and neonatal chicks and rats. *Immunology*, *9*, 67.

KATZ, D.H. and BENACERRAF, B. (1972). The regulatory influence of activated T cells on B cell responses to antigen. *Adv. Immunol.*, *15*, 1.

KAY, H.E.M., DOE, J. and HOCKLEY, A. (1970). Response of human foetal thymocytes to phytohaemagglutinin (PHA). *Immunology*, *18*, 393.

KENDRICK, J.W. and OSBURN, B.I. (1973). Immunologic response of the bovine foetus to inactivated infectious bovine rhinotracheitis - infectious pustular vulvovaginitis virus. *Am. J. vet. Res.*, *34*, 1567.

KIM, Y.B., BRADLEY, S.G. and WATSON, D.W. (1966a). Ontogeny of the immune response I. Development of immunoglobulins in germfree and conventional colostrum-deprived piglets. *J. Immun.*, *97*, 52.

KIM, Y.B., BRADLEY, S.G. and WATSON, D.W. (1966b). Ontogeny of the immune response II. Characterization of 19S γ G and 7S γ G immunoglobulins in the true primary and secondary responses in piglets. *J. Immun.*, *97*, 189.

KIM, Y.B., BRADLEY, S.G. and WATSON, D.W. (1968). Ontogeny of the immune response V. Further characterization of 19S γ G and 7S γ G immunoglobulin in the true primary immune response in germfree colostrum-deprived piglets. *J. Immun.*, *101*, 224.

KINCADE, P.W. and COOPER, M.D. (1971). Development and distribution of immunoglobulin-containing cells in the chicken. *J. Immun.*, *106*, 371.

KINCADE, P.W. and COOPER, M.D. (1973). Immunoglobulin A: site and sequence of expression in developing chicks. *Science*, *179*, 398.

KINCADE, P.W., LAWTON, A.R., BOCKMAN, D.E. and COOPER, M.D. (1970). Suppression of immunoglobulin G synthesis as a result of antibody-mediated suppression of immunoglobulin M synthesis in chickens. *Proc. natn. Acad. Sci. U.S.A.*, *67*, 1918.

KIROV, S.M. (1974). An anti-theta sensitive hapten-carrier response in nude mice. *Eur. J. Immunol.*, *4*, 739.

KOLB, C., DiPAULI, R. and WEILER, E. (1974). Induction of IgG by lipid A in the newborn mouse. *J. exp. Med.*, *139*, 467.

KONDA, S., STOCKERT, E. and SMITH, R.T. (1973). Immunologic properties of mouse thymus cells: membrane antigen patterns. *Cell. Immunol.*, *7*, 275.

KRANER, K.L. (1965). *Intra uterine* foetal surgery. *Adv. vet. Sci.*, *10*, 1.

LAMELIN, J.-P., LISOWSKA-BERNSTEIN, B., MATTER, A., RYSER, J.E. and VASSALLI, P. (1972). Mouse thymus-independent and thymus-derived lymphoid cells. I. Immunofluorescent and functional studies. *J. exp. Med.*, *136*, 984.

LANGMAN, R.E. (1972). The use of erythrocytes sensitized with flagellar antigens from Salmonella for the assay of antibody and antibody-forming cells. *J. Immunol. Methods*, *2*, 59.

LANGMAN, R.E., ARMSTRONG, W.D. and DIENER, E. (1974). Antigenic composition, not the degree of polymerization, determines the requirement for thymus-derived cells in immune responses to Salmonella flagellar proteins. *J. Immun.*, *113*, 251.

LAWTON, A.R., ASOFSKY, R., HYLTON, M.B. and COOPER, M.D. (1972a). Suppression of immunoglobulin class synthesis in mice I. Effects of treatment with antibody to μ-chain. *J. exp. Med.*, *135*, 277.

LAWTON, A.R., KINCADE, P.W. and COOPER, M.D. (1975). Sequential expression of germline genesis development of immunoglobulin class diversity. *Fedn. Proc.*, *34*, 33.

LAWTON, A.R., SELF, K.S., ROYAL, S.A. and COOPER, M.D. (1972b). Ontogeny of B-lymphocytes in the human foetus. *Clin. Immunol. Immunopath.*, *1*, 84.

LECKBAND, E. (1970). A minor population of immunocompetent cells among mouse thymocytes. *Fedn. Proc.*, *29*, 261.

LeFOR, W.M. and BAUER, D.C. (1970). Relative concentrations of IgM and IgG antibodies during the primary response. *J. Immun.*, *104*, 1276.

LESLIE, G.A. and MARTIN, L.N. (1973). Modulation of immunoglobulin ontogeny in the chicken: effect of purified antibody specific for μ-chain on IgM, IgY and IgA production. *J. Immun.*, *110*, 959.

LEVEY, R.H. and BURLESON, R. (1972). Studies on the isolation of lymphocytes active in cell-mediated immune responses II. Identification and recovery of an immunocompetent subpopulation of mouse thymocytes. *Cell. Immunol.*, *4*, 316.

LEVINE, B.B. and BENACERRAF, B. (1964). Studies on antigenicity: the relationship between *in vivo* and *in vitro* enzymic degradability of hapten-polylysine conjugates and their antigenicity in guinea pigs. *J. exp. Med.*, *120*, 955.

LIND, P.E. (1970). Influence of neonatal thymectomy on antibody production in the Lewis rat. *Int. Arch. Allergy app. Immun.*, *37*, 258.

LINNA, T.J., BACK, R. and HEMMINGSSON, E. (1971). Lymphoid cell migration. *Adv. exp. Med. Biol.*, *12*, 149.

LISCHNER, H.W., PUNNETT, H.H. and DiGEORGE, A.M. (1967). Lymphocytes in congenital absence of the thymus. *Nature (Lond.)*, *214*, 580.

LUDERITZ, O., STAUB, A.M. and WESTPHAL, O. (1966). Immunochemistry of O and R antigens of Salmonella and related enterobacteriaceae. *Bact. Rev.*, *30*, 192.

MANCINI, G., CARBONARA, A.O. and HEREMANS, J.F. (1965). Immunochemical quantitation of antigens by single radial immunodiffusion. *Immunochem*, *2*, 235.

MANNING, D.D. (1975). Heavy chain isotype suppression: a review of the immunosuppressive effects of heterologous anti-Ig heavy chain antisera. *J. Reticuloendothl. Soc.*, *18*, 63.

MANNING, D.D. and JUTILA, J.W. (1972). Immunosuppression of mice injected with heterologous anti-immunoglobulin heavy chain antisera. *J. exp. Med.*, *135*, 1316.

MARCHALONIS, J.J., ATWELL, J.L. and CONE, R.E. (1972b). Isolation of surface immunoglobulin from lymphocytes from human and murine thymus. *Nature (New Biol.)*, *235*, 240.

MARCHALONIS, J.J., CONE, R.E. and ATWELL, J.L. (1972a). Isolation and partial characterization of lymphocyte surface immunoglobulin. *J. exp. Med.*, *135*, 956.

MARTENSSON, L. and FUDENBERG, H.H. (1965). Gm genes and γG-globulin synthesis in the human foetus. *J. Immun.*, *94*, 514.

MARTIN, W.J. (1966). The cellular basis of immunological tolerance in newborn animals. *Aust. J. exp. Biol. med. Sci.*, *44*, 605.

MASON, J.H., DALLING, T. and GORDON, W.S. (1930). Transmission of maternal immunity. *J. Path. Bact.*, *33*, 783.

MASON, S. and WARNER, N.L. (1970). The immunoglobulin nature of the antigen recognition site on cells mediating transplantation immunity and delayed hypersensitivity. *J. Immun.*, *104*, 762.

McALPINE, J.G. and RETTGER, L.F. (1925). Serological studies on bovine infectious abortion. *J. Immun.*, *10*, 811.

McFARLANE, A.S. (1963). *In vivo* behaviour of ^{131}I-fibrinogen. *J. clin. Invest.*, *42*, 346.

MEUWISSEN, H.J., VAN ALTEN, P.T., BACH, F.H. and GOOD, R.A. (1968). Influence of thymus and bursa on *in vitro* lymphocyte function. *In* Immunological deficiency diseases in man (ed. Bergsma) p. 253.

MILER, I., TLASKALOVA, H., MANDEL, L. and TRAVNICEK, J. (1968). The phagocytic activity of reticuloendothelial system of newborn precolostral pigs to smooth and rough *Escherichia coli*. *Folia microbiol.*, *13*, 472.

MILLER, J.F.A.P. (1961). Immunological function of the thymus. *Lancet*, *2*, 748.

MILLER, J.F.A.P. (1962). Effect of neonatal thymectomy on the immunological responsiveness of the mouse. *Proc. R. Soc. (Ser. B).*, *156*, 415.

MILLER, J.F.A.P., MITCHELL, G.F. and WEISS, N.S. (1967). Cellular basis of the immunological defects in thymectomized mice. *Nature (Lond.)*, *214*, 992.

MILLER, J.F.A.P. and OSOBA, D. (1967). Current concepts of the immunological function of the thymus. *Physiol. Rev.*, *47*, 437.

MILLER, J.F.A.P. and WARNER, N.L. (1971). The immune response of normal, irradiated and thymectomized mice to fowl immunoglobulin G as detected by a haemolytic plaque technique. *Int. Arch. Allergy app. Immun.*, *40*, 59.

MILLER, H.R.P., TERNYNCK, T. and AVRAMEAS, S. (1974). A comparison of specific antibody and immunoglobulin synthesizing immunocytes in peroxidase stimulated lymph nodes. *Ann. Immunol. (Inst. Pasteur)*, *125c*, 231.

MILLER, H.R.P., TERNYNCK, T. and AVRAMEAS, S. (1975). Synthesis of antibody and immunoglobulins without detectable antibody function in cells responding to horseradish peroxidase. *J. Immun.*, *114*, 626.

MITCHELL, G.F., GRUMET, F.C. and McDEVITT, H.O. (1972). Genetic control of the immune response: the effect of thymectomy on the primary and secondary antibody response of mice to poly-L (try, glu)-poly-D, L-ala-poly-L-lys. *J. exp. Med.*, *135*, 126.

MOLL, L. (1906). *Ztschr. f. exp. Path. U. Ther.*, *3*, 325.

MOLLER, G. (1965). 19S antibody production against soluble lipopolysaccharide antigens by individual lymphoid cells *in vitro*. *Nature (Lond.)*, *207*, 1166.

MOLLER, E. (1974). B cell markers and subclasses. *Prog. Immunol. II.*, *3*, 308.

MONACO, A.P., WOOD, M.L. and RUSSELL, P.S. (1965). Adult thymectomy: effect on recovery from immunologic depression in mice. *Science*, *149*, 432.

MONTGOMERY, P.C. and WILLIAMSON, A.R. (1970). Molecular restriction of anti-hapten antibody elicited in neonatal rabbits. *Nature (Lond.)*, *228*, 1306.

MOORE, M.A.S. and OWEN, J.J.T. (1965). Chromosome marker studies on the development of the haemopoietic system in the chick embryo. *Nature (Lond.)*, *208*, 956.

MOORE, M.A.S. and OWEN, J.J.T. (1967). Experimental studies on the development of the thymus. *J. exp. Med.*, *126*, 715.

MOSIER, D.E. and JOHNSON, B.M. (1975). Ontogeny of mouse lymphocyte function II. Development of the ability to produce antibody is modulated by T-lymphocytes. *J. exp. Med.*, *141*, 216.

MOUTON, D., BIOZZI, G., BOUTHILLIER, Y. and STIFFEL, C. (1963). Phagocytosis of *Salmonellae* by reticuloendothelial cells of new-born piglets lacking natural antibody. *Nature (Lond.)*, *197*, 706.

NASH, D.R., CRABBE, P.A., BAZIN, H., EYSSEN, H. and HEREMANS, J.F. (1969). Specific and non-specific immunoglobulin synthesis in germ-free mice immunized with ferritin by different routes. *Experimentia, 25,* 1094.

NETER, E. (1969). Endotoxins and the immune response. *Curr. Top. Microb. Immunol., 47,* 82.

NIEDERHUBER, J.E., SHERMETA, D., TURCOTTE, J.G. and GIKAS, R.W. (1971). Kidney transplantation in the foetal lamb. *Transplantation, 12,* 161.

NOSSAL, G.J.V. and PIKE, B.L. (1972). Differentiation of B-lymphocytes from stem cell precursors. *Adv. exp. Med. Biol., 29,* 11.

NOSSAL, G.J.V., SZENBERG, A., ADA, G.L. and AUSTIN, C.M. (1964). Single cell studies on 19S antibody production. *J. exp. Med., 119,* 485.

NOSSAL, G.J.V., WARNER, N.L. and LEWIS, H. (1971). Incidence of cells simultaneously secreting IgM and IgG antibody to sheep erythrocytes. *Cell. Immunol., 2,* 41.

NOSSAL, G.J.V., WARNER, N.L., LEWIS, H. and SPRENT, J. (1972). Quantitative features of a sandwich radioimmuno-labelling technique for lymphocyte surface receptors. *J. exp. Med., 135,* 405.

OLINS, D.E. and EDELMAN, G.M. (1964). Reconstitution of 7S molecules from L and H polypeptide chains of antibodies and γ-globulin. *J. exp. Med., 119,* 789.

OPPENHEIM, J.J. (1968). Relationship of *in vitro* lymphocyte transformation to delayed hypersensitivity in guinea pigs and man. *Fedn. Proc., 27,* 21.

OSBORN, J.J., DANCIS, J. and JULIA, J.F. (1952). Studies of the immunology of the newborn infant. *Paediatrics, 9,* 736.

OSBURN, B.I. and HOSKINS, R.K. (1969). Antibody responses to *Brucella ovis* in maternal and foetal sheep. *J. infect. Dis., 119,* 267.

OSBURN, B.I. and HOSKINS, R.K. (1971). Infection with *Vibrio foetus* in the immunologically immature foetal calf. *J. infect. Dis., 123,* 32.

OSBURN, B.I., JOHNSON, R.T., SILVERSTEIN, A.M., PRENDERGAST, R.A., JOCHIM, M.M. and LEVY, S.E. (1971). Experimental viral-induced congenital encephalopathies II. The pathogenesis of blue tongue vaccine virus infection in foetal lambs. *Lab. Invest., 25,* 206.

OSOBA, D. and MILLER, J.F.A.P. (1963). Evidence for a humoral thymus factor responsible for the maturation of immunological faculty. *Nature (Lond.), 199,* 653.

OUDIN, J. and MICHEL, M. (1969). Idiotypy of rabbit antibodies II. Comparison of idiotypy of various kinds of antibodies formed in the same rabbits against *Salmonella typhi*. *J. exp. Med.*, *130*, 619.

OWEN, J.J.T. (1972). The origin and development of lymphocyte populations. *In* Ontogeny of acquired immunity (Ciba Fndn.) p. 35.

OWEN, J.J.T., COOPER, M.D. and RAFF, M.C. (1974). *In vitro* generation of B-lymphocytes in mouse foetal liver; a mammalian "bursa equivalent". *Nature (Lond.)*, *249*, 361.

OWEN, J.J.T. and RAFF, M.C. (1970). Studies on the differentiation of thymus-derived lymphocytes. *J. exp. Med.*, *132*, 1216.

OWEN, J.J.T. and RITTER, M.A. (1969). Tissue interactions in the development of thymus lymphocytes. *J. exp. Med.*, *129*, 431.

OXER, D.T. (1936). The transmission of antitoxic immunity from the ewe, vaccinated against entero-toxaemia, to the lamb. *Aust. vet. J.*, *12*, 54.

PAPIERNIK, M. (1970). Correlation of lymphocyte transformation and morphology in the human foetal thymus. *Blood*, *36*, 470.

PARISH, C.R. and STANLEY, P. (1972). Chemical and biological properties of bacterial flagellin following iodination and oxidation by chloramine -T. *Immunochem.*, *9*, 853.

PARISH, C.R., WISTAR, R. and ADA, G.L. (1969). Cleavage of bacterial flagellin with cyanogen bromide: antigenic properties of the protein fragments. *Biochem. J.*, *113*, 501.

PARKHOUSE, R.M.E. (1973). Assembly and secretion of immunoglobulin M (IgM) by plasma cells and lymphocytes. *Transplant. Rev.*, *14*, 131.

PARROT, D.M., DeSOUSA, M.A.B. and EAST, J. (1966). Thymus-dependent areas in the lymphoid organs of neonatally thymectomized mice. *J. exp. Med.*, *123*, 191.

PATON, D.N. and GOODALL, A. (1904). Contribution to the physiology of the thymus. *J. Physiol. (Lond.)*, *31*, 49.

PAUL, W.E. (1974). Ontogeny and differentiation of thymus-independent lymphocytes. *J. Reticuloendothl. Soc.*, *15*, 242.

PEARSON, L.D., SIMPSON-MORGAN, M.W. and MORRIS, B. (1976). Lympho-poiesis and lymphocyte recirculation in the sheep foetus. *J. exp. Med.*, "In Press".

PEDERSEN, N.C. and MORRIS, B. (1974). The rate of formation and the composition of lymph from primary and secondary renal allografts. *Transplantation*, *17*, 48.

PEGRUM, G.D., READY, D. and THOMPSON, E. (1968). The effects of phytohaemagglutinin on human foetal cells grown in culture. *Br. J. Haemat.*, *15*, 371

PEREY, D.Y.E. and BIENENSTOCK, J. (1973). Effects of bursectomy and thymectomy on ontogeny of fowl IgA, IgG and IgM. *J. Immun.*, *111*, 633.

PERNIS, B., COHEN, M.W. and THORBECKE, G.J. (1963). Specificity of reaction to antigenic stimulation in lymph nodes of immature rabbits. *J. Immun.*, *91*, 541.

PERNIS, B., FORNI, L. and AMANTE, L. (1970). Immunoglobulin spots on the surface of rabbit lymphocytes. *J. exp. Med.*, *132*, 1001.

PERNIS, B., FORNI, L. and AMANTE, L. (1971). Immunoglobulins as cell receptors. *Ann. N.Y. Acad. Sci.*, *110*, 420.

PHILLIPS, J.M. and DRESSER, D.W. (1973). Isoelectric spectra of different classes of anti-erythrocyte antibodies. *Eur. J. Immunol.*, *3*, 524.

PIERCE, C.W., SOLLIDAY, S.M. and ASOFSKY, R. (1972a). Immune responses *in vitro* IV. Suppression of primary γM, γG, and γA plaque-forming cell responses in mouse spleen cell cultures by class-specific antibody to mouse immunoglobulin. *J. exp. Med.*, *135*, 675.

PIERCE, C.W., SOLLIDAY, S.M. and ASOFSKY, R. (1972b). Immune responses *in vitro* V. Suppression of γM, γG, and γA plaque-forming cell responses in cultures of primed mouse spleen cells by class-specific antibody to mouse immunoglobulins. *J. exp. Med.*, *135*, 698.

PIERPAOLI, W., BARONI, C., FABRIS, N. and SORKIN, E. (1969). Hormones and immunological capacity II. Reconstitution of antibody production in hormonally deficient mice by somatotrophic hormone, thyrotrophic hormone and thyroxine. *Immunology*, *16*, 217.

PIERPAOLI, W., FABRIS, N. and SORKIN, E. (1970). Developmental hormones and immunological maturation. *In* Hormones and the immune response (Ciba Fndn.), p. 126.

PIERPAOLI, W. and SORKIN, E. (1967). Relation between thymus and hypophysis. *Nature (Lond.)*, *215*, 834.

PIERPAOLI, W. and SORKIN, E. (1969). A study on anti-pituitary serum. *Immunology*, *16*, 311.

PLAYFAIR, J.H.L. (1968). Strain differences in the immune response of mice I. The neonatal response to sheep red cells. *Immunology*, *15*, 35.

POSTON, R.N. (1974). A buffered chromic chloride method of attaching antigens to red cells: use in haemagglutination. *J. Immunol. Methods*, *5*, 91.

PRITCHARD, H., RIDDAWAY, J. and MICKLEM, H.S. (1973). Immune responses in congenitally thymus-less mice. *Clin. exp. Immuol.*, *13*, 125.

PROKESOVA, L. and REJNEK, J. (1971). Active synthesis of IgA in newborn precolostral piglets. *Folia Microbiol.*, *16*, 476.

PROKESOVA, L., REJNEK, J., STERZL, J. and TRAVNICEK, J. (1969). Isolation and characterization of immunoglobulins in the serum of precolostral pigs. *Folia Microbiol.*, *14*, 372.

PROKESOVA, L., REJNEK, J., STERZL, J. and TRAVNICEK, J. (1970). Ontogenetic development of immunoglobulins in pigs. *In* Developmental aspects of antibody formation and structure (ed. Sterzl and Riha - Czechoslovak Acad. Sci.) p. 757.

RABELLINO, E., COLON, S., GREY, H.M. and UNANUE, E.R. (1971). Immunoglobulins on the surface of lymphocytes I. Distribution and quantitation. *J. exp. Med.*, *133*, 156.

RAFF, M.C. (1970). Two distinct populations of peripheral lymphocytes in mice distinguishable by immunofluorescence. *Immunology*, *19*, 637.

RAFF, M.C. (1971). Evidence for subpopulations of mature lymphocytes within mouse thymus. *Nature (New Biol)*, *229*, 182.

RAFF, M.C. (1973). T and B lymphocytes and immune responses. *Nature (Lond.)*, *242*, 19.

RAFF, M.C. and CANTOR, H. (1971). Subpopulations of thymus cells and thymus-derived lymphocytes. *Prog. Immunol. I*, 83.

RAFF, M.C. and OWEN, J.J.T. (1971). The use of surface alloantigenic markers to study the differentiation of thymus-derived lymphocytes in mice. *Adv. exp. Med. Biol.*, *12*, 11.

RANK, W.R., DiPAULI, R. and FLUGGE-RANK, U. (1972). The lipid A immunity system I. Induction of heterophile antibodies by enterobacterial lipopolysaccharide and their lipid A component. *Eur. J. Immunol.*, *2*, 517.

READE, P.C. (1968). The development of bactericidal activity in rat peritoneal macrophages. *Aust. J. exp. Biol. med. Sci.*, *46*, 231.

READE, P.C. and JENKIN, C.R. (1965). The functional development of the reticulo-endothelial system I. The uptake of intravenously injected particles by foetal rats. *Immunology*, *9*, 53.

READE, P.C., TURNER, K.J. and JENKIN, C.R. (1965). The functional development of the reticulo-endothelial system IV. Studies of serum opsonins to *Salmonella typhimurium* in foetal and neonatal rats. *Immunology*, *9*, 75.

REJNEK, J. and PROKESOVA, L. (1973). Immunoglobulins and antibodies in pigs. *Cont. Top. Mol. Immunol.*, *2*, 117.

RICE, C.E. and SILVERSTEIN, A.M. (1964). Haemolytic complement activity of sera of foetal and newborn lambs. *Can. J. comp. Med.*, *28*, 34.

RICHARDSON, M., BECK, C.C. and CLARK, D.T. (1968). Prenatal immunization of the lamb to *Brucella:* dissociation of immunocompetence and reactivity. *J. Immun.*, *101*, 1363.

RICHARDSON, M., CONNER, G.H., BECK, C.C. and CLARK, D.T. (1971). Prenatal immunization of the lamb to *Brucella:* secondary antibody response *in utero* and at birth. *Immunology*, *21*, 795.

RITTENBERG, M.B. and PRATT, K.L. (1969). Antitrinitrophenyl (TNP) plaque assay. Primary response of Balb/C mice to soluble and particulate immunogen. *Proc. Soc. exp. Biol. Med.*, *132*, 575.

RODEY, G.E., DAY, N., HOLMES-GRAY, B. and GOOD, R.A. (1972). Ontogeny of the phytohaemagglutinin response of foetal piglet thymic and splenic lymphocytes. *Infect. Immun.*, *5*, 337.

RODEY, G.E. and GOOD, R.A. (1969). The *in vitro* response to phytohaemagglutinin of lymphoid cells from normal and neonatally thymectomized adult mice. *Int. Archs. Allergy app. Immun.*, *36*, 399.

ROELANTS, G.E. and GOODMAN, J.W. (1974). Kinetics and clonal restriction of the anti-dinitrophenyl antibody response to dinitrophenyl -6-amino-caproyl-L-tyrosine-azobenzene-p-arsonate in guinea pigs. *J. Immun.*, *112*, 883.

ROSZMAN, T.L., FOLDS, J.D. and STAVITSKY, A.B. (1971). A kinetic and quantitative analysis of antibody and protein synthesis during the primary response to Keyhole Limpet haemocyanin. *Immunology*, *20*, 1041.

ROUSE, B.T. and WARNER, N.L. (1972). Suppression of graft versus host reactions in chickens by pretreatment of leucocytes with anti-light chain sera. *Cell. Immunol.*, *3*, 470.

ROWE, D.S., CRABBE, P.A. and TURNER, M.W. (1968). Immunoglobulin D in serum, body fluids and lymphoid tissues. *Clin. exp. Immunol.*, *3*, 477.

ROWE, D.S., HUG, K., FORNI, L. and PERNIS, B. (1973b). Immunoglobulin D as a lymphocyte receptor. *J. exp. Med.*, *138*, 965.

ROWE, D.S., HUG, K., PAGE FAULK, W., McCORMICK, J.N. and GERBER, H. (1973a). IgD on the surface of peripheral blood lymphocytes of the human newborn. *Nature (New Biol.)*, *242*, 155.

ROWLANDS, D.T., BLAKESLEE, D. and ANGALA, E. (1974). Acquired immunity in opossum *(Didelphis virginiana)* embryos. *J. Immun.*, *112*, 2148.

RUBIN, E., COOPER, M.D. and KRAUS, F.W. (1971). Kinetics of cellular proliferation in the bursa of Fabricius. *Bacteriol. Proc.*, p. 67.

RUEBNER, B.H., KANAYAMA, R., BRONSON, R.T. and BLUMENTHAL, S. (1974). Meconium corpuscles in intestinal epithelium of foetal and newborn primates. *Archs. Path., 98,* 396.

SANDREUTER, A. (1951). Vergleichende Untersuchungen über die Blutbildung in der Ontogenese von Haushuhn und Star. *Acta. anat. (Supp. 14), 11,* 1.

SARVAS, H. and MAKELA, O. (1970). Haptenated bacteriophage in the assay of antibody quantity and affinity: maturation of an immune response. *Immunochem., 7,* 933.

SCHAFFNER, T., HESS, M.W. and COTTIER, H. (1974). A reappraisal of bursal function. *Ser. Haemat., 7,* 568.

SCHINCKEL, P.G. and FERGUSON, K.A. (1953). Skin transplantation in the foetal lamb. *Aust. J. biol. Sci., 6,* 533.

SCHLESINGER, M. (1970). Anti-θ antibodies for detecting thymus-dependent lymphocytes in the immune response of mice to SRBC. *Nature (Lond.), 226,* 1254.

SCHLESINGER, M. (1972). Antigens of the thymus. *Progr. Allergy, 16,* 214.

SCHLESINGER, M. (1974). The lymphocyte membrane. *Ser. Haemat., 7,* 427.

SCHLESINGER, M., GOTTESFELD, S. and KORZASH, Z. (1973). Thymus cell subpopulations separated by discontinuous BSA gradients: antigenic properties and circulation capacity. *Cell. Immunol., 6,* 49.

SCHNEIDER, L. and SZATHMARY, J. (1939). Ueber die Immunität des neugebornen Lammes. *Z. Immunitat. f. exp. Ther., 95,* 169.

SCHRADER, J.W. (1973). Specific activation of the bone marrow-derived lymphocytes by antigen presented in a non-multivalent form. *J. exp. Med., 137,* 844.

SCHRADER, J.W., (1974). The mechanism of bone marrow-derived (B) lymphocyte activation I. Early events in antigen-induced triggering in the presence of polymerized flagellin. *Eur. J. Immunol., 4,* 14.

SCHULTZ, R.D. (1973). Developmental aspects of the foetal bovine immune response: a review. *Cornell Vet., 63,* 507.

SCHULTZ, R.D., DUNNE, H.W. and HEIST, C.E. (1971b). Ontogeny of the bovine immune response. *J. Dairy Sci., 54,* 1321.

SCHULTZ, R.D., WANG, J.T. and DUNNE, H.W. (1971a). Development of the humoral immune response of the pig. *Am. J. vet. Res., 32,* 1331.

SHALABY, M.R. and AUERBACH, R. (1973). Studies on the maturation of immune responsiveness in the mouse I. The *in vivo* response to sheep red blood cells. *Differentiation, 1,* 167.

SHERWIN, W.K. and ROWLANDS, D.T. (1974). Development of humoral immunity in lethally irradiated mice reconstituted with foetal liver. *J. Immun., 113,* 1353.

SHIFRINE, M., SMITH, J.B., BULGIN, M.S., BRYANT, B.J., ZEE, Y.-C., and OSBURN, B.I. (1971). Response of canine foetuses and neonates to antigenic stimulation. *J. Immun., 107,* 965.

SILVERSTEIN, A.M. (1964). Ontogeny of the immune response. *Science, 144,* 1423.

SILVERSTEIN, A.M. (1972). Immunological maturation in the foetus: modulation of the pathogenesis of congenital infectious diseases. *In* Ontogeny of acquired immunity (Ciba Found.) p. 17.

SILVERSTEIN, A.M. and KRANER, K.L. (1965). Studies on the ontogenesis of the immune response. *In* Molecular and cellular basis of antibody formation (ed. Sterzl) p. 341.

SILVERSTEIN, A.M. and LUKES, R.J. (1962). Foetal response to antigenic stimulus I. Plasmacellular and lymphoid reactions in the human foetus to intrauterine infection. *Lab. Invest., 11,* 918.

SILVERSTEIN, A.M., PARSHALL, C.J. and UHR, J.W. (1966). Immunological maturation *in utero:* kinetics of the primary antibody response in the foetal lamb. *Science, 154,* 1675.

SILVERSTEIN, A.M. and PRENDERGAST, R.A. (1970). Lymphogenesis, immunogenesis, and the generation of immunological diversity. *In* Developmental aspects of antibody formation and structure (ed. Sterzl and Riha) p. 69.

SILVERSTEIN, A.M. and PRENDERGAST, R.A. (1971). The maturation of lymphoid tissue structure and function in ontogeny. *Adv. exp. Med. Biol., 12,* 37.

SILVERSTEIN, A.M. and PRENDERGAST, R.A. (1973). Immunosuppression of the thymectomized foetal lamb and its neonatal reconstitution. *Adv. exp. Med. Biol., 29,* 383.

SILVERSTEIN, A.M., PRENDERGAST, R.A. and KRANER, K.L. (1964). Foetal response to antigenic stimulus IV. Rejection of skin homografts by the foetal lamb. *J. exp. Med., 119,* 955.

SILVERSTEIN, A.M., THORBECKE, G.J., KRANER, K.L. and LUKES, R.J. (1963b). Foetal response to antigenic stimulus III. γ-globulin production in normal and stimulated foetal lambs. *J. Immun., 91,* 384.

SILVERSTEIN, A.M., UHR, J.W., KRANER, K.L. and LUKES, R.J. (1963a). Foetal response to antigenic stimulus II. Antibody production by the foetal lamb. *J. exp. Med.*, *117*, 799.

SINCLAIR, N.R.StC. (1967). A comparison of primary and secondary haemolysin responses to sheep erythrocytes in neonatally thymectomized, sham-thymectomized and normal Swiss mice. *Immunology*, *12*, 559.

SMEATON, T.C., COLE, G.J., SIMPSON-MORGAN, M.W. and MORRIS, B. (1969). Techniques for the long-term collection of lymph from the unanaesthetized foetal lamb *in utero*. *Aust. J. exp. Biol. med. Sci.*, *47*, 565.

SMITH, R.T. and BRIDGES, R.A. (1958). Immunological unresponsiveness in rabbits produced by neonatal injection of defined antigens. *J. exp. Med.*, *108*, 227.

SMITH, G.P., HOOD, L. and FITCH, W.M. (1971). Antibody diversity. *A. Rev. Biochem.*, *40*, 969.

SOLOMON, J.B. (1970). Unification of foetal and neonatal immunology. *Nature (Lond.)*, *227*, 895.

SOLOMON, J.B. (1971). Foetal and neonatal immunology. *Frontiers of Biology, Vol. 20.*

SPEAR, P.G. and EDELMAN, G.M. (1974). Maturation of the humoral immune response in mice. *J. exp. Med.*, *139*, 249.

SPEAR, P.G., WANG, A.-L., RUTISHAUSER, U. and EDELMAN, G.M. (1973). Characterization of splenic lymphoid cells in foetal and newborn mice. *J. exp. Med.*, *138*, 557.

SPRENT, J. (1973). Circulating T and B lymphocytes of the mouse I. Migratory properties. *Cell. Immunol.*, *7*, 10.

STEINER, L.A. and EISEN, H.N. (1967). The relative affinity of antibodies synthesized in the secondary response. *J. exp. Med.*, *126*, 1185.

STEINMULLER, D. (1961a). Transplantation immunity in the newborn rat I. The response at birth and maturation of response capacity. *J. exp. Zool.*, *147*, 233.

STEINMULLER, D. (1961b). Skin transplantation in newborn rats and mice. *In* Transplantation of tissues and cells (ed. Billingham and Silvers) p. 27.

STERZL, J., KOSTKA, J., MANDEL, L., RIHA, I. and HOLUB, M. (1962). Development of the formation of γ-globulin and of normal and immune antibodies in piglets reared without colostrum. *In* Mechanisms of antibody formation (Czechoslovak. Acad. Sci.) p. 130.

STERZL, J., MANDEL, L., MILER, I. and RIHA, I. (1965). Development of immune reactions in the absence or presence of an antigenic stimulus. *In* Molecular and cellular basis of antibody formation (ed. Sterzl) p. 351.

STERZL, J. and NORDIN, A. (1971). Demonstration of the common precursor for production of IgM and IgG antibodies. *Folia microbiol., 16,* 1.

STERZL, J., REJNEK, J. and TRAVNICEK, J. (1966). Impermeability of pig placenta for antibodies. *Folia microbiol., 11,* 7.

STERZL, J. and SILVERSTEIN, A.M. (1967). Developmental aspects of immunity. *Adv. Immunol., 6,* 337.

STEWARD, J.P. (1971). Immunologic response of neonatally thymectomized rats to sheep red blood cells and to flagellin from *Salmonella adelaide*. *Proc. Soc. exp. Biol. Med., 138,* 702.

STITES, D.P., WYBRAN, J., CARR, M.C. and FUDENBERG, H.H. (1972). Development of cellular immunocompetence in man. *In* Ontogeny of acquired immunity (Ciba. Fndn.) p. 113.

STOKES, H. and BODA, J.M. (1968). Immunofluorescent localization of growth hormone and prolactin in the adenohypophysis of foetal sheep. *Endocrinology, 83,* 1362.

STUTMAN, O. and GOOD, R.A. (1973). Thymus hormones. *Cont. Top. Immunobiol., 2,* 299.

SWIFT, B.L. and KENNEDY, P.C. (1972). Experimentally induced infection of *in utero* bovine foetuses with bovine para-influenza 3 virus. *Am. J. vet. Res., 33,* 57.

SYMONS, D.B.A. and BINNS, R.M. (1975). Immunoglobulin-bearing lymphocytes: their demonstration in adult sheep and ontogeny in the sheep foetus. *Int. Archs. Allergy. appl. Immun., 49,* 658.

TAUSSIG, M.J. (1973). Antigenic competition. *Curr. Top. Microbiol. Immunol., 60,* 125.

TAYLOR, R.B. and WORTIS, H.H. (1968). Thymus dependence of antibody response: variation with dose of antigen and class of antibody. *Nature (Lond.), 220,* 927.

THORBECKE, G.J. (1959). Some histological and functional aspects of lymphoid tissue in germ gree animals I. Morphological studies. *Ann. N.Y. Acad. Sci., 78,* 237.

THORBECKE, G.J. and KEUNING, F.J. (1953). Antibody formation *in vitro* by haemopoietic organs after subcutaneous and intravenous immunization. *J. Immun., 70,* 129.

THORBECKE, G.J., WARNER, N.L., HOCHWALD, G.M. and OHANIAN, S.H. (1968). Immune globulin production by the bursa of Fabricius of young chickens. *Immunology, 15,* 123.

THORBURN, G.D. and HOPKINS, P.S. (1972). Thyroid function in the foetal lamb. *In* Foetal and neonatal physiology (Camb. Uni. Press) p. 488.

TLASKALOVA, H., STERZL, J., HAJEK, P., POSPISIL, M., RIHA, I., MARVANOVA, H., KAMARYTOVA, V., MANDEL, L., KRUML, J. and KOVARU, F. (1970). The development of antibody formation during embryonal and postnatal periods. *In* Developmental aspects of antibody formation and structure (ed. Sterzl and Riha) VII, p. 767.

TRAININ, N. (1974). Thymic hormones and the immune response. *Physiol. Rev.*, *54*, 272.

TRAININ, N. and LINKER-ISRAELI, M. (1967). Restoration of immunological reactivity of thymectomized mice by calf thymus extracts. *Cancer Res.*, *27*, 309.

TRAININ, N. and SMALL, M. (1973). Thymic humoral factors. *Cont. Top. Immunobiol.*, *2*, 321.

TRAVNICEK, J. and MANDEL, L. (1971). The development of serum proteins in pig foetuses during prenatal period. *Folia microbiol.*, *16*, 92.

TRUEBLOOD, M.S., SWIFT, B.L. and BEAR, P.D. (1971). Bovine foetal responses to *Anaplasma marginale*. *Am. J. vet. Res.*, *32*, 1089.

TYAN, M.L., COLE, L.J. and HERTZENBERG, L.A. (1967). Foetal liver: a source of immunoglobulin producing cells in the mouse. *Proc. Soc. exp. Biol. Med.*, *124*, 1161.

UHR, J.W. (1960). Development of delayed-type hypersensitivity in guinea pig embryos. *Nature (Lond.)*, *187*, 957.

UHR, J.W., DANCIS, J., FRANKLIN, E.C., FINKELSTEIN, M.S. and LEWIS, E.W. (1962a). The antibody response to bacteriophage ϕ X 174 in newborn premature infants. *J. clin. Invest.*, *41*, 1509.

UHR, J.W., DANCIS, J. and NEUMANN, C.G. (1960). Delayed-type hypersensitivity in premature neonatal humans. *Nature (Lond.)*, *187*, 1130.

UHR, J.W., FINKELSTEIN, M.S. and BAUMANN, J.B. (1962b). Antibody formation III. The primary and secondary antibody response to bacteriophage ϕ X 174 in guinea pigs. *J. exp. Med.*, *115*, 655.

URBAIN-VANSANTEN, G. (1970). Concomitant synthesis, in separate cells, of non-reactive immunoglobulins and specific antibodies after immunization with tobacco mosaic virus. *Immunology*, *19*, 783.

VAN ALTEN, P.J. and MEUWISSEN, H.J. (1972). Production of specific antibody by lymphocytes of the bursa of Fabricius. *Science*, *176*, 45.

VAN FURTH, R., COHN, Z.A., HIRSCH, J.G., HUMPHREY, J.H., SPECTOR, W.G. and LONGEVOORT, H.L. (1972). The mononuclear phagocyte system: a new classification of macrophages, monocytes and their precursor cells. *Bull. W.H.O.*, *46*, 845.

VAN FURTH, R., SCHUIT, H.R.E. and HIJMANS, W. (1965). The immunological development of the human foetus. *J. exp. Med.*, *122*, 1173.

VARELA-DIAZ, V.M. and SOULSBY, E.J.L. (1972). Immunoglobulin synthesis in sheep: IgG_2 deficiency in neonatal lambs. *Res. vet. Sci.*, *13*, 99.

VENZKE, W.G. (1952). Morphogenesis of the thymus of chicken embryos. *Am. J. vet. Res.*, *13*, 395.

VITETTA, E.S., BAUR, S. and UHR, J.W. (1971). Cell surface immunoglobulin II. Isolation and characterization of immunoglobulin from mouse splenic lymphocytes. *J. exp. Med.*, *134*, 242.

VITETTA, E.S., BIANCO, C., NUSSENZWEIG, V. and UHR, J.W. (1972). Cell surface immunoglobulin IV. Distribution among thymocytes bone marrow cells, and their derived populations. *J. exp. Med.*, *136*, 81.

VITETTA, E.S., MELCHER, U., McWILLIAMS, M., LAMM, M.E., PHILLIPS-QUAGLIATA, J.M. and UHR, J.W. (1975). Cell surface immunoglobulin XI. The appearance of an IgD-like molecule on murine lymphoid cells during ontogeny. *J. exp. Med.*, *141*, 206.

de VOS-CLOETENS, C., MINSART-BALERIAUX, V. and URBAIN-VANSANTEN, G. (1971). Possible relationship between antibodies and non-specific immunoglobulins simultaneously induced after antigenic stimulation. *Immunology*, *20*, 955.

VOSS, E.W. and EISEN, H.N. (1968). Valency and affinity of IgM antibodies to the 2, 4, dinitrophenyl (DNP) group. *Fedn. Proc.*, *27*, 684.

WALTERS, C.S. and WIGZELL, H. (1970). Demonstration of heavy and light chain antigenic determinants on the cell-bound receptor for antigen. *J. exp. Med.*, *132*, 1233.

WATSON, J., EPSTEIN, R., NAKOINZ, I. and RALPH, P. (1973). The role of humoral factors in the initiation of *in vitro* primary immune responses II. Effect of lymphocyte mitogens. *J. Immun.*, *110*, 43.

WEBER, W.T. (1966). The *in vitro* response of thymic lymphocytes of the pig to phytohaemagglutinin. *J. Cell. Physiol.*, *67*, 285.

WEBER, W.T. (1967). Differences between medullary and cortical thymic lymphocytes of the pig in their response to phytohaemagglutinin. *J. Cell. Physiol.*, *68*, 117.

WIGZELL, H. (1970). Specific fractionation of immunocompetent cells. *Transplant. Rev.*, *5*, 76.

WIGZELL, H. (1974). On the relationship between cellular and humoral antibodies. *Cont. Top. Immunobiol.*, *3*, 77.

WILLIAMSON, A.R. (1971). Antibody Isoelectric spectra: analysis of the heterogeneity of antibody molecules in the serum by isoelectric focusing in gel and specific detection with hapten. *Eur. J. Immunol.*, *1*, 390.

WILLIAMSON, A.R. and ASKONAS, B.A. (1972). Senescence of an antibody-forming cell clone. *Nature (Lond.)*, *238*, 337.

WINKELSTEIN, A. and CRADDOCK, C.G. (1967). Comparative response of normal human thymus and lymph node cells to phytohaemagglutinin in culture. *Blood*, *29*, 594.

WHITTINGHAM, S. and MACKAY, I.R. (1973). Rosette formation by human thymocytes. *Cell. Immunol.*, *6*, 362.

WYBRAN, J., CARR, M.C. and FUDENBERG, H.H. (1972). The human rosette-forming cell as a marker of a population of thymus-derived cells. *J. clin. Invest.*, *51*, 2537.